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Kinam Park *Editors*

Cancer Targeted Drug Delivery

An Elusive Dream

Springer

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Preface

Cancer is something that nobody welcomes. Yet, it visits us randomly. Once cancer shows up on our doorstep, it just won't go away. It would be nice if we can figure out why and when cancer decides to visit any one of us. Cancer treatment has become more effective over the years, at least for some cancers, but overall cancer is seldom curable. Developing new effective drugs is important, but most drugs have side effects. The drug is useful as long as the benefit is larger than the side effects, but having the drug without side effects is the ultimate goal. One of the functions of drug delivery systems is to minimize the side effects while maximizing the pharmacological efficacy.

While current drug delivery systems have shown promising results in increasing the benefit/risk ratio, the technology has to be improved substantially. Thus, we ask a simple, yet fundamental, question: why is it so difficult to cure cancer? Cancer is not a single disease that can be eradicated by a single drug. Cancer occurs for a variety of reasons and no two cancers are identical. To improve our chances of treating cancers successfully, depending on how we define "successful," we need to understand cancer better. As Sun Tzu pointed out in his famous book, The Art of War, winning the battles requires knowing the enemy and ourselves. President Nixon declared the war on cancer, i.e., signed the National Cancer Act, in 1971, making cancer our enemy. This enemy is derived from our own cells, and unfortunately, is evasive as well as evolving; thus winning the battle becomes complicated. To know ourselves means knowing our current technologies on delivery of anticancer agents. One of our goals of editing this book was to define our enemy more clearly and understand our own ability of targeted drug delivery better.

Cancer Targeted Drug Delivery: An Elusive Dream begins with knowing the enemy through learning the history of our efforts on selected drug delivery to cancer cells, tumor physiology and the microenvironment around tumors, and tumor heterogeneity. To better understand ourselves, several chapters describe the current state-of-the art approaches of reaching cancer cells and developing improved preclinical models. Finally, this book discusses the current missing components and what can be done in the near future. It is the hope of the editors that the information in this book

can be used to stimulate scientists in the field to find better ways to manage cancers. The immediate goal is to find a way for the cancer patients to live a normal life without cancer recurrence for an extended period of time, hopefully the lifetime of the patients. The ultimate goal, of course, is to find a way to achieve our elusive dream of curing cancers.

The editors are indebted to Carolyn Honour at Springer who invited us to work on the topic of this book. We would also like to thank Renata Hutter who handled all administrative aspects of editing this volume. Our thanks go to all authors of *Cancer Targeted Drug Delivery*. The quality of the book is only as good as the quality of the authors, and we can confidently announce that the quality of this book cannot be higher. We hope that this book will serve as a valuable source of collective information on targeted drug delivery to cancers for scientists with all levels of background and experience.

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Part I
History of Cancer Targeting Efforts

Chapter 1
A Time Travel Journey Through Cancer Therapies

Ana S. Cravo and Randall J. Mrsny

Abstract The twentieth century was a landmark period in the history of cancer therapies; a time in which conventional cancer treatments such as surgery resection, radiotherapy and chemotherapy made tremendous advances and gave birth to strategies focused on greater selective targeting. Rational drug design allied to rational drug delivery, being led by novel small molecule anti-cancer compounds and monoclonal antibodies. Such approaches prolonged survival time, often without the horrific side effects of previous therapies, but rarely prevented ultimate disease relapse. Selective targeting of membrane transporters and receptors using prodrugs, polymer-cancer drug conjugates and antibody-toxin combinations has contributed to redefine the roadmap of cancer cell targeting. Current pharmacotherapies are still far from consistently delivering cures or sustained remissions. This chapter describes some of the historical events that brought about current cancer targeting strategies.

Introduction

Throughout their history, humans have battled cancer. From Hippocrates' times to the post-genomics era, numerous questions about cancer were asked with countless hypotheses formulated and tested; many questions are still unanswered and many hypotheses remain to be tested. Over the past one and a half centuries, a plethora of scientific advances in cancer research have identified the current options to treat cancer. The goal of this chapter is to highlight some of the major milestones for cancer therapies over the past 50 years in a timeline fashion. To guide the reader throughout this chapter and to better illustrate some of the breakthroughs in cancer research, a chronological timeline has been assembled (Fig. 1.1). Each event

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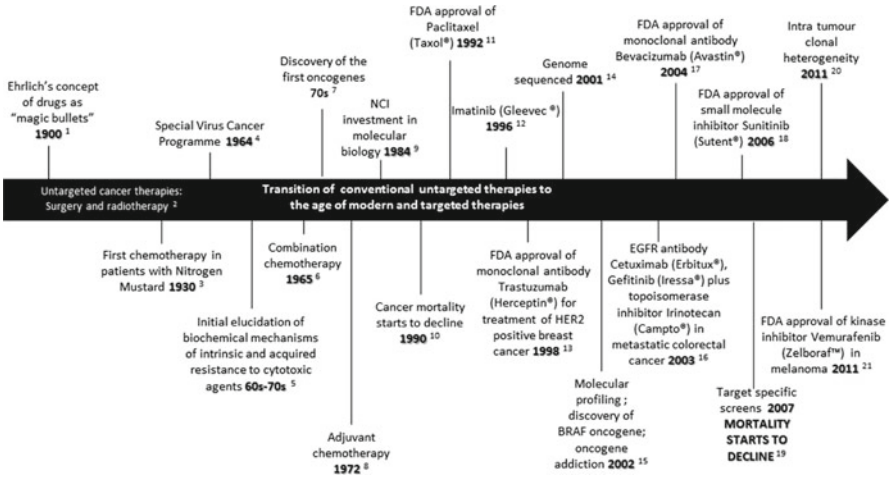

Fig. 1.1 A timeline representing a few of the main breakthroughs in cancer

represented in the timeline is numbered and matched up with the subsections where these events are discussed. The breadth of advances in cancer treatments, from early strategies to current approaches, will be shown using landmarks in each period. Which therapies were in place five decades ago, which therapies are available now, what did work and what failed to work are the primary aspects this chapter intends to address.

Conventional and Non-targeted Cancer Therapies

Surgery and radiotherapy monopolised the field of cancer therapies for a long time. Many solid tumours, when resected with clear margins, could be eradicated by a skilled surgeon. Follow-on radiation therapies were frequently included in an effort to kill any cancer cells missed by the surgeon's scalpel. All too often, however, most tumours were impossible to cleanly resect or were extensively disseminated at that time of presentation, and/or the remaining cancer cells were resistant to radiation. It became clear early in the twentieth century that cure rates were not significantly improving and this was mainly due to the unappreciated role of metastasis in tumour progression. In the early 1900, Paul Ehlrich coined the term "chemotherapy" and described it as the use of chemicals to treat disease (Fig. 1.1[1]). He was also responsible for proving the effectiveness of animal tumour models in screening chemicals to be used to treat disease [1]. The discovery of chemotherapy was a stepping stone for cancer therapies and opened up the possibility for targeting tumours more efficiently in conjunction with surgery and radiotherapy [2].

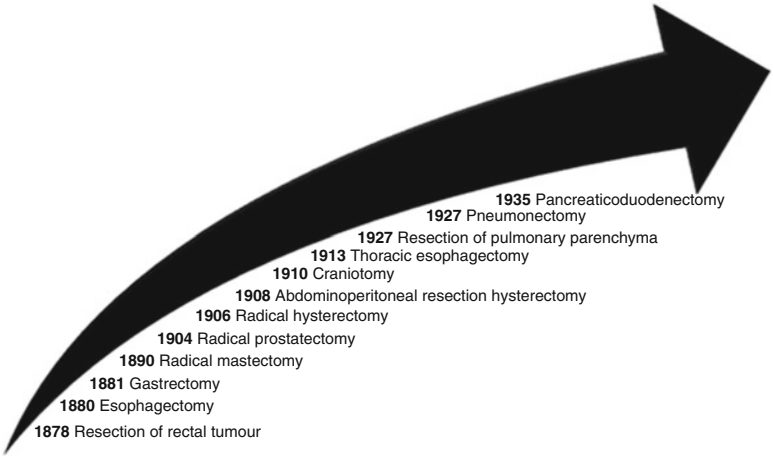

Fig. 1.2 Some early landmark advances in surgical oncology [6]

Surgical Resection

The use of surgery as a cancer therapy goes as far back as 1600 BC when the first known description of surgery in the treatment of cancer was recorded [3]. Hippocrates, the father of medicine, later described the stages of cancer and discerned the terms carcinoma as a "crab-leg" tumour and sarcoma as a "fleshy" tumour. Galeni, in approximately 200 AD, classified cancer as a systemic disease and came up with the terms primary tumour and metastasis [4]. By 1850 the first successful attempts to resect cancer were accomplished and standard surgical resection techniques started to be developed [5].

Until the nineteenth century cancer was regarded as incurable. However with the improvement of the anaesthetic techniques and the development of tissue histochemical techniques, surgery was implemented as a crucial part in the management of malignant disease (Fig. 1.1[2]). This led to the development of a medical speciality known as surgical oncology (Fig. 1.2).

The creation of multidisciplinary teams to treat cancer patients allowed medical oncologists to spearhead a team of specialists that included surgeons, pathologists, radiologists, epidemiologists and even clinical psychologists. This approach allowed therapies to be tailored according to cancer stage and adjusted during treatment for changes in patient status. Cancer staging, which is determined by a pathologist through the analysis of patient's biopsy samples retrieved by a surgeon, is one of the most important tools in aiding the design of a suitable treatment strategy that is planned according to the type and extent of tumour spread. Staging systems were developed to describe the status of a cancer in patients. This system serves several important functions: to tailor the treatment suitable to the patient, to indicate prognosis, to evaluate the treatment results, to assess the similarities between different cases and to help identify cancer trends [6].

A set of definitions to better describe a cancer in its various stages was started in the mid-1940s; Pierre Denoix came up with a staging system called TNM in which T described the size of the tumour, N referred to the extent of regional lymph node involvement and M defined the extent of metastasis. This system was able to determine the lymphatic spread of the tumour by measuring its size and extension, as well as the presence of metastases to classify cancer progression [7]. Subsequently, many staging systems have been created. Some are applied to all cancer sites while others are restricted to certain medical specialties, groups of patients, age brackets or tissue morphology.

Cancer staging is used to guide treatment decisions such as whether to perform surgical resection, which is currently the most common type of treatment for prostate, breast and colon cancer. Using staging as a guide to probable outcomes, the surgeon has a primary responsibility of deciding the likelihood of risk-to-benefit ratio. Additional factors that might affect the outcome and thus benefit of a surgery, such as diabetes, hypertension, nutritional status, congestive heart failure and immunosuppression, are also considered in this assessment. The main principles of surgical resection relate to the feasibility to obtain clean margins, the extent of manipulation required and the possibility of reconstruction [8]. Tumour removal inevitably carries risks depending on the type of procedure used and the possibility of misdiagnosis cannot be neglected. Other postsurgical complications include the risk of excessive bleeding, infection and damage to the adjacent tissues through the formation of strictures [8]. Despite the frequency of surgical resection and the remarkable methods used to remove as much of the cancer as possible, this approach in isolation is considered to have a negligible impact on reduction of cancer-related deaths over time [9].

Radiotherapy

For many years, surgery along with radiotherapy remained the only anti-cancer treatment that clinicians could offer for a long time (Fig. 1.1[2]) [9]. And in the case where surgical resection was not possible, radiotherapy was sometimes used independently. Differences between the role of surgery and radiotherapy in treating cancers can be appreciated by their mechanisms of action: surgery has the goal of eliminating 100 % of cancer cells in a single treatment; radiotherapy is designed to only target a portion of cancer cells with each treatment by affecting only those cells in the process of dividing. Thus, several rounds of radiotherapy are usually used since at any one time of radiation exposure, only a fraction of the cancer cells are in a radiation-sensitive phase of cell division. Collimators are often used to finely focus radiation to specific sites of the body to minimise damage to non-affected areas of the patient. Radiation therapy, with its recent advances, can be a highly effective cancer treatment strategy in some cases.

Radiation therapy has been used as an anti-cancer therapeutic strategy for more than 100 years, dating back to 1895 when Wilhelm Rontgen discovered X-rays.

It was not long after its discovery that deleterious effects of radiation started to become obvious. Numerous reports described swollen limbs and fingers, skin desquamation, severe dermatitis and dry itchy skin. A few years later, birth defects and carcinoma were reported as extremely serious side effects. It became clear that radiation had three main destructive effects: teratogenicity, mutagenicity and carcinogenicity. Along with these negative effects came a promising concept for cancer therapy. Two years after its discovery, X-rays were found to be useful in the treatment of abnormal cell masses [10, 11].

Although X-rays were found to be very promising in killing certain cancerous cells, they were indiscriminate on their actions towards noncancerous cells, particularly those that had even in normal conditions a rapid replication rate such as white blood cells. Efforts to maximise therapeutic benefit and minimise toxicity of radiotherapies became a priority. The problem of establishing a therapeutic radiation dose that could discriminate between cancerous and noncancerous tissue started to become solved through clinical experience [11]. In 1922, Regaud came up with a novel concept of fractionated radiotherapy as we know it today [11], several decades after Marie Curie discovered radioactivity [12]. He used an external beam to deliver a fraction of the complete radiation dose over many sessions to shrink or destroy the tumour. This principle was thought to be beneficial as it would allow time for normal cells to be able to repair themselves between treatments, protecting them from permanent injury or death. Unfortunately, this procedure also allows some of the cancerous cells to recover.

Major advances in radiotherapy occurred once a consistent and ready source of different radionuclides became available. The first cyclotron was built in 1930 by Ernest Lawrence and Stanley Livingston and extended to the generation of radionuclides for medical use by John Lawrence in 1936 [13]. Ready access to these different radionuclides propelled the establishment of computer-assisted tomography/magnetic resonance to improve the quality of radiotherapies and to target these energies locally to tumours [10, 11].

In the 1950s, Puck and Marcus demonstrated a gradual loss of colony-forming capacity of single mammalian cells in culture with increasing radiation exposure. These were exciting times for radiobiology that paved the way to the target-cell hypothesis formulated in 1987 [14]. Clinical expression of radiation damage was thought to involve two mechanisms: direct parenchymal loss and loss of vascular endothelial cells subsequently affecting cell survival [14]. Up to the mid-1990s, it was believed that the main effect of ionising radiation on cells and tissues was cell death leading to a much lower and functionally deficient cell population. Further studies, however, did not support this view. After irradiating single cells with directed and targeted micro-beams, cells exposed to radiation and cells in the vicinity of the radiation (not in direct contact with radiation) were both observed to die. This finding brought about the concept in radiation biology known as the “bystander effect” [15].

A few years later a remarkable finding about radiation-induced fibrosis changed the direction of radiotherapy as a cancer therapy [16]. It was reported that tissues exposed to radiation became fibrotic due to replacement of the epithelial cells by

mesenchymal cells. This resulted in a considerable accumulation of fibroblasts responsible for excessive collagen deposition [17]. Up to this point, lowering the dose of radiation given was the only way to minimise later side effects. What was not clear though was which cellular events were taking place between the exposure to radiation and the clinical expression of these subsequent side effects. In an attempt to solve this phenomenon referred to as the "silent interval", it was postulated that cells during this period would fail to undergo mitosis. This would lead to cell death and consequently to compromised tissue function. By this time, it was becoming clearer that there were some biologically relevant events occurring between irradiation and clinical expression of late tissue injury [16]. In 1995 it was suggested that soon after irradiation, cytokine cascades would induce collagen synthesis that resulted in fibrosis [18]. These findings introduced a new concept of radiotherapy, and a more biological view of radiation effects was adopted.

During the twentieth century, thorough studies of the biological effects of radiation demonstrated a clear relationship between irradiation and DNA damage [19]. Subsequently, improvements concerning the optimal dose of radiation given to patients and the balance between therapeutic benefit and toxicity have improved the safety to efficacy for patients. It is estimated that 50 % of all cancer patients could receive an adjusted dose of radiation either alone or in combination with other anticancer strategies such as chemotherapy or surgery [10]. The fact that radiation treatments are now organ-preserving and non-invasive, radiotherapy in combination with effective tumour de-bulking makes this cancer treatment strategy a viable option for many cancer patients [20].

Currently, the complex of cell responses induced by irradiation is thought to be primarily driven by DNA damage [19]. The most frequent molecular mechanisms prompted by radiation have been shown to include double or single strand breaks, oxidised base damage and apurinic/apyrimidinic sites. If left unrepaired, such lesions are believed to lead to a cascade of cellular events characterised by cell cycle arrest, mutation induction, transformation and ultimately cell death [21]. Patients, however, vary in their response to radiation. Phenotypic and genotypic interindividual differences have provided some insights into these variations and led to strategies to augment the use of radiation in the treatment of certain patients [19]. Specific biological factors associated with certain cancer cells and tumours can play a crucial role in the treatment outcome after radiotherapy. For example, tumour hypoxia and an intrinsic resistance to radiation can allow some surviving cancer cells to repopulate in 6–7 weeks after radiation exposure [19]. Oppositely, certain genetic factors and aspects of the tumour microenvironment, discussed in Chap. 5, can allow for the identification of complimentary approaches and the titration of radiation exposure that contribute to radiotherapy efficacy [10].

Over the last 90 years, radiotherapy has evolved into an important field of medicine and has an established and primary role in the treatment of many forms of cancer. The success of radiotherapy as a cancer treatment, however, is still variable. For example, NSCLC (non-small cell lung cancer) and early larynx cancer survival rates are high, whereas for advanced NSCLC, glioblastomas and sarcomas, they are not [10]. Strategies to improve radiation therapy techniques, however, have resulted

in a more accurate delivery of ionising radiation to cancer cells, reducing toxicity and side effects to the surrounding tissues. This has allowed the use of higher doses of radiation that otherwise would not be possible without a focused method.

Despite the benefits of radiotherapy observed for some tumours, several drawbacks still need to be addressed. The short wavelength energy of ionising radiation causes disruption of biomolecules and biological structures through the formation of reactive free radical ions that can affect the survival of noncancerous cells. Another downside of irradiation is that all cells in the path of the energy beam can be affected [10]. Thus, a positive clinical response assessed by tumour shrinkage may be related to the destruction of noncancerous cells present in or adjacent to the tumour and not necessarily the cancerous cells of the tumour. Finally, the side effects of radiation therapy are complex, often involving side effects that include skin erythema, peeling skin, nausea and diarrhoea, inflammation and ulceration of the mucous membranes at early stages and having later effects after latent periods of months to years that include vascular damage, atrophy, neural damage, fibrosis and a range of endocrine- and growth-related events [22].

Chemotherapy

It is difficult, if not impossible, to define where, when and how chemotherapy was first practised. The use of plants and herbal medicines as healing substances dates back to 1800 BC, and agents from these materials are currently being isolated and demonstrated to have anti-cancer activities [23]. Some components isolated initially from plants with noted anti-cancer activity, such as vincristine, are currently approved as chemotherapeutic agents [23]. One can clearly point to Paul Ehrlich as a pioneer in the concept of chemotherapy (Fig. 1.1[1]). Ehrlich explored the used of dyes that interacted strongly with tissues and cells, findings that led to the development of histological staining techniques and the concept of identifying agents that could bind to specific cellular components. Biology, chemistry and medicine were brought together by Ehrlich's research, and it was found that the effect(s) of a compound on a cell would depend not only on its chemical composition but also on the biology of the targeted cell. In 1900, Ehrlich introduced the receptor theory, through which drugs were described as "magic bullets" that would go straight to their specific targets in the cell (Fig. 1.1[1]). This new concept formed the basis for cancer-targeted medicine which was implemented only a few years later [2].

In 1930, the pharmacologists Louis Goodman and Alfred Gilman injected an analogue of nitrogen mustard called mustine in a patient with non-Hodgkin lymphoma, resulting in a striking anti-tumour effect. This was a landmark for chemotherapy as it was demonstrated for the first time that a chemical could induce tumour regression [24]. During World War II, this chemical warfare agent was found to cause myeloid and lymphoid suppression in humans. It was not long after the discovery that Goodman observed acquired tumour resistance to nitrogen mustard, a critical concept of chemotherapy: acquisition of resistance to a chemotherapeutic. In 1948,

Sidney Farber, a pathologist at Harvard Medical School, found that antagonists of folic acid could be used to disrupt the function of folate-dependent enzymes. Farber administered folic acid antagonists, first aminopterin and afterwards amethopterin, also known as methotrexate, to children with acute lymphoblastic leukaemia, and even though the remission was not very pronounced, the principle of suppressing proliferation of malignant cells was elucidated [25]. Ten years later, in 1958, Hertz and Li found that methotrexate could cure choriocarcinoma, a germ cell malignancy originated in the placenta [26]. This was the first successful attempt in curing a solid human tumour through drug therapy. This new approach revealed to be the practical application that small molecule inhibitors could in fact be used to target tumours providing evidence to Ehlrich's discoveries. The basis for cancer-targeted medicine was formed and a new era for cancer chemotherapy began [25].

Combination Chemotherapy

Before the 1970s, mechanisms for acquired cell resistance to cytotoxic drugs were outlined (Fig. 1.1[5]). This information led to the adoption of new strategies to improve cancer cell susceptibility to cytotoxic drugs. In 1965, a new and revolutionising concept of chemotherapy was created (Fig. 1.1[6]), and when this theory was taken into practice, clinical oncologists made a remarkable breakthrough. Combining different drugs with different mechanisms of action induced long-term remissions in children with acute lymphoblastic leukaemia [25]. By giving a combination of different drugs to the patient, it was noted that improved efficacy could be achieved at reduced doses relative to these agents administered individually. Three principles seemed to dictate the success of combination therapy: each drug needed to be active in the tumour when given alone, each drug had to have different mechanisms of action and there needed to be minimal overlapping toxicity profiles for each of the drugs used [9]. Administration of several drug combinations that fit these criteria was able to reduce side effects and overcome drug resistance. There were other issues, however, that also needed to be considered in this combination chemotherapy approach. These issues included the possibility that these drugs administered simultaneously did not have detrimental actions on the stability, distribution or delivery to the required site of action for one another [27].

Animal model studies in the 1990s predicted that a specific chemotherapy would be effective for only some types of cancer but not for all. It became clear that drugs were more active when used in small volume tumours and in combination with other chemotherapeutic agents. More recently, with the elucidation about the role of the intra-tumour clonal heterogeneity (Fig. 1.1[20]) in cancer progression as well as the discovery of many oncogenes (Fig. 1.1[7]), these mechanisms are still suggested as factors that contribute to the resistance in cancer cells towards cytotoxic agents [28]. Several non-invasive imaging techniques now partially rely on the specificity and complexity of combination chemotherapies [27]. While drug combinations are now a common and routine chemotherapy strategy, the possibility of additive

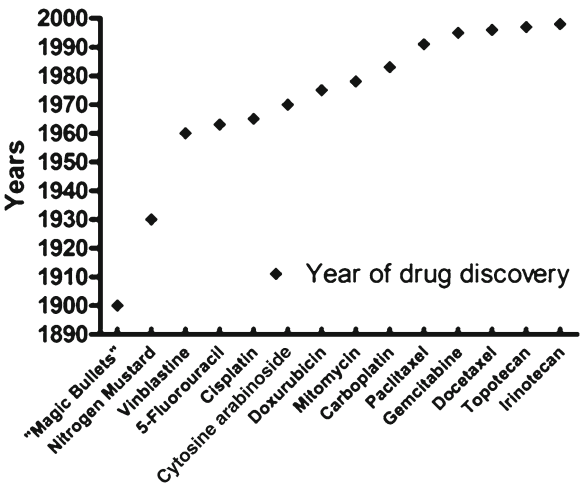

Fig. 1.3 Year of discovery of some anti-cancer drugs between 1900 and 2000 [1]

toxicity is a serious concern as every patient has the potential to respond uniquely to these agents. One example of additive on-target and off-target toxicity can be illuminated by data obtained when patients are treated with a combination of AKT and MEK inhibitors. Skin rash and diarrhea are two side effects that result from a potentiation effect when the two inhibitors are co-administered. Nonetheless, the creation of suitable drug modelling combination networks has allowed combinatorial chemotherapy to progress to more realistic and pragmatic approaches although it is still unclear whether it would be more beneficial to use a whole range of combinatorial agents instead of the administration of simpler combinations with fewer drugs involved [28].

Adjuvant Chemotherapy

The concept of combining chemotherapy with other therapeutic modality (surgery, radiation, etc.) was introduced in the early 1970s (Fig. 1.1[8]). In 1972, Emil Frei and colleagues demonstrated this concept to be useful when they showed that chemotherapy given after surgical removal of osteosarcoma could improve cure rates. This finding led to a period of huge investment by the pharmaceutical industry to look for novel agents that no longer had to function solely as a stand-alone chemotherapeutic, but instead could be administered in conjunction with some other therapeutic modality (Fig. 1.3). Two major limitations threatened this discovery process. The first was the problem in finding suitable screening approaches to identify promising leads from large libraries of compounds. The second was the difficulty of setting up clinical studies to test promising agents identified by these programmes [1].

Gordon Zubrod took the approach of screening natural products isolated from plant and marine sources to search for novel anti-cancer agents. As a result, taxanes and camptothecins were discovered in 1964 and 1965, respectively [29]. While they

were described as promising drug candidates, severe side effects and problems in their development did not allow these compounds to progress further into a clinical setting [29]. Paclitaxel, also known as Taxol, is a water-insoluble microtubule disruptor that caused hypersensitivity reactions in some patients when administered in a lipid emulsion. In 1987 a liposome-based formulation of Taxol was found to be effective in the treatment of ovarian cancer and approved by the FDA in 1992 (Fig. 1.1[11]). Despite a promising ability to inhibit topoisomerase I, an enzyme that controls DNA winding, and impede cancer cell growth, initial clinical studies with camptothecin demonstrated serious nephrotoxicity [29]. An unstable lactone ring in the molecule appeared to be the problem, but this issue was solved through formulation efforts. Long-term toxicities on the lungs, heart and reproductive organs were some of the other significant problems encountered during clinical development of camptothecin. However, in 1996 irinotecan, an analogue of camptothecin, was discovered and due to its enhanced stability was approved for colon cancer treatment in 1998 [30, 31].

The discovery of several oncogenes in the 1960s revolutionised the way scientists thought about cancer onset and progression. The identification of these genes led to the idea that metabolic pathway deregulation could function as the driving force of oncogenic conversion and metastasis. These studies drove a plethora of approaches to identify novel chemotherapeutics. An important aspect of this work was the observation that cancer cells required the continuous activity of these oncogenes, the so-called oncogene addiction state [28]. New molecular diagnostics, prognostics, pharmacogenomic biomarkers and pharmacodynamics endpoints all contributed to the generation of new treatments [32]. While this information provided the basis for a whole new stable of anti-cancer treatment strategies, it was soon realised that the processes being driven by the oncogenes were also active to a lesser extent in noncancerous cells, resulting in a lack of specificity and toxicities that were dose limiting, similar to previous chemotherapeutics. What was still needed was a way to focus the actions of all these chemotherapeutics to actively target cancer cells.

Radiotherapy and Chemotherapy

Approaches to reach individual cancer cells within a patient were explored in the 1960s [28]. This new approach was similar, in principle, to radiotherapy with the idea of using chemicals rather than radiation to interfere with DNA replication [1]. Further, it became clear that the combination of radiation with such drugs could provide additional benefit to the patient with possibly even better safety as the levels of each might be reduced. Three main mechanisms were thought to contribute for the overall success. Firstly, it was hypothesised that the interaction was likely to occur due to presence of the drug during radiation exposure. Secondly, it was believed that the drug could interfere with repair induced by radiation, and finally,

the complex radiation-drug actions could potentially have some differential effects on the proliferation kinetic rates of tumour and normal tissue.

Efforts to Target Cancer Therapies

Until 30 years ago, cancer chemotherapies were restricted to cytotoxic drugs that would kill rapidly dividing cells that did not necessarily discriminate between normal and cancer cells. Subsequent studies have moved from this cytotoxic approach towards cancer therapies where genetics and biology play a dominant role. Efforts have been made to develop compounds designed to interfere with a particular molecular pathway deregulated in the cancer cell, with particular interest in developing cancer medicines that preferentially block abnormal cell division, induce apoptosis and target the tumour microenvironment [28, 33]. Many of these modern anti-cancer strategies rely on the concept of specific molecular targeting. These small molecule inhibitors and monoclonal antibodies are based upon the "magic bullet" concept formulated by Ehlrich in 1900. One of the biggest challenges to selectively target cancer cells is to identity a way to readily discriminate them from the noncancer cells.

In 1964, a National Cancer Institute special programme was initiated to identify viruses associated with cancer as one way to possibly discriminate these cells, but this effort was without success (Fig. 1.1[4]). This programme was restarted in 1984, and instead of identifying cancer-associated viruses, it discovered a variety of oncogenes, tumour suppressors and signalling pathways associated with oncogenic conversion and metastasis (Fig. 1.1[9]). This discovery was a key factor in elucidating the cell signalling transduction pathways activated in cancer and culminated in the identification of a variety of drug targets as well as sequencing of the human genome. This led to a change of direction as cancer therapies started to become more driven towards a specific target [1]. The discovery of growth factors and the *ras* oncogene were two stepping stones that introduced new concepts of anti-cancer targeted therapies. Cell-cycle proteins, modulators of apoptosis, growth factors, signalling molecules and angiogenesis promoters now became potential anti-cancer targets. The characteristics of promising anti-cancer drugs improved through efforts to increase their stability, enhance their absorption and to reduce their toxicity: three requirements for a "recipe" of success [29].

In 2000, the establishment of six hallmarks of cancer postulated by Hanahan and Weinberg bridged the early discoveries made by Ehrlich. These concepts pointed to cancer as a genetic disease, identified molecular pathways hijacked in malignancy and fuelled the interest to discover small molecule inhibitors that targeted specific mutations that activated certain cell signalling transduction cascades. These hallmarks of cancer represented a landmark in the progressive elucidation of the genetic basis of cancer. Self-sufficiency in proliferative growth signals, resistance to apoptotic signals, insensitivity to antigrowth signals, sustained angiogenesis, tissue invasion and metastasis and limitless replicative potential were described as the main

Table 1.1 Examples of small molecule inhibitors and monoclonal antibodies FDA approved for cancer treatment

Small molecular inhibitors	Target	Drug
Target of molecular pathways	TK (EGFR)	Gefitinib (Iressa®)
	TKs (VEGFR, PDGFR, KIT, FLT3)	Sunitinib (Sutent®)
Target apoptosis regulation	28S protease	Bortezomib (Velcade®)
Monoclonal antibodies		
Target of molecular pathways	EGFR	Cetuximab (Erbitux®)
	HER2	Trastuzumab (Herceptin®)
	VEGF	Bevacizumab (Avastin®)

Abbreviations: *TK* tyrosine kinase, *EGFR* epidermal growth factor receptor, *VEGFR* vascular endothelial growth factor receptor, *FLT3* FMS-like tyrosine kinase 3, *HER2* human epidermal growth factor receptor

mechanistic capabilities acquired by most, if not all, types of cancers [34]. This new understanding of tumour biology and genetics allowed for the development of novel strategies that involved radical approaches to cancer therapy. Cancer drug discovery and the identification of novel molecular targets were facilitated by this new road map for cancer treatment strategies.

Modulation of Biological Processes

A variety of approaches have been taken to alter biological processes associated with cancers, particularly epithelial cancers that develop as solid tumours. The first issue of efficacy for these approaches is that of successfully reaching the tumour or cancer cells within the tumour. Large complexes designed to target tumours can be immunogenic and prone to uptake by the reticuloendothelial system. Tumours can take up particles of a certain size range due to unique vascular properties, discussed in Chap. 23. Antibodies, with a residence time in the body of several weeks, have now been shown to effectively target tumours and cancer cells. Compounds with a molecular weight less than 700 Da have demonstrated some advantages in treating cancers. These agents can rapidly penetrate most tumours to reach cancer cells, and their actions can be reversible because they can also be rapidly cleared [35]. Some examples of small molecule inhibitors and monoclonal antibodies FDA approved for cancer treatment have been tabulated (Table 1.1).

High affinity and specificity are key features for any agent that might be used effectively as a cancer chemotherapeutic [35]. Due to the vast number of potential interaction sites on the ~30,000 proteins expressed by the human genome, it is indeed very challenging to identify a small molecule that binds exclusively to a single site and has a discrete action. Some targets have active sites that, because of their structure, make the complex difficult to inhibit and small molecules may not

be sufficiently complex to selectively inhibit a specific protein-protein interaction. Additionally, the pathway targeted by small molecule chemotherapeutic must be sufficiently specific to the cancer and not one that is essential to normal cell function. Because of this, as Workman suggests, it might be of better value to identify new molecular targets by "walking down the pathway" of altered function in cancer cells to find better targets for selectively targeting cancers [32].

A rather prescribed series of steps were commonly taken to identify small molecule inhibitors that could be effective chemotherapeutics. Large chemical libraries were first screened to identify molecules that could activate or inactivate the function of a specific a gene. Combinatorial analysis and structure-activity relationship (SAR) studies were then used to optimise these initial lead compounds. Subsequently, a careful structural and mechanistic analysis was used to select a clinical candidate [35]. More recently, reverse chemical genetics have been used. After selection of a target protein, a library of small molecules with an inhibitory effect in vivo is screened to select a lead candidate with the desired phenotypic characteristics. Small molecules can frequently inhibit multiple targets, resulting in nonspecific actions. To overcome this lack of specificity, site-directed mutagenesis in the protein structure can be used to better define the nature of the inhibitor specificity and examine the SAR space [35].

The concept of monoclonal antibodies, referred to as "magic bullets" by Ehrlich, is essentially based upon on how the immune system establishes its exquisite selectivity to target structures on pathogens. Monoclonal antibodies designed to treat cancer are frequently targeted to cell-surface epitopes that are overexpressed in cancer and/or are involved in the uncontrolled growth properties of these cells. Often these antibodies are selected to not just bind to but to also block the function of these cell-surface targets. Recent technological advances have allowed for the engineering and production of monoclonal antibodies at sufficient scale to make clinical therapy feasible. As these engineered molecules are essentially equivalent to antibodies naturally produced by the body, they are well tolerated by patients and have durable actions due to the fact that antibodies have a circulation time of several weeks [36].

While most anti-cancer antibodies are targeted to antigens expressed on the cancer cell surface, another approach to treat cancers with a monoclonal antibody has involved the targeting of elements associated with the generation and support of vasculature associated with growing tumours [36]. By disrupting tumour blood supply, tumour shrinkage can be achieved. Monoclonal antibodies can also be engineered to deliver radiation or cytotoxic agents directly to cancer cells. It has been shown that radiation-linked monoclonal antibodies deliver a low level of radiation over a longer period of time, and this approach is believed to be as effective as radiotherapy [36]. In 2002, the first radioimmunoconjugate, Zevalin®, obtained full FDA approval (Fig. 1.4). Although it cannot be used for imaging, ^{90}Y-Zevalin is a murine, IgG1 kappa, monoclonal antibody directed against the B lymphocyte antigen CD20 [37].

A major limitation in using immunoconjugates of cytotoxic drugs targeted to cancer cells by attachment to a monoclonal antibody is the complexity involved in the delivery of a therapeutic concentration to the malignant cells, i.e. an insufficient

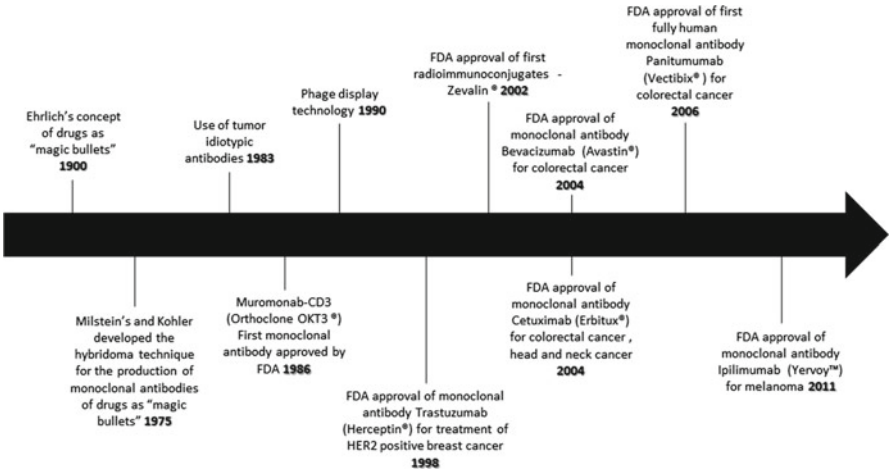

Fig. 1.4 Timeline representing the evolution in the discovery of monoclonal antibodies

amount of a chemotherapeutic is delivered by the few antibodies to selectively reach each cancer cell [38]. One approach to solve this problem involves using much more potent chemotherapeutics, those with 100–1,000-fold higher cytotoxicity that are too toxic to administer without some form of cancer cell targeting. Maytansine, for example, is a cytotoxic agent that kills cells by interfering with microtubule formation through tubulin polymerisation inhibition [39]. Due to its exceptional cytotoxic potency, maytansine was too toxic as a solo agent. When the compound is delivered in a targeted delivery conjugated form, however, the therapeutic index greatly increases as the systemic toxicity decreases. Immunoconjugation of maytansine and maytansinoid derivatives has been proven to be a beneficial anti-cancer strategy that involves the release of the drug at the target cell in its fully active form through a linker cleavage [38].

Remarkable efficacy to safety windows for monoclonal antibodies has driven extensive efforts to use this therapeutic approach more extensively. Initial efforts to develop monoclonal antibody therapies involved mouse proteins, which led to the development of humanised or human antibodies to reduce adverse reactions caused by the injection of a non-self protein. Decreasing the murine component in the monoclonal antibodies not only improved tolerability but also led to the development of antibody therapies with longer blood stream half-lives and the ability for these agents to engage human complement and other effector cells of the patient's immune system. Behaving in a similar manner to naturally occurring immunoglobulin and working in the same way as normal antibody-based immune response, current human monoclonal antibody therapies are an attractive approach in the field of molecular-targeted cancer therapeutics [40, 41].

Due to the enormous impact of genomics and proteomic technologies, cancer biology and therapeutics have recently been dramatically reshaped with two main outcomes resulting from these events. The first is the acquisition of an improved

understanding of the genetics that govern cancer cell machineries deregulated in cell signalling transduction pathways. The role of non-receptor tyrosine kinases are an example of newly appreciated mediators of cancer cell initiation and progression. Secondly, high throughput technologies have led to the discovery of new genetic elements involved in cancer formation and metastasis, identifying attractive new targets for new emerging cancer therapies [42]. In several cases, overexpressed receptor tyrosine kinases were identified as promising targets for the inhibition through binding of a monoclonal antibody to the surface of a cancer cell.

Protein tyrosine kinases are enzymes that catalyse the transfer of phosphate group from ATP to a protein substrate, functioning as an "on" or "off" switch in cellular processes that directly regulate signalling pathways mediating cell proliferation, differentiation, migration, metabolism, survival and communication between cells. Numerous studies demonstrated the role of mutations in both receptor tyrosine kinases and also non-receptor protein tyrosine kinase pathways in oncogenesis and metastasis. Thus, tyrosine kinase targets have become a very attractive class of molecular targets for cancer therapy. Some considerations need to be taken into account, however, before selecting a particular kinase as a valid and reliable anti-cancer target. First and foremost the protein tyrosine kinase should be identified in tumour biopsies used for the diagnosis and be involved in tumour progression. Secondly, the protein should not play a significant role in normal postnatal development or physiology. Finally, a thorough SAR analysis must be performed to develop a suitable inhibitor. This is critical to identify an optimal dose at which the drug can have a biological effect without reaching the maximal tolerated dose [43]. EGFR (epidermal growth factor receptor) and VEGFR (vascular endothelial growth factor receptor) are two prominent cell-surface receptor tyrosine kinases that have been targeted to achieve anti-cancer outcomes.

Targeting EGFR and HER2 Pathways

The EGFR (epidermal growth factor receptor) family is activated in many human epithelial-derived cancers and has shown to have a preponderant role in development and progression of human cancers. EGFR involvement in the survival of malignant cells can be due to EGFR overexpression, gene amplifications, mutations and increased production of ligands such as TGFα (transforming growth factor α) [44]. Several cancers have been linked to EGFR overexpression including colorectal cancer, NSCLC (non-small-cell lung cancer) and head and neck squamous cell carcinoma [44–46]. Specific EGFR inhibitors have been developed as anti-cancer agents, and many of them have already been approved in the clinic; however, the focus of this chapter will be on trastuzumab (Herceptin®), gefitinib (Iressa®) and cetuximab (Erbitux®).

Increased levels of HER2 have been associated with malignant mammary epithelial cell transformation [43]. There are four different receptors in this family: HER1 (also called erbB-1 or EGFR), HER2 (also named as erbB-2 or neu), HER3 and

HER4 (known as erbB-3 and erbB-4, respectively). The HER2 gene is amplified in 20–30 % of breast cancers, and as a consequence of this, EGFR is overexpressed in the cell membrane. Under normal conditions, the HER2 pathway is involved in controlling cell growth and division; however, when HER2 protein is overexpressed, cells start to divide uncontrollably and abnormal cell growth leads to tumour formation. In September 1998, a monoclonal antibody named trastuzumab gained FDA approval for the treatment of node-positive, HER2-positive breast cancer (Fig. 1.1[13]) [46, 47]. Trastuzumab, also known as Herceptin®, is an IgG antibody that binds selectively to HER2, effectively inhibiting the growth/survival of HER2 overexpressing cells [43]. In some types of breast cancer, HER2 may send signals without the binding of growth factors and trastuzumab does not appear to be effective in these cases [48]. Furthermore, a significant side effect with trastuzumab is congestive heart failure that is exacerbated by the presence of doxorubicin, which is an effective adjunct therapy to trastuzumab. Serious angioedema, anaphylaxis and pulmonary toxicity have also been reported within 24 h of its administration [49].

In 2003, FDA approved gefitinib (Iressa®), which binds to the ATP-binding site of EGFR and inhibits its signalling (Fig. 1.1[16]). Even though it caused partial remissions in 10–15 % of patients with NSCLC, gefitinib failed to demonstrate a chemotherapeutic activity in large randomised clinical trials. In a study done in 2004, it was found that gefitinib in combination with gemcitabine and cisplatin did not improve the efficacy over gemcitabine or cisplatin alone. As a result of this observation, it was suggested that further preclinical studies needed to be carried out in order to identify groups of patients that could effectively benefit more from this combination chemotherapy [50]. In the same year, cetuximab, or Erbitux®, a monoclonal antibody targeting EGFR, also won FDA approval in combination with chemotherapy for the treatment of colon cancer (Fig. 1.1[16]) [34]. Researchers realised that gefitinib and cetuximab, two drugs that target the same receptor but with unrelated mechanisms of action, were producing favourable chemotherapeutic effects in the same subset of patients. Later on, EGFR mutations and in-frame deletions were identified and reported to be responsible for different individual sensitivity to EGFR inhibitors, thus different treatment responses to this sort of chemotherapeutic strategy. This cause-effect observation represented an important milestone for cancer drug development strategy [51, 52].

Targeting the VEGFR

Vascular supply plays a crucial role in tumour development [53]. Targeting the tumour vasculature and angiogenesis, which will be discussed in more detail in Chap. 12, is a very attractive anti-cancer strategy from a therapeutic point of view. A signalling protein commonly involved in tumour neovascularisation is vascular endothelial growth factor (VEGF), which plays a key role in controlling the formation, growth and maturation of new blood vessels. In cancer, this signalling protein also plays a crucial role in the establishment and maintenance of blood vessels essential for tumour growth [54]. VEGF also induces vascular leakage by regulation

of vascular permeability [55]. VEGF belongs to a gene family that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and PLGF (placental growth factor). All of these elements have a unique role in controlling growth and differentiation of blood vessel and lymphatic endothelial cells [56]. Considering the central role played by VEGF family members in tumour neovascularisation, it is not surprising that there are now six VEGF inhibitors approved for cancer treatment.

Bevacizumab, also known as Avastin®, is a monoclonal antibody that blocks VEGF-A binding to its receptors; it was approved in 2004 as a treatment for colorectal cancer, NSCLC and recurrent glioblastoma (Fig. 1.1[17]). Many studies have been carried out in order to address the potency of bevacizumab as an inhibitor of the vasculature formation. One study reported that after a single dose of this compound given to six patients with colorectal cancer, a pronounced decrease in tumour perfusion, tumour vascular volume, interstitial fluid pressure and microvascular density was observed. These observations confirmed the rationale for blocking VEGF to inhibit tumour vasculature formation [57]. Two years later, a small molecule inhibitor of angiogenesis was approved for treating renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumour, making it the first cancer drug simultaneously approved for two different indications (Fig. 1.1[18]). Sunitinib, also called Sutent®, inhibits cellular signalling by targeting multiple receptor tyrosine kinases (RTKs) that include receptors for platelet-derived growth factor (PDGF-Rs) and vascular endothelial growth factor receptors (VEGFRs). Other small molecule RTK inhibitors that have also been approved for cancer treatment, especially for renal cell carcinoma, include sorafenib (Nexavar®), pazopanib (Votrient®) and axitinib (Inlyta®) [58].

Despite promising outcomes in the clinical development of a variety of VEGF inhibitors, there are some issues that limit the success of these compounds in the clinic. The existence of different pathways mediating the angiogenic cascade allied to the possible existence of signalling cascades involved in rescuing tumour cells when exposed to these agents is an important contributor that limits the anti-angiogenic activity of these compounds [54]. There is also increasing evidence that as disease progresses, VEGF-A might be replaced by other angiogenic pathways, making these cancers resistant to an angiogenesis therapy that blocks a specific pathway [59]. Other conceivable mechanisms for acquired resistance rely on a growth advantage of cells resistant to hypoxia with a reduced apoptotic potential. This inevitably makes these cells less dependent on angiogenesis and, as a consequence, the formation of stable vessels that are less responsive to anti-angiogenic drugs [59, 60]. VEGF can also induce the expression of two apoptosis resistance molecules, Bcl-2 and A1, in endothelial cells that highlights the interaction between VEGF and apoptosis regulation [61, 62].

Targeting Apoptosis Regulation

Apoptosis, also known as programmed cell death, plays a crucial role in controlling cell numbers in both embryonic and adult stages. This mechanism of selective cell death is required for organ formation during development, homeostasis, normal

function of the immune system and reproductive biology. Many diseases are linked to a defective apoptosis regulation and cancer is just one of them. In malignancy there is an impaired activation of the apoptotic cascade in contrast with ischemia, in which there seems to be an excessive activation of apoptosis [63]. The fact that the apoptotic machinery is frequently mutated in many human tumours makes the programmed cell death pathway a valid target for cancer treatment. It is the balance between pro-apoptotic and anti-apoptotic proteins that switches the "on" or "off" signals in apoptosis activation. In cancer cells, the ratio between cell survival and apoptosis induction is deregulated to favour the abnormal cell proliferation [64].

Central components of the apoptosis signalling cascade include BCL2 family of proteins, IAPs (intracellular anti-apoptotic proteins), TNF (tumour necrosis factor)-related apoptosis-inducing ligand (TRAIL) and the caspases. Core elements that constitute the cell death machinery, along with other triggers and regulators, represent promising "druggable targets" for pharmacological modulation of cell death and inflammation [64, 65]. Two major pathways can trigger apoptosis: the death receptor-induced extrinsic pathway and the mitochondria-apoptosome-mediated apoptotic intrinsic pathway. They both lead to the activation of apoptotic effector proteases called caspases and the cleavage of specific substrates. The death receptor-induced apoptotic pathway is formed by TNF gene superfamily members such as TNF, Fas ligand (FasL) and Apo2L/TRAIL [64]. The use of TNF and FasL as apoptotic inducers is compromised by significant off-target effects that induce leukopenia, thrombus formation, hypotension and liver function abnormalities [66].

The trimer molecule Apo2L/TRAIL is able to activate apoptosis selectively in many transformed cells, and mutations in the TRAIL pathway have been detected in different human tumours, which can be indicative of its role in tumour onset and progression [67]. A monoclonal design to target TRAILR1 has already been developed and it recognises the R1 protein expressed at the cell surface of many solid tumours. This monoclonal antibody has the capacity of killing different human cancer cell lines and it has been shown to be effective in breast, colon and uterine cancers [68]. Despite the fact that TRAIL preferentially targets pro-apoptotic activity in cancer cells, a recent study has suggested that it can also induce apoptosis in human hepatocytes. Nevertheless, some studies have shown that other chemotherapeutic agents such as imatinib are able to potentiate TRAIL activity in vitro, suggesting that these agents might hold greater promise when used in combination with other cancer treatments such as irradiation [69].

BCL-2 represents a family of proteins that regulate mitochondria-apoptosome-mediated apoptotic pathways. Some proteins have crucial roles in suppressing apoptosis, while others promote cell death. BCL-2 is an example of an apoptotic suppressor; in malignancy this protein is commonly overproduced when induced by anti-cancer drugs and radiation. It has also been suggested that the cytotoxicity caused by Taxol might be mediated by phosphorylation and functional inhibition of BCL-2 [64, 70]. BCL-2 antisense therapy is well tolerated [64] and G3139, an antisense phosphorothioate oligodeoxynucleotide able to suppress BCL-2 expression, can potentiate anti-tumour effects when co-administered with docetaxel and taxanes. It is not yet clear whether these agents can act specifically on tumour cells

or if they would be more useful as a monotherapy [64, 71]. Further, the ability to efficiently deliver siRNA to every single tumour cell is a rather daunting process. Thus, strategies to produce small molecule inhibitors targeting BCL-2 family members have attracted a great deal of attention. BH3 domains are known to be crucial for the function of BCL-2 protein family members, and due to their small size, approximately ten amino acids long, they are ideal candidates for this approach [65].

Defects in apoptosis underpin drug resistance mechanisms as most of anti-cancer drugs exert their cytotoxic effect through apoptosis induction [71]. Thus identifying some of the key regulators involved in the apoptotic machinery either at the transcription or expression level will certainly allow the development of new strategies for the enhancement of the anti-tumour effect. The proteasome is an enzyme complex involved in degrading proteins that control the cell cycle and a variety of cellular processes. Blocking proteasome function puts rapidly dividing cells in stress and makes them susceptible to the induction of apoptosis. Bortezomib is an example of a small molecule and a selective inhibitor of the proteasome. Bortezomib has shown to have anti-tumour activity in solid tumours of the prostate, pancreas and colon. Nonetheless, apoptotic drugs still show some cytotoxicity in normal cells [72]. Different cancers can vary in their apoptotic response to chemotherapeutic agents; carcinomas are more responsive than sarcomas. Adding to this inter-tumour heterogeneity, there is also substantial intra-tumour heterogeneity in apoptosis. Tumours are not composed of uniform populations of cells; instead they are formed by multiple clonal subpopulations of cancer cells. Thus, the tumour microenvironment can have an enormous impact on tumour biology and consequently in cancer therapy.

Efforts to Target the Tumour Microenvironment

Cancer cell growth is dependent upon its interaction with the local microenvironment (Fig. 1.1[20]). Indeed, the term "tumour heterogeneity", discussed in Chaps. 13 and 14, not only refers to differences in the morphology of cancer cells in different areas of the tumour but also to the immediate environment produced by noncancerous cells such as tumour infiltrating leukocytes (TILs) and cancer-associated fibroblasts (CAFs). Noncancerous cells of a solid tumour can have an impact on local pH, oxygen tension and other physiological factors that affect behaviours such as growth and migration. Thus, within a single solid tumour, there could be multiple microenvironments, all with different levels of vascularity and thus accessibility for various therapeutic agents. Additionally, various microenvironments could drive the differentiation of distinct clonal populations from a single cancer initiator, each population having different properties of growth and differentiation. The coexistence of different subpopulations in the same tumour will influence a variety of parameters associated with cancer treatment such as cell-surface antigen expression, metastatic behaviour, growth characteristics, survival under hypoxic conditions

and sensitivity to apoptosis. Thus, tumour heterogeneity can pose multiple problems for the development of successful chemotherapeutic agents [73].

All too often, a cancer drug compound with promising Phase II clinical trial outcomes fails to reach its endpoints in a Phase III study. It is not unrealistic to suggest that as cancer patient populations enlarge in these bigger trials, the breadth of tumour heterogeneity encountered by the drug being tested also increases. Thus, inter- and intra-tumour heterogeneity could realistically be confounding factors for the successful development of anti-cancer therapies that are founded on sound biology that can be clearly demonstrated in appropriate preclinical models and in limited patient sets. There is relatively little known about how tumour heterogeneity might contribute to these late-stage clinical trial failures; tumours have started being regarded as clonal heterogeneous masses only recently [58]. Tumour stromal cells are now recognised for their involvement in aiding in the survival of cancer cells and actively contributing to chemotherapy resistance [58]. One aspect of stromal involvement relates to high interstitial tumour pressures along with hypoxia which are critical factors that contribute to the vicissitudes of events that drive tumour heterogeneity [74]. Targeting of stromal cells in solid tumours will be discussed in Chap. 6.

The tumour microenvironment can be regarded as sophisticated weaponry in the battle waged by cancer cell to evade the cytotoxic actions of anti-cancer agents. Given this, there is a need for greater insight regarding the various microenvironments within tumours with the goal of improving our ability to select the most efficient anti-cancer therapy [75]. Tumour heterogeneity issue suggests that a combination of therapies should be considered in the development of therapeutic strategies, especially when dealing with chemotherapy resistance [73]. Pancreatic carcinoma is an example where stromal involvement is significant, with most tumours demonstrating extensive desmoplasia that helps creating a complex microenvironment capable of promoting cancer cell growth, invasion, metastasis and ultimately resistance to chemotherapy [76]. This microenvironment functions as a physical barrier to chemotherapy events, preventing an efficient pancreatic drug perfusion [77]. Clearly, there is need to refine preclinical models to also reflect tumour heterogeneity and stromal involvement, although it may be essentially impossible to recreate the human condition outside of patients.

Therapies Involving RNA Interference

Gene expression patterns have been assumed to define normal and abnormal cell function with analysis of these differences performed either at the RNA level (the transcriptome) or by functional assessment of an encoded protein (the proteome). Due to technology constraints and complexities of mechanisms involved in regulating protein function, the transcriptome is more often used to define cancer cell characteristics [32]. Changes in transcriptional properties have often provided a strategy to identify an anti-cancer agent using small molecule or biopharmaceutical strategies.

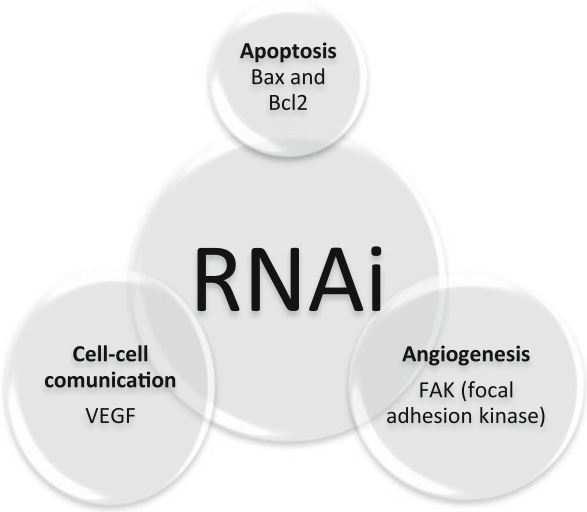

Fig. 1.5 Cancer-related genes targeted by RNAi [81]

Recently, a more direct approach to use transcriptional information has been identified. RNA interference (RNAi) is an endogenous mechanism in cells that regulates gene expression with exquisite specificity and constitutes a defence mechanism against viruses and transposable elements [78]. RNAi has now been used in the treatment of human disease using two types of small RNAs to perform gene silencing: microRNA (miRNA) and small interfering RNA (siRNA). Most siRNAs are double stranded with each strand being 20–24 nucleotides in length. Remarkably, siRNAs can be designed to target almost any gene of interest and they work through endogenous enzymatic mechanisms to degrade a specific mRNA [79]. Thus, anti-cancer strategies have used RNAi to silence the expression of oncogenes [80], and RNAi-based therapies can be used to target genes involved in cell cycle, progression or even angiogenesis (Fig. 1.5).

Aptamers, oligonucleotide sequences that exhibit secondary and tertiary structures, have recently been suggested as a possible targeting tool for the delivery of siRNAs. The first application of an aptamer-delivered siRNA was for prostate cancer. An aptamer was designed to target the prostate-specific membrane antigen (PSMA) receptor expressed at the cell surface of malignant prostatic cells. After intra-tumour injection, the complex was internalised to silence a target mRNA. These studies were carried out in a murine xenograft model, and as a result of this, through the addition of a 20-KDa PEG moiety to the 50-terminus of one of the siRNA strands to improve in vivo stability, tumour growth was inhibited [80]. Other vectors such as adenoviruses, AAVs (adeno-associated viruses) or rAAVa (recombinant adenoviruses) have been developed as possible carriers to deliver small molecule siRNA into cancer cells [78]. While these results are promising, there are several issues related to RNAi-based therapies that limit the usefulness of this approach: delivery to the correct cell/tissue and durability of RNAi activity [79], as well as the potential for some sequences to activate innate immune system elements such as the interferon response and NF-kB-mediated inflammation through Toll-like receptors [81].

Table 1.2 List of some transporter systems able to shuttle specific anti-cancer drugs

Transporter system name	Endogenous substrate	Type of transport	Anticancer drug
Organic anion: OATP1A2	Bile salts, organic anion and cation	Facilitated transport	Methotrexate
Organic cation: OCT1	Organic cation polyspecific	Facilitated transport	Oxaliplatin, Glivec®
CT2	Carnitine, betaine	Facilitated transport	Doxorubicin
Folate	Folate	Exchanger/OH^-	Methotrexate, Tomudex®, edatrexate
Glucose: GLUT1	Glucose	Facilitated transport	18-FDG
Peptide: PepT1	Oligopeptides	Cotransporter/H^+	Bestatin

Abbreviations: *18-FDG* 18 fludeoxyglucose

Using Membrane Transporters to Target Delivery

All cells express surface proteins capable of selectively transporting materials that are otherwise impermeable to the plasma membrane. Since many of the materials brought into cells via these transporters are nutrients essential for cell survival, many of these integral membrane proteins are constitutively expressed at the surface of most cells in the body. A few transporters, however, can be restricted to discrete cell types and/or cells under specific conditions. Also, some of these proteins are capable of transporting anti-cancer drugs or anti-cancer agents in a prodrug form. Thus, the use of specific transporters as molecular targets in cancer therapy has been proposed for tissue selective drug delivery as a means to reduce systemic toxicity (Table 1.2) [82].

The receptor for folate (FR type α) has attracted a great deal of attention, as it is overexpressed in epithelial lineage tumours such as ovarian cancer [83]. Due to the restricted number of receptor sites in a specific target tissue, FR is a particularly "smart" approach for directing chemotherapeutics into the cell. Many advantages have been reported in the use of folate as a targeting ligand over monoclonal antibodies. First of all, the fact that the receptor is overexpressed in certain types of solid tumours and not as much in normal tissues represents a wide range of tumour targets along with tumour tissue specificity. The small size of the targeting ligand allows for a favourable pharmacokinetic profile and a less likely immunogenicity scenario. This opens up the possibility for repeated administration. Another important advantage in the use of folate as a targeting ligand is the possibility of cytosolic delivery of chemotherapeutics through the induction of the receptor/ligand complex to internalise the therapeutic agents via endocytosis [84]. Liposomes have been a very popular carrier used for drug delivery. Over the years many efforts have been made towards the coupling of monoclonal antibodies to liposomes; however, major limitations underpin the use of immunoliposomes for drug delivery. Besides the immunogenicity of the targeting ligand, the covalent attachment of a protein to a liposome is technically challenging [84]. For efficient drug delivery,

the carrier system should not only assist in tumour localisation but should also allow for intracellular access. The use of FR can fulfil all of these requirements [85]. Doxorubicin entrapped in folate-PEG-liposomes has shown cancer cell uptake and cytotoxicity that was 45- and 86-fold higher, respectively, than the free drug [86]. The deficient expression of FRs in normal tissues as well as its differential and tissue selectiveness in tumour tissue makes this family of receptors selective targets for drug delivery or possibly as prognostic markers [83]. The difficulty of using folate to target cancer cells is that the amount of folate receptor per cell may be increased in certain cancers, but the total number of folate receptors throughout the body is much greater than that expressed in a tumour. Thus, much of the dose of a drug-folate conjugate would not reach the cancer cells, producing systemic toxicity.

Another nutrient uptake transporter system, known as hPepT1 (human oligopeptide transporter 1), has also been suggested as a promising molecular target in cancer therapy [87]. Expression of hPepT1 is normally restricted to the apical surface of the small intestine and kidney epithelial cells where it functions to absorb di- and tripeptides. Previous studies have reported a high hPepT1 expression in cells derived from pancreatic cancer, gastric cancer, osteosarcoma, bladder cancer and cholangiosarcoma [88, 89]. These findings have raised interest in adopting hPepT1 as a possible route of drug delivery into these types of cancers. Indeed, a labelled peptide substrate (Gly-Sar) transported by hPepT1 has been used to detect tumours in vivo [90], and a specifically constructed cell line expressing hPepT1 has demonstrated transporter substrate accumulation [91].

Conjugation of certain non-PepT1 substrates with a single amino acid can render these conjugates sufficiently similar to a di- or tripeptide to allow this prodrug to be transported by hPepT1 [92]. Amino acid ester conjugates of gemcitabine (a drug commonly used for pancreatic cancer) acquire the capacity for transport by hPepT1 [93], and an amino acid ester prodrug of the anti-cancer compound fluxoridine has enhanced cytotoxic activity on pancreatic cancer cells and a prolonged systemic circulation [94]. Thus, a delivery system targeted to a specific drug transporter can be accomplished by using an amino acid-prodrug approach [95]. Once inside the cancer cell, it is assumed that cytosolic peptidases would cleave the anti-cancer agent from the amino acid component of the prodrug (Fig. 1.6).

Targeting cancer cells can theoretically reduce side effects and increase clinical efficiency [82], with novel prodrug chemotherapeutics functionally directed to nutrient uptake pathways showing some promise [89]. Nonetheless, there are some pitfalls that need to be considered. It is becoming clearer; even though some cancer cells overexpress certain cellular markers, it is not known if these receptors are functional or not. Also, it cannot be discarded the fact that such up-regulation could be a result of the clonal expansion of the cell line when cultured in vitro, instead of representing the original tumour conditions [96]. Further, anti-cancer compound uptake could focus potential secondary toxicities to specific organs where these cellular markers are normally expressed. With this is mind, it could be useful to understand what drives such up-regulation in cancer cells. By understanding the mechanism involved, a selective therapeutic approach could then be evaluated. Finally, because

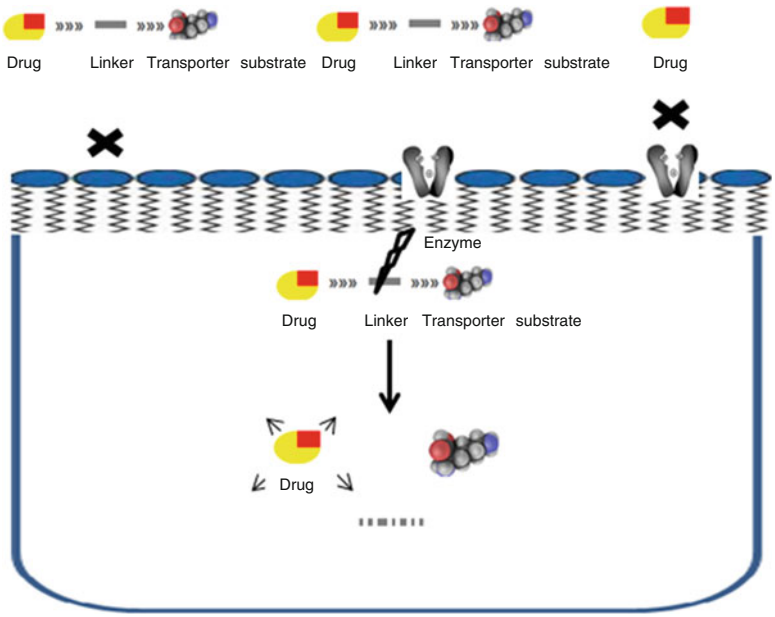

Fig. 1.6 Schematic representation of hPepT1 transporter-targeted prodrug uptake

there are specific drivers that drive the expression of these transporter systems in cancer cells, it is not known if the same drivers are also up-regulating efflux pathways, such as P-gp (P-glycoprotein). Needless to say, an up-regulated efflux system could limit the effectiveness of anti-cancer compounds delivered to these cancer cells [96].

The importance of drug transporters in cancer biology and chemotherapy highlights the pivotal role of these molecules in the redesign of modern and targeted therapies. A more rational and sensible use of these molecules, with unique patterns of expression, will certainly underline the optimisation of individualised cancer medicines. Targeting membrane transporters and the introduction of polymer-drug conjugates are two emerging areas in the discovery of new cancer therapies. The use of polymer conjugates applied to the formulation of new drug delivery systems is the focus of the next subsection in this chapter.

Polymer Drug Conjugates

Polymers provided a platform where targeting ligands can be coupled to achieve the focused delivery of chemotherapeutics conjugated to or captured within a material [97, 98]. This approach builds upon the well-established field of polymer chemistry and

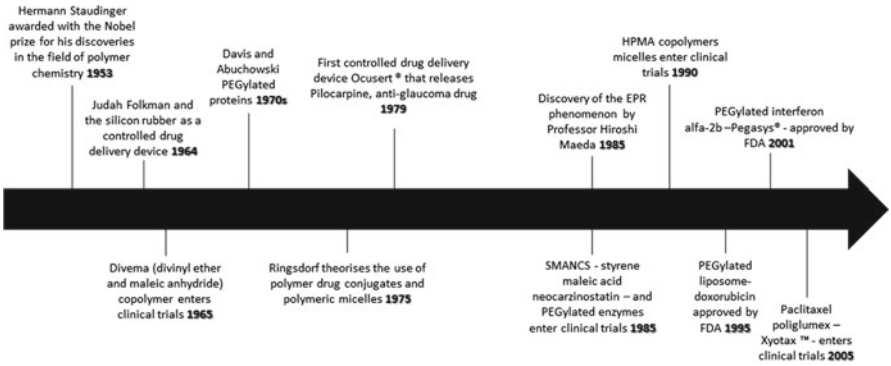

Fig. 1.7 Some milestones in the field of anti-cancer polymer therapeutics

materials already approved for use in humans for a variety of applications. The origins of controlled drug delivery go back to the mid-1960s. Judah Folkman circulated rabbit blood inside a silicone rubber (Silastic®) implanted as an arteriovenous shunt and discovered that after exposing the tubing to external anaesthetic gases, the rabbits would fall asleep (Fig. 1.7) [98]. He then theorised that short and impenetrable fragments of such silicon rubber tube containing a drug could be implemented in the body. Provided that the material would not change in size or composition, the implant would become a constant rate drug delivery device [99]. In the 1970s and 1980s subcutaneous or intramuscular implants, topical patches and even mucosal inserts were tested as controlled drug delivery devices. Subsequently, degradable polymer systems were examined for the delivery of drugs from small polymer structures [100].

While work with liposomes represents some of the earliest efforts to use nano-carriers to target chemotherapeutics to cancer cells, targeted polymer nano-carriers as drug delivery started the studies of Ringsdorf in 1975 (Fig. 1.7). In this approach, water-soluble polymers were described as efficient platforms to deliver drugs to the right disease sites [101]. Since then a wide range of nano-carriers, including polymer coated liposomes, have been described as potential "smart" drug delivery approaches [102]. The field of polymer therapeutics, as we know it today, primarily involves the use of polymer-drug conjugates or polymeric micelles in which the drug is covalently entrapped [103]. The rational design of polymer-drug conjugates is now well understood, and these macromolecular entities have three essential elements: a water-soluble polymeric carrier; a biodegradable linker between the polymer and the drug and a bioactive anti-tumour agent.

These three main constituents of rationally designed polymer-drug conjugates allow for tumour-enriched delivery through the enhanced permeability and retention effect (EPR). The EPR effect was first elucidated in 1985 by Hiroshi Maeda and describes the property of solid tumours that result in the selective accumulation of certain-sized particles in tumour tissue compared to normal tissues [104]. Tumour vessels support tumour growth and as a consequence cancer cells start to become

dependent on blood supply for their nutritional and oxygen supply. These newly formed vessels are normally abnormal in form and architecture, being characterised by wide fenestrations, lack of smooth muscle layer and defective lymphatic drainage. Due to this impaired intra-tumour lymphatic drainage, macromolecules and nano-carriers are retained in the interstitium, leading to an effective tumour targeting [102, 105]. The high molecular weight of polymer carriers does not allow the elimination of the drug complex through renal filtration, ensuring that the drug is retained in the body prolonging its systemic circulation [106]. Owed to the EPR effect, the clearance of the polymer-drug complex is slower, leading to a higher accumulation and maximised anti-tumour activity [107].

Several advantages have been linked to the use of polymer-drug conjugates in cancer therapy, and some of them are fewer side effects, enhanced therapeutic efficacy, ease of drug administration and improved patient compliance. Some polymer-drug conjugates are in clinical trials [108]. HPMA (*N*-(2-hydroxypropyl) methacrylamide) copolymer-doxorubicin was the first synthetic polymer-anti-cancer drug conjugate to enter the clinic. Doxorubicin is a potent cytotoxic agent widely used for the treatment of solid tumours. In an attempt to reduce its toxicity, doxorubicin was conjugated to a water-soluble polymer, HPMA, and this allowed the release of the active drug into the tumour site, through the action of lysosomal enzymes. Preclinical studies also suggested that the complex was more potent than the free parental drug [102, 109]. HPMA copolymer-camptothecin is another example of a polymer-anti-cancer drug conjugate. Camptothecins are a family of potent anti-cancer agents that inhibit topoisomerase I activity. Lack of water solubility and instability of the lactone form, due to the preferential binding of the carboxylate to serum albumin, are two main factors that compromise the therapeutic efficacy of these compounds in humans. Nonetheless, conjugation of camptothecins to water-soluble polymeric carriers results in an increased water solubility and enhanced stability of the lactone ring [102]. Alongside HPMA, PEG (polyethylene glycol) can also be used as a drug carrier; however, there are potential limitations to this approach. The presence of only two reactive groups per polymer chain leads to an intrinsically low drug payload, limiting therefore the efficacy of the polymer-drug conjugate [108]. Yet, there are at least two PEG polymer-conjugated cancer drugs on clinical trials. Prothecan also known as PEG-camptothecin is a prodrug conjugate of the DNA-damaging agent and PEG-irinotecan is on Phase II studies. Both of these compounds seem quite promising as they are showing an extended pharmacokinetic profile compared to the free drug [110].

Polymer therapy holds great promise. Many advantages have been showed by a wide range of polymer-drug conjugates in relation to the corresponding parental drugs. In the near future, nano-carriers need to be optimised with modulated rates of degradation, polymerisation methods that will accurately control the polymer molecular weights and their distribution, and finally some flexible conjugational chemistry needs to be identified to improve site-specific attachment for targeting moieties [108].

Conclusion

It is common sense that cancer is part of normal aging. Statistics are clear and show that cancer has a much higher incidence rate in an aged population compared to a young and healthy one. It is common knowledge as well that the best way of treating cancer is prevention as well as early detection. Being a heterogeneous disease, cancer is the perfect substrate for its own evolution and clonal selection. While it is difficult to treat it, it is also difficult to diagnose. Cancer diagnosis is frequently based upon vague and unclear symptoms without a clear pattern. We now understand that this difficultly in cancer diagnosis is due to the complex nature of how cancers form and how they interact with noncancerous cells of the body.

The human body is made of a highly complex and hypermutable cell network. It is estimated that each of its $\sim 10^{13}$ cells experience thousands of DNA-damaging events per day [111]. Mother Nature, however, bequeathed the human body with the possibility of repairing most of these genetic alterations but also to self-destruct if critical genetic errors occur. Cancer cells take selective advantage of these processes to ensure survival without self-destruction due to genetic errors. As a genetic disease specific to an individual, each cancer is distinct. It can be appreciated the complexities involved in developing effective anti-cancer therapeutic methods for a general population suffering from individual cancers.

Thirty years ago, cancer treatments had a myopic focus overtly killing cancer cells, and histological classification was regarded as the main diagnostic tool to outline cancer treatment strategies. Extensive investments by government funding agencies and pharmaceutical companies have led to a much greater understanding of cancer causes and progression. This has changed the search for effective cancer therapies from empirical approaches in which cytotoxicity played a crucial role to a more hypothesis-driven science where cancers are being looked at as a set of potentially chronic diseases. Further, rational design of drugs has introduced an improved drug safety and tolerability profile. Notwithstanding the advances and the incorporation of genetics and biology into cancer research, the prognosis for a number of malignancies remains poor.

Surgery, radiotherapy and chemotherapy were reviewed in this chapter as the available core cancer treatments available. There has always been a continuous search for efficacy and usefulness in cancer therapies. While surgery was the first treatment strategy that revealed some promise, it has been proven to be insufficient. The discovery that radiation could kill cancer cells represented a leap forward towards the cure, although this approach has also failed to provide consistent cures. Chemotherapy came along with the concept of cell-damaging events similar to radiation but with the hope of selective cell killing akin to very precise surgery. Thus, these ideas have pushed the concept that local, selective targeting of cancer therapies without achieving this goal. While this aim appears to be feasible, it will require expertise from many different areas including molecular and cellular biology, structural biology, medical oncology, biomarkers specialists, medicinal chemists and pharmacokinetic and pharmacodynamics modellers [74].

Greater precision of what causes cancer and an improved understanding of mechanisms involved in cancer cell survival should lead to more astute methods of preventing and treating the many forms of this disease. We have already observed a shift from conventional, non-targeted cancer therapies to current efforts that are attempting to selectively deliver anti-cancer agents to the right cell, for the right duration, and at the right concentration to be optimally effective and maximally safe. A more in-depth knowledge about metabolic pathway networks involved in cancer and how to effectively and selectively target them will improve our approach to cancer therapies. Combined with efforts to effectively screen for very early-stage cancers, it is realistic to assume that improved prevention and treatment of cancers will be achieved in the near future.

Oncogene-directed treatments seem promising and it looks that incipient opportunities for personalised medicine are emerging. The idea of looking at each cancer as a unique disease for tailoring specific cancer therapy to each patient individually is gaining progressive strength. Soon, the concept of individualised medicine will become a reality. For instance, a patient with breast cancer will no longer need to go to a breast cancer clinic but instead may go to a clinic specialising in HER2 amplification or EGFR activation. Pre-screening patients as possible responders or nonresponders to a particular treatment and increasing the speed at which non-effective and effective therapies can be determined through biomarkers should also improve patient outcomes.

Remarkable advances in cancer research have been achieved over the past 50 years and some cancer therapies are successful in prolonging patient's survival, but we are still far away from consistently achieving complete cures. For the past five decades, cancer therapies had focused on treating the individual gene in understanding cancer. However in the twenty-first century, cancer needs to be viewed with a broader vision in order to better understand its biology. Adoption of cross-disciplinary strategies to understand the nature of cancer cells in the complex environment of the body will likely be essential to achieve this goal.

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Chapter 2
Nanotechnology for Cancer Treatment: Possibilities and Limitations

Joseph W. Nichols and You Han Bae

Abstract Drug delivery to solid tumors is one of the seminal challenges to developing more effective cancer therapies. A well-designed drug delivery system can potentially improve the efficacy of a treatment by enhancing drug accumulation in the tumor and combining synergistic effects into a single package. It may also reduce negative side effects by limiting drug access to sensitive noncancerous tissue. The most common drug delivery design is to package small molecule drugs with a nanoparticle. Nanotechnology provides a versatile platform onto which many functions can be added. Nanoparticles are widely considered to have superior biodistribution and efficacy when compared to free drug particles, but this expectation has not matched clinical results. One reason for the disappointing clinical outcomes of nano-sized drug carriers is the numerous barriers to drug delivery encountered by the nanoparticle on route from the administration site to tumor interior. These barriers are encountered along the entire delivery pathway and can severely limit the total effective amount of drug in the tumor.

Introduction

Interest in nanomedicine and drug delivery has increased exponentially in the last several decades. As with many newly developed technologies, the ability to manipulate matter at the nanoscale to create unique structures has generated creativity,

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enthusiasm, and a burst of funding. Biology may hold the most intriguing prospects for nanotechnology as it allows access to those scales at which most biological functions take place. The field of nanomedicine has indeed seen an increase of activity and continues to grow, as seen in the rise of both patents and publications [1, 2]. This rise represents a growth of research encompassing many facets of medicine including biomaterials, active implants, in vivo imaging, in vitro diagnostics, therapeutic materials, and gene and drug delivery.

Worldwide funding for all nanotechnology is expected to exceed $1 trillion by 2015 and, perhaps more importantly, market revenues for nanotechnology is thought to be close to $3 trillion worldwide [3, 4]. Nanomedicine research is also receiving a growing amount of funding, with public funding research reaching nearly $1 billion in the United States, $600 million in Japan, and $400 million in Germany. The level of funding for nanomedicine is indicative of the tremendous enthusiasm for the field. Recently, as much as 50 % of biomedical advances were estimated be related to nanotechnology [3].

The field of nanomedicine was originally conceived with fantastic visions of future capabilities. Nobel Prize winning physicist Richard Feynmann envisioned building nanorobots by employing manufacturing robotics to make another series of robotics at a smaller scale and following this sequence in series until the near atomic scale is reached [5, 6]. This concept was later seized upon and expanded to envision submarine-like nanomachines capable of independently performing numerous tasks, from supplementing immune function to eradicating cancer. Theoretically such machines could protect and prolong life by rebuilding damaged tissues, repairing virus-damaged cells, supporting or reconstructing damaged limbs and organs, and even reversing aging [7].

Though such visions of nanotechnology are clearly many decades, if not centuries, away (if physically possible at all), nanomedicine has found many applications and is still rightly hailed as potentially revolutionary. Nanotechnology has been applied to diabetes research for glucose sensors and nano-pancreases [8]; to tuberculosis and other respiratory diseases [9, 10]; in neurological diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis [11]; for hemophilia [12]; to bone healing and osteoporosis [13]; and even for hair growth [14].

Perhaps the most explored application of nanomedicine is in cancer chemotherapy methods. Compared to regular chemotherapeutic treatments, nanoparticle drug carriers are presumed to have improved tumor specificity, fewer side effects, improved efficacy, and more flexibility in treating the highly diverse cancer types. These advantages, in conjunction with the seemingly limitless versatility of nanoparticles in both composition and surface chemistry, have led to an explosion of designs [15–18]. These designs attempt to address the various challenges facing drug delivery to solid tumors, which are present from the point of initial blood contact until the drug action occurs within the tumors. Effective therapy is further challenged by the development of drug resistance mechanisms and intratumoral heterogeneity.

Improving the efficacy of cancer therapy requires that the drug carrier adequately address the challenges of drug delivery so that sufficient drug can be brought against the tumor to eradicate it fully without causing excessive toxicity in the patient. Unfortunately, although the drug delivery designs currently coming out of labs

around the world have shown abundant potential in literature and preclinical studies, they have largely failed to make a significant impact in the clinic. Of the thousands of patents and publications filed in the field of cancer therapy, only a few carriers have found their way on the market in the United States. Doxil® and Abraxane® are two of the most successful drug carriers. Both are simple in concept and design and showed little to no improved efficacy compared with traditional chemotherapy [2, 19, 20].

New and dramatically improved therapies are needed if we are to meet the growing challenge of cancer in the future. In the US, cancer rates are expected to increase nearly four times as fast as population growth through 2030 [21]. This growth is largely attributed to changing demographics and an aging population that has benefitted greatly from the lifesaving and life-extending advances in other fields of medicine, but is now at greater risk of cancer. This places a greater burden on cancer researchers to design new treatments to extend and improve life for this growing group. Many designs are being tested, but the translation to the clinic is failing. Not only must new solutions to drug therapy be found, but improved methods of testing these solutions must be developed.

Challenges to Drug Delivery

Nanoparticles come in an almost infinite variety of sizes, shapes, and compositions, with more diversity of form and function to be found in the ability to modify the surface in myriad ways [22] (see Fig. 2.1). This versatility allows the nanoparticle to become a blank canvas, reflecting the creativity and skill of the researcher and exhibiting a wide range of unique behaviors. Nanoparticles can be designed with multiple functionalities to aid cancer therapies. Targeting moieties can be grafted onto the surface to aid cell uptake [23]. Synergistic drug types can be loaded into the same carrier to improve overall efficacy and combat the development of multiple drug resistance (MDR) [24, 25]. Imaging and therapy can be combined, allowing the progress of a treatment to be monitored in real time and aid clinicians in making appropriate treatment decisions [26]. Nanoparticles can even be designed to respond to outside stimuli, giving doctors the ability to very specifically target the release of drug or other therapeutic effects to a specific region in the body [27–29]. This versatility, however, can be more than matched by the impressive mechanisms the body and tumor employ to guard against potentially dangerous substances.

The body possesses numerous defense mechanisms to protect itself against foreign substances including viruses, bacteria, protein toxins, and other chemicals [30–32]. Nanoparticles are no exception and are actively cleared by the body [33]. The body has multiple strategies to prevent or mitigate tissue damage and maintain cell viability. These defense mechanisms are in place at every level of organization, systemic, organ, tissue, cellular, and intracellular (see Fig. 2.2). These barriers can be accentuated by the development of MDR phenotypes. MDR is associated with poor clinical outcomes and can apply to a wide variety of drugs. Heterogeneity

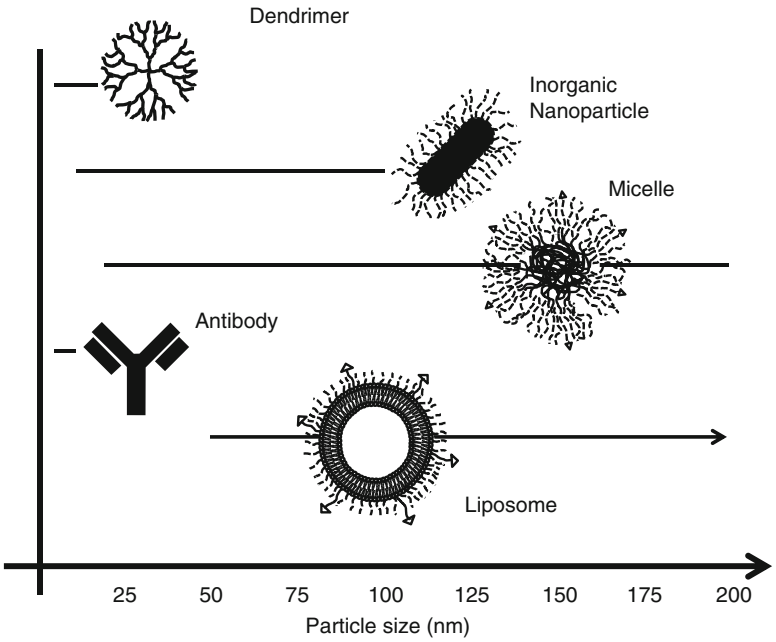

Fig. 2.1 Size of representative nanoparticles. There is a vast range of sizes and compositions of nanoparticles. This diversity gives researchers a great deal of versatility in designing drug delivery strategies

within the tumor presents a final challenge to successful cancer therapy. Tumor heterogeneity encompasses the genotypic variances among cancerous cells as well as the diversity of cell types within the tumor ecosystem that can make the tumor a more robust and resilient organ than a more isotropic model would suggest.

Most chemotherapeutic drugs are cytotoxic agents which have specific targets for action inside a cancerous cell [34]. These drugs are typically introduced into the body intravenously and must then complete a perilous journey through the circulatory system until it can encounter and enter the tumor. While in circulation the drug or drug carrier must avoid the many routes of clearance used by the body to protect against foreign substances, including renal clearance, liver metabolism, and the mononuclear phagocyte system (MPS). Long lasting particles may circulate long enough to encounter the tumor microvasculature and some of those may successfully diffuse out of the blood vessels to enter the tumor interstitial space. The drug that has made it to this point must then diffuse through the tumor, encounter a target cell, cross the lipid bilayer membrane, and finally localize in the cellular compartment relevant to the drug's mechanism of action in sufficient concentrations to cause cell death. The vast majority of drug administered to the patient does not complete all phases of this journey and becomes at best ineffective, and at worst toxic to the patient [35].

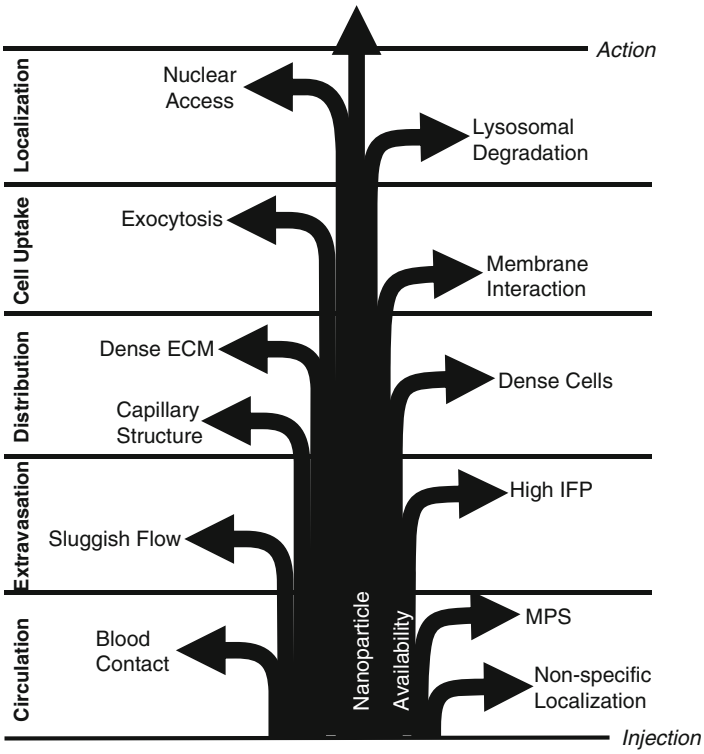

Fig. 2.2 Barriers to drug access. The journey of a nanoparticle from the intravenous injection site to the site of action in the tumor includes numerous obstacles. Each of these obstacles reduces the total quantity of nanoparticles potentially capable of treating the tumor. Successfully treating the tumor requires that enough of the drug navigates all obstacles to kill the tumor cells

Systemic Barriers

One of the primary purposes of drug carriers is to solubilize and protect its drug cargo from clearance and degradation until it reaches its target site. For most nanoparticles the first challenge to that purpose comes immediately on blood contact after administration. Nanoparticles have a very high ratio of surface to bulk atoms, which tends to result in high surface energies and unusual behaviors [36]. These behaviors include aggregation which can impact the polydispersity and biodistribution of the particles. The high surface energies may also result in strong binding of blood proteins to the nanoparticle surface [37]. These bound proteins can serve as a signal for MPS macrophages to engulf circulating nanoparticles, causing them to accumulate outside the tumor [36, 38]. MPS, also known as the reticular endothelial system (RES), is a system of tissue embedded macrophages that clear foreign substances from the blood and tissues. It is most prominent in the white blood cell rich spleen which sees a significant portion of administered nanoparticles in most studies. The liver also collects a very large quantity of nanoparticles due

both to MPS activity and to the unique porous sinusoid structures in the liver which help it to filter and clean blood [32]. Nanoparticles are generally large enough to avoid renal clearance [39].

Currently, the predominant strategy to minimize protein adsorption and MPS uptake is to densely graft a hydrophilic polymer, most commonly poly (ethylene glycol) (PEG), to the surface of the nanoparticle in a process known as PEGylation. The grafted polymer extends from the surface and forms a brush-like barrier that limits access to the nanoparticle surface and slows the rate of binding. PEGylation can significantly slow the kinetics of protein binding and MPS clearance of nanoparticles [35], allowing the circulation time of most nanoparticles to increase by several orders of magnitude compared to unmodified nanoparticles [33, 38].

Long circulating drug carriers are expected to show improved biodistribution and increased intratumoral accumulation in comparison to conventional treatments. This expectation is driven by the enhanced permeability and retention (EPR) of nanoparticles in the tumor, and when EPR leads to improved efficacy of treatment it is known as the EPR effect. EPR is actually a result of two separate phenomena, enhanced permeability and enhanced retention. Both are related to physiological abnormalities resulting from the rapid growth of the tumor and the way it modifies the local microenvironment.

Rapid and uncontrolled cell growth is one of the chief hallmarks of cancer [40]. The division and growth of cells within a confined space can cause cells to become very tightly packed and the resulting compressive stress can crush native blood and lymph vessels [41]. Lacking intact blood vessels the tumors must rely on simple diffusion to deliver oxygen and nutrients and to remove waste from the tumor center. Once the tumor diameter reaches approximately one millimeter, hypoxic conditions become dominant in the core causing hypoxic cells to release factors promoting angiogenesis [42]. Angiogenesis proceeds rapidly to supply the tumor resulting in tortuous, chaotic, and disorganized vasculature. The vessel walls of the newly formed vasculature are similarly disorganized, leaving large gaps or fenestrations through which large particles such as proteins and nanoparticles can diffuse [43–45]. The ability for nanoparticles to diffuse into the tumor more readily than in normal tissues with organized, coherent vascularization is the primary mechanism for the enhanced permeability of EPR.

The enhanced retention of nanoparticles in a solid tumor results largely from the destruction of lymph vessels due to solid compressive stress [41]. Without functioning lymph vessels, fluid must flow out the periphery of the tumor before it can be cleared. The rate of flow to the tumor exterior can be slowed by the hydraulic resistance from the tightly packed cells and dense collagen matrix. This fluid retention has several effects related to drug delivery that will be discussed later, but among them is the tumor's limited ability to clear drug carriers from the tissue [46].

EPR provides what is perhaps the primary advantage to cancer nanotherapies over traditional chemotherapy. The discovery of EPR in the mid-1980s brought a great deal of attention to nanotechnology in search of the next major breakthrough in cancer treatment [47]. Nanotechnology seemed to hold the promise of improved treatment efficacy, combined with a means of controlling or even eliminating nonspecific toxicity [48].

EPR is a form of microenvironmental targeting, attacking the pathogenic tumor lymph and blood vessels to achieve tumor specificity [49]. This reliance on the tumor microenvironment to deliver effective drug doses can be problematic for treating potentially metastatic cancers. Metastasized cells or colonies too small to have created a microenvironment are unlikely to be affected by nanotherapies making adjuvant therapy with traditional chemotherapeutic drug cocktails necessary to prevent cancer spreading and relapse. Nanotechnology is thus unlikely to form the basis of a stand-alone cancer therapy.

Long circulation and EPR do not guarantee the drug carriers will reach the tumor site. Most tumors are only a few centimeters in diameter, a small fraction of the total size of the patient [50]. The administered drug is carried indiscriminately throughout the body via the circulatory system, meaning that a given drug particle will rarely encounter the tumor much less extravasate into it, and even under the best of circumstances only a tiny fraction of the injected dose will enter into the tumor where it can be effective. Whatever drug does not enter the tumor does not contribute to the drug's efficacy, but instead causes the dangerous side effects for which chemotherapy is famous. Loading the drug into a carrier can help limit drug access to certain tissues and improve the overall toxicity profile, but the overwhelming majority of the injected drug is incorporated into tissues other than the tumor [19].

The EPR effect predicts that long circulating nanoparticles should accumulate in higher concentrations in the tumor relative to surrounding tissue, thus improving the overall efficacy of the treatment. There is a great deal of evidence showing this effect in animal models and limited clinical evidence that limited preferential tumor accumulation does take place, but the clinical benefit has yet to be seen [19, 51, 52]. The discrepancy between preclinical and clinical success exposes the inadequacy of the models used to study cancer nanomedicine. While these models are both necessary and useful for designing and testing drug carriers, they are at best flawed representations of reality. Tumor models developed in mice are generally grown much more quickly than naturally occurring tumors, a condition which accentuates the rapid angiogenesis leading to the disorganized vasculature presaging EPR [53, 54]. Additionally, murine model tumors are grown to as much as 10 % of the total mouse weight, compared to a human tumor which generally only grows to a tiny fraction of a percent of body weight. A circulating nanoparticle in a mouse will thus encounter the tumor exponentially more often than in a human and is more likely to extravasate into it.

Extravasation

Due to the relatively small size of a tumor compared to the rest of the patient and the effectiveness of the body at clearing foreign material from the blood, a large portion of nanoparticles will never encounter the tumor and thus have no opportunity to provide a therapeutic effect. Of those nanoparticles that do encounter the tumor vasculature, most pass straight through and back into the larger circulatory system, eventually causing unwanted side effects in distant organs. Moving a drug carrier

from the blood compartment and into the tumor interstitial space is a significant problem for drug delivery researchers.

Blood flow through the tumor can be sluggish and intermittent due to the disorganized, chaotic nature of the hastily formed vasculature and can result in poor or intermittent delivery of blood-borne drug to the vascularized areas [28, 44, 45]. This uneven supply can have an important impact on the spatial distribution of drug in the tumor as a whole, leaving large regions of the tumor untreated or undertreated.

When nanoparticles do pass through the microvasculature, they are expected to diffuse out of the capillary and into the tumor interstitial space via the large fenestrations or openings in the capillary wall [55]. In healthy capillaries, movement across the capillary wall is described by the Starling equation, which expresses the balance of hydrostatic and oncotic pressures across the wall [56]. At the arterial side of the capillary, the hydraulic pressure provided by the heart exceeds the tissue interstitial pressure, which tends to drive bulk fluid flow out of the vessel. Waste-bearing fluid is returned to the venous side by osmosis. The osmotic potential in the blood is generally higher than in the interstitial fluid due to the exclusion of blood proteins such as albumin from the interstitial space.

In cancerous tissue this balance is disrupted as a result of the pathological structure of the vessel walls. The large fenestrae in tumor vasculature are not only permeable to nanoparticles, but to all blood-borne macromolecules including albumin and other large proteins [57]. The free flow of large solutes across the capillary wall results in equal osmotic potentials both inside and outside the capillary. The combination of high osmotic pressure, lack of lymphatic drainage, and high hydraulic resistance in the tumor results in a tumor interstitial fluid pressure (IFP) that approaches the microvascular pressure [41, 58]. With no pressure differentials across the capillary wall, the driving force for bulk fluid exchange is negligible and extravasation out vessel fenestrae must rely almost entirely on diffusion [55]. Extravasation then becomes dependent on the probability that a particle encounters a fenestration by random motion. Once extravasated, there is also no flow gradient to prevent the particle from passing back into the capillary rather than diffuse deeper into the tissue.

Reducing tumor IFP to restore bulk flow across the capillary wall is one potential strategy to improve particle extravasation. One way to accomplish this is to normalize the tumor vasculature [54]. This can be done by blocking the proangiogenic factors released by the tumor, thus slowing the rate of angiogenesis and giving the nascent blood vessels time to organize [44]. Tumor IFP may also be temporarily reduced by degrading the collagen mesh that makes up the tumor extracellular matrix (ECM). The dense collagen mesh gives the tumor a high hydraulic resistance and prevents fluid from draining out. Degrading this mesh may allow more drainage and reduce IFP [59]. Both of these strategies would seem to offset some of the natural advantages of nanotherapies gained from EPR. Normalizing the vasculature to restore normal pressure gradients would reduce the fenestration size in the capillaries and negate the enhanced permeability of nanoparticles to the tumor. Improving tumor drainage may hurt nanoparticle retention in the tumor and also increase metastatic potential as cells are brought outside the tumor mass.

More specific methods of promoting extravasation include attaching the drug carrier to a tumor penetrating peptide such as iRGD [60]. The mechanism of these

peptides is currently unclear, but it appears to improve transcytosis in tumor tissues by binding to α_v integrins on the tumor endothelial cells. Some studies have shown significant improvements in tumor accumulation when using the tumor penetrating peptides compared with controls [61, 62]. Localized hyperthermia may also be used to enhance nanoparticle penetration in a tumor-specific manner by increasing vascular permeability in a targeted area. This method relies on good imaging methods so that doctors can see exactly where to direct heating stimuli [63].

Most efforts to improve extravasation and intratumoral accumulation have been focused on lengthening the particle circulation time, giving circulating particles more opportunity to encounter the tumor. PEGylation has proven to be the most successful method of lengthening circulation time, with coated particles lasting more than 40 fold longer than uncoated particles [64]. However, some studies have indicated that extending circulation time beyond a certain point does not significantly improve treatment efficacy, but does contribute to worsening side effects [65]. This may be due to the limited mobility of extravasated nanoparticles, preventing them from moving away from the fenestrae. These nanoparticles can then become a barrier preventing subsequent nanoparticles from extravasating. Particle extravasation may thus be partially limited by the rate of diffusion away from the fenestrae after passing out of the capillary. Some evidence suggests that extremely long circulation times may also result in greater toxicity than shorter circulating drug carriers [65, 66]. Very long circulation times allow the kinetically slow extravasation of nanoparticles into skin and other tissues to become much more significant, leading to painful side effects such as foot and hand syndrome [66].

Intratumoral Distribution

Nanoparticles may have to diffuse relatively huge distances to reach a large portion of tumor cells. The chaotic nature of the vasculature can leave large regions of the tumor underserved and difficult to access, especially for large, relatively immobile nanoparticles [67]. These regions also tend to be hypoxic and select for highly resistant and potentially dangerous cells [68]. Killing these cells may be critical to the long-term success of a therapy. The distance a drug carrier must travel to reach these cells, however, becomes even more daunting in light of the many barriers to oppose the already weak diffusion driving force (see Fig. 2.3).

The difficulty of diffusion through a tumor can be compounded by the dense ECM. The structure of the collagen matrix can limit or halt the movement of large particles [69]. The densely packed cells of the tumor can be another impediment to nanoparticle motion [70]. Cells are very large compared to most nanoparticles; for example, if the nanoparticles were the size of a soccer ball, the cell would be approximately the size of the field. Navigating a mess of such relatively huge obstacles can significantly increase the effective path length the drug carrier must travel to diffuse within the tumor [71]. These physical barriers can be greatly exacerbated by interactions with either the ECM components or the cell membrane [72]. Many particles are designed to interact with markers on the cancer cell membrane to

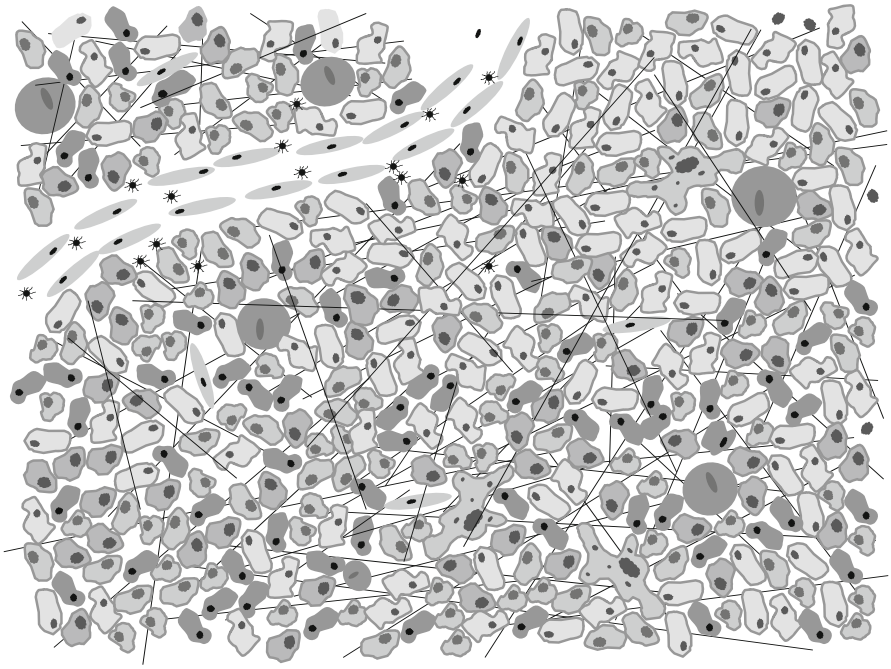

Fig. 2.3 Tumor composition and distribution barriers. Effective therapy requires that the drug carrier extravasate from the blood vessel to the tumor interstitial space and then diffuse throughout the whole tumor. This diffusion is made very difficult by the tense tumor cells and ECM

improve cell uptake and specificity. This may result in the development of a "binding site barrier" in which the drug carriers get caught on the first cells encountered after extravasation and fail to penetrate more deeply [73].

The large size of nanoparticles, relative to small molecule drugs, is a major liability for the intratumoral distribution portion of drug delivery. Improving intratumoral distribution would hugely benefit the efficacy of treatment. Limiting interactions with the ECM may be the most important strategy to improve distribution. Fortunately PEGylation appears to be effective at limiting these interactions and can dramatically increase diffusivity in some circumstances [72]. Even PEG coated particles are much too large to diffuse readily through the tumor environment. Recognizing this, some drug carriers are designed to degrade in the tumor microenvironment, leaving the small drug cargo to diffuse the remainder of the way [74].

Unfortunately, opportunities to increase diffusivity by modifying the nanoparticles are limited leaving many researchers to attempt to modify the tumor microenvironment to be more conducive to particle distribution. One such strategy is coadministration of the nanoparticle with collagenase enzymes to degrade the ECM [75]. Breaking up the collagen matrix should allow more space for diffusion to occur, though this benefit may be somewhat offset by remaining debris [76]. This method also carries the potential risk of metastasis from cells that have become more mobile in the degraded matrix. As discussed above, degrading the collagen

matrix has the added benefit of reducing intratumoral IFP and potentially improving drug extravasation into the tumor [59].

Cancers should not be considered an isotropic mass of identical cells, but may be more accurately thought of as an organ whose primary function is growth and achieves that objective by acting as a parasite on other tissue [77, 78]. As with other organs, the tissue contains both primary cells and cells serving secondary support functions, including epithelial cells, fibroblasts, endothelial cells, perivascular cells, mesenchymal stem cells, and immune cells, all in addition to the primary cancer cell type [78].

The diversity of cell types in the tumor present both a challenge and an opportunity for cancer therapy. The support functions performed by the secondary cells render the tumor more robust than the isotropic model would indicate. However, the tumor also depends on these cells to perform important functions to maintain viability and thus may represent a target for therapy. Targeting the vascular endothelial cells, for example, eliminates the distribution barrier because the cells are immediately accessible from the vasculature. VEGF inhibition slows angiogenesis and may lead to more normal blood vessels capable of distributing drugs [54]. Attacking the blood vessels may also be used as a method to starve the tumor by restricting its blood supply [79].

Tumor associated macrophages (TAMs) also present a potentially inviting target. TAMs may aid drug distribution by collecting drugs then leaking it as it travels through the tumor [80, 81]. They also play a role in some critical functions such as angiogenesis, metastasis, and tumor progression [82, 83]. Therapies targeting secondary cells have shown impressive clinical potential but generally must be administered in conjunction with traditional therapies to effectively combat cancer [79].

Cell Uptake

The lipid bilayer membrane is designed to serve as a selectively permeable barrier to a wide range of substances. Only small, hydrophobic molecules are capable of diffusing through the membrane without assistance from protein channels or active uptake mechanisms. Most small molecule chemotherapeutic drugs diffuse directly across the membrane to access the cytoplasm [84]. Large hydrophilic molecules are not capable of diffusing across the membrane and do not have uncontrolled access to the cell [85].

Drug resistance is among the major problems facing cancer drug delivery, and one of the primary mechanisms of MDR is in the cell membrane. P-glycoprotein (Pgp) is a membrane embedded active pump responsible for removing a wide variety of toxins from cells. It is a member of a broad family of protein pumps known as the ATP-binding cassette (ABC) pump family, which are commonly found in cells frequently exposed to toxic environments such as in the liver, jejunum, and skin [86–88]. Pgp is also significantly upregulated in MDR cancer cells, protecting the cell against a wide variety of cytotoxic drugs. It mops up these substances and then pumps them to the cell exterior, hydrolyzing ATP in the process. Pgp can maintain significant concentration gradients across the membrane, meaning that to

achieve lethal concentrations inside the cells by passive means, unsustainable doses must be used [89, 90].

Bypassing Pgp mediated MDR is critical to treating many of the most lethal cancers. One solution to resolve the Pgp obstacle is to co-deliver the drug with a Pgp modulator. These modulators use various mechanisms to compete or block Pgp activity, allowing small molecule drugs to diffuse more easily across the membrane [91]. Early modulators had problems with specificity, inhibiting other ABC pumps and causing harmful drug interactions. New modulators are promising better specificity and fewer negative reactions, though the safety of these modulators is as yet unproven [92, 93]. The systemic toxicity caused by these inhibitors can limit the maximum tolerated dose of a treatment regime [94].

A nanoparticle may also circumvent the Pgp barrier entering the cell interior intact while carrying the drug. Nanoparticles are not able to enter the cell by diffusion and thus must gain access almost exclusively via an active form of endocytosis [84]. There are several mechanisms by which endocytosis can take place. Pinocytosis is one such mechanism in which the cell randomly samples the surrounding fluid while other methods are generally mediated through particle-membrane interactions and cell receptors. Increasing nanoparticle interaction with those receptors is one method to improve overall cell uptake [95].

Equipping nanoparticles with ligands for cancer-specific cell receptors can theoretically improve the drug internalization rate and is thought to simultaneously enhance specificity, though the claim is controversial [96–99]. Achieving tumor cell specificity requires the presence of cancer-specific markers, which are extremely difficult to find. Cancer is born of our own biology, so nearly all proteins in cancer serve a role somewhere in the body. At a minimum, similar, if not identical, proteins will be present rather abundantly in the body compared to the total expression in the tumor. The cumulative effect of the lower affinity interactions elsewhere in the body may still lead to a great deal of nonspecific toxicity. The relevant interactions also take place on the scale of a few nanometers or less, so the nanoparticle cannot be actively guided to the tumor by receptor-ligand targeting [100].

Targeting strategies are popular in drug delivery research but have thus far failed to provide much clinical benefit. Nearly 30 years of intensive research has yielded only a handful of clinically available nanotechnology based cancer therapies (see Table 2.1). Most of these treatments are antibody therapies, but of the dozen that have gained clinical approval, only trastuzumab is indicated to directly attack the cells of solid tumors [101]. Other clinically approved cancer nanotherapeutic designs are even rarer. The two most popular formulations are Doxil® and Abraxane® which are both FDA approved to treat solid tumors [19, 20]. However, these therapies rely wholly on passive targeting, rather than active receptor-ligand targeting, and are not representative of the complexity of drug carrier designs seen in literature.

The lack of clinical success for these treatments is surprising, given the promising preclinical results. The discrepancy may again be largely due to problems with the models used to test these formulations. Many tests are conducted in two dimensional Petri dish models with cultured cells [95]. While useful in proof-of-concept studies, these models suffer from two important shortcomings. First, they give the drug formulation unhindered access to the cells, free of any physical barriers such

Table 2.1 Cancer nanotherapies currently in clinical use. NYA = Not yet approved in U.S. but in clinical use elsewhere

Antibody Formulations				
Trade Name	*Formulation*	*Target*	*Indication*	*Approval Date*
Rituxan	Rituximab	CD20	Non-hodgkin Lymphoma-Leukemias	1997
Herceptin	Trastuzumab	HER2	Metastatic breast cancer, adjuvant for gastric cancers	1998
Campath	Alemtuzumab	CD52	Leukemia	2001
Zevalin	90Y-ibritumomab	CD20	Non-hodgkin lymphoma	2002
Bexxar	131I-tositumomab	CD20	CD20+ Non-hodgkin lymphoma	2003
Erbitux	Cetuximab	EGFR	Head and neck, some colon; adjuvant with radiation	2004
Avastin	Bevacizumab	VEGF	Metastatic colon and rectal cancers; antiangiogenic	2004
Vectibix	Panitumumab	EGFR	Colon and Rectal cancer with traditional therapy	2006
Arzerra	Ofatumumab	CD20	Chronic lymphocytic leukemia	2009
Yervoy	Ipilimumab	CTLA-4	Melanoma	2011
Adcetris	Brentuximab vedotin	CD30	Anaplastic large cell lymphoma (ALCL), Hodgkin lymphoma	2011
Kadcyla	Trastuzumab emtansine	HER2	Metastatic breast cancer	2013
Mylotarg	Gemtuzumab ozogamicin	CD33	Acute myeloid leukemia (AML)	2000–2010
Liposomal Formulations				
Trade Name	*Formulation*	*Drug*	*Indication*	*Approval Date*
Doxil	PEGylated liposome	Doxorubicin	Secondary treatment for ovarian cancer	1995
DaunoXome	Citrate liposome	Daunorubicin	Karposi's sarcoma	1996
DepoCyt	Cytarabine liposomal	Cytarabine	Lymphomatous meningitis, leukemia	1999
Myocet	Non-PEGylated liposome	Doxorubicin	Metastatic breast cancer with cyclophosphamide	NYA
Nanoparticle Formulations				
Trade Name	*Carrier Type*	*Drug*	*Indication*	*Approval Date*
Abraxane	Albumin	Paclitaxel	Secondary treatment for breast cancer	2005
Genexol-PM	Polymeric micelle	Paclitaxel	Metastatic breast cancer	NYA

as those previously discussed. This allows a much greater than normal portion of nanoparticles to come within the nanometer range required for specific interactions. Second, the cultured cells used typically lack the genetic diversity of natural tumors and may thus overpredict the actual presence of the relevant markers. These cultured cell lines are also inoculated into animals to generate tumor models that lack

the genetic diversity of natural tumors and may not realistically reflect the composition of surface receptors or the presence of secondary cell types [102].

Finding a silver bullet for cancer targeting remains an elusive goal. A great deal of research has gone into finding cancer-specific markers to be used as drug targets [103, 104]. These studies have revealed a better understanding of cancer biology, but few new therapies. Part of the difficulty of translating newly discovered markers to new treatments is the intratumoral diversity of marker expression. Her2 is a good example of the genetic diversity of cancer cells. Her2 is a protein receptor overexpressed in some breast cancers and targeted by the antibody trastuzumab under the brand Herceptin®. The American Society of Clinical Oncology has established guidelines used to determine eligibility for Herceptin® treatment. A sample of the tumor is biopsied and stained for Her2 expression and based on the degree of staining the tumor is assigned an immunohistochemistry (IHC) score ranging from 0 to 3+. The highest score (3+) is given to tumors in which 30 % or more of cells strongly stain for Her2 and the tumor is considered Her2 positive (score of 2+) if only 10 % of cells show definite staining [105, 106]. If 30 % staining represents nearly an ideal case in a clinically proven receptor, then any targeted drug formulation should acknowledge that targeting gaps will exist in other receptors as well. Furthermore, samples drawn for biopsy are small and IHC scoring can vary spatially as well as temporally [107].

Other receptors are also used to target cancer in various studies. The folate receptor is a longstanding and popular target for cancer treatment. It is strongly expressed in the pulmonary, endocrine, gastrointestinal, and genitourinary systems as well as tumors derived from those sources [108, 109]. The folate receptor was the target of one of the earliest chemotherapeutic treatments for leukemia [110]. The transferrin receptor is another target commonly overexpressed in tumors, but because it plays an important role in iron transport between blood and tissue, it is found in almost all cells [111]. Though these receptors are considered to be overexpressed in many tumors, overexpression and specificity should not be conflated. These markers are abundant throughout the body, and intratumoral expression can vary both spatially and temporally according to the microenvironmental conditions surrounding the cell.

An alternative to receptor-ligand targeting is to equip the nanoparticles with nonspecific peptides that are exposed only in the appropriate environmental conditions, such as the relatively acidic extracellular pH found in most tumors. TAT is a peptide sequence used by some viruses to penetrate the cell membrane and gain access to the cytoplasm [112]. It works to enhance cell uptake on all cells but can be shielded using pH-sensitive polymers until it reaches the tumor [113]. This method alleviates the problem of intratumoral heterogeneity by targeting the environment rather than the cells individually.

Intracellular Distribution

Gaining access to the cell is still not sufficient to guarantee treatment efficacy. The drug must still be delivered intact to whatever region of the cell it is designed to attack. The first barrier to drug carriers that entered the cell by endocytosis is

avoiding drug degradation by lysosomal digestion. Most active uptake mechanisms include a digestion phase to break down the endocytosed material into usable components and destroy potentially pathogenic substances before the material is given access to the cell [114, 115]. The lysosome is an acidified organelle filled with proteases optimized to function near pH 4.5. Conditions within the lysosome may be harsh enough to degrade or deactivate many drugs, rendering them ineffective against the cancer cell [116].

Avoiding lysosomal degradation may be critical in delivering an effective drug dose to the tumor cell and may be achieved in a number a ways. One strategy is to avoid the lysosome by utilizing endocytotic pathways that do not undergo cellular digestion. Caveolae-mediated endocytosis appears to bypass the lysosomal phase and may be activated by the TAT peptide [117, 118]. Particles uptaken by different pathways may require a strategy to escape the vesicle during the endosomal phase before the lysosome can form. This can be done by releasing the drug from the nanoparticle during the endosomal phase, allowing the small molecule drug to diffuse out into the cytoplasm before the lysosome forms. Drug carriers may also be designed to rupture the endosome and release the contents. The proton sponge effect is a popular strategy to disrupt the endosome and avoid lysosomal degradation [119]. The proton sponge effect works by sequestering excess protons, usually by a polymer such as polyethylenimine (PEI) which contains unsaturated amino groups that can act as a buffer. This forces additional counter ions and water to be pumped into the endosome which may eventually cause it to swell and rupture, releasing the contents directly into the cytoplasm. The reality of the proton sponge effect is still somewhat controversial, but the improved transfection efficiency of PEI-based gene delivery systems provides some evidence of its utility [119].

Once it is in the cytoplasm, most drugs must proceed to a specific target within the cell. The location of the target depends on the drug type and mechanism of action. Most taxanes act on the microtubules that are ubiquitous in the cytoplasm. Cisplatin and related drugs indiscriminately alkylate proteins and nucleotides, but is most effective in the nucleus. Doxorubicin and its derivatives work by intercalating with DNA and must enter the nucleus to be effective.

Nuclear entry is one of the most formidable challenges to intracellular localization. Nuclear access is typically regulated at the nuclear pore complex (NPC) [120]. As with the cell membrane, the nuclear envelope is soluble to small hydrophobic compounds, but diffusion across the membrane can be limited by the presence of Pgp, giving the nuclear envelope high drug resistivity [121]. Nuclear access through the NPC can be aided by co-delivering the drug with compounds that dilate it from free flowing channels between the two compartments [122]. Mitosis also provides an opportunity for drugs to interact without the nuclear envelope present [123]. The nuclear envelope must disassemble during prophase to allow the chromatids to separate and is reassembled during telophase. Compounds that associate with the DNA during this window may be incorporated into the nucleus upon reassembly.

The individual cell has other mechanisms to protect itself and mitigate damage from cytotoxic compounds that must be considered when designing a drug delivery strategy. Sequestering the drug away from sensitive areas of the cell is one such mechanism. MDR cancer cells may overexpress acidic vesicles which can

concentrate and sequester a variety of slightly basic drugs until it can be metabolized or exocytosed [124]. Lung resistance related proteins (LRP) or vault proteins are another mechanism for sequestering and exocytosing drugs that are commonly seen in MDR lung cancers [125].

Cells may also modify certain chemical pathways to mitigate or compensate for damage done to the cell by a drug. Upregulating pathways that metabolize toxic substances can speed the breakdown of cytotoxic drugs and mitigate the damage done to the cell. Glutathione is a key protein in many cells' detoxification pathway and can be overexpressed in resistant cells [126, 127]. Further resistance can be conferred by modifying certain pathways to raise the threshold for apoptosis and cell death. Pro-apoptotic factors such as p53 are mutated or suppressed, while pro-survival factors such as Bcl-2 are inhibited [128, 129]. Repair mechanisms can be upregulated to repair damage done by DNA targeting drugs [130]. There are few specifically designed methods with which nanomedicine can combat these resistance mechanisms. Most often the best that can be done is to achieve sufficiently high intracellular drug concentrations to neutralize the cell in spite of its resistance.

Conclusion

Cancer is a formidable foe. It is born as a "distorted version of our own selves" having wriggled free of the remarkable cooperative system of the body to pursue its own objectives [131]. It takes advantage of the natural defenses by which the body protects itself against diverse pathogens and dangers. Nanomedicine is a remarkable tool to approach the difficult task of treating a so elusive disease. The nanoparticle's large size may confer it with inherent advantages, specifically the ability to target the tumor vasculature via EPR. Nanoparticles are also extremely diverse, encompassing many sizes, shapes, surfaces, and compositions. This versatility gives it the capability of stretching to accommodate the creativity of the researcher. Our ability to design and manufacture nanoparticles is continuing to grow and will provide even more capability in the future.

However, nanomedicine should not be looked on as a panacea or miracle cure, it carries inherent disadvantages to go along with its advantages. Distribution through the tumor is severely limited by the relatively large size of the nanoparticle which slows diffusion and can become trapped in the ECM. Nanoparticle entry into the cell is restricted to specific pathways, often relying on unreliable interactions between cell receptors and ligands and introducing the nanoparticle and drug to the lysosome digestion process. Drug carriers are also subject to MPS clearance and other mechanisms the body uses to clear nonself particles from blood and tissue.

The sheer quantity of barriers to effective drug delivery turns it into a game of attrition, in which progressively more particles are sheered away at each obstacle until little or none is left to treat the tumor. Failure at any point in the drug delivery pathway may irreparably harm the ability of the drug to sufficiently treat the tumor.

However, attempting to build specific mechanisms to bypass each of these barriers can quickly become cumbersome and overcomplicated. Imagine a bare nanoparticle to which PEG is added to prolong circulation and limit unwanted protein or ECM interactions, tumor penetrating peptides are included to promote extravasation by transcytosis, collagenases are inserted to degrade the collagen matrix, reducing tumor IFP and improving diffusion, targeting moieties are attached to promote cell uptake and improve cancer cell specificity, pH-sensitive polymer with buffering capacity is included to increase environmental sensitivity and aid endosomal escape, and numerous compounds are co-delivered with the drug to inhibit angiogenesis, aid nuclear entry, and limit cellular resistance. Designing a drug delivery system in such a way would quickly become onerous, possibly much too complicated to be effective and certainly expensive.

To reach the promise of nanomedicine, it is necessary to take a step back and look at the problems facing drug delivery as a whole rather than designing around only one or two obstacles. Incremental designs may not be sufficient to accomplish the task of treating cancer effectively. Instead, a revolution in concept is needed; one that incorporates a healthy respect for the complexity of both body and tumor and the ability of each to protect itself from harm.

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Part II
Targeting Issues

Chapter 3
Vascular Targeting Approaches to Treat Cancer

Joseph W. Wragg and Roy Bicknell

Abstract Anticancer therapeutics have historically been targeted against malignant cells directly. These approaches often have limited efficacy, particularly in advanced disease, due to poor drug infiltration into the tumour. In recent years increasing interest has been focused on the development of alternative targeted therapies, which inhibit tumour development by disrupting the stromal cells that support it. This chapter explores the development of tumour vascular targeting therapies, the successes and setbacks and the encouraging potential of this approach to potentiate the effect of other anti-tumour therapeutics.

Introduction

Specific targeting of therapeutics to the tumour has long been a quest in cancer research. Systematic cytotoxics do not target cancer cells or the pro-tumourigenic environment selectively and so lead to adverse side effects and provide limited anti-tumour effect, particularly in advanced cases. Most chemotherapeutics do not accumulate preferentially in the tumour site; indeed the drug dose is often 10–20 times higher in normal tissue than in the tumour [1, 2]. This poor drug infiltration is thought to be due to irregular tumour vasculature and high interstitial pressure [3, 4]. Therefore an improved approach to targeting the tumour is warranted.

The vasculature is thought to be an ideal candidate for targeted anticancer therapies.

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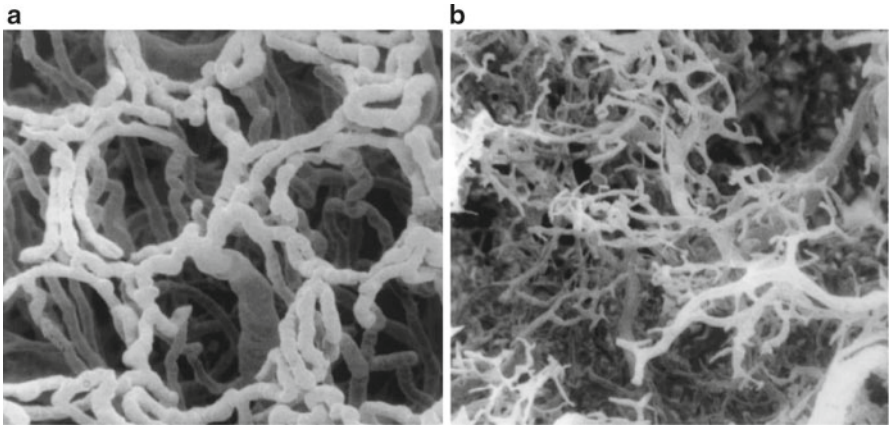

Fig. 3.1 Abnormal tumour vasculature from colorectal carcinoma. Microvascular corrosion casts of human (**a**) normal ascending colon and (**b**) colorectal carcinoma [18]

It is a key part of the tumour microenvironment required for the delivery of nutrients and removal of toxic waste products and so is essential for tumour growth and metastasis [5]. The tumour vasculature is directly accessible to drugs via the circulation and composed of endothelial cells thought to be genetically stable and less adaptive than tumour cells; therefore they are less likely to acquire drug resistance [6, 7]; however this idea has been challenged by Hida and Klagsburn [8]. They found that some endothelial cells in solid tumours are aneuploid and so inherently genetically unstable [8]. In addition it is estimated that up to 100 tumour cells are fed from a single endothelial cell [2, 9], and so a therapeutic targeting the vasculature could achieve a considerably more potent anticancer effect than targeting the tumour cells directly.

The tumour acquires a vascular network by the formation of neovessels (angiogenesis), the incorporation of existing blood vessels (vessel co-option), the splitting of existing vessels into daughter vessels (intussusception) and even by mimicking the vasculature by forming blood vessel like tubes lined with tumour cells (vascular mimicry) [10]. Tumour angiogenesis is essential for the survival and development of a tumour greater than 2 mm in size [5, 11]. However tumour vascularisation invariably lags behind the expanding tumour mass [12] resulting in highly abnormal tumour vasculature that morphologically and functionally differs from the vasculature of normal tissues [13–15]. It is highly chaotic in nature composed of tortuous, highly dilated and elongated vessels with blind ends, bulges, leaky sprouts and considerable variability in diameter [16] (Fig. 3.1). The tumour vascular endothelial cells themselves have been found to be highly abnormal expressing several cell surface markers barely detectable on normal or quiescent vascular endothelial cells [17]. The abnormality of the tumour vasculature is often associated with

treatment failure, due to poor drug infiltration [3, 4]; however it could offer an opportunity for targeted anticancer therapy if these differences can be used to specifically target therapies against the tumour vasculature.

To this end many anti-tumour-vasculature therapeutics have been developed over the last 20 years with a number reaching the clinic. There is a wide variety of different therapeutics, but they can be very broadly divided into vascular disrupting agents (VDAs), which target and occlude the existing tumour vasculature, and angiogenesis inhibitors (or antiangiogenics, AIs), which inhibit neovascularisation of the tumour. Collectively they aim to devascularise and starve the tumour, thus achieving tumour regression [5]. This chapter will explore the development of these therapies, the successes achieved and the hurdles still to be faced by this promising anti-tumour treatment direction.

Angiogenesis Inhibitors

Tumours generally arise from a single cell in which several genetic events have occurred, allowing the cell to escape the normal growth control mechanisms in operation in the tissue. Initially the cancer cell can proliferate and develop into a tumour while receiving sufficient oxygen and nutrients by diffusion from surrounding normal vessels. However as the mass increases, the tumour rapidly reaches a point whereby the cancer cells furthest from the nearest vessel do not receive sufficient oxygen and nutrients to survive. Further expansion of the tumour is restricted and the tumour remains localised and dormant [5].

To develop further and metastasise, the tumour must develop its own blood supply. The vascularisation of a tumour, known as angiogenesis, is a complex and multistep process, driven primarily by a combination of tumour-associated hypoxia and cellular transformation [19, 20]. Oncogene activation in tumour cells can result in the secretion of a number of pro-angiogenic growth factors including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [20], which recruit endothelial cells and promote their proliferation and migration to the source of the angiogenic signals, where they form into blood vessels [21]. However it is not only cancerous cells that promote angiogenesis in the tumour; when stimulated by PDGF from the tumour cells, stromal cells and pericytes are known to produce growth factors that support angiogenesis, such as angiopoietin-2 (Ang-2) [22]. In addition tumour-associated hypoxic conditions can lead to the activation of the transcription factor hypoxia-inducible factor-1a (HIF-1α) [23] in multiple cell types including tumour-associated macrophages, leading to the expression of multiple angiogenic factors such as VEGF and PDGF [24]. Besides this, in the tumour environment, both pro- and anti-angiogenic factors have been found to emanate from endothelial cells, blood and even the extracellular matrix [19].

Folkmann was the first to champion the idea of using therapeutics to inhibit tumour angiogenesis and thus maintain the tumour in a dormant, avascular state [5, 25].

Approaches targeting VEGF have shown the most promise. Indeed considerable success has been achieved by using therapeutics developed to block VEGF pro-angiogenic signalling in the tumour. These therapeutics are broadly split into three types: blocking antibodies (Table 3.1), soluble decoy receptors and small-molecule inhibitors (Table 3.2).

Blocking Antibodies

Anti-VEGF Antibodies: Bevacizumab

Bevacizumab (Genentech), marketed as Avastin, is a humanised monoclonal antibody that is designed to bind to VEGF, blocking its association with VEGF receptors on endothelial cells and thus block angiogenesis [26, 27].

In practice bevacizumab has been shown to have three potential mechanisms of action: firstly, an antiangiogenic mechanism, based on preclinical human tumour xenograft models in which bevacizumab showed effective tumour growth reduction as a monotherapy [28]; secondly, an anti-hematopoietic progenitor cell mechanism, by which it inhibits the colonisation of the tumour vasculature by circulating endothelial progenitor cells [29]; and thirdly, by a process of vascular normalisation [30, 31].

In the clinic, vascular normalisation appears to be the primary mechanism of action of bevacizumab. Normalisation is achieved by pruning and remodelling the tumour vasculature to more closely resemble normal vasculature [31]. Bevacizumab has shown most effectiveness when in combination with traditional chemo- or radiotherapy, as vascular normalisation enhances blood flow and oxygenation in the tumour, improving the delivery of chemotherapeutics to the heart of the tumour and rendering the tumour cells more chemo- and radiosensitive [32, 33].

In 2004 bevacizumab, in combination with irinotecan, fluorouracil and leucovorin, showed efficacy against metastatic colorectal carcinoma in a phase III trial [34]. Patients treated with bevacizumab benefited from a 4.7-month increase in overall survival and the result caused the US Food and Drug Administration (FDA) to approve the use of bevacizumab for the treatment of colorectal cancer. Similar results against other cancers meant that bevacizumab was approved for the treatment of non-small cell lung cancer in 2006, renal cancer in 2007, breast cancer in 2008 and glioblastoma multiforme in 2009. However, its indication in breast cancer was subsequently revoked by the FDA in 2011 because of clinical trial data showing that bevacizumab neither prolonged overall survival nor slowed disease progression sufficiently to outweigh its risk of side effects, which include hypertension, proteinuria, bleeding, thrombotic events and in very rare cases pulmonary embolisms [35].

Table 3.1 Antiangiogenic blocking antibodies in the clinic

Antibody	Company	Antibody type	Target	Indications/clinical trials
Bevacizumab (Avastin)	Genentech	Humanised IgG1	VEGF-A	First- and second-line metastatic colorectal carcinoma First-line advanced non-squamous non-small cell lung cancer Metastatic renal cell cancer Recurrent glioblastoma multiforme Phase III ovarian cancer FDA approval revoked for metastatic breast cancer
Ramucirumab (IMC-1121b)	ImClone Systems	Fully human IgG1	VEGFR-2	Phase III: breast cancer, metastatic gastric adenocarcinoma, non-small cell lung cancer, hepatocellular carcinoma, metastatic colorectal carcinoma Phase II: stomach, oesophageal, renal, bladder, prostate, ovarian cancers, melanoma and glioblastoma multiforme
CDP-791	Celltech	PEGylated humanised DiFab	VEGFR-2	Phase II: non-squamous non-small-cell lung cancer
IMC-18F1	ImClone Systems	Fully human IgG1	VEGFR-1	Phase II: breast, colorectal and renal cancers
2C5	ImClone Systems	Fully human IgG1	VEGFR-3	Phase III: pancreatic cancer Phase I: colorectal cancer

Information retrieved from: http://clinicaltrials.gov and http://www.cancer.gov (Accessed 2 Oct 2012)

Table 3.2 Antiangiogenic small-molecule inhibitors in the clinic

Inhibitor	Alternative names	Company	Activity	Indications/clinical trials
Sunitinib	Sutent, SU11248	Pfizer	VEGFR-2, PDGFRα and β, c-kit, Flt3, RET	First- and second-line treatment for metastatic renal cell carcinoma Gastrointestinal stromal tumours Progressive, unresectable, neuroendocrine pancreatic tumours Phase III: breast, colorectal and lung
Sorafenib	Nexavar, BAY 43-9006	Bayer HealthCare Pharmaceuticals and Onyx Pharmaceuticals	VEGFR-2 and 3, Raf, PDGFRβ, Flt3 and c-kit	Unresectable hepatocellular carcinoma Advanced renal cell carcinoma Phase III: non-small cell lung carcinoma and melanoma
Pazopanib	Armala, GW786034	GlaxoSmithKline plc	VEGFR-1–3, PDGFRα and β, c-kit	Renal cell carcinoma Soft tissue sarcoma Phase II: non-small cell lung carcinoma, ovarian cancer Phase I: colorectal cancer
Axitinib	AG-013736 Inlyta	Pfizer	VEGFR-1–3, PDGFRβ, c-kit	Second-line treatment for renal cell carcinoma Phase III: pancreatic cancer Phase II: lung, gastrointestinal, thyroid and breast cancer
Vandetanib	Zactima, ZD6474	AstraZeneca	VEGFR-1–3, PDGFRβ, EGFR, RET	Medullary thyroid cancer Phase III: non-small cell lung carcinoma
Cediranib	Recentin, AZD2171	AstraZeneca	VEGFR-1–3, PDGFRα and b, c-kit	Phase III: recurrent glioblastoma, colorectal cancer Phase II/III: non-small cell lung carcinoma

Vatalanib	PTK787, ZK222584 Caprelsa	Novartis	VEGFR-1–3, PDGFRα and b, c-kit	Phase III: colorectal carcinoma Phase II: metastatic neuroendocrine tumours, brain and central nervous system tumours, sarcoma
Brivanib alaninate	BMs-582664	Bristol-Myers Squibb	VEGFR-1–3, FGFR1–3	Phase III: colorectal cancer, hepatocellular carcinoma Phase II: renal cell carcinoma, multiple tumour types
BIBF 1120	Vargatef	Boehringer Ingelheim	VEGFR-1–3, PDGFRα and β, FGFR1–3, Flt3, Src, Fyn, Lck	Phase III: non-small cell lung cancer (NSCLC), ovarian cancer Phase II: breast cancer, prostate cancer, acute myeloid leukaemia, glioblastoma, hepatocellular carcinoma, colorectal cancer, high-grade glioma
Tivozanib	KRN951, AV-951	AVEO Pharmaceuticals, Inc.	VEGFR-2, PDGFR-β, c-Kit	Phase II: NSCLC, colorectal cancer, renal cell carcinoma Phase I: breast cancer, solid tumours

Information retrieved from: http://clinicaltrials.gov and http://www.cancer.gov (Accessed 2 Oct 2012)

Anti-VEGFR-2 Antibodies: DC101, Ramucirumab and CDP-791

VEGFR-2 is the major pro-angiogenic VEGF receptor [36]. A number of monoclonal antibodies raised against the extracellular domain of VEGFR-2 have been designed to block the binding of VEGF to its receptor and thus inhibit angiogenesis. DC101 was the first drug of this type to show antiangiogenic effectiveness [37]. The rat anti-mouse monoclonal antibody (ImClone) has been shown to be a potent antagonist to VEGF binding, VEGFR-2 signalling and endothelial cell proliferation in in vitro models [37]. In animal models DC101 has been shown to have potent antiangiogenic, anti-tumour and antimetastatic activity [37–39].

Studies using DC101 led to the development of Ramucirumab (IMC-1121b) (ImClone), a fully human anti-VEGFR-2 monoclonal antibody, and CDP-791 (Celltech, UCB), a PEGylated, humanised anti-VEGFR-2F(ab′)2 fragment. Ramucirumab has shown positive results in a number of phase II trials [40, 41] and is now under investigation in several phase III clinical trials for the treatment of breast cancer, metastatic gastric adenocarcinoma, hepatocellular carcinoma, metastatic colorectal carcinoma and non-small cell lung cancer.

CDP-791 has reached phase II clinical trials against non-small cell lung cancer in combination with carboplatin and paclitaxel; however its development is currently on hold, as progression-free survival was not improved by CDP-791 treatment in this study [42].

Soluble Decoy Receptors

Aflibercept

Aflibercept, also known as ziv-aflibercept or ZALTRAP (Sanofi-Aventis and Regeneron), is a fusion protein incorporating the second binding domain of the VEGFR-1 receptor and the third domain of the VEGFR-2 receptor [43]. It binds VEGF with high affinity ($K_d \sim 1$ pM) [44] and so acts as a potent competitive inhibitor of VEGFR binding. Aflibercept has been shown to be a potent angiogenesis inhibitor [44] and is highly effective against neovascularisation related to macular degeneration [45]. Aflibercept is the subject of clinical trials against a number of cancers. A phase III clinical trial involving aflibercept in combination with docetaxel and dexamethasone for the treatment of non-small cell lung cancer reported that aflibercept improved progression-free survival but not overall survival relative to placebo [46]. However a phase III trial involving aflibercept in combination with irinotecan, 5-fluorouracil and leucovorin for the treatment of metastatic colorectal cancer reported that aflibercept improved overall survival relative to placebo [47]. Because of this trial, in 2012 FDA approval was given for this drug to be used against metastatic colorectal cancer.

A subsequent phase III clinical trial involving aflibercept for the treatment of metastatic colorectal cancer also showed statistically significant improvements in

overall survival, progression-free survival and overall tumour response rate relative to placebo [48]. This trial provides further support for the use of aflibercept against metastatic colorectal cancer.

Small-Molecule Inhibitors

The small-molecule inhibitors are an alternative form of antiangiogenic therapy. Rather than blocking the binding of VEGF to its receptor, they block angiogenesis by inhibiting downstream signalling from the activated pro-angiogenic receptors in the endothelial cells. Drugs of this class are receptor tyrosine kinase inhibitors. They bind to and inhibit the kinase activity of certain receptor tyrosine kinases (RTKs) such as the VEGFRs. VEGFRs are not the only receptor tyrosine kinases involved in cancer (or normal cellular functions) however. Unlike targeted anti-VEGF signalling therapies discussed previously, this therapeutic class additionally blocks other pathways involved in angiogenesis and tumourigenesis and thus has anti-tumour, as well as antiangiogenic activity [49]. Additionally small-molecule inhibitors are orally available and so are cheaper to manufacture and administer to patients than many anti-VEGF signalling therapies.

Five antiangiogenic small-molecule inhibitors have received FDA approval for use against various malignancies. Sorafenib (Bayer and Onyx pharmaceuticals), sunitinib, axitinib (both Pfizer), pazopanib (GlaxoSmithKline) and vandetanib (AstraZeneca) are collectively approved for the treatment of renal cell carcinoma, gastrointestinal stromal tumours, pancreatic tumours, hepatocellular carcinoma, soft tissue sarcoma and medullary thyroid cancer. These drugs and those of their class are also undergoing many clinical trials in other tumour types.

The mechanism of action of small-molecule inhibitors is less well established than for bevacizumab. A number of drugs of this class have shown significant single-agent activity in the form of tumour growth inhibition and devascularisation in both clinical and preclinical settings. Indeed sorafenib, sunitinib, pazopanib and axitinib are licensed as monotherapies. This suggests that they have direct antiangiogenic effectiveness. However, like bevacizumab, certain small-molecule inhibitors (cediranib and tivozanib) have been shown to cause vascular normalisation [50, 51]. Because of this several clinical trials are under way to investigate the effectiveness of cediranib and tivozanib, among others, in combination with tumour cell-directed therapy (e.g. chemotherapy and radiotherapy).

Antiangiogenic Therapy Resistance

Antiangiogenic therapies have had great success in treating a number of solid tumours as demonstrated by FDA approval for their use against nine different malignancies. However they are primarily used in advanced settings and so rarely

provide long-term survival. There is little evidence that they provide any survival benefit in early-stage cancer where there is the potential for cure. Two large phase III clinical trials, where bevacizumab in combination with chemotherapy was used in patients with early-stage colorectal carcinoma, showed no benefit in progression-free survival (PFS) compared to chemotherapy alone [52, 53].

Long-term progression-free survival on antiangiogenic drugs is prevented by the almost inevitable acquisition of resistance to the treatment. Additionally tumours often return with a more aggressive, invasive and metastatic phenotype. Because of this the survival benefits provided by antiangiogenic treatments in the clinic are often measured in months [54–56].

There is a considerable call for research into why certain tumours are unresponsive to antiangiogenic treatments and why initially sensitive tumours frequently then progress after a short period of stasis or shrinkage [57–59]. The current hypothesis is that tumours adapt to the therapeutic blockade of angiogenesis by acquiring new mechanisms to functionally evade it [54, 60]. Current experimental evidence suggests five distinct adaptive mechanisms employed by the tumour and surrounding tissue to evade inhibition.

Evasive Resistance by Up-regulation of Alternative Pro-angiogenic Signalling Pathways Within the Tumour

An elegant example of adaptation to evade the actions of antiangiogenesis agents was observed in a preclinical trial of a monoclonal antibody (DC101) that specifically blocked VEGFR signalling in a mouse model of pancreatic neuroendocrine cancer (*Rip1-Tag2*) [61]. The antibody initially elicited a response in the form of tumour stasis and reduced vascularity. However this responsiveness was short-lived; after 10–14 days the tumour regrew and dense vasculature was restored. This response suggested a reinitiation of tumour angiogenesis bypassing the monoclonal VEGFR blockade. The investigators found that the relapsing tumours had heightened expression levels of a number of angiogenic factors, including several members of the fibroblast growth factor family, ephrin A1 and A2 as well as angiopoietin-1 [61]. To confirm that these expression changes were responsible for cancer relapse, tumours were first treated with the monoclonal antibody, but after the 10–14-day responsive phase had elapsed, a second-line treatment consisting of an FGFR-FC fusion protein, shown to suppress signalling through the FGF ligands, was used to treat the tumour. The investigators found that tumour growth was slowed and revascularisation attenuated relative to untreated controls. This result indicated that FGF signalling played a part in the tumour relapse [61] and that dual targeting of the VEGF/FGF pathways could slow the onset of resistance. Preclinical evaluation of brivanib alaninate, a dual inhibitor of VEGFR and FGFR, showed it to be effective both as a first-line therapy and as a second-line therapy against tumours that had relapsed on sorafenib or DC101 treatment [62]. Brivanib alaninate is currently undergoing phase III clinical trials for patients with hepatocellular carcinoma that has relapsed under sorafenib treatment.

Another study showed that the angiogenic capability of tumours deficient in a key inducer of VEGF expression, hypoxia-inducible factor 1a (HIF1α), could be saved by inducing the expression of the pro-angiogenic cytokine interleukin-8 (IL-8) [63] suggesting over-expression of IL8 could be one of the mechanisms used by tumours to evade the VEGF signalling blockade.

In agreement with this, a study showed that tumours in which growth was initially halted by ectopic expression of the angiogenesis inhibitors, thrombospondin, tumstatin and endostatin, quickly bypassed growth inhibition by up-regulating a number of pro-angiogenic factors such as PDGF, FGF and VEGF [64].

Recruitment of Pro-angiogenic Cells

Cells under hypoxic stress caused by antiangiogenic therapy-induced vessel regression can recruit various bone marrow-derived cells (BMDCs) that elicit a pro-angiogenic response, fuelling the tumour [65, 66]. These pro-angiogenic BMDCs consist of vascular progenitors and vascular modulatory cells, either differentiating into new endothelial cells and pericytes or expressing various cytokines and growth factors that promote vascular development [65, 66].

Rapid Vascular Remodelling by Recruitment of Pericytes

Several groups have observed that although there is a substantial reduction in vascularity in tumours treated with VEGF signalling inhibitors, a small population of functional vessels remain, which are morphologically distinctive from untreated tumour vasculature. These vessels are far thinner, less dilated and far more densely covered in pericytes [67, 68]. These observations suggest that endothelial cells recruit pericytes to protect against VEGF signalling blockade-induced death. In support of this suggestion, it has been observed that vessels with low pericyte coverage are more sensitive to VEGF inhibition [67, 69].

Increased Invasion and Metastasis Mitigating the Need for Neovascularisation

It has been widely observed that some angiogenically inhibited tumours become more invasive and metastatic than they were before treatment [55, 56]. This increased invasive phenotype was first observed in orthotopic mouse models of glioblastoma multiforme (GBM), in which angiogenesis was thwarted either by deletion of a number of angiogenic factors, VEGF, HIF1α and matrix metalloproteinase 9 (MMP9), or by treatment with the VEGF inhibitor semaxanib. It was observed in these models that the tumours adapt to the angiogenic inhibition by becoming more invasive, extensively infiltrating into the brain [70–72]. It was suggested that the glioblastoma cells manage to maintain vascular sufficiency, and so

continue to grow, by dispersing throughout the brain and forming many small colonies. This response was also observed in clinical trials of bevacizumab therapy in GBM [73, 74].

Selection of Resistant Endothelium

There is increasing interest in the role endothelial cells play in mediating antiangiogenic resistance. A recent study showed that endothelium extracted from hepatocellular carcinoma and cultured was behaviourally quite different from normal liver endothelium. They had an increased rate of migration and proliferation, were far less dependant on growth factors for their survival and were more resistant to antiangiogenic and other chemotherapeutic treatments [75].

Insensitivity to Antiangiogenic Therapy

A significant minority of patients enrolled in clinical trials, where the efficacy of antiangiogenic drugs are tested, are documented to fail to respond at all to treatment. There are no discernable beneficial effects from treatment, no tumour shrinkage or stasis and no improvement in quality of life or survival [59]. It is possible that the tumours of these patients adapted to antiangiogenic treatment before any benefit could be observed, but a more plausible explanation is that these tumours have intrinsic resistance to antiangiogenic therapy [54]. The mechanism of resistance could be quite similar to that of tumours that gain resistance; however the requisite changes have been driven by pressures from the intrinsic tumour microenvironment rather than by pressures from antiangiogenic treatment [54]. VEGF is not the only growth factor capable of mediating angiogenesis in the tumour. Some tumours may be intrinsically resistant to VEGF targeted antiangiogenic therapies because their vasculature has developed to be dependant on a different angiogenic growth factor, such as FGF or IL-8.

Given the considerable proportion of people who don't respond or respond very poorly to antiangiogenic therapies, there appears to be a need for predictive biomarkers of response, so that those for whom antiangiogenic therapy would be effective can be identified. A few potential biomarkers have been identified from clinical trials, such as VEGF polymorphisms or pharmacodynamic changes induced by antiangiogenic treatment [76], but further analysis is warranted.

Animal Models Versus Clinical Trials for the Study of Antiangiogenic Therapy Resistance

There are question marks over the reliability of animal models to predict antiangiogenic therapy-resistant mechanisms [77, 78]. There are inherent differences between the tumour development, including vacularisation, of spontaneous human tumours

and artificially induced or implanted tumours in animal models. A human tumour can take many years to develop gradually incorporating a blood supply over time, whereas an implanted murine tumour can grow, vascularise and metastasise over a period of a few weeks. The advent of spontaneous tumour modelling in mice, driven by specific genetic mutations, does promise improved accuracy and sophistication [78]. However to date few well-characterised tumour models of this type have been developed; they are expensive and inappropriate for the evaluation of humanised therapies, such as bevacizumab.

The preclinical-clinical development of bevacizumab epitomises this issue with mouse tumour modelling. In preclinical animal models, bevacizumab was shown to be a potent antiangiogenic agent, causing tumour devascularisation and regression as a monotherapy [28]. When bevacizumab progressed to clinical trials, however, it was found to have very little clinical activity in isolation and was only effective when combined with traditional anticancer therapies [32, 33]. This is because the primary mechanism of action of bevacizumab is very different in human tumours, where it normalises the vasculature by reducing VEGF activity down to normal tissue levels [10, 32, 33], and animal tumour models, where it deprives the tumour of VEGF pro-angiogenic activity and inhibits its vascularisation [28].

Many of the mechanisms of antiangiogenic resistance suggested to date have been identified with the aid of animal modelling. While these studies are useful for predicting the form that antiangiogenic therapy resistance may take, the mechanisms at work in human spontaneous tumours may differ. More clinical trial-centred studies into the development of resistance are therefore warranted.

Vascular Disrupting Agents

It has long been known that the disruption of the blood supply to a tissue causes rapid and extensive cell death by ischemia and hemorrhagic necrosis. This phenomenon was first described in 1852 in relation to testicular torsion [79]. The testicular torsion condition is caused when the spermatic cord, which carries blood to the testicles, becomes twisted, reducing blood flow and causing necrosis in the affected testicle. It was not until 1923 however that the potential of vascular disruption to starve a tumour was realised. In a seminal paper, William Woglam described how the disruption of the blood supply to a tumour could cause regression and suggested the potential for novel therapies to achieve this. He did however observe that the difficulty posed by this treatment would be effective targeting, so that the vessels of the tumour are disrupted but no others [80]. It took another 60 years for the idea to be seriously considered and investigated. Juliana Denekamp and her group demonstrated that the physical obstruction of the blood supply to transplanted tumours in mice, using D-shaped metal clamps, caused tumour cell death directly proportional to the length of the clamping [81]. Denekamp over the subsequent years championed the idea of tumour vascular disruption as an anticancer treatment.

As William Woglam explained many years ago, the key to the use of vascular targeting therapies is to achieve maximum tumour endothelium disruption while leaving normal endothelium unaffected. To this end therapeutics have been developed that take advantage of the many differences between normal and tumour endothelium. As discussed earlier, these approaches attempted to achieve specificity in tumour endothelium targeting and disrupt the tumour vasculature, while leaving normal vasculature unaffected. Such tumour-specific disrupting agents can be broadly divided into two types, small molecule and ligand based. The small-molecule class exploits physiological differences between tumour and normal vasculature to destroy the vessels. These include microtubule-destabilising agents and N-cadherin antagonists. Ligand-based VDAs use antibodies, peptides, or growth factors that specifically bind to the tumour vasculature and deliver agents that destroy the vessels.

Small-Molecule VDAs

Microtubule-Destabilising Agents

Tumour endothelium is immature in nature and highly proliferative and thus is dependant on a tubulin cytoskeleton to maintain cell shape [82–84]. Tubulin is also essential for cell motility, invasion, attachment and proliferation [82]. Mature quiescent vasculature, which supplies most normal tissues, has a far more established actin cytoskeleton and so is far less dependant on the tubulin cytoskeleton for cellular functions. Microtubule-destabilising agents act by disrupting the tubulin cytoskeleton. This has the dual effect of inhibiting spindle formation, leading to mitotic arrest in tumour cells and causing tumour vascular collapse, reducing blood flow. Therefore, drugs that block tubulin function can have both antimitotic and anti-vascular effects [85]. In practice the dominant mechanism of action for these drugs is to cause mitotic arrest with anti-vascular activity only seen at close to the maximum tolerated dose (MTD) [86].

Combretastatin A-4 Disodium Phosphate

Combretastatin A-4 disodium phosphate (also known as CA4P, fosbretabulin, Zybrestat; Oxigene) was the first microtubule-destabilising agent observed to have anti-vascular effects below the MTD [87]. It is delivered as a prodrug, which is cleaved to its natural form by endogenous phosphatases. CA4P binds to tubulin inhibiting its polymerisation.

In experimental tumour models, CA4P causes extensive vascular damage with haemorrhagic necrosis within 1 h of treatment coupled with subsequent tumour growth delay [87–90]. The effect of CA4P on the tumour is considerably greater than its effect on the normal tissue [91]. CA4P is the subject of a number of clinical trials for advanced anaplastic thyroid cancer, non-small cell lung cancer, gynaecological

cancers and high-grade glioma (Table 3.3). A recent phase II/III clinical trial for advanced anaplastic thyroid cancer showed considerable survival benefit in patients treated with CA4P in combination with chemotherapeutics; 26 % of patients treated with CA4P survived 1 year compared to 9 % treated with chemotherapy alone. The clinical trial was however halted early due to lack of funding [92].

Combretastatin Derivatives (Oxi 4503, Ombrabulin)

The success of CA4P has led to the development of a number of derivative drugs. Oxi 4503 (Oxigene), the prodrug form of combretastatin A-1, has been reported to have more potent anti-vascular and anti-tumour effects than CA4P [93]. Oxi 4503 has been shown to be effective in solid tumours such as metastatic colorectal carcinomas [94] and in blood cancers, including acute myelogenous leukaemia (AML) when given in combination with the antiangiogenic drug bevacizumab [95]. Oxi 4503 is the subject of a number of phase I clinical trials in both solid tumours and blood cancers (Table 3.3).

Another combretastatin derivative, ombrabulin (AVE8062) (Aventis Pharma), has been shown to rapidly shut down tumour blood flow and cause extensive tumour core necrosis in experimental models [85, 96–100]. A recent mouse model of head and neck squamous cell carcinoma demonstrated that ombrabulin treatment augmented the anti-tumour effectiveness of the standard treatment regimen of radiation and cisplatin or cetuximab [101]. Ombrabulin is in phase III trials for the treatment of soft tissue sarcoma, in phase II trials in combination with taxane and platinum drugs (docetaxel and cisplatin or paclitaxel and carboplatin) for the treatment of patients with metastatic non-small cell lung cancer (NSCLC) and in a number of phase I trials in combination with other drugs in solid tumours.

N-Cadherin Antagonist

The adhesive interactions between endothelial cells are essential for the maintenance of functional integrity of the vasculature [102, 103]. N-Cadherin is a cell surface protein involved in mediating these interactions. A cyclic peptide named ADH-1 competitively inhibits N-cadherin homotypic binding and has been shown to reduce blood flow and cause haemorrhage necrosis in animal tumour models [104–106]. ADH-1 has been investigated in phase I/II trials as a monotherapy and phase I in combination with chemotherapeutics in a range of tumours (Table 3.3). It has been well tolerated and shows modest anti-tumour effect [107, 108].

Toxicity

Toxicity is an important issue limiting the clinical development of small-molecule VDAs. ZD6126 is a phosphate prodrug of the tubulin-binding agent *N*-acetylcolchinol. The drug disrupts the tubulin cytoskeleton of endothelial cells causing endothelial

Table 3.3 Small-molecule VDAs in the clinic

Agent	Company	Type	Clinical trials
CA4P (fosbretabulin, Zybrestat)	Oxigene	Microtubule-destabilising agent	Phase II/III: advanced anaplastic thyroid cancer Phase II: non-small cell lung cancer, fallopian tube cancer, ovarian cancer and peritoneal cavity cancer Phase I: glioma
Oxi4503	Oxigene	Microtubule-destabilising agent	Phase I: acute myeloid leukaemia, liver and other solid tumours
AVE8062 (Ombrabulin)	Sanofi-Aventis	Microtubule-destabilising agent	Phase III: soft tissue sarcoma Phase II: metastatic non-small cell lung cancer, recurrent ovarian cancer
Dolastatin-10	National Cancer Institute	Microtubule-destabilising agent	Phase II: lymphoma, macroglobulinemia, lymphocytic leukaemia, colorectal adenocarcinoma, melanoma, soft tissue sarcoma, breast, liver, prostate, pancreatic, ovarian, renal and non-small cell lung cancer (*not presently in clinical trials*)
ZD6126	AstraZeneca/Angiogene pharmaceuticals	Microtubule-destabilising agent	Phase II: metastatic renal cell carcinoma and colorectal carcinoma (*not presently in clinical trials*)
CYT997	Cytopia	Microtubule-destabilising agent	Phase II: glioblastoma (terminated) and myeloma (terminated) (*not presently in clinical trials*)
NPI 2358	Nereus	Microtubule-destabilising agent	Phase II: non-small cell lung cancer (*not presently in clinical trials*)
MPC-6827 (Azixa)	Myriad	Microtubule-destabilising agent	Phase II: metastatic melanoma and glioblastoma multiforme Phase I: brain metastases
MN 029 (Denibulin)	Medicinova	Microtubule-destabilising agent	Phase I: solid tumours

BNC-105	Bionomics	Microtubule-destabilising agent	Phase II: renal cell carcinoma and ovarian cancer
EPC-2407	Epicept	Microtubule-destabilising agent	Phase II: anaplastic thyroid cancer
DMXAA (Vadimezan, ASA404)	Novartis	Cytokine-inducing agent	Phase II: metastatic prostate cancer, non-small cell lung cancer and urothelial carcinoma (*not presently in clinical trials*)
ADH-1 (Exherin)	Adherex Technologies	N-cadherin antagonist	Phase I/II incurable solid tumours (*not presently in clinical trials*)

Information retrieved from: http://clinicaltrials.gov and http://www.cancer.gov (Accessed 2 Oct 2012)

cell detachment. In vivo, ZD6126 was shown to cause endothelial cell retraction, extensive endothelial cell loss [109], a reduction on tumour blood flow [110], and reduced vascularisation [111]. ZD6126 was also shown to cause extensive tumour necrosis in a range of animal xenograft models [109, 112] and inhibit metastasis from lung adenocarcinomas [113]. When ZD6126 progressed to clinical trials, however, it was observed to have severe side effects at the clinically required dose. Phase II clinical trials involving ZD6126 for the treatment of metastatic renal cell carcinoma and metastatic colorectal cancer had to be halted in 2006 due to excessive cardiotoxicity [114] and no subsequent clinical trials have been arranged (Table 3.3).

Likewise, almost half of the small-molecule VDAs that have reached clinical trials have subsequently had their development halted, often due to insufficient efficacy at dosages with an acceptable level of side effects [114]. Clinical trials involving these drugs show side effects that are consistent with anti-vascular activity and include transient hypertension, myocardial infarction, and cardiac ischemia [115]. This type of toxicity suggests that small-molecule VDAs are having an effect on normal vasculature as well as other normal cell types, and therefore the drugs are insufficiently selective for the tumour endothelium. Experimental models have shown that antihypertensive therapy can prevent some of the side effects of tubulin-targeting drugs in particular, without reducing the clinical efficacy of the therapy [115]. Another idea that has been suggested to improve the selectivity of this therapy involves incorporating ligands specific for tumour endothelium in order to better target these small-molecule vascular disrupting therapies [116].

Ligand-Directed VDAs

Ligand-directed VDAs act directly on the vasculature. Therapeutics of this class are made up of two components joined by chemical cross-linkers or peptide bonds; a ligand, such as an antibody, peptide or growth factor, which binds specifically to the tumour vasculature and an effector, which once delivered to the tumour vasculature, destroys it. These effectors are bioactive molecules, which include coagulation-inducing proteins, toxins, cytokines, apoptosis-inducing agents, cytotoxic agents and radioisotopes (Table 3.5).

Burrows and Thorpe [117] were the first to demonstrate that ligand-directed approaches for disrupting the vasculature of tumours could be effective. They set up subcutaneous neuroblastoma tumours in nude mice. The tumours were engineered to express interferon gamma, which induced the vasculature of the tumour to express MHC class II. They targeted the tumour vasculature with a high-affinity antibody to mouse MHC class II, coupled with the toxin, ricin. This approach destroyed the tumour vasculature, causing rapid tumour shrinkage, while leaving the vasculature of the normal tissue unaffected [117] (Fig. 3.2).

A key requirement for success using this treatment strategy is the discovery of appropriate targets on the tumour vasculature for ligands to bind to and deliver their effector component. A number of cell surface molecules have been found to be

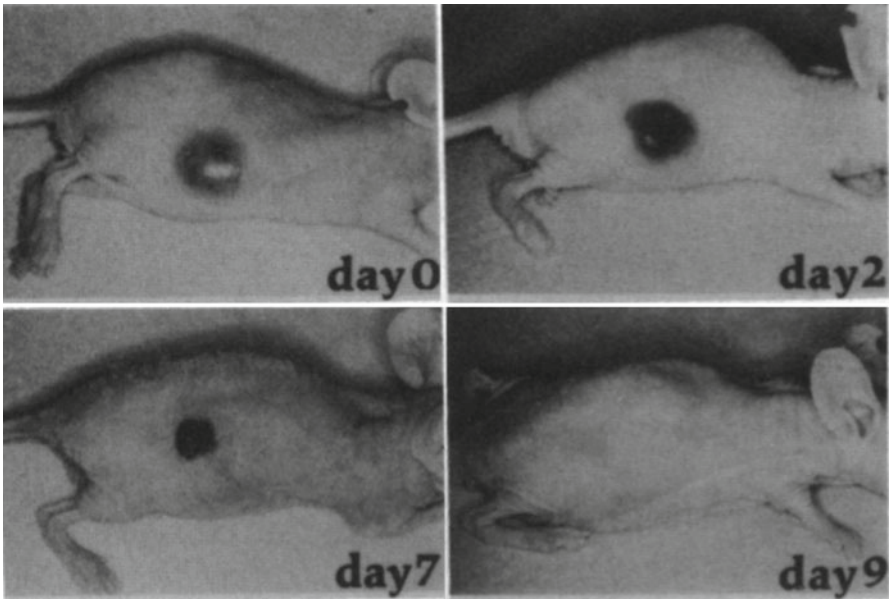

Fig. 3.2 Ligand-directed VDA-induced tumour necrosis. Gross appearance of subcutaneous neuroblastoma tumours treated with an anti-MHC class II immunotoxin. At day 0 the tumours are pink/purple (highly vascular). Two days after treatment, the tumour is blackened (indicating massive intratumoural haemorrhage). At day 7 the tumour has collapsed into a scab-like plug and by day 10 there is no visible living tumour [117]

up-regulated on the tumour vasculature when compared to normal vasculature and the discovery of additional cell surface targets is a continuing quest in this field.

The Search for New Tumour Endothelial Markers

The search for new, more specific or more selectively expressed tumour endothelial markers (TEMs) is ongoing. The aim is to find markers that allow drugs to be efficiently targeted to the tumour endothelium with appropriate specificity so as the effector dosage at the tumour is sufficient to cause vascular destruction while ensuring the effector dosage in the normal vascular bed is below threshold levels for a destructive response. To this end several techniques have and are being used to identify differentially expressed genes on tumour endothelium.

Historically, the first markers of tumour endothelium were discovered through extensive immunohistochemical profiling with monoclonal antibodies. For example, the discovery that tumour fibronectin contains an extra-domain B (ED-B) domain not found in most normal tissues occurred due to the observation that antibodies specific to ED-B-containing fibronectin stain blood vessels in many cancer types but do not stain normal tissue, with the exception of certain vessels in the endometrium, ovaries and placenta [118–124].

In addition, in vivo phage display analysis has been used to identify endothelial targets. Vast numbers of phage, each expressing a different protein, are injected into animals [125] or terminally ill patients [126]. After a period of time, tumour and normal tissue are removed, the endothelium is recovered and phage specifically localised to the tumour endothelium are isolated and analysed. This method used on breast tumours was used to identify an aminopeptidase as a target on breast tumour vasculature [127]. Similarly, phage display analysis has been used on laser microdissected tissue sections [128] and on tumour-associated endothelial cells in culture [129].

A number of groups have extracted endothelial RNA from clinical samples of various tumours together with paired normal host tissue and conducted serial analysis of gene expression (SAGE) [6, 130] and microarray analysis [131–133] to identify differentially expressed genes between the endothelium derived from the host normal tissue and the tumour. These approaches have led to the discovery of several tumour endothelial markers (TEMs)

In silico techniques have also been used to predict tumour endothelial markers, which can then be further validated. One group has developed a subtractive algorithm to screen publically available sequence tag expression data as a method to identify novel endothelial-specific genes [134]. The expression of these genes was then screened by in situ hybridisation, which identified ROBO4 and EndoPDI as markers of active angiogenesis and tumour endothelium [135, 136]. Another group analysed a vast number of cDNA libraries of various cell lines to identify endothelial-specific genes. This gene list was then used to identify what endothelial-specific genes are up-regulated in cDNA libraries derived from bulk tumour of various types versus normal tissue. This approach identified 27 genes as being tumour endothelial markers (TEMs) in multiple malignancies [137]; these included known TEMs such as Robo4 and novel ones such as ECSCR.

A number of groups have used direct vascular labelling techniques to identify differentially expressed cell surface TEMs. One group perfused tumour-bearing rodents with silica beads, which stripped the membrane proteins from the surface of the tumour endothelium. From subsequent proteomic analysis of beads perfusing to tumour and normal cells, a number of cell surface proteins were found to be enriched on tumour endothelium [138]. Other groups have chemically labelled vascular proteins with biotin [139]. Biotinylated cell surface proteins of tumour vasculature were purified on a streptavidin column and subjected to proteomic analysis to quantitate expression differences between hundreds of cell surface proteins that were identified. A similar approach has also been used ex vivo to analyse surgically resected cancerous human kidney and colon [140, 141].

Ligand-Directed Drugs Under Investigation

A number of the targets identified by these analyses have subsequently been validated for vascular targeting (Table 3.4) and when coupled to bioactive effector molecules (Table 3.5) have shown promise in various endothelial and tumour models.

Table 3.4 Tumour vascular specific targets

Class	Examples	References
Angiogenesis/vascular remodelling	Fibronectin ED-A and ED-B domains	[145, 150–152]
	Endoglin	[153]
	Extra domains of Tenascin-C	[154, 155]
	Prostate-specific membrane antigen	[146]
	Robo 4	[135]
	TEM7	[6]
	CD44-related antigen (TES-23)	[156]
	MMP2	[157]
	MMP9	[157]
Cell adhesion	Integrins ($\alpha_v\beta_3$)	[158]
	VCAM-1	[144]
	E-selectin	[159]
	CLEC14A	[160]
Thrombosis	Phosphatidylserine phospholipids	[161]
	Tissue factor	[162]
Inflammatory modulation	Annexin A1	[138]

Table 3.5 Effector molecules used in vascular targeting

Class	Examples	References
Coagulation-inducing proteins	Tissue factor	[143–146]
Toxins	Gelonin	[149]
	Ricin	[117]
	Diphtheria toxin	[147, 148]
Cytotoxic agents	Doxorubicin	[163]
	Paclitaxel	[164]
Cytokines	Interleukin-2	[150]
	Interleukin-12	[151]
	Tumour necrosis factor-α	[165]
Apoptosis-induction	*RAF-1* gene	[166]
	Mitochondrial-membrane disrupting peptide	[167]
Radioisotopes	Iodine-131	[168]
	Actinium-225	[169]
	Bismuth-213	[170]

The potential of targeting agents to disrupt vascular development was demonstrated using an immunotoxin known as TEC-dgA, which was composed of the deglycosylated ricin A chain coupled to the endothelial proliferation marker endoglin. TEC-dgA showed considerable effectiveness in binding to proliferating HUVEC selectively and inhibiting HUVEC proliferation [142].

Several groups have investigated the potential of targeting the extracellular domain of tissue factor, a coagulation-inducing protein that can bind to tumour vasculature. This approach has been shown to induce rapid tumour-specific vessel

Table 3.6 Ligand-directed VDAs in the clinic

Agent	Company	Target	Description	Clinical trials
EndoTAG-1	Medigene	Negatively charged EC membrane	Cationic lipid-directed cytotoxic	Phase II: advanced pancreatic cancer, triple negative breast cancer, liver tumours
L19-Il2	Philogen	ED-B of fibronectin	Antibody fragment directed Il-2	Phase II: metastatic melanoma, advanced pancreatic cancer Phase I/II: advanced renal cancer
131I-L19	Philogen	ED-B of fibronectin	Antibody fragment directed radioisotope	Phase II: brain tumours Phase I: non-small cell lung cancer
111In-J591	Weill Medical College of Cornell University	Prostate-specific membrane antigen	Antibody-directed radioisotope	Phase II: prostate cancer
NGR-hTNF	MolMed	CD13, integrin	Peptide-directed TNF	Phase III: pleural mesothelioma Phase II: soft tissue sarcoma, non-small cell lung cancer, ovarian cancer, colorectal cancer, hepatocellular carcinoma

Information retrieved from: http://clinicaltrials.gov and http://www.cancer.gov (Accessed 2 Oct 2012)

thrombosis, which was capable of destroying much of the tumour core over a period of 72 h. Specific targeting was achieved by using antibodies and peptides specific for a variety of tumour-specific markers, including MHC class II [143], VCAM-1 [144], fibronectin [145] and prostate-specific membrane antigen [146].

Other successful studies have used VEGF-A, rather than a specific antibody, to target toxins such as diphtheria toxin [147, 148] or gelonin [149] to the tumour vasculature, resulting in tumour regression in mice.

Ligand-Directed Therapies in the Clinic

A few ligand-directed vascular targeting therapies have progressed to clinical trials (Table 3.6). EndoTAG-1 (Medigene) is composed of the cytostatic drug paclitaxel combined with cationic lipids. The positively charged lipids allow EndoTAG-1 to bind to newly developed, negatively charged endothelial cells that make up the

tumour vasculature and selectively deliver paclitaxel to this site. EndoTAG-1 has been successful in two proof-of-concept clinical trials. In a phase II clinical trial for pancreatic cancer, EndoTAG-1 in combination with gemcitabine significantly increased survival rates compared to gemcitabine therapy alone [171]. A phase II clinical trial involving EndoTAG-1 for the treatment of triple negative breast cancer also showed promising results; a phase III clinical trial for this indication is planned [172].

Another ligand-directed vascular targeting therapy to progress to clinical trials is L19-IL-2. This molecule is an immunoconjugate consisting of a human single chain Fv antibody fragment directed against fibronectin ED-B (known to be up-regulated in proliferating tumour vasculature) and a recombinant form of the cytokine IL-2 (known to locally induce a T cell cytotoxic immune response). L19-IL-2 has shown promising results in mouse models of cancer [150, 173] and has progressed to human trials, showing clinical activity against advanced solid tumours, including renal cell carcinoma and metastatic melanoma [174–176]. Phase II clinical trials for the treatment of advanced pancreatic cancer and metastatic melanoma are ongoing.

Ligand-Directed Gene Therapy

An intriguing approach to specifically destroy tumour endothelium involves the engineering of retroviruses, where they are coated with antibodies specific for a tumour endothelial marker. These targeted viruses can specifically deliver toxic or antiangiogenic genes to tumour endothelium [177]. One group has generated an adenovirus conjugated to polyethylene glycol and CGKRK tumour vascular homing peptide. This adenovirus was tumour vascular specific and showed promising anti-tumour effects in mouse models [178].

An intriguing related strategy is to use tumour cell-specific cytotoxic T lymphocytes to deliver a retroviral vector, containing a gene construct encoding a VEGF-toxin fusion protein, to tumour cells. The fusion protein should then be synthesised and secreted by the tumour cells, interact with adjacent tumour endothelium and destroy it, thus devascularising the tumour [179].

Anti-vascular DNA Vaccines

Another approach to target cancers is to vaccinate patients against factors specifically expressed on tumour endothelium. One group has generated a DNA vaccine against aquaporin-1 (AQP-1), a water channel protein highly expressed in tumour and proliferating endothelium. This DNA vaccine profoundly inhibited the growth of B16F10 melanoma, CT26 colon and MBT/2 bladder tumours in mouse models. Microvessel density decreased, and the ratio of blood vessel to tumour area was reduced in immunised mice, challenged by a tumour, versus controls [180].

Future Directions for Ligand-Based Vascular Targeting

A major obstacle to the success of ligand-based vascular targeting approaches is the potential for nonresponse or acquired resistance to therapy due to marker heterogeneity. It has been observed that tumours can modulate marker expression on adjacent endothelia, leading to heterogeneity in the expression of tumour vessel markers [60]. A potential method to circumvent this issue would be to use combinations of VDA or bispecific VDAs with ligand that recognise differently regulated tumour-specific genes. This approach could not only reduce the effect of marker heterogeneity but also reduce normal tissue pathology as normal endothelial cell might express one marker but not the other [116].

Vascular Targeting Agents in Combination with Conventional Cancer Therapy

A number of vascular disrupting agents have now progressed into clinical trials but most are accompanied with conventional anticancer therapies. It is widely believed that these VDAs will find optimal utility when administered in combination with other treatment modalities. By their very nature, VDAs are indirect in their anticancer effect. They can be a highly effective method of damaging tumour blood vessels but are incapable of eradicating small pockets of tumour cells on the extremities of the tumour (the viable rim), which derive their nutrition from surrounding normal blood vessels [181]. Traditional cytotoxic drugs on the other hand are generally most effective at killing the well-oxygenated and vascularised cells on the extremities of the tumour. Therefore, to achieve complete tumour kill and prevent relapse, an additional direct anti-tumour approach must be used [116, 182].

Antiangiogenic therapies have also found increased efficacy when used in combination with other treatment modalities. The greatest benefit of antiangiogenic therapies is their ability to control tumour growth, but as has been discussed previously, this is only achieved for a comparatively short period before tumours become refractory. The primary effect of some antiangiogenic drugs such as bevacizumab is to normalise the blood flow within the tumour and so improve the infiltration of other blood-bourne drugs [30].

The complexity and variety of pathways available for neovascularisation can limit the efficacy of antiangiogenic drugs that target a single pathway. Such approaches are unlikely to adequately disrupt tumour angiogenesis. It is therefore likely that combinations of differently targeted antiangiogenic drugs will provide improved clinical outcomes. In support of this suggestion, angiogenesis co-inhibition via treatment with VEGFR-2 and bFGF inhibitors significantly slowed islet cell carcinoma growth in an animal model [183]. In addition, co-treatment with the VEGF inhibitor bevacizumab and the HIF-α inhibitor topotecan resulted in a

considerable synergistic anti-tumour outcome in a U251 glioma model; tumours showed considerable devascularisation and shrinkage [184].

Combinations of antiangiogenic therapies with vascular disrupting therapies may provide the best outcomes. Both the integrity of the existing vascular network and the creation of new vessels are critical for the survival of tumours. As discussed previously, vascular disrupting agents have been shown to be highly effective at destroying the highly abnormal vasculature in the core of tumours [87–90] but far less effective at destroying the comparatively normal vessels at the extremities. Antiangiogenic therapies on the other hand are only effective at disrupting the production of neovessels at the extremities of the tumour where angiogenesis is most active. Additionally, VEGF is up-regulated in hypoxia and inhibited by many antiangiogenics, suggesting it to be a key regulator of tumour revascularisation after VDA-induced vascular shutdown [185]. Concordant therapies involving both VDAs and VEGF-targeting antiangiogenics are therefore a promising treatment direction, have been shown to be effective in preclinical studies [186–188] and are currently in clinical testing. Recently completed phase I and II clinical trials involving CA4P (VDA) in combination with the bevacizumab (antiangiogenic) and traditional cytotoxics for the treatment of various malignancies suggest this therapeutic combination is well-tolerated with additive clinical activity, but larger trials are needed to verify these findings [189, 190].

The rational for concordant treatment schedules involving cytotoxics, antiangiogenics and VDAs is clear. The treatments are complementary, targeting different regions or pathways within tumours, with the strengths of each treatment overlapping the weaknesses of the others. Additionally these classes of drugs are synergistic in their activity against tumours. There are a multitude of factors that lead to the clinical failure of cytotoxic therapies, such as poor drug infiltration or metastatic spread, which are caused by the microenvironment of tumours. VDAs and antiangiogenics induce changes in tumour microenvironment (such as vascular normalisation), which may improve the effectiveness of cytotoxic therapies. Additionally, the effectiveness of certain cytotoxic drugs, such as melphalan, is potentiated by the hypoxia and low pH caused by a number of VDAs and antiangiogenics [191].

Concluding Remarks

In conclusion, a variety of therapeutics have been developed to target and modulate the tumour vasculature with quite disparate mechanisms and effects on the tumour, from normalising the vasculature, to inhibiting the production of neovessels, to destroying existing vessels. A large number of these therapies have been brought to clinical trials and some to the clinic indicated against nine different tumour types. Effectiveness has been shown in both monotherapy and co-therapy settings, but it is likely that in combination with other vascular targeting or tumour targeting drugs, optimal utility will be achieved.

Lessons to Be Learnt and Future Directions

Antiangiogenic Therapies

As previously discussed, the issues of acquired and innate resistance have curtailed the impact of antiangiogenic therapies as a treatment direction for cancer. This phenomenon is unsurprising if we consider the multitude of factors that contribute to the development of a human tumour. Each malignancy is as unique as the human in which it arises. Each angiogenic tumour will develop its blood supply in a distinct way, modulated by and dependant upon a different combination of angiogenic factors.

In this context it is naive to think that one therapeutic or even a family of closely related therapeutics will be effective against all solid malignancies or even a single form of malignancy, based on current classifications (e.g. renal cell carcinomas). Therefore the future of antiangiogenic therapy lies in the identification of subpopulations sensitive to specific drugs. In-depth analysis of clinical trial data, to identify predictive biomarkers of robust response, is therefore warranted.

Vascular Disrupting Therapies

The vascular disrupting agents that have progressed to clinical trials have almost uniformly had issues with toxicity. The success of drugs of this type has often had as much to do with the acceptability of their side effects as with their anti-tumour effectiveness (which is generally quite dramatic at least in the short term).

The issue therefore curtailing the success of this treatment direction is the selectivity of its therapeutic effect. Small-molecule VDAs are quite non-specific in their effects, targeting cellular components, such as tubulin, not only vital for tumour endothelial development but also important for the integrity of many normal tissue components.

Ligand-directed therapies offer hope for more selective tumour vascular disruption, with fewer side effects. This therapeutic class is in its infancy however, and its development is complicated by the need to find valid tumour endothelial-specific markers against which the therapies can be targeted.

As has been discussed previously, if ligand-directed cancer therapies can be made to work, the tumour vasculature would be an ideal target. The issue is getting it to work. There is considerable heterogeneity even in tumour vasculature. Therefore either a target must be found that is constitutively and selectively expressed on tumour endothelium or some form of individual tumour analysis or identification of biomarkers must be conducted to allow the use of specific targets for specific patients or patient groups.

The viability of vascular disrupting therapies rests heavily on whether good targets can be found.

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Chapter 4
Tumor Immunotherapy by Utilizing a Double-Edged Sword, Chemokines

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Abstract Both innate and adaptive immune responses have an essential role in protection against tumor cells. Various types of immune cells such as dendritic cells and lymphocytes contribute to the establishment of immune responses to tumor cells. Chemokines, a family consisting of more than 40 related chemoattractant proteins, have a crucial role in the control of the recruitment of immune cells needed for the induction and activation of tumor immunity. Based on these properties, several chemokines have been utilized in preclinical models to augment tumor immunity by enhancing the migration and activation of immune cells. Paradoxically, tumor tissues use chemokines to evade immunosurveillance by attracting immune suppressive cells. Moreover, chemokines can mediate survival and migration of tumor cells and promote new blood vessel formation, thereby leading to tumor progression and metastasis. Thus, a number of therapeutic strategies have been proposed to target chemokines, in order to reduce tumor progression and metastasis, although these strategies have not yet been translated to clinical situations. Here, we will briefly summarize the preclinical results obtained by using and/or targeting chemokines to combat tumors and discuss the potential efficacy of these methods.

Introduction

Chemokines are heparin-binding proteins characterized by the presence of four cysteine residues in the conserved positions [1]. Two intermolecular disulfide bonds are formed between the first and third cysteines and between the second and fourth

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cysteines, and these bonds result in the formation of triple-stranded β-sheet structures, while the carboxyl-terminal region forms an α-helix form [2]. Thus, although overall sequence similarities are not high among chemokines, they exhibit a similar three-dimensional structure. Chemokines exert their biological activities by binding their cognate receptors, which belong to G-protein-coupled receptor (GPCR) with 7-span transmembrane portions [1]. Thus, the target cell specificity of each chemokine is determined by the expression pattern of its corresponding receptor. At high concentrations, chemokines tend to dimerize by forming hydrogen bonds between their β-sheet structures [3]. The current consensus is that monomeric forms of chemokines are sufficient for receptor binding to induce cell migration. It still remains elusive on the functions of dimerized chemokines, although the dimer is assumed to be associated with other complex functional roles [3]. Moreover, through the carboxyl-terminal region with the capacity to bind heparin, chemokines can bind to proteoglycans and glycosaminoglycans with a high avidity. Consequently, most chemokines are produced as secretory proteins, but upon their secretion, they can be immobilized on endothelium cells and in extracellular matrix by interacting with proteoglycans and glycosaminoglycans [2]. The immobilization facilitates the generation of a concentration gradient, which is crucial for inducing the target cells to migrate in a directed way.

Based on their structure, chemokines are classified into four subgroups, namely, CXC, CC, CX_3C, and C [1] (Table 4.1). The first two cysteines are separated by one and three amino acids in CXC and CX_3C chemokines, respectively, while the first two cysteines are adjacent in CC chemokine. The C chemokine lacks the second and the fourth cysteines. Systematic chemokine nomenclature is based on their cysteine subclass roots, followed by "L" for "ligand" [4]. The numbers correspond generally to the same number used in the corresponding gene nomenclature. Because most chemokine receptors can bind to a single chemokine subclass, the nomenclature system of chemokine receptors is rooted by the chemokine subclass specificity, followed by "R" for "receptor" and the number [4] (Table 4.1). The CXC chemokines are further grouped based on the presence or the absence of a 3-amino acid sequence, glutamic acid–leucine–arginine (the "ELR" motif), immediately preceding the CXC sequence [5]. In general, CXC chemokines with the ELR motif can bind CXCR1 and/or CXCR2 and exhibit an angiogenic and a neutrophil chemotactic activity [5].

Chemokines can be classified as inflammatory, homeostatic, or both, based on their expression pattern [6]. Various types of inflammatory stimuli induce the expression of inflammatory chemokines, which have a crucial role in the infiltration of inflammatory cells including granulocytes and monocytes/macrophages. Representative inflammatory chemokines are CXC chemokines with ELR motif and CCL2. On the contrary, homeostatic chemokines are expressed constitutively in specific tissues or cells. They are involved in organogenesis of various organs including lymph nodes, as they have key roles in stem cell migration. Moreover, most homeostatic chemokines can regulate the trafficking of immune cells such as lymphocytes and dendritic cells and eventually adaptive immunity.

Table 4.1 The human chemokine system

Chemokine receptor	Chemokines	Receptor expression in		
		Leukocytes	Epithelium	Endothelium
CXCR1	CXCL6, 8	PMN	+	–
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8	PMN	+	+
CXCR3	CXCL4, 9, 10, 11	Th1, NK	–	+
CXCR4	CXCL12	Widespread	+	+
CXCR5	CXCL13	B	–	–
CXCR6	CXCL16	Activated T	+	–
CXCR7	CXCL12, CXCL11	Widespread	+	+
Unknown	CXCL14 (acts on monocytes)			
CCR1	CCL3, 4, 5, 7, 14, 15, 16, 23	Mo, Mϕ, iDC, NK	+	+
CCR2	CCL2, 7, 8, 12, 13	Mo, Mϕ, iDC, NK activated T, B	+	+
CCR3	CCL5, 7, 11, 13, 15, 24, 26, 28	Eo, Ba, Th2	–	+
CCR4	CCL2, 3, 5, 7, 22	iDC, Th2, NK, T, Mϕ	–	–
CCR5	CCL3, 4, 5, 8	Mo, Mϕ, NK, Th1 activated T	+	–
CCR6	CCL20	iDC, activated T, B	+	–
CCR7	CCL19, 21	mDC, Mϕ, naïve T activated T	+	–
CCR8	CCL1, 4, 17	Mo, iDC, Th2, Treg	–	–
CCR9	CCL25	T	+	–
CCR10	CCL27, 28	Activated T, Treg	+	–
Unknown	CCL18 (acts on mDC and naïve T)			
CX3CR1	CX3CL1	Mo, iDC, NK, Th1	+	–
XCR1	XCL1, 2	T, NK	–	–
Miscellaneous	Scavenger receptors for chemokines			
Duffy antigen	CCL2, 5, 11, 13, 14 CXCL1, 2, 3, 7, 8			
D6	CCL2, 3, 4, 5, 7, 8, 12 CCL13, 14, 17, 22			

Leukocyte anonyms are as follows: *Ba* basophil, *Eo* eosinophil, *iDC* immature dendritic cell, *mDC* mature dendritic cell, *Mo* monocyte, *Mϕ* macrophage, *NK* natural killer cell, *Th1* type I helper T cell, *Th2* type II helper T cell, *Treg* regulatory T cell

The human and mouse genomes contain over 44 and 38 different chemokine genes, respectively [7]. There is a difference in gene numbers with some ambiguities of orthologous relationship between the human and mouse chemokine family. These observations would indicate that the chemokine gene family has been rapidly evolving, resulting in species-specific expansions and contractions. A notable difference has been found in one of the major chemokine, CXCL8, and its receptors, CXCR1

and CXCR2. Mice and rats do not possess a homolog of the *CXCL8/IL-8* gene, which is present in other species including humans, rabbits, cats, and dogs [7]. Moreover, the *CXCR1* and *CXCR2* genes encode functional receptor proteins in humans, whereas there still remains a question on the presence of functional *CXCR1* in mice or rats [8]. Different expression patterns between humans and mice were observed also on other chemokine receptors such as CCR1 [9]. These observations should be taken into consideration when the findings obtained with mouse models are extrapolated to human conditions.

Chemokine Receptor Signaling (Fig. 4.1)

Approximately 20 signaling chemokine receptors have been identified as well as several non-signaling receptors (Table 4.1) [10]. The presence of a DRY motif in the second transmembrane region is responsible for the ability of chemokine receptors to signal upon ligand binding, and non-signaling receptors lack this motif. Chemokine receptors are coupled with heterotrimeric Gαβγ proteins bound to intracellular loops. The Gα subunit contains a GTPase domain involved in binding and hydrolysis of GTP. In the inactive state, the Gα subunit binds GDP and interacts directly with the intracellular loop of chemokine receptors and with Gβ subunit, which in turn forms a tight complex with Gγ subunit. A two-step model has been proposed for activation of the receptor [2]. In the first step, a chemokine specifically recognizes and binds the receptor. Consequently, the amino-terminus of the chemokine interacts with the receptor, leading to the activation of the receptor. Simultaneously, ligand binding induces internalization of the chemokine receptor by using the clathrin-mediated pathway or the lipid raft/caveolae-dependent internalization routes [11]. Internalized receptors are recycled and reappear on the cell surface quickly. However, it still remains controversial on the necessity of internalization and recycling for chemokine-mediated signaling and chemotaxis.

The activation induces dissociation of GDP from Gα and replacement of GTP. Gα-GTP eventually dissociates from the receptor and the Gβγ heterodimer, and both complexes activate a series of downstream effectors (Fig. 4.1). Generated Gβγ heterodimer recruits and activates phosphatidylinositol 3-kinase-γ (PI3K-γ), which in turn generates phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [12]. PIP_3 activates protein kinase B (Akt) as well as small GTPase such as Rac and Rho (Fig. 4.1). In addition, active Gα and Gβγ facilitate the polarization of the cells with the leading edge (pseudopodium) in the front and the formation of a trailing tail (uropod) at the back. PI3K and Rac accumulate at the leading edge to induce actin polymerization and F-actin formation [13]. Simultaneously, Rho and its effector molecules accumulate at the trailing edge to facilitate actomyosin contraction and tail retraction, thereby leading to the migration of the cells.

GPCR-mediated signals can be downregulated by regulators of G protein signaling (RGS) proteins. RGS proteins are a family consisting of 20 members and can activate GTPase activities. RGS proteins directly interact with GTP-bound Gα

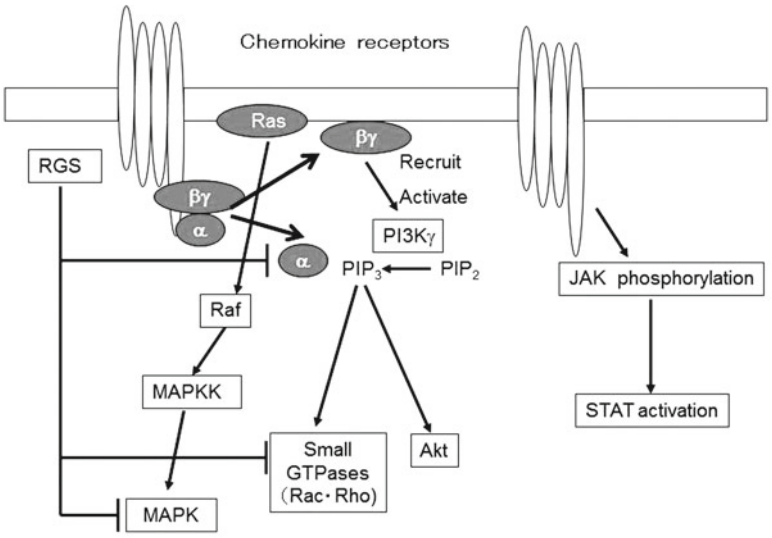

Fig. 4.1 Intracellular signaling pathway of chemokines

subunit to catalyze GTP hydrolysis and G protein downregulation and eventually decrease the half-life of the active GTP-bound state of Gα. RGS1, RGS3, and RGS4 attenuate CXCL8-mediated signals in neutrophils [14], while RGS1 and RGS13 reduce CXCL12- and CXCL13-mediated signals in B cells [15, 16].

The binding of a chemokine to its corresponding receptor exposes the tyrosine residue in DRY motif in the second transmembrane region [17, 18]. This exposure allows access of Janus kinase, which activates the receptor by tyrosine phosphorylation. Simultaneous activation of Janus kinase leads to the recruitment of STAT (signal transducers and activators of transcription) and eventually STAT-mediated expression of the target genes [17, 18] (Fig. 4.1). Moreover, this pathway requires ligand-induced homodimerization of chemokine receptors, as observed on other GPCRs that can frequently exist as dimers and/or high-order oligomers [19]. In the case of CCR5, Ile52 in transmembrane region-1 (TM1) and Val150 in TM4 are key residues in the interaction surface between CCR5 molecules [20]. Moreover, mutation in these residues generates nonfunctional receptors that cannot dimerize or trigger signaling. Similar regions in CCR2 receptor are required for CCL2-induced homodimerization and subsequent activation [18].

It is widely accepted that even distantly related GPCRs can form heterodimers [19]. Indeed, heterodimerization is also observed among several chemokine receptors including CCR2, CCR5, CXCR2, and CXCR4 [21]. For example, the heterodimerization of CCR2 with CCR5 cooperates to trigger calcium influx at concentrations 10- to 100-fold lower than the threshold for either CCL2, a ligand for CCR2, or CCL5, a ligand for CCR5 [22]. However, it recruits a dissimilar signaling pathway such as $G\alpha_{/11}$ association and delays activation of PI3-K. The consequences are

triggering of cell adhesion rather than chemotaxis. In the case of CCR2/CXCR4 heterodimers, specific antagonists of one receptor inhibit the binding of chemokines to other receptor both in recombinant cell lines and primary leukocytes [23]. This results in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. Thus, chemokine receptor antagonists can regulate allosterically the functions of receptors, which they do not directly bind. These observations may have important implications for the effects of these antagonists.

Ras and its downstream signaling pathway, mitogen-activated protein kinase (MAPK)/Erk kinase pathway, can be activated by several chemokine receptors including CXCR1, CXCR2 [24], and CXCR4 [25] (Fig. 4.1). The activation is frequently observed in tumor cells and leads to gene expression and cell proliferation. Moreover, activation of CXCR4 stimulates ovarian cancer cell growth through transactivation of the epidermal growth factor receptor [26]. The activation of these signaling pathways may favor tumor cell proliferation.

Effector Cells in Tumor Immunity and Chemokines

Accumulating evidence indicates the presence of cytotoxic T lymphocytes (CTLs) that can specifically recognize tumor-associated antigens (TAA) and attack tumor cells in humans as well as in mice [27] (Fig. 4.2). In this immunological approach

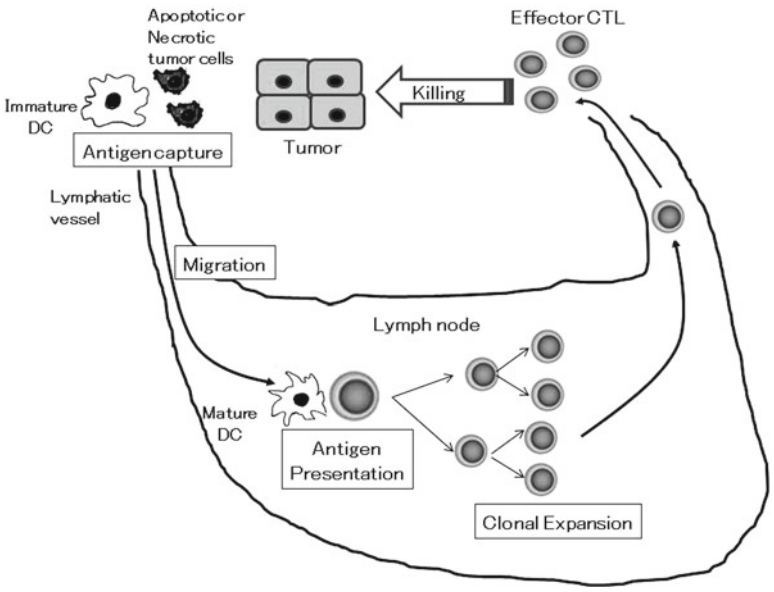

Fig. 4.2 Tumor immunity generation

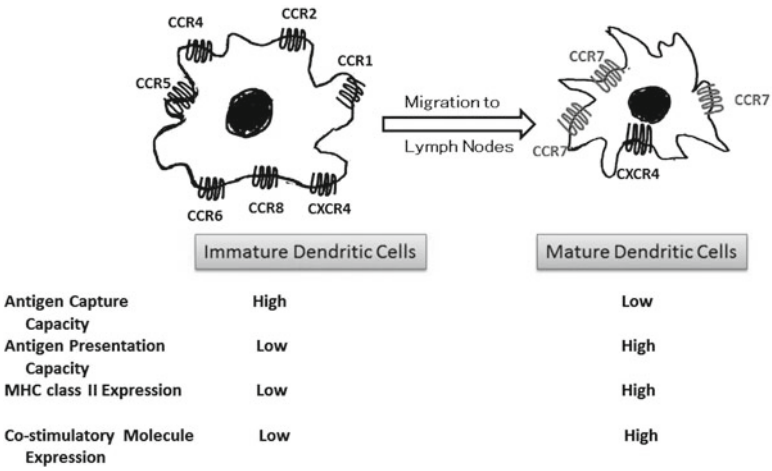

Fig. 4.3 Maturation stages of dendritic cells

to cancer, antigen-presenting cells can deliver TAAs and prime TAA-specific T cells. Dendritic cells (DCs) are professional antigen-presenting cells and can express on their cell surface major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules, all of which assist in T cell activation [28]. DCs are widely distributed over peripheral tissues, and DCs in peripheral tissues are in an immature state and have a high capacity to endocytose various materials [29]. In periphery, DCs capture exogenous and endogenous antigens including tumor cell-derived antigens (Fig. 4.2). When DCs capture antigens in the absence of inflammatory cues such as Toll-like receptor-mediated signals, they fail to increase the expression of co-stimulatory molecules and to present antigens efficiently. On the contrary, when DCs capture antigens in the presence of inflammatory stimuli, they change to a mature state with a loss of endocytosis ability and start to migrate into the T cell areas of regional lymph nodes via afferent lymphatic venules under the guidance of chemokines (Figs. 4.2 and 4.3). Mature DCs process the antigens into the peptides presented on MHC molecules, exhibit enhanced expression of co-stimulatory molecules, and induce primary immune responses through antigen presentation to T cells in the regional lymph node [29] (Fig. 4.3). Immature DCs in peripheral tissues express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR6, CCR8, and CXCR4, whereas mature DCs express a limited set of chemokine receptors, CCR7 and CXCR4 [29] (Fig. 4.3).

CCR7 and its ligands, CCL19 and CCL21, have a pivotal role in DC migration to lymph nodes in both steady state and inflammatory conditions [30] although the contribution of another chemokine receptor, CCR8, cannot be excluded [31]. Antigen-pulsed $CCR7^{+/+}$ but not $CCR7^{-/-}$ DCs migrate efficiently to the draining lymph nodes when an antigen is injected intravenously [32]. Moreover,

DC migration is markedly enhanced when intranodal CCL21 expression is augmented by pretreatment with interleukin (IL)-1 or tumor necrosis factor (TNF). Furthermore, the magnitude and quality of T cell response is proportional to the number of antigen-carrying DCs in the lymph nodes [32]. Furthermore, DCs can produce the chemokines which affect the trafficking and functions of natural killer (NK) cells, a main executor of innate immunity-mediated tumor cell killing [29].

Once generated in the regional lymph nodes, TAA-specific CTLs should migrate to tumor sites to kill tumor cells (Fig. 4.2). Numerous clinical studies have indicated that the presence of $CD3^+$ or $CD8^+$ tumor-infiltrating lymphocytes (TILs) has a positive prognostic influence on survival [33]. Most TILs are deemed to possess cytotoxic activities against tumor cells. Evidence is accumulating to indicate that several chemokines regulate the migration of CTLs into tumor sites. CXCR3 is deemed to be a major chemokine receptor expressed by TILs. In a mouse model, increased expression of ligands for CXCR3, CXCL9, and CXCL10 can elicit antitumor response accompanied with an enhanced infiltration of $CD4^+$ and $CD8^+$ lymphocytes [34]. In line with this observation, in human gastric and colorectal cancer, TILs express CXCR3 [35–37]. Moreover, high levels of CXCL9 and CXCL10, ligands for CXCR3, are produced by stromal cells, mainly macrophages [36]. $CD8^+$ TILs also express CCR5 [35, 37]. Concomitantly, $CD8^+$ TIL numbers correlate well with the expression of CCL5, a ligand for CCR5, by tumor tissues [37]. TILs express other chemokine receptors, CX3CR1, and the expression of its ligand, CX3CL3, is elevated in tumor cells in colorectal cancer tissues [38]. Furthermore, the expression level of CXCL16 also correlates with $CD4^+$ and $CD8^+$ TIL numbers with a better prognosis although cells expressing CXCR6, a receptor for CXCL16, are not identified [39]. Thus, CXCL9, CXCL10, CXCL16, CCL5, and CX3CL1 can be used to efficiently mobilize CTLs from regional lymph nodes to tumor tissues with an objective to enhance CTL-mediated tumor destruction.

NK cells are unconventional lymphocytes and were initially identified as a leukocyte to kill tumor cells without any antigen stimulation [40]. Mouse and human NK cells can in vitro kill a broad range of tumor cells of both hematopoietic and non-hematopoietic origin by utilizing perforin and secreting interferon (IFN)-γ [40]. Moreover, in vivo, mouse NK cells can eliminate many transplantable and spontaneous tumors. Distinct sets of chemokine receptors are utilized for NK cell trafficking (Table 4.1). NK cells migrate to lymph nodes mainly by utilizing CXCR3 and CCR7, while their migration to the inflamed tissues including tumor sites involves CCR1, CCR2, CCR5, CXCR3, and CX3CR1 [41]. Thus, the ligands for these receptors can regulate NK cell trafficking and augment their functions. However, in colorectal tumor tissues, NK cells are scarce despite a significant lymphocyte infiltration, even in the presence of high levels of CXCL9, CXL10, CCL3, CCL4, CCL5, and CX3CL1 [42]. These observations suggest that NK cell migration into tumor tissues is impaired early during tumor development by the mechanism that does not affect TIL trafficking.

Chemokine-Mediated Enhancement in Tumor Immunity

As discussed above, the establishment of tumor immunity is a process consisting of multiple steps: migration of DCs to tumor sites, capture of tumor antigens by DCs, migration of DCs to regional lymph nodes, antigen presentation to effector cells by DCs in regional lymph nodes, and migration of effector cells to tumor sites (Fig. 4.2). Chemokines have profound effects on tumor immunity, particularly migration steps.

The appearance of apoptotic cells induces the migration of immature dendritic cells to the tumor tissues. Accumulated immature dendritic cells capture TAAs and migrate to draining lymph nodes, where DCs present antigens to induce specific CTLs (Fig. 4.2). Tumor-infiltrating DCs expressed CCR1 and CCR5, and a ligand for these receptors, CCL3, was abundantly detected in mouse bearing hepatocellular carcinoma (HCC) [43]. Moreover, DCs in tumor sites and lymph nodes and subsequent cytotoxicity generation were reduced in CCR1-, CCR5-, or CCL3-deficient mice [43]. These observations may mirror the capacity of CCL3 to mobilize immature DCs to peripheral blood from bone marrow by interacting with CCR1 or CCR5 [44]. Actually, systemic administration of CCL3 increased the numbers of DCs in peripheral blood and tumor tissues and concomitantly augmented antitumor effects after radiofrequency ablation of murine HCCs [45]. These observations suggest that CCL3 may be effective to enhance tumor immunity by inducing the migration of immature DCs through peripheral blood to dying tumor cells.

The interaction between CCR7 and its ligands, CCL19 and CCL21, regulates DC migration to lymph nodes for antigen presentation to naïve T cells, which also utilize CCR7-mediated mechanisms to enter T cell zone [30]. Moreover, CCL19 and CCL21 can attract NK cells to the lymph node. These observations suggest the potency of these chemokines to enhance acquired and innate immunity against various antigens including TAAs. Indeed, when CCL21 was injected into a regional lymph node of SV40-transgenic mice that developed bilateral multifocal lung adenocarcinomas, it increased $CD4^+$ and $CD8^+$ lymphocytes as well as DCs at lymph nodes and tumor sites and eventually led to a marked reduction in tumor burdens with enhanced survival [46]. Similar results were also obtained when CCL19 was injected intranodally into SV40-transgenic mice [47].

Ex vivo generated DC have a very limited capacity to move from the injected sites to locally draining lymph nodes [48]. This limitation may account for a clinical weakness in DC-based vaccines. The capacity of CCL19 and CCL21 to effectively induce DC migration prompted the use of these chemokines to modify ex vivo generated DCs. Intratumoral injection of *CCL21* gene-modified DCs resulted in tumor growth inhibition that was significantly better than unmodified control DCs [49], together with intratumoral accumulation of DCs and T cells [50]. Moreover, even when *CCL21* gene-modified DCs were pulsed with tumor lysates and subsequently injected subcutaneously to tumor-free sites in tumor-bearing mice, it elicited an antitumor response [49]. These promising preclinical results have led to ongoing phase I clinical trials [51].

Intratumoral administration of *CCL21* gene-modified DCs reduced tumor burden in spontaneous murine lung carcinoma, accompanied with extensive T cell infiltration, and the enhanced elaboration of IFN-γ, IL-12, CXCL9, and CXCL10 [52]. Moreover, in vivo depletion of either CXCL9 or CXCL10 significantly reduced the antitumor efficacy of *CCL21* gene-modified DCs. This may mirror the fact that CXCR3 is highly expressed by activated effector $CD8^+$ T cells and Th1-type $CD4^+$ T cells [53]. CXCL10 gene transduction into tumor cells had few effects on in vitro tumor cell proliferation but in vivo elicited a potent T cell-dependent antitumor response [54]. Likewise, tumor cells expressing CXCL10 induced the infiltration of tumor-specific cytotoxic T cells into the tumor site [55]. Moreover, tumor cells induced these cytotoxic T cells to proliferate and to produce high level of IFN-γ, while CXCL10 expanded these tumor-specific T cells. Gene transduction of another ligand for CXCR3, CXCL11, into tumor cells also retarded in vivo tumor growth accompanied by intratumoral infiltration of $CD8^+$ cells [56]. As T cells rapidly acquire CXCR3 expression upon activation with IL-2 [53], combined strategy of systemic IL-2 with intratumoral CXCL9 administration was proven to be more efficacious than either cytokine alone, for augmenting tumor-associated immunity [34]. Thus, CXCR3-binding chemokines can be utilized to redirect the migration of effector T cells to tumor sites.

Muthuswamy observed that colorectal tumors with reduced accumulation of $CD8^+$ effector cells express low levels of CXCL10 and CCL5, the chemokine with potent chemoattractant activities for $CD8^+$ effector cells [37]. They demonstrated that a combination of IFN-α and a TLR3 ligand, poly-I:C, can uniformly enhance the production of CXCL10 and CCL5. Moreover, these effects can be optimized by the further addition of cyclooxygenase (COX)-2 inhibitors. Of interest is that this triple combination also consistently suppresses the production of a ligand for CCR4, CCL22, a chemokine associated with Treg infiltration. Thus, this strategy can enhance the intratumoral trafficking of $CD8^+$ effector T cells and can simultaneously reduce that of Treg cells, thereby augmenting local tumor immunity.

CCL2 protein was initially isolated as a factor which can augment monocyte-mediated tumor cytostatic activity and can exhibit monocyte chemotactic activity [57]. Indeed, tumor formation was suppressed in vivo but not in vitro when the tumor was genetically engineered to express *CCL2* gene [58]. CCL2-expressing cells elicited a predominantly monocytic infiltrate at the site of injection, suggesting the roles of infiltrating monocytes in tumor rejection process [58]. In addition to monocytes/macrophages, a receptor for CCL2, CCR2, is expressed by additional types of leukocytes such as NK cells (Table 4.1). *CCL2* gene transduction into tumor cells retarded tumor growth in vivo by inducing NK infiltration into tumor sites [59]. Moreover, NK cell infiltration was associated with elevated Th1 response in tumor sites [60], suggesting that CCL2 can regulate the infiltration and activation of Th1 cells in tumor sites through NK cell recruitment and activation.

Tumor formation was also suppressed in vivo when mouse lymphoma cell lines were transduced with the gene of another chemokine, CX3CL1 [61]. This antitumor response was abolished in NK cell-deficient beige mice but not in T- and B-cell-deficient $Rag1^{-/-}$ mice, indicating the indispensable roles of NK but not T cells.

Gene therapy using *CX3CL1* gene could activate T cells as well as NK cells to exert its antitumor responses [62, 63]. Moreover, intratumoral injection of a DNA plasmid coding for a chimeric immunoglobulin presenting CX3CL1 chemokine domain provided strong antitumor activity [64]. The administration of this fusion protein with tumor antigens induced a strong in vivo antigen-specific T cell proliferation and effector function, accompanied with myeloid DC accumulation [64]. Thus, CX3CL1 can redirect T cells and DCs as well as NK cells, thereby augmenting adaptive immunity to tumor antigens.

In order to enhance the capacity to move to tumor sites by utilizing the chemokine(s) produced by tumor cells, several groups genetically engineered T cells to express the corresponding chemokine receptor. The Reed–Sternberg cells of Hodgkin lymphoma predominantly produce CCL17 and CCL22, which preferentially attract CCR4-expressing Th2 and Treg cells [65]. On the contrary, effector $CD8^+$ T cells lack CCR4. When $CD8^+$ cells were forced to express CCR4, these cells migrated more efficiently to Hodgkin lymphoma site. Moreover, tumor formation was more effectively inhibited by the administration of T lymphocytes expressing CCR4 and a chimeric antigen receptor directed to the Hodgkin lymphoma-associated antigen CD30 [66]. Similarly, CCL2 was highly secreted by malignant pleural mesothelioma cells, but CCR2 was minimally expressed on activated human T cells transduced with a chimeric antibody receptor (CAR) directed to mesothelioma tumor antigen, mesothelin (mesoCAR T cells) [67]. *CCR2* gene-transduced mesoCAR T cells exhibited enhanced antitumor responses accompanied with augmented T cell infiltration into tumor sites, when they were given intravenously [67]. This novel gene therapy technology using a chemokine receptor can effectively enhance the migration of adoptively transferred T cells into tumor sites, where a corresponding chemokine is expressed abundantly.

Reversal of Suppressor Cell-Mediated Immune Suppression by Targeting Chemokines

Tumor immunity can frequently induce immune suppressive mechanisms to dampen the "immunity to self." Thus, tumor immunity can be reduced by the action of several negative immunoregulatory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and the programmed death receptor-1 (PD-1)–PD ligand-1 (PD-L1) axis. Indeed, evidence is accumulating to indicate that the antagonizing monoclonal antibodies to CTLA-4, PD-1, or PD-L1 are effective against various types of cancer even at advanced stages [68, 69]. These observations indicate that targeting tumor-induced immune suppression can be effective to enhance tumor immunity.

Tumor tissues contain the leukocytes that can diminish tumor immunity. The most predominant subset is tumor-associated macrophages (TAMs) [70]. Circulating monocytes are mostly the precursor of these TAMs and are attracted into tumor sites, by chemotactic factors including CCL2, CCL5, CCL7, CCL8,

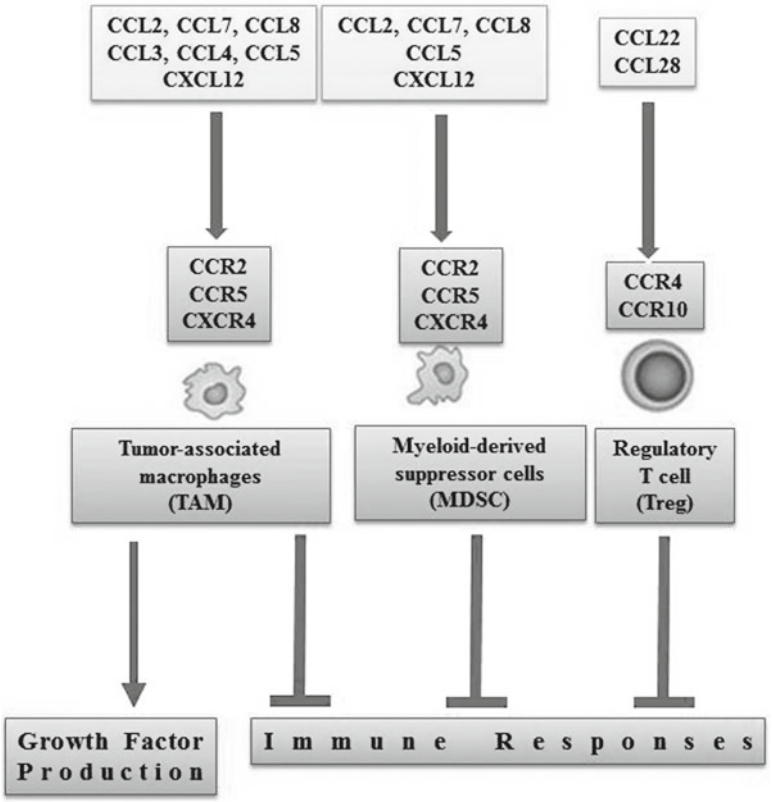

Fig. 4.4 Biological effects of chemokines on suppressive leukocytes in tumors

CXCL12, and macrophage colony stimulating factor (M-CSF), which are produced in tumor tissues [70] (Fig. 4.4). In human colorectal cancer tissues, macrophage accumulation increases with tumor stages and correlates with CCL2 expression in tumor sites [71]. Thus, CCL2-induced TAM infiltration can have a pro-tumorigenic activity.

Hypoxia in tumor microenvironment induces TAMs to produce abundantly vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), two potent angiogenic factors [70]. Moreover, a fraction of TAMs can be incorporated into tumor vasculature [72]. TAMs are frequently polarized into M2 phenotypes under the influence of various factors present in tumor microenvironment, such as IL-4, IL-10, and prostaglandins (PGs) [73]. M2 phenotype is characterized by the expression of arginase (Arg)-1 and inducible NO synthase (iNOS), the enzymes responsible for the generation of reactive oxygen species (ROS), which can inhibit CTL proliferation [73]. TAMs can additionally produce IL-10 and TGF-β to promote the generation of other immunosuppressive cells, regulatory

T cells (Treg) [70], while they can also produce CCL22 to induce intratumoral Treg migration [74]. Moreover, a fraction of TAMs express B7-H4 on their surface to inhibit CTL proliferation [75]. These properties endow TAMs with an immunosuppressive capacity. Thus, TAMs can promote tumor progression by inducing angiogenesis and suppression of adaptive and innate antitumor immunity (Fig. 4.4).

Systemic delivery of neutralizing anti-CCL2 antibody attenuated tumor burdens in human prostate cancer-bearing mice although its effects of TAMs have not been examined [76]. Combined treatment of azoxymethane and repeated dextran sodium sulfate solution ingestion caused multiple tumors in murine colons, together with a massive infiltration of monocytes/macrophages expressing COX-2, an enzyme crucially involved in colon carcinogenesis [77]. CCL2 was abundantly detected in colon tissues and induced CCR2-positive COX-2 expressing monocytes/macrophages to infiltrate colon tissues and blocking CCL2 retarded tumor progression with reduced macrophage infiltration [77]. CCL2 also recruited monocytes to pulmonary metastatic sites of murine breast cancer [78]. As a consequence, infiltrated monocytes promoted the extravasation of tumor cells, a prerequisite step for metastasis, in a process that required monocyte-derived VEGF and CCL2 blockade markedly reduced lung metastasis.

Myeloid-derived suppressor cells (MDSCs) are an additional type of cells characterized by a strong ability to suppress various T cell functions [79]. MDSCs represent a heterogenic population of immature myeloid cells that consists of precursors of macrophages, granulocytes, and dendritic cells. In mice, MDSCs are characterized by the co-expression of two distinct myeloid-cell lineage differentiation antigens, Gr-1 and CD11b in mouse [79]. In humans, MDSCs are defined as $CD14^{-}CD11b^{+}$ cells or as cells that express the common myeloid marker CD33 but lack the expression markers of mature myeloid and lymphoid markers. Similarly as TAMs do, MDSCs express Arg-1 and iNOS and produce immunosuppressive cytokines such as TGF-β1 and IL-10, thereby inhibiting T cell response [79] (Fig. 4.4). CCL2 recruits MDSCs in several types of mouse cancer including Lewis lung carcinoma, MethA sarcoma, melanoma, and lymphoma [80]. Moreover, CCL2-mediated MDSC accumulation can negatively regulate the entry of adoptively transferred activated $CD8^{+}$ cells into tumor sites [81]. However, CCR2 deficiency caused conversion of the MDSC phenotype to neutrophil lineage without affecting tumor growth [82], probably because MDSC contains a subset of immature neutrophils [83]. CXCL5 and CXCL12 also induced MDSC infiltration in mouse mammary adenocarcinoma [84]. In ascites isolated from human ovarian cancer patients, PGE_2 induced CXCL12 production and the expression of its receptor, CXCR4, and the CXCL12–CXCR4 axis subsequently induced the accumulation of MDSCs [85]. Due to the heterogeneity of MDSCs [79], it remains elusive on the relevance of this observation.

Treg cells are characterized by the expression of CD4 and CD25 on their cell surface with the expression of a transcription factor, Foxp3 [86]. Treg cells are polarized from $CD4^{+}$ naïve T cells in thymus or periphery and are physiologically engaged in the maintenance of immunological self-tolerance. A large number of Treg cells often infiltrate into tumors and systemic removal of Treg cells enhances

natural as well as vaccine-induced antitumor T cell immunity [86]. Intratumoral $CD8^+$/$Foxp3^+$ ratio but not absolute $Foxp3^+$ cell numbers correlated inversely with survival [33]. Thus, the relative ratio of Treg to $CD8^+$ CTL but not absolute Treg number can have impacts on immune tolerance to tumor cells.

Treg cells express CCR4 and its ligand, CCL22, mainly regulates intratumoral Treg infiltration in various tumors [86] (Fig. 4.4). Indeed, intratumoral CCL22 expression correlated well with Foxp3 expression in colorectal carcinoma tissues [37]. Hypoxia induced the expression of another chemokine, CCL28, in colorectal tumor cells [87]. CCL28 seemed to utilize mainly CCR10 to induce Treg migration into tumor sites (Fig. 4.4) although CCL28 was reported to utilize both CCR3 and CCR10 as its receptors (Table 4.1). Moreover, infiltrating Treg cells can produce VEGF to promote tumor neovascularization [87]. Furthermore, anti-CCL2 antibody augmented cancer immunotherapy against non-small cell lung cancer in mice when it was administered in combination with a tumor vaccine [88]. This enhanced tumor immunity was associated with reduced intratumoral Tregs and increased numbers of intratumoral $CD8^+$ cells that are more activated and more antitumor antigen specific. These observations illustrate that targeting these chemokines can reduce intratumoral Treg cells, resulting in the enhancement of tumor immunity.

Adult T cell leukemia (ATL) cells are characterized by robust expression of CCR4 and can migrate in vitro to CCL17 and CCL22, ligands for CCR4 [89]. By using genetic engineering methods, humanized monoclonal antibody to CCR4 has been defucosylated to exert more potent antibody-dependent cytotoxicity (ADCC) [90]. The resultant antibody is capable of removing CCR4-expressing ATL cells in peripheral blood and bone marrow mainly by ADCC. Thus, this antibody may also be effective to reduce intratumoral Treg cell numbers in solid tumors, thereby augmenting T cell-mediated cytotoxicity against tumor cells.

Recently, CCR1-expressing $CD34^+$ immature myeloid cells have been detected in murine intestinal tumors with SMAD4 deficiency [91]. These cells expressed abundantly MMP-9 and MMP-2 and were involved in invasion. Moreover, a CCR1 antagonist suppressed colon cancer liver metastasis by blocking accumulation of $CD34^+$ immature myeloid cells [92].

Other Strategies of Antitumor Therapy Targeting Chemokines

Chemokines were originally identified as factors affecting leukocyte migration and activation [93]. Subsequent studies revealed that chemokines have effects on non-leukocytic cells including tumor cells and endothelial cells (Fig. 4.5). Indeed, several chemokines can directly induce cancer cells to express pro-tumorigenic genes and to proliferate. CXCL8 can induce the proliferation of human gastric cancer cells [94], esophageal cancer cells [95], and melanoma cells [96]. CXCR4 activation also caused the proliferation of various cancer cells including ovarian, glioma, melanoma, lung, renal, and thyroid cancer cells [97]. Likewise, CCR6 and CXCR6 can promote the proliferation of colorectal cancer cells [98] and prostate cancer cells

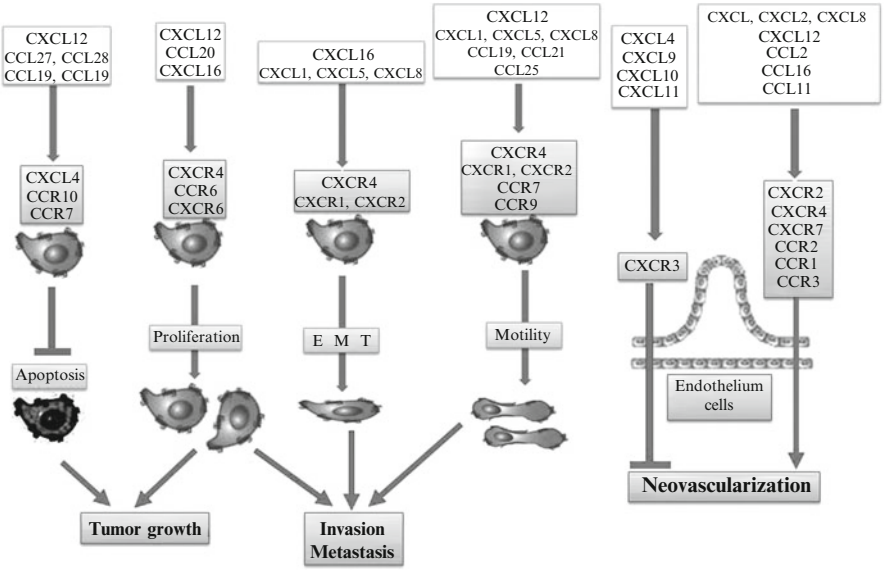

Fig. 4.5 Biological effects of chemokines on tumor and endothelial cells

[99], respectively. Furthermore, the activation of CXCR4, CCR10, or CCR7 axis delivered surviving signals to various types of malignant cells [100–104]. Thus, the inhibition of these chemokines may directly reduce in vivo tumor cell proliferation.

Metastasis is a complicated process wherein cancer cells extravasate from the original tissues, move inside bloodstream and/or lymphatics, and invade to and grow in distant organs. The first step of metastasis, extravasation from the original tissues, requires epithelial–mesenchymal transition (EMT) [102]. Accumulating evidence indicates the crucial roles of CXCL12 [102] and CXCL8 in EMT [105]. Moreover, when tumor cells enter circulation, tumor cells are prone to anoikis, which is a form of cell death arising from the lack of the support from extracellular matrix and is a major block in the metastatic spread of various types of cancer cells. CXCL12 and a CCR7 ligand, CCL21, can reduce the sensitivity of cancer cells to anoikis by regulating pro-apoptotic Bmf and anti-apoptotic Bcl-xL proteins [106].

CXCR4, CCR7, CCR9, CXCR1, and CXCR2 were detected in tumor cells and their ligands induced the chemotaxis of the corresponding receptor-expressing cells [107–111]. Specific chemokine receptor-expressing tumor cells may migrate to organs with high expression levels of respective chemokines along a concentration gradient [107]. However, there remains a question on the presence of a concentration gradient between primary and metastatic sites. Alternatively, cancer cells themselves are actively promoting their own metastasis and tropism by producing chemokines [112]. Moreover, the arrival of tumor cells in a specific organ is passive and chemokine receptor expression provides tumor cells with an advantage to

survive and grow in another ligand-rich metastatic microenvironment [113]. Nevertheless, several chemokines can serve as inducers of metastasis to distant organs and therefore, may be a good target for controlling metastasis.

Neovascularization is crucial for tumor growth, progression, and metastasis [114]. The ELR motif-positive CXC chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, can directly promote the migration and proliferation of endothelial cells and eventually neovascularization, mainly interacting with CXCR2, but not CXCR1 [115] (Fig. 4.5). Indeed, the administration of anti-CXCL8 reduced the tumor sizes of human non-small cell lung cancer cells which are injected into severe combined immune-deficient (SCID) mice in advance [116]. The reduction in tumor size was associated with a decline in tumor-associated vascular density and was accompanied by a decrease in spontaneous lung metastasis.

CXCL12 is not an ELR-positive CXC chemokine but exhibits potent angiogenic effects [117]. In addition, three CC chemokines, CCL2, CCL11, and CCL16, have also been implicated in tumor neovascularization [118–120]. Indeed, CCR2, a specific receptor for CCL2, was expressed by endothelial cells and CCL2 exerted its angiogenic activity in a membrane type 1 (MT1)-MMP-dependent manner [118] (Fig. 4.5). TAMs and MDSCs are recruited at tumor sites mainly by CCL2 and promote angiogenesis by producing a wide variety of angiogenic factors such as VEGF, TGF-β, CXCL8, platelet-derived growth factor (PDGF), and MMP such as MMP-2 and MMP-9. Moreover, recruited TAMs and MDSCs may acquire endothelial cell phenotypes and can be incorporated into the newly formed vascular structure [121]. Thus, targeting these chemokines may be effective to control tumor neovascularization.

CXCL4 and interferon-inducible ELR motif-negative CXC chemokines such as CXCL9, CXCL10, and CXCL11 inhibit the angiogenesis induced by ELR motif-positive CXC chemokines, VEGF, and bFGF [122, 123]. The anti-angiogenic effects of these chemokines are mediated by a common receptor, CXCR3 (Fig. 4.5), and targeted expression of CXCL9 or intratumoral CXCL9 administration retarded in vivo tumor growth by inhibiting tumor-derived angiogenesis [34, 124]. Thus, these chemokines can be effective for tumor therapy by inhibiting neovascularization as well as inducing CXCR3-expressing cytotoxic T cell infiltration.

Perspective

Chemokines regulate the trafficking of leukocytes including immune cells in the presence of a concentration gradient and have a crucial role in the control of the recruitment of immune cells needed for the induction and activation of tumor immunity. As we described above, based on these properties, several chemokines have been utilized in preclinical models to augment tumor immunity by enhancing the migration and activation of immune cells. However, trafficking of a particular type of immune cells is regulated simultaneously by several distinct chemokines in a

redundant manner (Table 4.1). Thus, it still remains to be investigated which chemokine(s) is the most suitable for inducing the trafficking of the targeted immune cells, to exert efficient immune response to tumors.

Moreover, it is embarrassing that the same chemokine can induce tumor progression as well as protection against a tumor. One representative chemokine is CCL2, which can destroy tumor tissues when administered to tumor tissues by using gene therapy technology. It, however, exhibits a wide variety of actions involved in promotion of tumor progression and metastasis, and targeting CCL2 was proven to be effective for reducing tumor burdens and metastasis in several murine models. This paradox may be explained by the assumption that endogenously produced CCL2 can act on the cells present in tumor tissues but cannot cause a concentration gradient sufficient to attract immune cells from outside of the tumor tissues. Otherwise, the responsiveness of immune effector cells to CCL2 may be much lower than that of immune suppressive cells, endothelial cells, and tumor cells. Thus, we should also clarify the local concentration of the chemokine, which is required for the responsiveness of immune effector cells but not that of immune suppressive cells, endothelial cells, and tumor cells. Based on the information, we should devise a method to sustain a local chemokine concentration sufficient to attract immune effector cells to elicit immune response to tumor. Alternatively, genetic modification with a chemokine receptor gene can confer a capacity to respond more efficiently to a chemokine on immune effector cells. Thus, this may be an attractive maneuver to change chemokine-mediated pro-tumorigenic environments, where a particular chemokine is present abundantly, into effective immune surveillance situations, where the abundantly expressed chemokine can attract immune effector cells to exert immune responses.

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Part III
General Tumor Physiology and Microenvironment Issues

Chapter 5
Cancer Biology: Some Causes for a Variety of Different Diseases

Abood Okal, Shams Reaz, and Carol S. Lim

Abstract Advances and integration of biochemistry, cell biology, molecular biology, and genetics have led to a better fundamental understanding of cancer biology and the causes for many types of cancer. Cancer is now thought to originate following either the "cancer stem cell hypothesis" or the "stochastic clonal model." The pathways that lead to cancer have been delineated genetically and epigenetically. In addition, posttranslational players such as miRNA are now known to have a significant role in cancer diagnosis. To meet the high demands of rapidly proliferating cancer cells, alterations of nutrient and metabolic pathways are required. Accordingly, tumor physiology and the cancer microenvironment have been extensively studied due to their significant role in malignancy. This chapter will discuss these topics and provide a detailed investigation of cancer biology including identification of many of the genes, proteins, signals, and other factors involved in tumorigenesis.

Introduction

The oldest identification and description of cancer dates back to 1600 BC in Egypt [1]. Since then, different theories have been proposed to explain the origins for cancer development and survival. While the word *cancer* itself refers to the blood vessels that feed the tumors, cancerous cells develop from normal cells that eventually acquire the ability to proliferate aberrantly and grow uncontrollably into tumors that can metastasize [2]. From the most common initiating events that lead to malignant transformation, the diverse modifications in tumor metabolic pathways that give

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cancerous cells a clear proliferative advantage, and terminating in the dynamics of the cancer microenvironment, this comprehensive cache of knowledge can be used for effective drug delivery of existing and novel cancer therapeutics.

Origins of Cancer

Cancer Stem Cells

Based on data obtained from both in vitro and in vivo studies, only a small population of tumor cells are capable of self-renewal, commonly identified as cancer stem cells (CSCs) [3, 4]. Characterized by their ability to proliferate indefinitely, these tumorigenic cells drive malignancy in a similar manner to the way normal stem cells construct organs. As with normal organs and tissues, tumors are formed from heterogeneous populations of cells with different levels of differentiation and proliferation capacities. Therefore, tumors have been viewed as aberrant organs that originate from cancer stem cells that have acquired mutations allowing them to proliferate abnormally [4]. However, cancer stem cells are only a small subset of cells in a given tumor. For instance, 1–4 % of leukemic cells were capable of forming spleen colonies when introduced in vivo [5, 6], and only 0.0001–0.01 % of leukemic mouse myeloma cells, separated from normal hematopoietic cells, were able to form colonies in vitro [7]. Although cancer stem cells are very similar in nature and function to normal stem cells, cancer stem cells are not necessarily aberrant counterparts of normal stem cells [8]. In certain cases, genetic modifications of normal stem cells can lead to their transformation into cancer stem cells (Fig. 5.1). For example, it is well accepted that the reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] in chronic myelogenous leukemia (CML), which leads to the formation of the Bcr–Abl oncogene, can transform normal hematopoietic stem cells into cancer stem cells that propagate in the bone marrow [9, 10]. However, it is often difficult to establish a link between normal stem cells and cancer stem cells. It is well accepted now that CSCs can also originate from progenitors that have acquired the ability to self-renew as well as from normal stem cells (Fig. 5.1). Therefore, identifying proper markers and techniques to isolate a homogenous population of CSCs remains challenging and is considered to be the rate-limiting step in understanding the nature and function of CSCs. In addition, proper isolation of CSCs could lead to identification of the specific molecular characteristics of such cells for cancer targeted therapy [11].

Some success has been achieved in isolating CSCs often in a heterogeneous mixture with normal stem cells. For instance, it has been demonstrated that cells defined by the phenotype $CD34^{+}/CD38^{-}$ contain a subset of cells that were capable of initiating human acute myeloid leukemia (AML) when transplanted into murine models [13]. Table 5.1 illustrates some of the well-established definitions of CSCs in different cancer types and their origins [8].

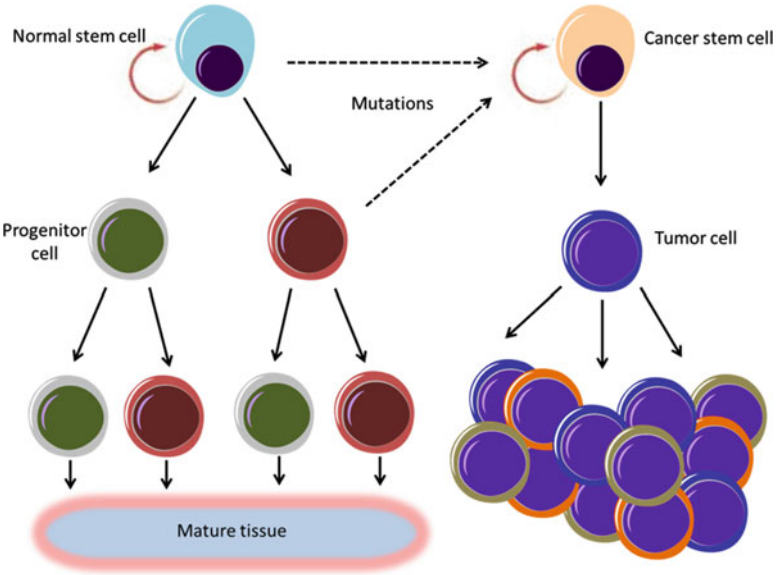

Fig. 5.1 Certain genetic changes or mutations can transform normal stem cells or progenitor cells into cancer stem cells. Such an event allows malignant tumors to divide and differentiate indefinitely. Adapted from [12]

Table 5.1 Markers of cancer stem cells in different cancers (adapted from [8])

Cancer	Definition	Fraction (%)	Origin	References
AML	$CD34^+$ $CD38^-$	0.2–1	Myeloid progenitors	[14]
B-ALL (p190 BCR–ABL1)	$CD34^+$ $CD38^-$ $CD19^+$	1.1	B progenitors	[15]
Medulloblastomas	$CD133^+$	6–21	Stem cells/ progenitors	[16]
Glioblastomas	$CD133^+$	19–29	Stem cells/ progenitors	[16]
Ependymomas	$CD133^+$ $Nestin^+$ $RC2^+$ $BLBP^+$	0.001–1.5	Radial glia cells	[17]
Breast cancer	ESA^+ $CD44^+$ $CD24^{-/low}LIN^-$	0.5–5	Stem cells/ progenitors	[18]
Melanomas (metastatic)	$CD20^+$ $MCAM^+$	20.0	N/A	[19]

Similar work has led to the identification of cancer stem cells in breast cancer [18], gliomas [20, 21], melanoma [19], prostate cancer [22], and osteosarcoma [23]. These observations have led to an increased interest in the "cancer stem cell hypothesis" [4] due to the therapeutic potential that could be translated clinically upon proper identification of CSCs.

Cancer Stem Cell Hypothesis vs. Stochastic Model

While the "cancer stem cell hypothesis" suggests a hierarchical organization in which (a) tumors originate in cancer stem cell niches or their progeny through dysregulation of self-renewal process and (b) tumors contain a subset of cells that have stem cell characteristics [24], other models have been suggested to describe tumor development. One such model is the *stochastic* model for cancer origins in which tumors are thought to develop as a consequence of random somatic mutations and develop the capability to self-renew and differentiate similar to stem cells. According to the stochastic model, any cell has the potential to activate carcinogenesis implying that tumor initiation is no longer exclusive to a rare subset of cells. In other words, every cell in the tumor bulk will have an equal probability to develop cancer stem cell-like characteristics and recapitulate the tumor [25, 26]. Some of the major arguments supporting the stochastic model are the high heterogeneity, genomic instability, and epigenetic alterations observed in tumors [27]. Nevertheless, it is well established now that not all cancers follow one model or another. Even though in vivo studies suggest that leukemias [20, 28], breast cancer [29–31], and squamous cell carcinoma [32] in mice follow the cancer stem cell model, it remains dangerous to generalize that cancers in mice follow a hierarchical CSC model rather than a stochastic clonal evolution model for tumor development.

Pathways That Lead to Cancer

Following vast advances in the field of genetics, the stochastic genetic model for cancer development has predominated other models, supported by the discovery of dominantly acting oncogenes, recessively acting tumor suppressor genes, and diverse molecular changes observed in cancer that lead to the highly heterogenic nature of the disease [33]. Nevertheless, recent correlations between cancer development and the pathological epigenetic changes commonly observed in cancers such as global DNA methylation, chromatin alterations, and genomic imprinting suggest that such events can serve as surrogates for genetic mutations [33, 34]. In this section, an overview of the main changes that take place during cellular transformation in cancer cells will be discussed. In addition, a summary of the main pathways that lead to cancer cell survival will be presented.

Genetic Changes

First postulated by Carl O. Nordling in 1953, the multiple-hit hypothesis offered a clonal approach for explaining cancer development [35]. Nordling suggested that accumulation of mutations in the DNA of cells leads to malignant development. In addition, Nordling emphasized that cancer susceptibility in industrialized nations can be correlated to the sixth power of age, implying that for cancer to develop, six

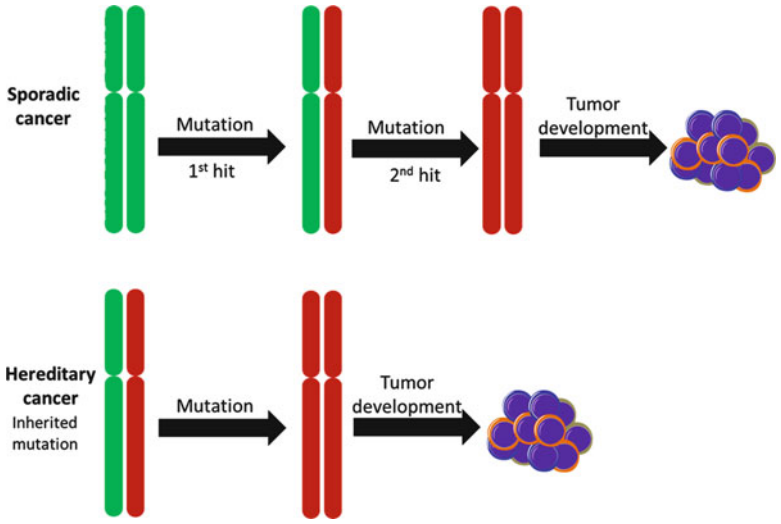

Fig. 5.2 According to the "two-hit hypothesis," both alleles of a tumor suppressor gene must be mutated prior to malignant transformation. In sporadic cancers, two mutations must be acquired before a complete inactivation of a tumor suppressor gene. In hereditary cancers, an inherited mutation exists and only one more "hit" is required before tumors develop

mutations in the DNA must be acquired. However, Nordling's explanation for cancer development did not agree well with the fact that cells possess several defense mechanisms against acquiring mutations via the expression of tumor suppressors. Therefore, it was not until 1971 when the geneticist Alfred Knudson developed the concept of a "two-hit hypothesis" based on several studies of retinoblastoma patients. Between 1944 and 1969, Knudson studied 48 patients that had retinoblastoma due to either somatic or germ-line mutations. Interestingly, Knudson showed that for patients with somatic mutations, tumors took longer to develop in the eye compared to patients who inherited a mutation [36]. He concluded that for retinoblastoma to develop, a subject has to acquire a mutation in both alleles of a tumor suppressor gene (TSG) that encodes for the retinoblastoma protein (pRb). Thus, subjects who inherited a mutation in one of the two copies of the RB1 gene were more susceptible to developing tumors, since only one more mutation in the second copy of the tumor suppressor needed to be acquired [37]. Knudson's hypothesis (Fig. 5.2) forms the basis for understanding how mutations in tumor suppressor genes correlate to tumor development, where, for a tumor suppressor to become inactivated, both alleles need to be mutated or "hit" before a tumor can develop.

Tumor Suppressor Genes in Cancer

Following Knudson's findings, aberrant function of tumor suppressor genes emerged as the leading cause for cancer development. It is well documented now that

activation of a proto-oncogene will not lead to cancer unless accompanied by an inactivation event of both alleles of a TSG. Therefore, it is important to examine tumor suppressor genes and understand the mechanisms by which they prevent uncontrolled cellular growth in normal cells. In this section, several key prototypical tumor suppressor genes will be discussed.

pRb

The retinoblastoma protein (pRb) was one of the first tumor suppressors to be identified during extensive studies on cancer-prone families in the 1940s [38]. Even though pRb is important in all cells, its inactivation usually corresponds in most cases to tumor development specifically in the eye. Similar to other tumor suppressors, the pRb inhibits tumor growth by interfering directly with cell cycle progression, leading to arrest in G1 phase. To exert its tumor suppressor function, pRb inhibits the E2F transcription factor family known to be essential for transactivating a cohort of genes involved in DNA replication in S phase. Consequently, pRb prevents replication of damaged DNA commonly found in cancer cells. During pathogenesis and tumor progression, pRb function can be impaired via several mechanisms. In retinoblastoma, small cell lung carcinoma, and bladder carcinoma, the tumor suppressor gene is mutated leading to a loss of function of pRb [39]. In cervical carcinomas, the human papillomavirus E7 oncoprotein can bind the active pocket of pRb causing the tumor suppressor protein to become incapable of binding E2F transcription factors [40]. Finally, in esophageal, breast, and squamous cell carcinomas, cyclin D is frequently overexpressed which then destabilizes the pRb–E2F complex by phosphorylating pRb resulting in the inhibition of the tumor suppressor [41, 42]. Figure 5.3 summarizes the effects of pRb on the cell cycle.

p53

Encoded by the TP53 gene mapped on the short arm of chromosome 17, p53 is commonly referred to as the "guardian of the genome" [44]. Its tumor suppressor function can be divided into two main categories: the first being a sensor for DNA damage that activates the DNA repair machinery in the cells and the second being a "policeman" for oncogenic signaling and activation [44]. Known to be mutated in more than 50 % of human cancers, the tumor suppressor p53 is a major player in several signaling pathways that are dependent on the context of the stimuli such as DNA repair, cell cycle progression, angiogenesis inhibition, and programmed cell death (apoptosis). Similar to the tumor suppressor gene RB1, both alleles of TP53 must be inactivated for the protein to lose its function. According to Knudson's "two-hit hypothesis," subjects with an inherited mutation in one of the two copies of TP53 are predisposed to develop tumors in early adulthood since only one mutation needs to be acquired in the second copy of the gene to completely inactivate the tumor suppressor. This condition is rare and is known as Li–Fraumeni syndrome.

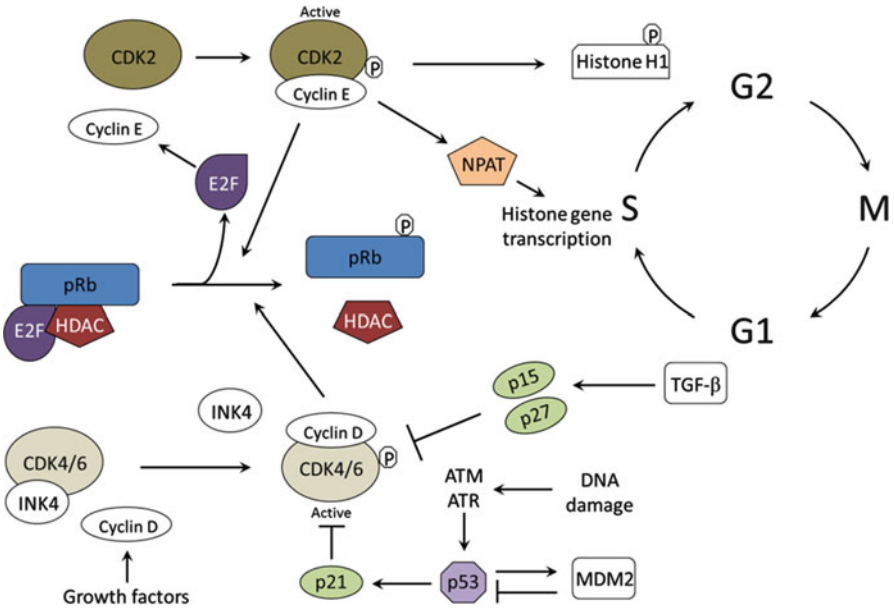

Fig. 5.3 Retinoblastoma tumor suppressor function in cell cycle progression (adapted from [43])

Once activated in response to various cellular stimuli such as DNA damage (via UV, IR, or chemotherapy), hypoxia, or oncogene activation, the tumor suppressor, in its tetrameric form, acts as a transcription factor that regulates the expression of several genes involved in different cellular signaling events. This activity is mainly mediated by nuclear p53 which activates both the extrinsic and intrinsic apoptotic pathways. Furthermore, p53 can activate the extrinsic apoptotic pathway via the mitochondria as well [45]. One of the most important signaling pathways controlled by p53 is DNA repair and its ability to induce apoptosis if cellular damage is too extensive to be repaired. Two of the main factors that control p53 activation upon DNA damage are ataxia–telangiectasia-mutated (ATM) and ataxia–telangiectasia and Rad3-related (ATR) protein kinases. ATM and ATR are capable of rapidly degrading MDM2 [46], the main negative regulator protein of p53, as well as influencing the outcome of the p53 response to DNA damage by inducing various posttranslational modifications on the tumor suppressor [47, 48]. This is depicted in Fig. 5.3, bottom right. In addition, p53 can be activated via the tumor suppressor ARF signaling pathway in response to sustained cellular proliferation. Similar to ATM and ATR, ARF can act as an inhibitor of MDM2 leading to the accumulation of p53 in the nucleus [49]. Another major pathway by which p53 can regulate cell growth is via transactivation of the CDKN1A gene that leads to the expression of p21/WAF1 protein. As a member of the cyclin-dependent kinase inhibitor (CKI) family, p21 can inhibit the cyclin–CDK2 complex required for entry into G1 phase, causing an arrest in cell cycle progression [50]. Therefore, p53 inactivation can be crucial during malignant transformation due to its ability to inhibit tumor growth via many different mechanisms.

PTEN

PTEN is another tumor suppressor gene that is commonly found to be mutated in several human cancers. Although the PTEN gene product helps regulate cell cycle progression in a similar fashion to p53, this protein functions as a dual protein/lipid phosphatase. One of the major substrates for PTEN is PIP3, a crucial protein involved in the AKT/PKB signaling pathway. Upon dephosphorylation by PTEN, PIP3 can no longer recruit AKT to the cell plasma membrane, therefore disrupting the AKT/PKB pathway. This results in increased proliferation and activation of the growth regulatory factor mTOR [51]. Similar to other tumor suppressor genes, PTEN can be inactivated by inheriting a mutation or by acquiring sporadic mutations that can render the protein inactive. Although PTEN knockout models in vivo showed embryonic lethality, the tumor suppression functions of PTEN have been validated using heterozygous ($PTEN^{+/-}$) murine models where animals developed tumors in several organs [52, 53].

Proto-oncogenes and Oncogenes in Cancer

As part of the malignant transformation process, oncogene activation lies at the heart of tumor-initiating mechanisms. Proto-oncogenes are normal genes involved in controlling cellular growth in normal cells that upon alteration (usually mutation or overexpression) can lead to the production of oncogenes, whose protein products induce cancer formation [38]. Certain alterations of proto-oncogenes during tumorigenesis result in oncogene activation that allows cancer cells to exhibit abnormal proliferation. It has been suggested that the main mechanisms of proto-oncogene activation are (a) point mutations that lead to gain of function, (b) gene amplification resulting in growth advantage of cancer cells, and (c) chromosomal rearrangement and fusion that can result in aberrant expression of growth regulatory proteins [54]. Since tumor suppressor proteins such as p53 respond rapidly and efficiently to oncoprotein activity in normal cells, it is frequently observed that oncogene activation is accompanied by inactivation of tumor suppressor genes. In this section, some examples of oncogenes and their activation mechanisms will be examined in the context of malignant progression.

Ras Mutations

The Ras family of proto-oncogenes (H-ras, K-ras, N-ras, and others) is known to be mutated in approximately 20–30 % of human cancers [55]. K-ras is mutated in about 30 % of lung cancers, 50 % of colon carcinomas, and 90 % of pancreatic carcinomas [56]. N-ras is known to be mutated in acute myeloid leukemias [57]. The full function of Ras proteins remains elusive. However, Ras proteins are known to be monomeric membrane G proteins that can be activated in response to several extracellular stimuli that control cellular proliferation and differentiation [58]. Upon mutation, Ras proteins are locked in the GTP-bound active state, resulting in constitutive activity. Consequently, oncogenic Ras continuously activates downstream effectors such as

the MAP-kinase Raf-1, which further activates the MEK/ERK gene regulation pathway that governs proliferation, differentiation, and survival of cancer cells [59].

Myc Amplification

The myc family of proto-oncogenes encodes transcription factors that are involved in several cellular pathways that control cell cycle, cell growth, differentiation, apoptosis, and angiogenesis [60]. C-myc is a myc family member that is commonly overexpressed in several carcinomas. Studies show that the gene-encoding c-myc is amplified in about 20–30 % of breast, ovarian, and squamous cell carcinomas [61]. N-myc is another member of the myc family that is amplified in neuroblastomas, where up to 300 copies of the gene can be found in a single cell [62, 63]. Upon overexpression of myc, several genes involved in cellular proliferation are altered. For example, cyclins (which promote cell cycle progression and division) [64] and ribosomal RNA and proteins (which increase global protein synthesis needed for cell division) [65] are upregulated, and p21 (a key factor in causing cell cycle arrest) is downregulated [66]. Similarly, the proto-oncogene erbB is another example in which amplification of a normal gene that regulates cellular growth can contribute significantly to tumor development [67].

Bcr–Abl Chromosomal Translocation

Chromosomal rearrangements are another mechanism by which proto-oncogenes can be activated during pathogenesis. Such events are frequently detected in hematologic cancers and to a lesser extent in some solid tumors [68, 69]. In chronic myelogenous leukemia, a reciprocal translocation between chromosome 9 and 22 results in the formation of the Philadelphia chromosome that encodes the Bcr–Abl oncogene. Capable of autophosphorylation (i.e., autoactivation), the Bcr–Abl oncogene does not require activation by other cellular signals which allows it to retain constitutive activity [70]. As a result, Bcr–Abl can drive malignant transformation by activating several pro-survival signaling pathways such as Ras–Raf–ERK, JAK–STAT, and PI(3)K pathways [71].

There are many other proteins that have been implicated in or have been used as biomarkers in certain cancers. The relation of these and other proteins in certain subtypes of cancers are listed in Table 5.2, as well as the risk factors contributing to these cancer subtypes.

Epigenetic Alterations

Epigenetic alterations in cancers are mitotically and meiotically heritable phenotypes caused by changes in the gene expression profile of cells and not dependent on alteration of the primary DNA sequence in the nucleus. Such alterations during

Table 5.2 Examples of biomarkers, causes/risk factors, and subtypes of certain types of cancers

Type of cancer	Biomarkers (genes/proteins)	Cause/risk factors	Main subtypes	References
Ovarian	BRCA1, BRCA2, FBXW7, PKB2, HER2, TGFBR1, TGFBR2, STK11, DDR1, CLDN3-7, KLK, LRRN5, CELSR2, PCDH7, APOL1, AGR, FOLR1, MUC, TACSTD2, NPR1, SCNN1A	Unknown	Serous, mucinous, endometrioid, clear cell	[72, 73]
Brain	TP53, ATM, NBS1, PI3K, WRN	Association with exposure to vinyl chloride, radiation exposure, hereditary, compromised immune system	Acoustic neuroma, astrocytoma, chordoma, CNS lymphoma, craniopharyngioma, oligodendroma, ependymoma, medulloblastoma	[73, 74]
Breast	TP53, BRCA1, BRCA2, SMAD2, FBXW7, PKB2, PI3K, CCND1, Her2, NTRK1,3; MAP2K4	Alcohol (more than 1 or 2 glasses), radiation exposure, hormone replacement therapy	Ductal carcinoma, lobular carcinoma	[73, 75]
Colon	APC, AXIN 2, MSH2, MLH1, MSH6, PMS2, MUTYH, CTNNB1, NRAS, FGFR1-3, FES, SMAD2, TGFBR1, TGFBR 2, BAX, KRAS 2, N-Ras, TP53, BRAF, PI3K, MAP2K4, PTNP1	Polyps, hereditary, diet (high-fat or low-fiber consumption), obesity, inflammatory bowel's disease (Crohn disease), prior breast cancer	Adenocarcinoma, leiomyosarcoma, lymphoma, melanoma	[73, 76]
Lung	MYC, MYCN, MYCL1, KRAS2, NRAS, EGFR	Smoking, asbestos, exposure to cancer-causing agent (such as uranium, beryllium, vinyl chloride, nickel chromates, coal products, mustard gas, chloromethyl ethers, gasoline, and diesel exhaust), hereditary, high levels of air pollution, high levels of arsenic in drinking water, radiation therapy to lungs, radon gas, products using chloride and formaldehyde	Adenocarcinoma (squamous cell carcinoma, large cell carcinoma, small cell carcinoma, small cell combined carcinoma)	[73, 77]
Liver	AFP, AFP L3, GP 73 (GOLPH2), DCP	Cirrhosis (from alcohol abuse, autoimmune disease, hepatitis B or C infection, chronic inflammation, hemochromatosis)	Hepatocellular carcinoma, intrahepatic bile duct carcinoma (most common subtypes)	[78–80]

Pancreatic	PEAK1, CA 19-9	Unknown, but increased risk in patients with diabetes, chronic pancreatitis, and in smokers	Pancreatic ductal adenocarcinoma (PDA) most common; ampullary cancer; pancreatic neuroendocrine tumors	[81–83]
Prostate	PSA, AMACR, methylated GSTP1, TMPRSS2-ETS, MUC-1, $NCOA_2$ (oncogene), $FOXP_1$, RYBP, and SHQ_1 (tumor suppressors)	Higher risk in males over 60, with a father or brother with prostate cancer, in African-American males, exposure to agent orange, alcohol abuse, farmers, high-fat diet (esp. animal fat), tire plant workers, painters, cadmium exposure	95 % are adenocarcinomas; also small cell carcinomas and squamous cell carcinomas	[84–87]
Kidney	VHL, MET, FLCN, TSC1/2, FH, SDH	Unknown, but increased risk in patients with kidney disorders (requiring dialysis, horseshoe kidney, polycystic kidney disease), with a family history of the disease, high BP, smokers, and VHL disease	Renal cell carcinoma (RCC) most common type; common RCC subtypes: clear cell RCC (70–75 %), papillary RCC (10–15 %), chromophobe RCC (5 %)	[73, 88, 89]

AFP alpha fetoprotein; *Apc* adenomatous polyposis coli; *AXIN 2* axis-inhibition protein 2; *ATM* ataxia–telangiectasia mutated; *APOL1* apolipoprotein L1; *AGR* anterior gradient homolog; *AMACR* α-methylacyl-coenzyme A racemase; *BRCA1* breast cancer type 1 susceptibility protein; *BRCA2* breast cancer type 2 susceptibility protein; *CA 19-9* cancer antigen 19-9; *CLDN3* claudin 3; *CCND1* cyclin D1; *CELSR2* cadherin EGF LAG seven-pass G-type receptor 2; *CTNNB1* catenin (cadherin-associated protein), beta 1; *Bax* BCL2-associated X protein; *DCP* des-gamma-carboxyprothrombin; *DDR1* discoidin domain receptor family, member 1; *FBXW7* F-box/WD repeat-containing protein 7; *FOLR1* folate receptor alpha; *FLCN* folliculin; *FGFR1-3* fibroblast growth factor receptor 1; *FES* feline sarcoma oncogene; *$FOXP_1$* forkhead box transcription factor; *FH* fumarate hydratase; *GP 73* golgi protein 73 (also known as golgi phosphoprotein 2); *methylated GSTP1* glutathione *S*-transferase π; H/ACA RNP-specific assembly factor; *VHL* von Hippel–Lindau; *HER2* human epidermal growth factor receptor2; *KLK* kallikrein; *KRAS2* V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *LRRN5* leucine-rich repeat neuronal protein 5; *MUC* mucin; *MUTYH* mutY homolog; *MYC* myelocytomatosis viral oncogene homolog; *MYCN* myelocytomatosis viral-related oncogene, neuroblastoma derived; *MYCL1* myelocytomatosis viral oncogene homolog 1 (lung carcinoma derived); *MAP2K4* mitogen-activated protein kinase kinase 4; *MSH2* mutS homolog 2; *MLH1* mutL homolog 1; *MSH6* mutS homolog 6, *$NCOA_2$* nuclear receptor coactivator$_2$; *NRAS* neuroblastoma Ras viral (v-ras) oncogene homolog; *NTRK1* neurotrophic tyrosine kinase receptor type 1; *NBS1* Nijmegen breakage syndrome gene; *NPR1* natriuretic peptide receptor 1; *PEAK1* pseudopodium-enriched atypical kinase 1; *PSA* prostate specific antigen; *PI3K* phosphatidylinositol 3-kinases; *PMS2* postmeiotic segregation increased 2; *PTNP1* protein tyrosine phosphatase-1B gene; *PKB2* protein kinase B2; *PCDH7* protocadherin-7; *RYBP* ring and YY1-binding protein; *SHQ_1MET* MNNG Hos transforming gene; *STK11* serine/threonine kinase; *SCNN1A* amiloride-sensitive sodium channel subunit alpha; *SMAD2* mothers against decapentaplegic homolog2; *TMPRSS2-ETS* transmembrane protease serine 2-E-twenty-six transcription factor gene rearrangement; *TSC* tuberosclerosis associated; *SDH* serine dehydratase; *TGFBR* transforming growth factor beta receptor; *TACSTD2* tumor-associated calcium signal transducer2; *TP53* tumor protein 53; *WRN* Werner syndrome

carcinogenesis involve events such as DNA methylation, histone modifications, and gene silencing. Unlike genetic changes during carcinogenesis, most of the epigenetic alterations that take place in cancer cells are clinically reversible by directly targeting the moieties that contribute to carcinogenesis [90].

It has become clear that epigenetic silencing of certain genes contributes significantly to pathogenesis. For instance, an alternative mechanism to gross or intragenic deletions and point mutations that lead to inactivation of tumor suppressor genes could be epigenetic silencing of the promoters that drive the expression of those genes. Such an event can play a major role during malignant transformations since inactivation of one tumor suppressor allele via genetic alterations combined with silencing of the second allele can lead to a complete loss of function of the tumor suppressor. Although modifications of other parts of the genes can contribute to gene silencing, the main cause for epigenetic changes in the expression profiles of genes is methylation patterns in the promoter regions of those genes. DNA methylation in mammalian cells usually takes place on cytosine bases on cytosine–guanine (CpG) dinucleotide pairs [91]. Approximately half of all genes contain CpG-rich regions, commonly referred to as CpG clusters or islands, in their promoters [90]. It is estimated that up to 70 % of the CpG pairs are methylated in humans. In addition to gene silencing in cancer, methylation of DNA can prompt point mutations in DNA and lead to general genomic instability in tumors. For instance, the "hot spot" mutations of the tumor suppressor gene TP53 at residues 248 and 273 are caused by cytosine to thymine transition following the deamination of m^5C, the methylated form of cytosine [92]. Parallel to hypermethylation of cytosine residues in the CpG-rich regions of many gene promoters is the global genomic hypomethylation phenotype observed in cancer cells. DNA hypomethylation is responsible for upregulating several genes such as the MDR1 (multiple drug resistance 1) gene [93]. It has been well established that tumors demonstrate large-scale loss of DNA methylation [94–96]. Furthermore, it has been suggested that DNA hypomethylation can reactivate silenced genes in normal cells (mostly genes involved in cell proliferation and survival signaling) and the transcription of intragenomic parasitic DNA (such as viral DNA) [97].

Additionally, chromatin methylation and histone modifications are another set of epigenetic alterations that are frequently observed in many cancers. Histones are proteins around which the DNA wraps itself, and understanding histone modifications and how they alter the gene expression profile in cancer cells remains a major barrier in the field of epigenetic characterization of cancer [98]. However, since histones represent the core building blocks for chromatin structures which can regulate gene expression, the variable posttranslational modifications that take place on histones can contribute to gene silencing or reactivation by changing chromatin structure depending on which amino acids are undergoing modification [99–101]. Therefore, studies on epigenetic alterations in cancer have focused on validating the significance of the synergy between genetic and epigenetic alterations during malignant transformation, rather than attempting to prove whether cancer is a genetic or an epigenetic disease [102].

miRNA in Cancer Diagnosis

MicroRNAs (miRNAs) are short biological molecules (23 ribonucleotides on average) that are involved in interference with messenger RNAs (mRNAs) leading to posttranslational repression [103]. The rapid discovery and identification of new microRNAs have led to a better understanding of the complexity of cancers and the different biological processes underlying the disease. Indeed, the ability to detect miRNAs has required the development of sensitive and high-throughput screening methods such as microarrays that can screen hundreds of miRNA expression profiles at once. In the last few years, miRNA expression profiles have become one of the most powerful tools in cancer diagnosis. For instance, detection of miR-221 is considered to be a very specific and accurate diagnosis for human prostate cancer [104]. Another example for using miRNA as a signature for cancer diagnosis is the overexpression of miR-155 and downregulation of miR-141 in 97 % of patients with renal malignancy [105]. In addition, cancer-related miRNAs (known as oncomiRs) can serve as biomarkers not only for diagnosing malignant development but also for progression, metastasis, and response to therapy [106]. In gastric cancer, it is currently established that downregulation of miR-451 correlates with poor prognosis. In gastric cancer, upregulation of miR-451 leads to repression of the oncogene MIF (macrophage migration inhibitory factor), leading to inhibition of cell growth and sensitization of cancer cells to radiotherapy [107]. Furthermore, miRNA expression profiles can serve as biomarkers for anticipating survival rates. In pancreatic cancer, patients that demonstrate overexpression of miR-155, miR-203, miR-210, and miR-222 have a sixfold higher fatality rate from pancreatic malignancy compared to patients with lower levels of the miRNAs [108]. Table 5.3 summarizes some of the well-defined miRNAs in different cancers. Finally, miRNAs have also been found to influence malignant transformation due to the fact that they can function as tumor suppressors (e.g., miR-15a and miR-16-1) or oncogenes (e.g., miR-17-92 cluster) [109]. However, the utility of miRNAs in cancer diagnosis remains a largely underutilized field that requires more research before it can be implemented efficiently in the clinic.

Nutrients and Metabolic Characteristics of Cancer

One of the major issues with anticancer therapies is their lack of tumor specificity. Therefore, a fundamental understanding of the unique physiological properties of cancer is needed. In normal cells, microvasculature develops with order and exhibits regular blood flow. But, in tumors, these vessels are tortuous, leaky, and often sluggish with irregular blood flow, resulting in poor drug delivery due to high interstitial pressure [126]. Selective cytotoxic studies revealed that cancer cells are highly heterogeneous with hypoxic regions that reduce tumor sensitivity to radiation therapy. Furthermore, necrosis (premature cell death) is more common in cancer than in

Table 5.3 A summary of some of miRNAs in different cancers (adapted from [106])

miRNA	Alteration in cancer	Molecular mechanisms	Diagnostic biomarkers	References
miR-21	Upregulated in breast, bladder, laryngeal cancers, and tongue squamous cell carcinoma	Maintains a low level of BTG2 and inhibits apoptosis partly via TPM1	Overexpression indicates poor survival of tongue squamous cell carcinoma	[110–112]
miR-155	Upregulated in Hodgkin lymphoma, breast, pancreatic and clear cell renal cell cancers	N/A	Overexpression indicates poor prognosis in pancreatic cancer	[113–117]
miR-203 miR-210 miR-222	Upregulated in pancreatic cancer	Overexpression of any of these three miRNAs correlate with poor prognosis	N/A	[115]
miR-452	Upregulated in urothelial carcinoma	Overexpression correlates to poor prognosis	N/A	[118]
miR-29c	Downregulated in CLL, neuroblastoma, brain tumor, and sarcoma	Low expression correlates to poor prognosis in CLL	N/A	[119, 120]
miR-34 family	Downregulated in CLL and acute myeloid leukemia	Low expression of miR-34a correlates with impaired DNA damage response, apoptosis resistance, and chemotherapy refractory disease	miR-34b alters the cell cycle and lowers anchorage independent growth	[121–123]
miR-129	Downregulated in bladder cancer	Low expression is correlated with poor prognosis in bladder cancer	Inhibits cell growth and induces cell death	[124, 125]
miR-451	Downregulated in gastric cancer	Low expression correlates with poor prognosis in gastric cancer	Inhibits cell proliferation and sensitizes cells to radiotherapy	[107]

normal cells [127]. Understanding the causes underlying each of these distinctive features, together with cancer-associated metabolic pathways, may help to develop more cancer-specific therapeutics.

The most obvious feature that separates tumor cells from normal cells is their accelerated metabolic rate, resulting in uncontrolled growth [128] and proliferation [129]. The elevated metabolism of cancer cells can be tracked back to their unique ability to reprogram the traditional mechanisms of energy production, which leads to cellular stress [130]. Because metabolic pathways are connected, and therefore interdependent, alteration of a single pathway can have a significant impact on the regulation of others. These reprogrammed pathways not only widen the options to consume energy in different forms but also facilitate utilization of inorganic nutrients to augment proliferation. Even though cancer cells are notorious for chaos and instability, metabolically they are very well organized to satisfy their need for growth and support [128].

Organic Players: How Tumors Feed and Grow

Carbohydrates

Sugars such as glucose, fructose, and sucrose are primary sources of fuel for any cell. Moreover, glucose can coordinate gene transcription, hormone secretion, enzyme activity, and glucoregulatory neurons [131, 132].

The Warburg Effect

Cancer cells develop several unconventional mechanisms to employ glucose and its downstream metabolites to dominate their functions for amplified cell growth. Warburg effect is one of the prime cancer reprogramming models associated with glucose and respiration. In the presence of adequate oxygen, normal cells produce energy by breaking down glucose into carbon dioxide and water, a process called aerobic respiration. Aerobic respiration begins with the conversion of glucose to pyruvate via glycolysis in the cytosol, followed by oxidative phosphorylation to yield a maximal amount of ATP. When an adequate supply of oxygen is not available, cells break down glucose to lactate by fermentation at the expense of ~18-fold less energy production than aerobic respiration [128]. However, the same rule does not apply to cancer cells. In the early nineteenth century, a German scientist named Otto Warburg first noticed an aberrant biochemical characteristic in cancer cell metabolism [130]. Warburg observed that even under aerobic conditions, rat sarcomas and human tumors were generating copious amounts of lactate compared to normal cells [133]. This finding together with others confirmed that cancer cells restrict their metabolic energy largely to glycolysis, leading to a condition termed "aerobic glycolysis," more commonly known as the Warburg effect. The Warburg effect is depicted in Fig. 5.4.

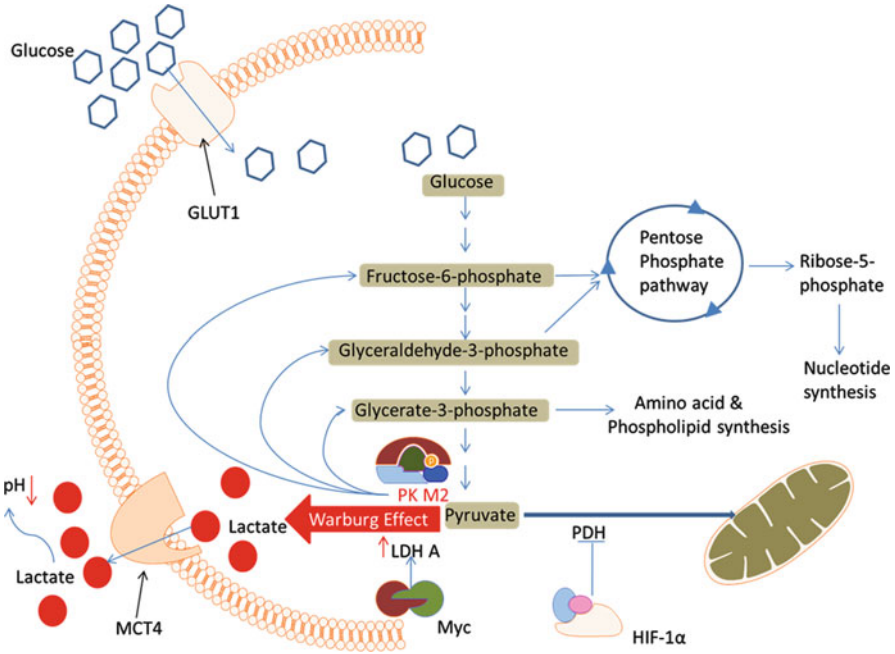

Fig. 5.4 Reprogramming carbohydrate metabolism in cancer. Overexpression of glucose transporter 1 (Glut1) facilitates import of excess glucose for accelerated glycolysis. Pyruvate kinase M2 (PK M2) favors accumulation of biosynthetic precursors fructose-6-phosphate and glyceraldehyde-3-phosphate for nucleotide synthesis and glycerate-3-phosphate for amino acid and phospholipid synthesis. The oncoprotein myc upregulates lactate dehydrogenase A (LDH A) to alleviate Warburg effect, and hypoxia-inducible factor 1, alpha subunit (HIF-1α) inhibits pyruvate dehydrogenase (PDH) to prevent cytosolic pyruvate export to the mitochondria. Monocarboxylate transporter 4 (MCT 4) exports lactate out of the cell to lower the surrounding pH for acidification

In 1931, Warburg won the Nobel Prize in Physiology or Medicine for the discovery of the hydrogen-transferring function of flavine and nicotinamide [130]. Warburg's finding changed the scientific perspective of cancer with his first step that distinguishes tumor metabolic features from normal cells. From this, numerous questions arise: Do cancer cells consume more glucose than normal cells? If they do, which proteins facilitate the high uptake of glucose? How does this overconsumption affect neighboring normal cells? Is glucose the only essential nutrient required for tumor growth and survival? What other metabolic characteristics can be unraveled to specify malignant transformation?

Glucose and Its Transporters

To detect glucose uptake, positron emission tomography (PET) has been widely used to diagnose tumor staging and to monitor treatment progression [134]. 2-Fluoro-2-deoxy-D-glucose (FDG) labeled with ^{18}F serves as a molecular probe for

PET imaging. Once inside the cell, FDG is catalyzed by hexokinase and is converted to a 6-phosphate derivative which is chemically stable and resistant to further catalysis [135]. Signal from the resultant compound correlates directly to the amount of glucose uptake in the cell. PET diagnosis of cancer patients reported increased uptake of FDG at the cancer site, confirming that cancer cells indeed consume more glucose than normal cells [136].

Because of limited passive diffusion through membranes, cells largely rely on specific transporters for the exchange of vital substances such as glucose to achieve high glucose uptake [137]. The uptake of glucose into cells is facilitated by the Glut family of transporters. Among the 14 members of this family, the Glut1 isoform is the most studied transporter due to its critical role in providing nutrients for cancer cells. Glut1, encoded by solute-linked carrier gene family member SLC2A1, is a facilitated glucose transporter ubiquitously expressed in human tissues [138]. Since the brain depends solely on glucose as the energy source, Glut1 is more highly expressed in the blood–brain barrier compared to all other tissues [139]. Cancer cells mimic the same strategy to meet their excessive glucose requirement by upregulating Glut1 expression via Ras and SRC oncogenes [140]. Thus, Glut1 can be signified as a metabolic marker for malignant transformation. Clinical reports showed that Glut1 expression correlates reciprocally with cancer patient survival [141–143] and directly to tumor aggressiveness [144]. With further advancement in modern technologies, overexpression of several other Glut family members in cancers has been identified, including Glut3 in cervical cancers [145] and Glut4 in thyroid carcinoma and alveolar rhabdomyosarcoma [140].

Pyruvate and Its Regulatory Enzyme, Pyruvate Kinase M2

Besides glucose, pyruvate is at the heart of cellular metabolism. Despite being the final product of glycolysis, pyruvate plays a central role in interconnecting biosynthetic pathways. In cancer cells, HIF-1α inactivates pyruvate dehydrogenase (PDH) to prevent mitochondrial matrix import, confining pyruvate to the cytosol [146]. At the same time, oncogenic myc upregulates lactate dehydrogenase A (LDH A) expression for the conversion of cytosolic pyruvate to lactate, securing NAD^+ regeneration for tumor propagation and avoiding pyruvate-related cytotoxicity [147] as illustrated in Fig. 5.4.

For the last decade, the pyruvate kinase M2 (PK M2) isoform has received attention as an indicator of malignant transformation. Pyruvate kinase is the rate-limiting enzyme in glycolysis for pyruvate generation and therefore a major regulator of pyruvate-linked pathways. Depending on the tissue, different isoforms of PK are expressed to perform designated tasks. For instance, PK M1 is mostly found in brain and muscle for instant energy generation, whereas PK M2 is present mainly in multiplying cells such as embryonic cells, which require constant nucleic acid synthesis. Not surprisingly, tumor cells replace PK M1 with PK M2 for rapid cell proliferation [148, 149]. Being the key glycolytic enzyme, PK M2 dominates glycolysis either directly or indirectly. By generating pyruvate, PK M2 favors

pyruvate–alanine conversion via a glutamate intermediate to synthesize purines and pyrimidines [150] and simultaneously controls the glutaminolysis cycle (truncated Krebs cycle) for ATP production [151]. Conversely, limiting pyruvate production results in accumulation of preceding metabolites such as fructose-6-phosphate, glyceraldehyde-3-phosphate, and glycerate-3-phosphate (precursors of biosynthetic pathways). PK M2 canalizes glyceraldehyde-3-phosphate and fructose-6-phosphate to the non-oxidative pentose phosphate cycle for ribose-5-phosphate production, necessary for nucleic acid synthesis. PK M2 also channels glycerate-3-phosphate to amino acid and phospholipid synthesis as depicted in Fig. 5.4 [149]. Further, the role of PK M2 is not limited to its glycolytic function in the cytosol. With the help of a nuclear localization signal (NLS) located at the C-terminus, PK M2 can translocate to the nucleus upon epidermal growth factor (EGF) receptor activation [152]. Once inside the nucleus, PK M2 phosphorylates Stat3 for MEK5 transcription, leading to cell proliferation [153].

Since PK M2 is predominantly expressed in cancer cells, targeting it may provide tumor specificity with minimal cytotoxicity to normal cells. Theoretically, inhibition of PK M2 should deplete pyruvate production in cancer cells. Therefore, this precludes subsequent anabolic pathways such as amino acid and phospholipid biosynthesis and hence could manifest anticancer activity.

Lactate and Its Transporter MCT

Lactate, the final product of "aerobic glycolysis," is primarily associated with biosynthesis, metastasis, and immune suppression. Similar to pyruvate and glucose, lactate requires an efficient shuttling system to augment tumorigenic effects. Monocarboxylate transporters (MCT), encoded by SLC family genes, are transmembrane proteins responsible for lactate and pyruvate transport [154]. Unlike glucose transporters, MCT isoforms are involved in both import and export of lactate and therefore expressed selectively in different cell types. Recent studies have established that cancer cells upregulate the proton-coupled MCT4 isoform in a HIF-1α-dependent fashion [155] for the export of excess lactate and epigenetically suppress sodium-coupled MCT1 (SMCT1) to prevent lactate import inside the cell [156]. Moreover, MCT shuttles lactate to fibroblasts and imports pyruvate back to the cancer cell. This conserves a high lactate to pyruvate ratio, which is proposed to be essential for tumor survival [157]. Besides MCT, several other lactate-shuttling proteins have been identified over the past few decades, such as the lactate–alanine shuttle for amino acid synthesis [158, 159].

Release of excessive lactate into the extracellular milieu decreases the pH of the surroundings as shown in Fig. 5.4 and thereby causes acidification of adjacent normal cells. Removal of normal cells reduces competition for nutrients and provides additional space for tumors to grow. This explains how overconsumption of glucose by cancer cells can be pernicious to normal cells. Moreover, reduction in pH facilitates angiogenesis and metastasis through upregulation of EGFR and HIF-1α [160, 161] and inhibits T-cell proliferation via blockade of lactate efflux thus evading the immune response.

Protein: *mTOR*

Mammalian target of rapamycin, also known as mechanistic target of rapamycin (mTOR), is an atypical serine/threonine kinase at the border of cell growth and starvation. In presence of a plethora of nutrients, mTOR is advantageous to cells with uncontrolled growth and deregulated metabolism such as cancer cells. However, mTOR is unaffordable to nutrient-deprived cells due to its high energetic requirements. Since the first discovery in yeast [162], mTOR has gained a considerable reputation throughout the years for its reprogrammed expansive signaling array to fuel cancer cell growth, proliferation, survival, metabolism, and transcription [163]. Structurally, mTOR has two distinct catalytic domains, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which may exist as dimers [164, 165]. Regulatory-associated protein of mTOR (RAPTOR) and rapamycin-insensitive companion of mTOR (RICTOR) serve as a scaffold for mTORC1 and mTORC2, respectively, to form complexes with their substrates and regulators.

Growth factors and amino acids such as insulin regulate mTORC1 activation. Another key protein, GTPase RHEB, when loaded with GTP can activate mTORC1 as well. In fact, overexpression of RHEB can maintain mTORC1 kinase activity even under starvation conditions [166]. However, for mTORC2 regulation, emerging evidence suggests that the oncogene Ras may be necessary to link growth factors to mTORC2 [167].

Substrates of mTORC1 mainly S6 kinase (S6K) and initiation factor 4E-binding protein 1 (elF4E-BP1) are involved in strict regulation of mRNAs. Upon phosphorylation by mTORC1, elF4E-BP1 dissociates from elF4E, allowing incorporation of translation factors to initiate anti-apoptotic protein synthesis [168, 169]. When phosphorylated by mTORC1, S6K binds to multiple proteins, including nuclear cap-binding protein (CBP) for mRNA translation initiation and progression [170]. In addition, phosphorylated S6K initiates transcription of rRNA polymerase 1(RNAP I), signifying that mTORC1 actively upregulates rRNA synthesis [171], which may contribute to oncoprotein translation.

mTORC1 is also involved in autophagy, a process of self-degradation of damaged cells through the lysosomal machinery. mTORC1 phosphorylates the enzyme Atg-13, preventing autophagic action [172]. Increasing evidence indicates that autophagy facilitates tumor suppression; thus, autophagic evasion implies mTORC1 may favor tumorigenesis [173].

Unlike mTORC1, mTORC2 directly activates a group of signaling pathways that are already known for tumorigenesis. The primary substrates of mTORC2, predominantly Akt, serum- and glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC), are responsible for cell cycle progression and cell survival [174, 175].

Lipids

To date, the majority of cancer metabolic research has focused on the catabolic process of glycolysis. Because cancer cells can proliferate faster than normal cells,

they seemingly must have a shifted anabolic rate as well. For rapidly proliferating cells such as cancer cells, lipid synthesis can be vital for new membrane formation, energy storage, hormone production, and growth factor regulation. Studies of ^{14}C-labeled glucose show that most of the esterified acids are derived from de novo synthesis [176, 177], confirming that a higher rate of lipid biosynthesis takes place in cancer cells. Two key enzymes, ATP citrate lysate (ACL) and most importantly fatty acid synthase (FAS), have been identified as mandatory supporters for increased lipid synthesis [178]. To stimulate lipid production, the P13/Akt pathway inhibits breakdown of fatty acids by blocking β-oxidation and activates ACL [179] to channel oxaloacetate for lipid synthesis. Fatty acid synthase (FAS), encoded by the FASN gene, is downregulated in most normal tissues but highly upregulated in cancer cells, which makes it a possible candidate as a therapeutic biomarker. Functions of FAS include energy storage in liver and adipose tissue, reproduction, and lactation. As the name suggests, FAS synthesizes palmitate (16C) from acetyl-CoA (2C) [178] to serve as a precursor for longer fatty acid synthesis. Inhibition of FAS in promyelocytic leukemia caused cell accumulation in G1 phase, followed by reduction in cell proliferation [180], demonstrating the possible role of FAS in cell cycle regulation. Another lipid-associated marker protein, Spot 14, was found to be overexpressed in breast cancer cells, and its expression level correlates with aggressiveness of the disease state [181]. However, the role and mechanistic pathway of Spot 14 in lipid synthesis are still unknown [179]. Recently, Nomura and colleagues reported that monoacylglycerol lipase (MAGL) drives tumorigenesis through lipolytic release and remodeling of free fatty acids. Inhibition of overexpressed MAGL in vitro impaired cell migration and invasiveness [182]. If MAGL is proven to be specific for cancer, this finding will add another enzyme to the existing list of lipid players in cancer.

Inorganic Compounds

Biological systems are dominated by organic molecules ranging from substrates to products. However, trace amounts of inorganic molecules exist in humans, which are crucial for maintaining genomic stability and for regulating most organic macromolecules such as enzymes.

Selenium

Selenium, an essential trace element, mediates metabolic pathways in conjunction with proteins, collectively called selenoproteins. So far, 25 selenoproteins have been discovered in humans [183]. Most selenoproteins are involved in antioxidant function, including glutathione peroxidase-1 (GPX1) [184]. GPX1 knockout mice exhibited increased susceptibility to H_2O_2-induced apoptosis [185] and accelerated accumulation of mutations [186], suggesting a potential role of selenium in genomic

stability. When in excess, selenium metabolites can stimulate selenite-induced apoptosis and cell cycle arrest via the p53-dependent pathway [187, 188]. Moreover, inorganic selenium sensitizes cancer cells to apoptotic inducers such as TRAIL through the p53-mediated mitochondrial pathway [189].

Copper

The balance of copper is important to maintain regular cell function. Cu^{2+} deficiency can cause myeloneuropathy, a fatal developmental disease [190]. Conversely, the elevated serum level of copper observed in cancer patients [191] is caused by an excess of free Cu^{2+} radicals, which promotes oxidative stress, leading to genomic instability [192]. Surprisingly, when Cu^{2+} binds to its enzyme, CuZnSOD, the resultant complex reduces oxidative stress by eliminating O_2^- directly from mitochondria, preventing oxidative DNA damage [193].

Zinc

More than 300 enzymes and proteins require zinc as a cofactor for functional activity, including the DNA repair protein, p53. Under oxidative stress, the zinc finger domain of p53 responds to DNA damage and assists in sequence-specific recognition of DNA repair machinery [194]. Nevertheless, above a certain threshold, zinc inhibits DNA repair proteins such as DNA ligase I [193], which allows for the propagation of genomic mutations.

Iron

Most iron found in the body is present in hemoglobin in red blood cells or in myoglobin of muscle tissue. In terms of intracellular signaling, iron found in the cytochrome of mitochondria is directly involved in ROS formation, which can lead to oxidative stress. On the other hand, release of cytochrome c from the mitochondria activates the caspase cascade which leads to apoptosis. Additionally, iron is a cofactor of several DNA repair proteins, including α-ketoglutarate-dependent DNA repair enzyme (A1KB) that showed reduced binding affinity to its substrate upon iron replacement with copper [195].

Calcium

Calcium is the most abundant metal in biological systems. Ca^{2+} is a highly versatile intracellular signaling molecule that ensures different cellular processes can respond precisely to diverse stimuli. For example, Ca^{2+} signals presynaptic neurons to release neurotransmitters in response to a nerve impulse, and the same Ca^{2+} also regulates

actin for muscle contraction. The elaborate role of Ca^{2+} connects the entire signaling network, which makes it a perfect target for cancer to reprogram many metabolic pathways. Ca^{2+} plays a significant role in the metastatic behavior of cancer cells. In order to be invasive, cancer cells require focal detachment and proteolysis of the extracellular matrix for migration. Ca^{2+} binds to myosin light-chain kinase (MLCK) for myosin II phosphorylation and degrades focal adhesion proteins, resulting in focal detachment for migration [196]. Moreover, upon binding to S1004A (EF-hand calcium-binding protein), Ca^{2+} exposes the interacting domain to interact with cytoskeleton proteins, which has been implicated to be important for cell migration and epithelial-to-mesenchymal transition (EMT) [197, 198].

The Cancer Microenvironment

As mentioned, cancer develops after two initiating events in succession (the "two-hit hypothesis" of tumor suppressor mutation [36]) or after a promoting event, which leads to genetic modifications [199] usually in tumor suppressor genes (see Fig. 5.5a). Growth and invasion of the cancer are promoted by the tumor microenvironment, which develops as a result of cross talk among different cell types. The microenvironment is formed and controlled by the tumor itself but also consists of the tumor stroma, blood vessels, inflammatory cells, and other associated cells [200] (including cancer-associated fibroblasts, vascular and lymphatic endothelial cells).

The Stroma and Its Components

The stroma is the surrounding matrix that supports the tumor [201]. The tumor stroma consists of the extracellular matrix (ECM) and the surrounding noncancerous cells [202]. One of the most important types of cells in the stroma is cancer-associated fibroblasts (CAFs, also known as activated fibroblasts or myofibroblasts). CAFs are spindle-shaped, mesenchymal cells with stress fibers and fibronexus [203] and may arise from epithelial cells through the epithelial-to-mesenchymal transition (EMT) [204]. CAFs synthesize the ECM by producing fibrous proteins such as collagens and fibronectin which are embedded in a glycosaminoglycan gel [205]. CAFs not only secrete growth factors that impact cell motility but also contribute to ECM remodeling by secreting matrix metalloproteinases [204]. This may allow cancer cells to get across tissue boundaries and create cancer cell niches and initiate angiogenesis [205]. CAFs also secrete laminin and type IV collagen to make up the basement membrane (also known as the basal lamina).

Infiltrating inflammatory cells in the tumor microenvironment include those that mediate adaptive immunity, including tumor-infiltrating T lymphocytes, dendritic cells, and B cells (to a lesser extent), and those that mediate innate immunity, including tumor-associated macrophages, polymorphonuclear leukocytes (PMLs), and rare natural killer (NK) cells [200]. Many tumor-infiltrating T lymphocytes are specific

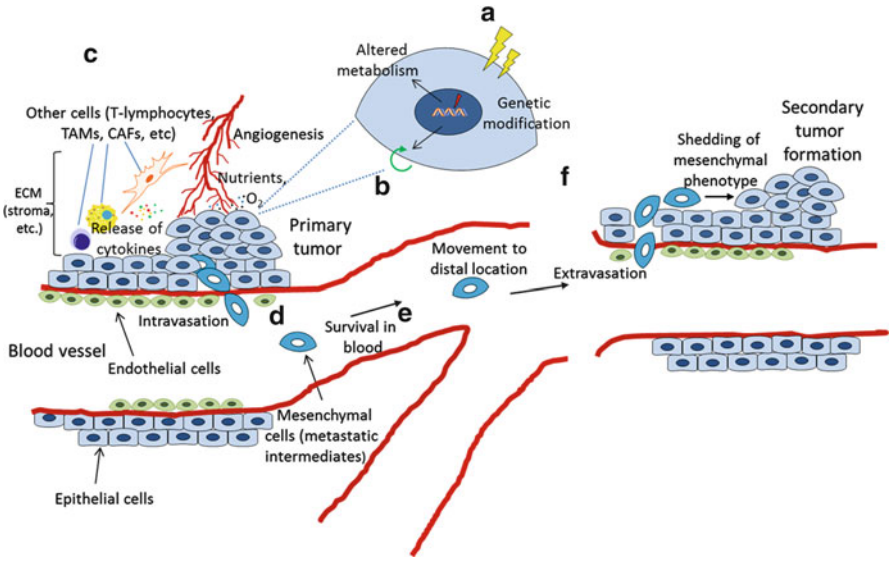

Fig. 5.5 The major steps in cancer. (*a*) Initiating events. (*b*) Uncontrolled cell division. (*c*) Tumor microenvironment. (*d*) EMT. (*e*) Cancer invasion and progression. (*e*) Metastasis

for tumor-associated antigens, implying host immune surveillance, but are incapable of halting tumor growth [200]. Tumor-associated macrophages (TAMs) are reprogrammed to inhibit lymphocyte functions (by the release of inhibitory cytokines) [206]. A version of immature dendritic cells known as myeloid suppressor cells (MSCs) produces arginase 1, which facilitates tumor growth and suppresses immune cell functions [207]. Important mediators of cancer are cytokines (and their cognate receptors), which may promote or inhibit cancer, and in general regulate immunity and inflammation [208]. Chemokines are chemoattractant cytokines that play important roles in allowing cells to traffic in and out of the tumor microenvironment. The chemokine system is subjugated by cancer cells for this purpose [209, 210]. Interestingly, the chemokine receptor CXCR4 has been found to be overexpressed on many cancer cell types including breast, prostate, and pancreatic cancers; melanomas; and certain leukemias [209]. Overall, the tumor subverts inflammatory cells which leads to tumor growth and evasion of the host immune system, allowing the tumor to proliferate.

Cells that form the tumor-associated vasculature (vascular endothelial cells) may have altered characteristics compared to normal vasculature, including differences in gene expression profiles and cell surface markers. On the other hand, the role of lymphatic endothelial cells (that form lymphatic vessels) is poorly understood in terms of tumor growth. Lymphatic vessels in the tumor itself are often collapsed and nonfunctional, while lymphangiogenesis is occurring on the periphery of the tumor and on adjacent normal cells. This suggests that these lymphatics form channels that allow seeding of metastasis [128]. Regardless, studies of proteins and factors involved in either vascular or lymphatic endothelial cells lead to identification of

new therapeutic targets (anti-angiogenic or anti-metastatic therapies). One key protein secreted by endothelial cells is SPARC (secreted protein acidic and rich in cysteine, also termed osteonectin), involved in cell–cell and cell–matrix interaction without participating structurally in the ECM [211]. SPARC can modulate focal adhesion and metalloproteinase expression and interact with growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [212] to determine cell shape, cytoskeleton architecture, and proliferation [213]. Histological studies have also validated overexpression of SPARC [214] and its importance in epithelial–mesenchymal transition for metastasis [215, 216]. In addition to endothelial cells, pericytes are another type of cell that wrap around the endothelium of blood vessels. Pericytes secrete antiproliferative signals, produce vascular endothelial growth factor (VEGF), and work with endothelial cells to stabilize vessel walls [217, 218]. In tumors, pericytes help support the tumor endothelium and are thus another target for pharmacological intervention [128]. Some of the components of the tumor microenvironment are depicted in Fig. 5.5c.

Paramount to tumor malignancy is the process of angiogenesis [219], where new blood vessels form to supply nutrients/oxygen to the existing tumor and allow for the removal of waste products (Fig. 5.5c). Folkman and colleagues demonstrated the need for inducing and sustaining angiogenesis in tumors [128, 219, 220]. In cancer, the angiogenic switch can be activated by altering the balance of angiogenic inducers and inhibitors. Angiogenic inducer and inhibitor prototypes include VEGF-A (vascular endothelial growth factor-A) and TSP-1 (thrombospondin-1), respectively [128, 221]. In the last decade, many other angiogenic factors have been identified and summarized [222]. Angiogenic inhibitors are being actively pursued for cancer therapy, with the concept of cutting off the blood supply to the tumor [222, 223] (Table 5.4).

The Invasion–Metastasis Cascade

Further cancer progression into metastases involves the tumor cells' ability to (1) invade through the ECM and stromal cells, (2) intravasate into the blood vessel lumen, (3) survive in the bloodstream, (4) seed at an organ site, (5) extravasate into these organs, (6) survive and form micrometastases, and (7) form metastatic colonies [199, 225, 226]. The end result is the spread of cancer to new sites/organs, or metastasis [227] (Fig. 5.5).

Epithelial-to-Mesenchymal Transition

For the invasion through the ECM and stroma, tumor epithelial cells must undergo an epithelial-to-mesenchymal transition [228] which allows for an increased capacity to migrate, an enhanced resistance to apoptosis, an increased invasiveness, and an ability to remodel the extracellular matrix [229–231]. Of the two types of EMTs,

Table 5.4 Pro- and anti-angiogenic factors adapted mostly from [222–224]

ECM proteins and derived fragments	Proteinases
Fibronectin (+)	MMPs (+)
Collagens (+)	uPA/tPA (+)
Thrombospondin(TSP) (−)	uPAR (−)
Fibrin (+)	*Adhesion molecules*
Endostatin (−)	Integrin αVβ3 (+)
Tumstatin (−)	Integrin α5β1 (+)
CYR61/CCN1 (+)	N-cadherin (+)
Angiostatin (−)	VE-cadherin (+)
Growth factors	JAM-C (+)
VEGF (+)	*Signaling molecules*
FGF (+)	PKA (−)
Wnt (+)	PKB/AKT (+)
TGFα/β (+ or −)	PKC (+)
PDGF (+)	mTOR (+)
SDF1 (+)	COX2 (+)
PlGF (+)	Rac1 (+)
HGF (+)	Ras–Raf–MAPK (+)
Growth factor receptors	*Transcription factors*
VEGFR1 (Flt1), 2, 3 (+)	HIF-1α (+)
FGFR (+)	Hox D3/B3 (+)
Fzd (+)	Id1/2 (+)
TGFα/β R (+ or −)	KLF2 (−)
PDGFR (+)	FOXO1, 3a (−)
CXCR4 (+)	NFkβ
Morphogenic and guidance molecules	Egr1 (+)
Ang/tie (+ usually)	Sox (+)
Eph/ephrins (+)	CoupTFII (+)
Netrins/DCC/UNC (+ or −)	
Semaphorin/collapsin (+)	
Slits/roundabouts (+)	
DII4 (Notch family) (−)	

Pro-angiogenic factors are indicated by (+); anti-angiogenic, (−), if known

CYR61 or CCN1 cysteine-rich angiogenic inducer 61; *VEGF* vascular endothelial growth factor; *FGF* fibroblast growth factor; *TGF* transforming growth factor; *PDGF* platelet-derived growth factor; *SDF1* stromal cell-derived factor 1; *PlGF* placental growth factor; *HGF* hepatic growth factor; *VEGFR* vascular endothelial growth factor receptor; *FGFR* fibroblast growth factor receptor; *Fzd* frizzled receptor (interacts with Wnt); *TGFα/β R* transforming growth factor α or β receptor; *PDGFR* platelet-derived growth factor receptor; *CXCR4* CXC chemokine receptor (receptor for SDF1); *Ang* angiopoietin; *Tie* tyrosine kinase receptors for angiopoietin; *DCC* deleted in colorectal cancer (receptor for netrin4); *UNC C. elegans* homolog of DCC; *DII4* delta-like 4; *MMP* matrix metalloproteinase; *uPA/tPA* urokinase/tissue-type plasminogen activator; *uPAR* urokinase plasminogen activator receptor; *JAM-C* junctional adhesion molecule C; *PKA, PKB, PKC* protein kinase A, B, C; *mTOR* mammalian target of rapamycin; *COX2* cyclooxygenase 2; *Rac1* Ras-related C3 botulinum toxin substrate 1 (Rac1); *MAPK* mitogen-activated protein kinase; *HIF* hypoxia-inducible factor; *Hox* homeobox; *Id* inhibitor of differentiation; *KLF* Kruppel-like factor; *FOXO* forkhead box O; *NFkβ* nuclear factor kappa B; *Egr1* early growth response transcription factor 1; *CoupTFII* chicken ovalbumin upstream promoter-transcription factor

type 3 EMT is associated with cancer progression and metastasis [230]. EMT may be the activating factor for acquisition of malignancy for epithelial cancers. These cancer cells appear to have a mesenchymal phenotype; express typical mesenchymal markers such as vimentin, desmin, FSP1 (fibroblast-specific protein 1), and α-SMA (smooth muscle α-actin) [232]; and appear at the invasive front of tumors. After the invasion–metastasis cascade, to form a secondary tumor, these cells have to shed their mesenchymal phenotype and return to their epithelial phenotype [230] (Fig. 5.5f). It is thought that EMT-inducing signals (including HGF; EGF, epidermal growth factor; PDGF; TGF-β) may emanate from tumor stroma, resulting in a complex cascade, starting with transcription factor activation (of Snail; Slug; ZEB1, zinc finger E-box-binding homeobox 1; Twist; Goosecoid; FOXC2, Forkhead 1) followed by further signal transduction (by Ras; c-Fos; LEF, lymphoid enhancer factor; ERK, extracellular signal-regulated kinase; MAPK; PI3K; Akt; Smads; RhoB; β-catenin). Cell surface proteins (integrins) are also activated which disrupt several cell–cell or cell–ECM junctions [230]. Lastly, E-cadherin loss is central in the EMT program [233]. TGF-β exposure induces transcription factors Snail, Slug, SIP1 (Smad-interacting protein 1), and E12 (E2A transcription factor family member), which in turn repress E-cadherin expression. Lack of E-cadherin correlates with increased tumorigenicity and metastasis in some models [234]. Finally, microRNAs miR-200 and miR-205 increase E-cadherin expression and help maintain the epithelial phenotype, while miR-21 upregulation facilitates TGF-β-induced EMT [235]. The role of the EMT in cancer progression is depicted in Fig. 5.5d.

Entering and Surviving the Circulation

Intravasation of cancer cells into the lymphatic lumen represents the main mechanism of dispersion of such cells [236]. This process is facilitated by changes that allow these cancer cells to cross the pericytes and endothelial cells that make up the vessels [226]. Tumor-associated blood vessels (neovasculature) are leaky and are continuously being reconfigured. These weak interactions between the endothelial cells and pericytes facilitate intravasation [237]. Once in the bloodstream, these circulating tumor cells (CTCs) are thought of as “metastatic intermediates” [226] and use particular signaling pathways to avoid anoikis (a form of apoptosis caused by anchorage loss). These CTCs must also avoid damage by hemodynamic shear and attack by the immune system. They do so by using the blood coagulation process, forming microemboli, a process likely mediated by L- and P-selectins expressed by the CTCs [238]. See Fig. 5.5d, e.

Seeding, Extravasation, Micrometastasis, and Metastatic Colonization

CTCs may either be physically trapped in microvessels or are “predetermined” to lodge in certain organs/tissues. Predetermined lodging is based on formation of

specific adhesive interactions between the CTC and the organ. After honing to a specific organ, CTCs may form microcolonies that disrupt the surrounding vessels or extravasate by entering the vessel by penetrating the endothelial and pericytes in the stroma. To facilitate this process, primary tumors may secrete a number of factors that disrupt distant metastatic sites and induce permeability at these distant sites [226]. Cancer cells may establish a "premetastatic niche" [239] where primary tumors secrete systemic signals that induce fibronectin expression from specific organs, which leads to mobilization of VEGFR-1 and its ligand and secretion of MMPs, integrins, and other ECM factors, prior to the arrival of the CTCs [226]. Further stimulation of signaling allows the cells to survive in this foreign environment. Finally, colonization of large macrometastases occurs following Paget's "seed and soil" hypothesis where the "soil" represents a hospitable tissue environment for the "seed" (micrometastases) to form [240]. Recently, gene expression of factors that help metastatic colonization has been identified for bone, lung, liver, and brain [226]. An example of the implication of this is that breast cancer cells that metastasize to the bone use different mechanisms for colonization than those that metastasize to the lung. The ability of cells to undergo high self-renewal (e.g., tumor-initiating cells) is more likely to undergo metastatic colonization. Several transcription factors (EMT-inducing and those involved in inhibition of cell differentiation) have been implicated in this self-renewal process. The process of metastasis is depicted in Fig. 5.5f. Factors involved in the metastatic process are indeed being actively pursued as cancer therapeutics as well.

Conclusion

In the late 1800s, since the "two-hit hypothesis" of cancer initiation was postulated, our understanding of cancer initiation and progression has truly evolved. A basic summary of events occurring in cancer is depicted in Fig. 5.5. Initiating events trigger DNA damage, leading to genetic modification, and changes in the cell including altered metabolism (Fig. 5.5a). Uncontrolled cell division leads to cell proliferation (Fig. 5.5b). The tumor microenvironment depicts recruitment of other cells and angiogenesis that occurs when tumors form. Nutrients, cytokines, etc. are released (Fig. 5.5c). EMT is a program where proliferating cells undergo an epithelial-to-mesenchymal transition and intravasate out of the primary locale (Fig. 5.5d). Cancer invasion and progression continue, and cells extravasate (Fig. 5.5f). Finally, metastasis occurs when cells extravasate to a new site, shed their mesenchymal phenotype, and form secondary tumors (Fig. 5.5f). A fundamental understanding of tumor development has come from detailed investigation of cancer biology and has identified many of the genes, proteins, signals, and other factors involved in cancer. With this arsenal of knowledge, scientists will continue to find ways to halt cancer in its tracks. Paramount to this will not only be the discovery of novel drug targets but approaches to deliver new drugs specifically to tumor cells.

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Chapter 6
Cancer Stromal Targeting (CAST) Therapy and Tailored Antibody Drug Conjugate Therapy Depending on the Nature of Tumor Stroma

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Abstract In spite of recent success of monoclonal antibody (mAb) drug conjugate (ADC) therapy in patients with hypervascular and special tumors recognized by a particular mAb, there are several issues to be solved for ADC counted as universal therapy for any types of cancer. Especially most human solid tumors possess abundant stroma that hinders the distribution of ADC. To overcome these drawbacks, we developed a unique strategy that the cancer stromal targeting (CAST) therapy by cytotoxic immunoconjugate bound to the collagen IV or fibrin network in the tumor stroma from which the payload is released gradually and distributed throughout the tumor, resulting in the arrest of tumor growth due to induced damage to tumor cells and tumor vessels.

In addition to the CAST therapy, we clarified the appropriate combination of targeting antibody and conjugate design of antitumor immunoconjugate depending on a quantity of tumor stroma. Hence, we selected two types of conjugate linker, ester bond and carbamate bond. It was found that combination of stromal targeting mAb and a linker composed of ester bond to release drug outside the cells was effective against the stroma-rich cancer. Conversely, cancer cell targeting via carbamate bond to release drug inside the cells was effective against stroma-poor cancer. It seemed that outcome of ADC therapy against each individual tumor having distinct stromal structure was dependent on the selection of conjugation design, as well as targeting mAb.

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Introduction

There are two main concepts in DDS, active targeting and passive targeting. Active targeting involves monoclonal antibodies (mAb) or ligands to tumor-related receptors which can target the tumor by utilizing the specific binding ability between the antibody and antigen or between the ligand and its receptor. The passive targeting system can be achieved by the EPR effect, that is, the enhanced permeability and retention effect [1–3]. Small molecules easily leak from normal vessels in the body, which gives small molecules a short plasma half-life. On the other hand, macromolecules have a long plasma half-life because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system (RES) in various organs. Solid tumors generally possess several pathophysiological characteristics: hypervasculature, secretion of vascular permeability factors stimulating extravasation of macromolecules within a tumor, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues.

Macromolecules and lipids in the interstitial tissue are known to be recovered via the lymphatics in normal tissues [4]. The limited recovery from the lymphatic system in tumor tissues may be attributed to poor development of the lymphatics in tumor tissues, which has been demonstrated by using lipid lymphographic agents [5].

Although there is no clear anatomical proof that tumor lymphogenesis is implicated in the drainage of extravasated macromolecules in human, some studies have indicated that the growth of lymphatic vessels is actively involved in tumor dissemination [6].

This inconsistency regarding tumor lymphogenesis may be due to differences between mice and humans, or differences among tumor types. These characteristics of solid tumors are the EPR effect. Based on the EPR effect, several formulations categorized in passive targeting have been developed and some of them such as Doxil [7] and Abraxane [8] have been approved in clinical use, and anticancer agents (ACAs) incorporating micelles and polymer-conjugated ACAs are now under preclinical and clinical evaluation [9–11].

Monoclonal antibody, which can target the tumor cell actively by the specific binding ability against corresponding antigen, easily extravasates from leaky tumor vessels but not from normal vessels, is long retained in the tumor by utilizing active targeting and passive targeting based on the EPR effect. Therefore, numerous mAbs have been developed and conjugated with ACAs or toxins to create "ADC, immunoconjugate strategy" [12–15]. Recent examples of the conjugates include anti-CD33-calicheamicin and anti-CD20-radiolabeled immunoconjugate and were effective to hematological malignancy such as malignant lymphoma and leukemia [12]. Very recently, the phase 3 trial showed that T-DM1 appeared to have a significant survival benefit in HER2-positive breast cancer that is a representative of hypervascular cancers [16]. Heterogeneity of the cancer cells, however, prevents development of the ADC based on cell-specific antigen [17–20]. Moreover, conventional ADCs depend on cleavage of conjugation site with intracellular biochemical (enzymatic) process after the cell uptake of the conjugate [21–24]. In addition to

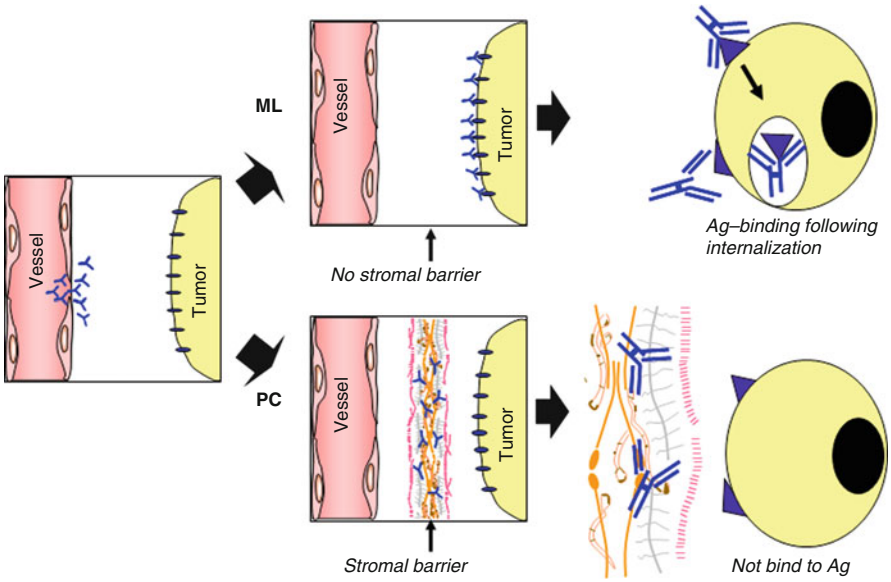


Fig. 6.1 The schema of antibody delivery into the tumor cells. In the tumor having no stromal barrier like malignant lymphoma (ML), antibodies were delivered into the cancer cells, can be internalized after antigen-binding. However, many human solid tumors including pancreatic cancer (PC) possess stromal barrier hindering the distribution of the immunoconjugates into cancer cells such that antigen-binding following antibody-intrernalization never occur. Ag, Antigen

such annoying characteristics of cancer cells themselves, most human solid tumors such as pancreatic cancer and gastric cancer possess abundant stroma that hinders the distribution of mAbs (Fig. 6.1) [25–28]. To overcome these drawbacks, we developed a unique strategy that the cancer stromal targeting (CAST) therapy by cytotoxic immunoconjugate bound to the collagen IV or fibrin network in the tumor stroma from which the payload is released gradually and distributed throughout the tumor, resulting in the arrest of tumor growth due to induced damage to tumor cells and tumor vessels [29, 30]. However, the merit and demerit of anti-stromal targeting immunoconjugate therapy in relation to the conjugate design and the amount of tumor stroma were not yet fully elucidated.

In this context, it is important to clarify the appropriate combination of targeting antibody and conjugate design of antitumor immunoconjugate depending on a quantity of tumor stroma. Hence, we selected two types of conjugate linker, ester bond and carbamate bond. We hypothesized that combination of anti-stromal targeting mAb and a linker composed of ester bond to release ACA outside the cells would be effective against the stroma-rich cancer. Conversely, anticancer cell targeting via carbamate bond to release ACA inside the cells would be effective against stroma-poor cancer. It seemed that outcome of immunoconjugate therapy against each individual tumor having distinct stromal structure was dependent on the selection of conjugation design, as well as targeting mAb.

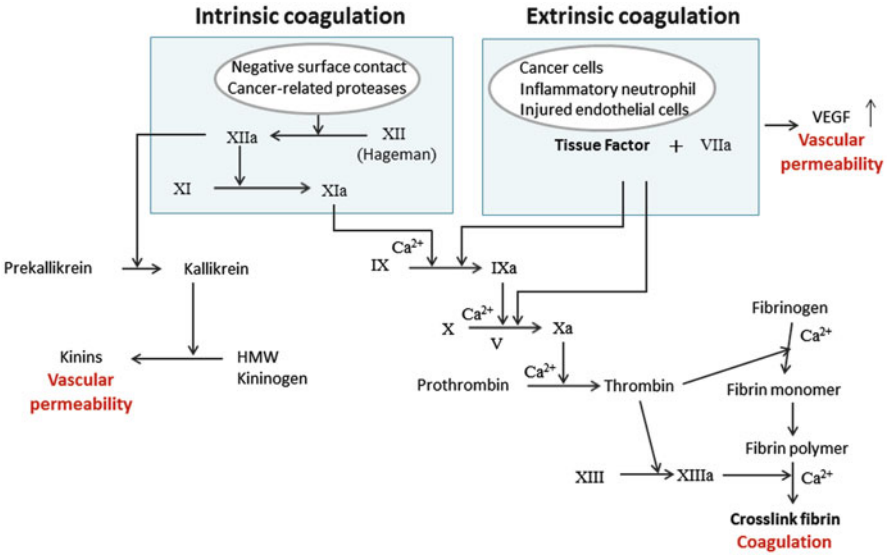


Fig. 6.2 Tumor-induced blood coagulation cascade. Both intrinsic and extrinsic coagulation factors may be involved in tumor vascular permeability

Cancer Stroma

The increased tumor vascular permeability is the most important event for the EPR effect. At the time we proposed the EPR effect, we also succeeded in purifying two types of kinin (bradykinin and hydroxyprolyl^3-bradykinin) from the ascitic fluid of a patient with gastric cancer [1, 31]. We also clarified that this kinin generation system was triggered by the activated Hageman factor, an intrinsic coagulation factor XII [32].

Meanwhile, Dvorak et al. discovered that vascular permeability factor (VPF) was involved in tumor vascular permeability [33]. Later, it was found that VPF was identical to vascular endothelial growth factor (VEGF) [34]. Recently, an extrinsic coagulation factor, namely a tissue factor (TF), appeared to activate VEGF production [35]. So, both intrinsic and extrinsic coagulation factors may be involved in tumor vascular permeability as well as tumor-induced blood coagulation (Fig. 6.2).

In the nineteenth century, French surgeon Armand Trousseau described thrombophlebitis in patients with stomach cancer for the first time [36]. Today, a large body of clinical evidence supports the conclusion that abnormal coagulation occurs in a variety of cancer patients [37]. It is now known that TF is highly expressed on the surface of almost all human cancer cells, and alternatively spliced soluble TF is also produced by many types of tumor [35]. Therefore, TF may be involved in tumor-related abnormal blood coagulation.

Above all, any malignant tumor can erode the surrounding normal tissue, and the more erosive types of cancer have more destructive actions. If these cancer clusters erode adjacent normal or tumor vessels, microscopic hemorrhage may occur at any

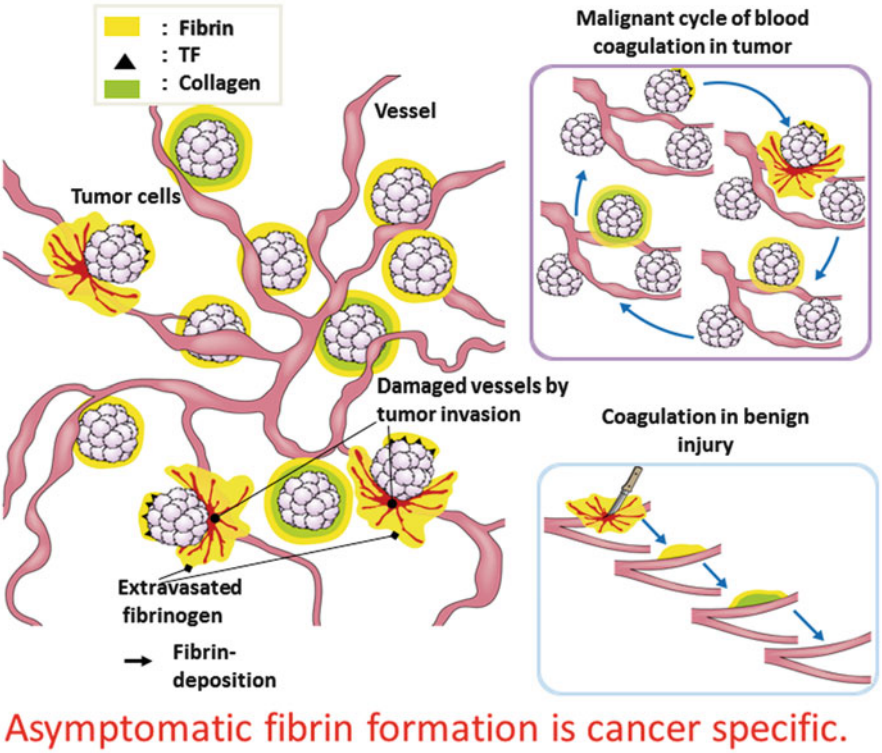


Fig. 6.3 A diagram of the 'malignant cycle of blood coagulation' in cancer tissue

place and at any time within or adjacent to cancer tissues, and fibrin clots immediately form in situ to stop the bleeding. The fibrin clots are subsequently replaced by collagenous stroma in a process similar to that in normal wound healing and other nonmalignant diseases. Fibrin clot formation in nonmalignant disorders such as cardiac infarction, brain infarction, injuries, and active rheumatoid arthritis should form only at the onset or active state of disease and subsequently disappear by plasmin digestion or replacement with collagen within a few weeks and is accompanied by some symptoms. On the other hand, the fibrin clot formation in cancer lasts for as long as the cancer cells survive in the body and occurs silently. Therefore, we call this "malignant cycle of blood coagulation" (Fig. 6.3). In fact, tumor invasion and metastasis progress without symptoms (which is why imaging instruments are needed). When any symptoms accompanying cancer such as pain, intestinal obstruction, or macroscopic bleeding occur, the cancer is likely to involve the sensory nerves and destruction of the bones and larger blood vessels and to occupy the whole lumen of a particular place of the intestine. Usually, patients with an advanced stage of cancer receive chemotherapy and it is worth noting that oncologists never treat such patients if they suffer from existing acute thrombotic complications, bleeding by injury, or active inflammation. Therefore, we conclude that cancer-induced blood

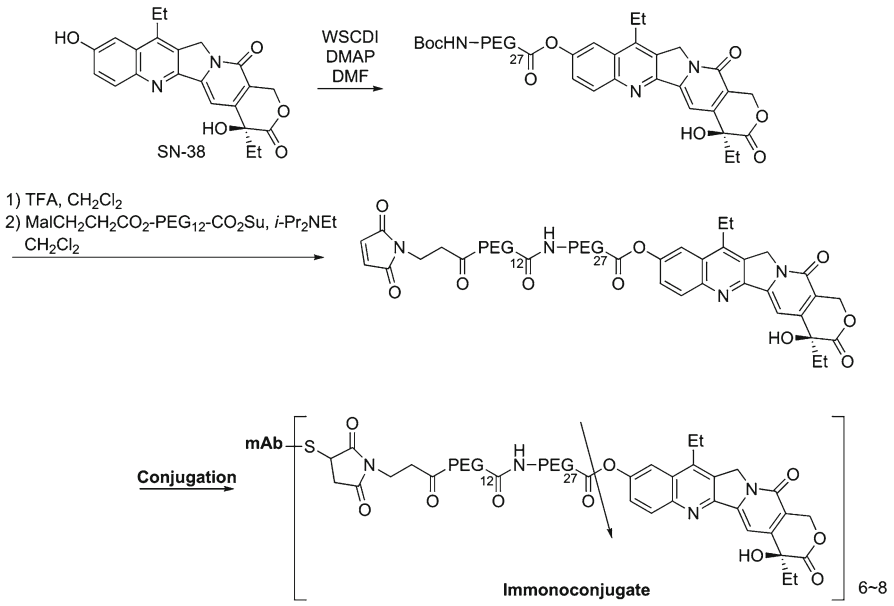


Fig. 6.4 Synthetic scheme of the immunoconjugate. The arrow indicates the cleavage site for releasing free active SN-38. PEG, Polyethylene glycol

coagulation may be an origin of tumor stroma and that fibrin clots in cancer tissues of patients who can receive chemotherapy are actually tumor specific.

CAST Therapy

CAST Therapy Using Anti-collagen 4 mAb

SN-38 is a topoisomerase 1 inhibitor and an active component of CPT-11 which is used clinically for colorectal, lung, and other cancers. For the mAb conjugation to phenol-OH in SN-38, an ester bond was selected. In our design, polyethylene glycol (PEG) was combined close to the bond (Fig. 6.4). PEG is known to evade nonspecific capture by RES. The drug (SN-38)/mAb ratio (the number of drugs attached to a mAb) of each immunoconjugate ranged from 6.7 to 8.4.

Antitumor activities of immunoconjugates with ester bond SN-38 were evaluated in mice bearing human pancreatic tumor genografts. CPT-11 and three immunoconjugates showed significant antitumor activities compared to results in mice treated with saline, in mice bearing either PSN1 (EpCAM positive and stroma poor) or SUIT2 (EpCAM positive and stroma rich) tumors. In SUIT2 tumors, while the tumor continued to increase in mice treated with CPT-11, anti-CD20 immunoconjugate (as a nonspecific control), and anti-EpCAM immunoconjugate, the tumor in

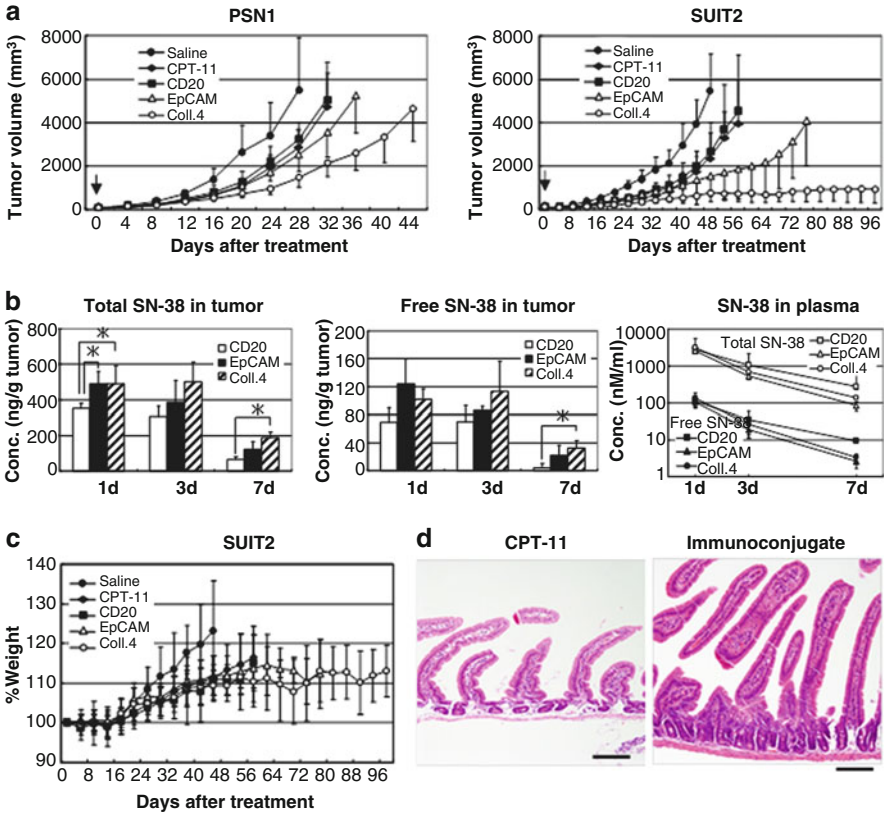


Fig. 6.5 Anti-tumor effects, pharmacokinetics and drug toxicities of anti-CD20, EpCAM and collagen 4 immunoconjugates. **(a)** Anti-tumor activities in vivo were examined. In animal models of PSN1 and SUIT2, the 3 immunoconjugates or saline as control, were administered to separate groups of mice by intravenous bolus injection on day. Arrows indicate day of administration and the curves illustrate the effects of the treatments on tumour size. P<0.05 (Saline or CD20 vs. EpCAM in PSN1), P<0.01 (Saline vs. CPT11 or EpCAM in PSN1, CPT11 or CD20 vs. EpCAM in SUIT2), P<0.001 (Saline vs. CPT11 or CD20 or EpCAM in SUIT2, Saline or CPT11 or CD20 or EpCAM vs. Collagen 4 in PSN1 or SUIT2). Bar=SD. **(b)** Tumor concentrations of total (bound and unbound) SN-38 (upper) and free (unbound) SN-38 (middle), and plasma concentrations (lower) were determined using HPLC analysis. The concentrations on days 1, 3 and 7 are shown. *P<0.05, Bar=SD. **(c)** Changes in the % body weight of saline, CPT-11, CD20, EpCAM and Collagen 4 in the same treated SUIT2 group (A) were shown Bar = SD. **(d)** Pathologic mucosal change of jejunum from mouse treated with CPT11 (upper) or anti-collagen 4 immunoconjugate (lower). Scale bar: 1mm. Coll.4,Collagen 4; Conc.,Concentration

mice treated with anti-collagen IV immunoconjugate stopped growing by about 1 month and never resumed up to 3 months (Fig. 6.5a). In mice bearing PSN1 tumors (stroma poor), differences were present but less marked. Thus, anti-collagen 4-SN-38 immunoconjugate exerted the most potent antitumor activity as compared with anti-CD20 or anti-EpCAM immunoconjugates and CPT-11 (Fig. 6.5a). In both

tumor models, anti-EpCAM immunoconjugate exerted superior antitumor effect compared to CPT-11 and anti-CD20 immunoconjugate, but inferior antitumor effect to anti-collagen IV immunoconjugate.

Significantly higher concentrations of free and total SN-38 were detected in tumor tissues of mice treated with the anti-collagen 4 immunoconjugate compared to the anti-CD20 immunoconjugate (Fig. 6.5b). The concentration of free and SN-38 in the tumor treated with anti-EpCAM immunoconjugate was intermediate among them, but not significant (Fig. 6.5b). There was no significant difference in body weight changes among saline groups, CPT-11, and immunoconjugate groups (Fig. 6.5c). In the small intestinal mucosa of mice, widespread villous atrophy and decreased crypt density were observed by the treatment of free unbound CPT-11 which is well known to have severe intestinal toxicity in clinics. On the other hand, the small intestinal mucosa of mice in groups treated with all immunoconjugates did not show any pathological change (Fig. 6.5d).

The most important observation from a therapeutic standpoint was that only SUIT2 tumors treated with anti-collagen IV immunoconjugate stopped growing about 1 month after treatment and remained dormant for more than 3 months. It may be concluded that the strategy of orchestrating slow sustained release from a scaffold erected on the stable inert structural components of the tumor stroma is most effective. We histologically compared this nongrowing tumor with a size-matched, growing, control tumor and found that both tumors showed central necrosis due to decreased blood flow, which is often observed in a murine xenotransplant model [38, 39]. The striking difference was that large confluent necrotic zones and dense fibrotic capsule formation were observed only in the treated tumor (Fig. 6.6a, b). In addition, CD31-positive endothelial cells, which may be tumor-feeding vessels in the peripheral part of the tumor, were never observed in the treated tumor compared with the untreated control. Instead, many collagen 4-positive round profiles corresponding to traces of destroyed vessels were observed in the peripheral area of the treated tumor (Fig. 6.6c).

CAST Therapy Using Anti-fibrin mAb

Chemically induced mouse cutaneous cancer was selected as an appropriate experimental model for evaluating the therapeutic effects of our immunoconjugate chemotherapy, because this spontaneous carcinogenetic model has remarkable fibrin deposition and abundant interstitial tissue as in human cancer (Fig. 6.7a), unlike human tumor xenografts, which have less fibrin clots and interstitial tissue [40, 41]. In addition, the spontaneous tumor is very slow in tumor growth that is also more similar to the general human cancer as compared to the xenografts. Using systemic in vivo imaging, anti-fibrin IgM, anti-fibrin chimeric IgG, and anti-fibrinogen IgG were delivered and retained in the tumor until day 3, utilizing leaky tumor vessels [1–3]. However, accumulation of anti-fibrin IgM and anti-fibrinogen IgG was weak

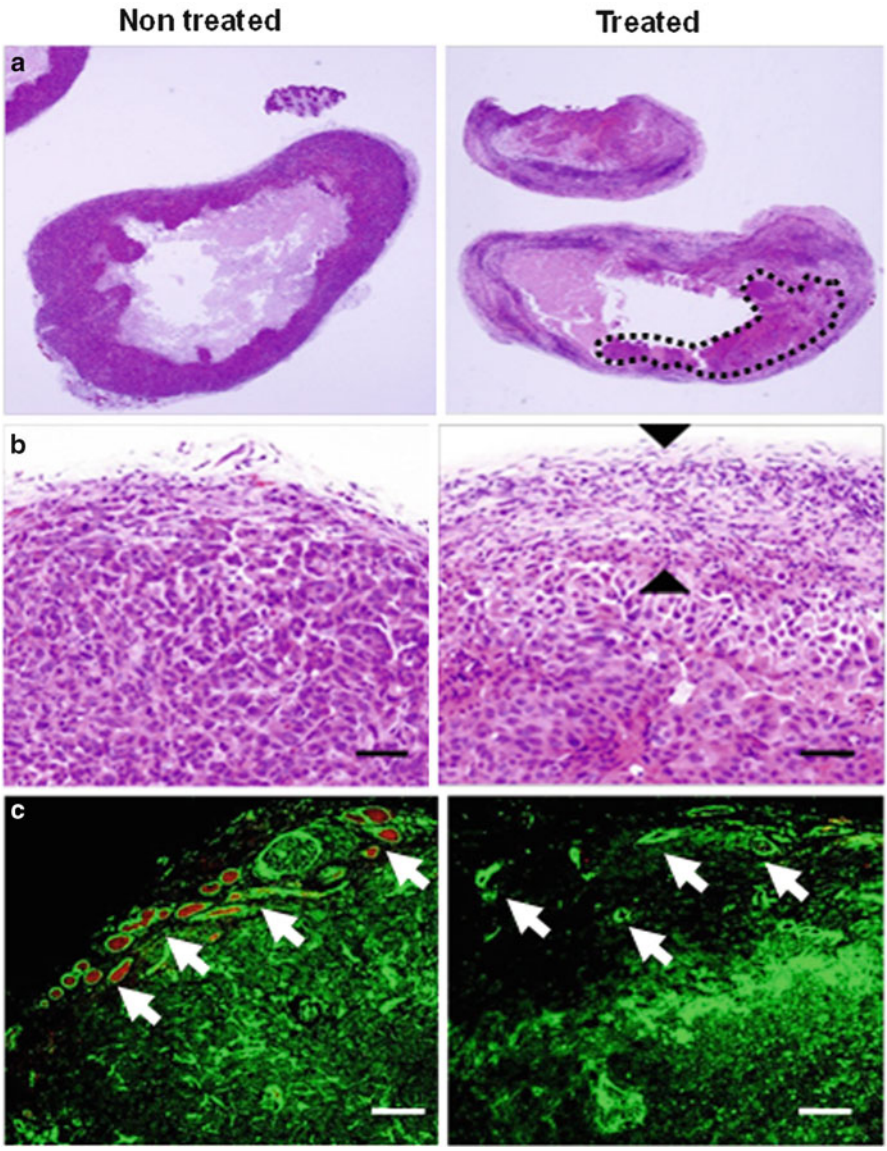


Fig. 6.6 Histopathological features of SUIT2 tumors after anti-collagen 4 immunoconjugate treatment.**(a)** Hematoxylin and eosin staining of non-treated (left) and immunoconjugate-treated (right) SUIT2-tumors. A non-necrotic viable lesion in the treated tumor is enclosed by a dotted line. **(b)** The fibrotic capsule width in the treated tumor is indicated between black arrowheads. **(c)** Tumor vessels were examined by the CD31 (red) collagen IV (green) double-staining techniques. White arrows indicate tumor vessels or their traces in the boundary area. Scale bar: 100μm

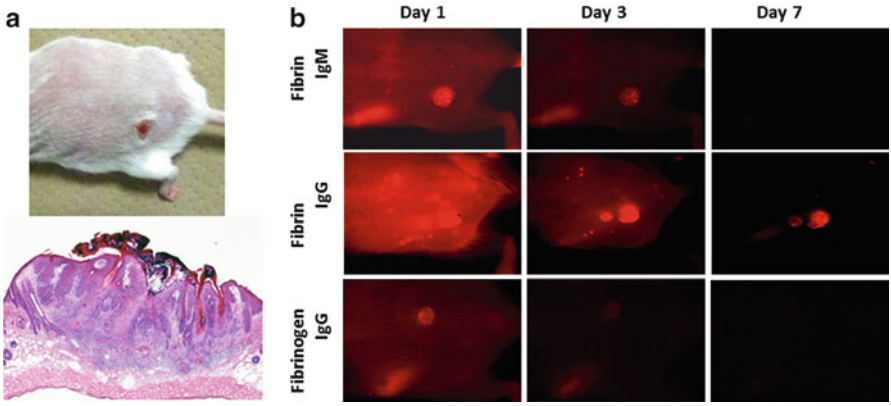
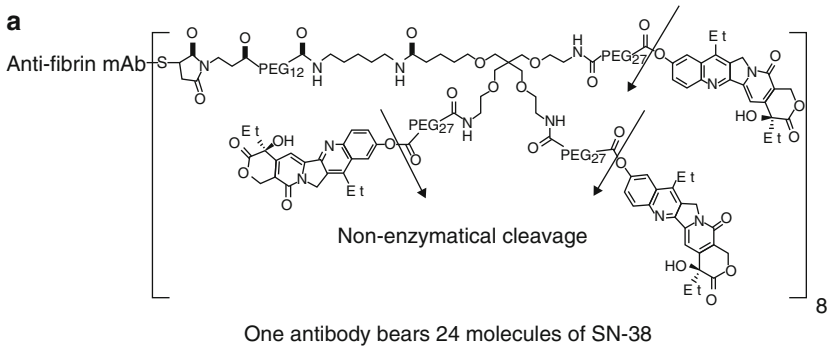


Fig. 6.7 **(a)** Chemical skin carcinogenesis. Mouse bearing a tumor (upper) and hematoxylin-eosin staining (lower) were shown. **(b)** In vivo systemic imaging analysis of Alexa-647-labeled anti-fibrin IgM (upper), Anti-fibrin chimeric mAb (middle) or anti-fibrinogen mAb (lower) on Days 1, 3 and 7 after injection. Arrows indicate each tumor position

and was eliminated by day 7, but the chimeric IgG was still highly retained (Fig. 6.7b). The use of human chimera is beneficial for clinical application to avoid human anti-mouse neutralizing antibodies (HAMA) and allergic reaction in human. In addition, because of the rapid blood clearance and low penetration of IgM compared with IgG [42], IgM is not suitable as a drug delivery vehicle. The branched composition had one maleimide for attachment of mAb, one PEG_{12} spacer, and three PEG_{27} ester bonds for attachment of three SN-38 molecules (Fig. 6.8a). There were approximately eight thiol residues able to react with the maleimide in the reduced mAb. The calculated drug (SN-38)/mAb ratio of the immunoconjugate was about 24. This immunoconjugate exerted significantly stronger antitumor activity compared with CPT-11 (Fig. 6.8b). Although treatment-related body weight loss was observed in mice treated with each drug, there was no significant difference between control groups and CPT-11 or the immunoconjugate treatment group. After injection of the immunoconjugate, the concentration of total SN-38 (antibody bound and unbound form) and free SN-38 (unbound form) in plasma gradually declined within a week, whereas CPT-11 showed rapid clearance (Fig. 6.9a). Significantly high concentrations of total and free SN-38 were detected in tumor tissues treated with the immunoconjugate for a long time compared to CPT-11 (Fig. 6.9b). The second significant observation of the treatment was a change in the gross tumor color from reddish to white (Fig. 6.9c). There was no clear change of fibroblast or macrophage, which plays an important role for tumor progression [43, 44]. It was found that discontinuation and irregularity comprising a mixture of narrowness and enlargement of the tumor vessels were manifested after treatment with the immunoconjugate (Fig. 6.9d, e).



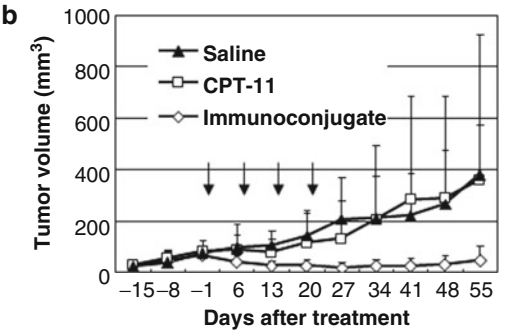


Fig. 6.8 Drug design, anti-tumor effect of anti-fibrin immunoconjugate **(a)** Drug design of immunoconjugate; anti-fibrin mAb-PEG-three branched PEG-(SN-38)3 via ester bond. One antibody bears 24 molecules of SN-38. The arrow indicates the cleavage site for releasing free active SN-38. **(b)** Anti-tumor activity in vivo was examined. Immunoconjugates, CPT-11 or saline, were administered to mice bearing chemical-induced cutaneous cancer via intravenous injection on Day 0, 7, 14, and 21. Arrows indicate day of administration and the curves illustrate the effect of treatment on tumor size. P =0.0005 (CPT-11 vs. immunoconjugate), P < 0.0001 (saline vs. immunoconjugate). Bar = SD

We have made clear that our newly developed tool is not a simple cytotoxic immunoconjugate. Our strategic concept of cancer stromal targeting (CAST) therapy is unique as follows.

1. Newly developed cytotoxic immunoconjugate can extravasate from the leaky tumor vessels selectively and forms a scaffold as it is captured by the tumor stromal network.
2. The immunoconjugate allows the effective sustained release of anticancer agent from the scaffold, and this released anticancer agent is distributed throughout the tumor.

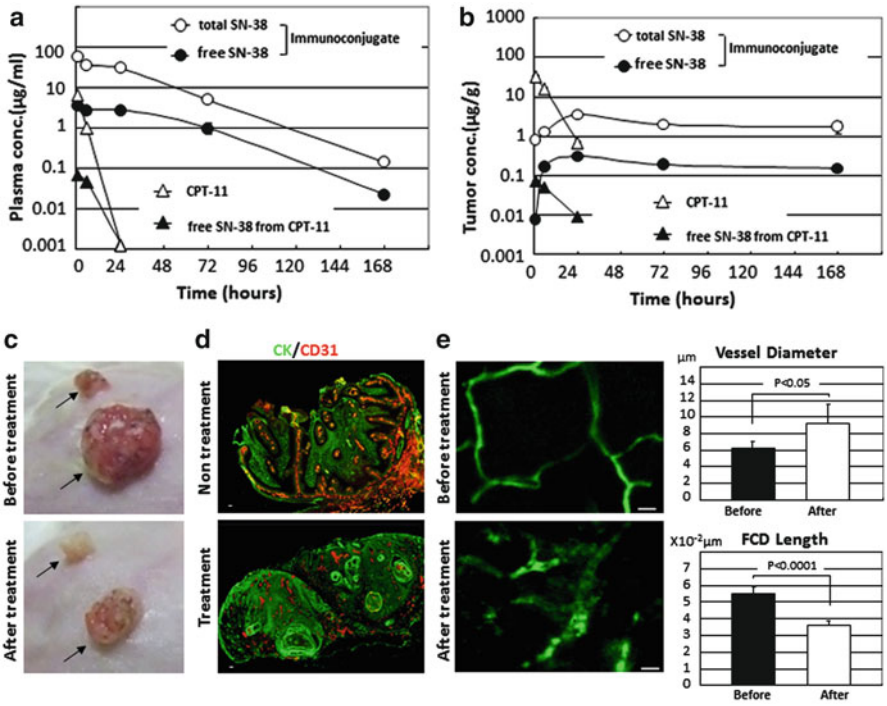


Fig. 6.9 Drug distribution and anti-vascular activity of anti-fibrin mAb conjugated with SN-38 **(a)** Plasma concentration of total SN-38 (bound and unbound form) or CPT-11 and free SN-38 (unbound form) released from the immunoconjugate or converted from CPT-11 was determined using HPLC analysis 1, 6, 24, 72, and 168 h after the injection. **(b)** Tumor concentration of total SN-38 (bound and unbound form) and free SN-38 (unbound form) released from the immunoconjugate, CPT-11 and free SN-38 converted from CPT-11 was determined using HPLC. **(c)** Tumor color changed from reddish to white at 5 days after injection of the immunoconjugate but not CPT-11. Arrows indicate each tumor position. **(d)** Tumor vessels after the injection of the immunoconjugate (Treatment) were examined using CD31 (red) and CK (cytokeratin, green). Untreated mouse (Non treatment) was used as control. bar: 100 μm. **(e)** Tumor vessels before and after the injection were visualized using FITC-dextran by in vivo fibered confocal fluorescence microscopy (left). Quantified vessel diameter and functional capillary density (FCD) length are shown (right). Bar: 20 μm

Consequently, the strategy described above was highly effective in causing arrest of tumor growth due to induced damage to tumor cells and tumor vessels without exerting the drug adverse effect (Fig. 6.10). Cancer stromal targeting therapy, utilizing a cytotoxic agent conjugated to a mAb directed at a specific inert constituent of the tumor stroma, is thus validated as a highly effective new modality of oncological therapy [45].

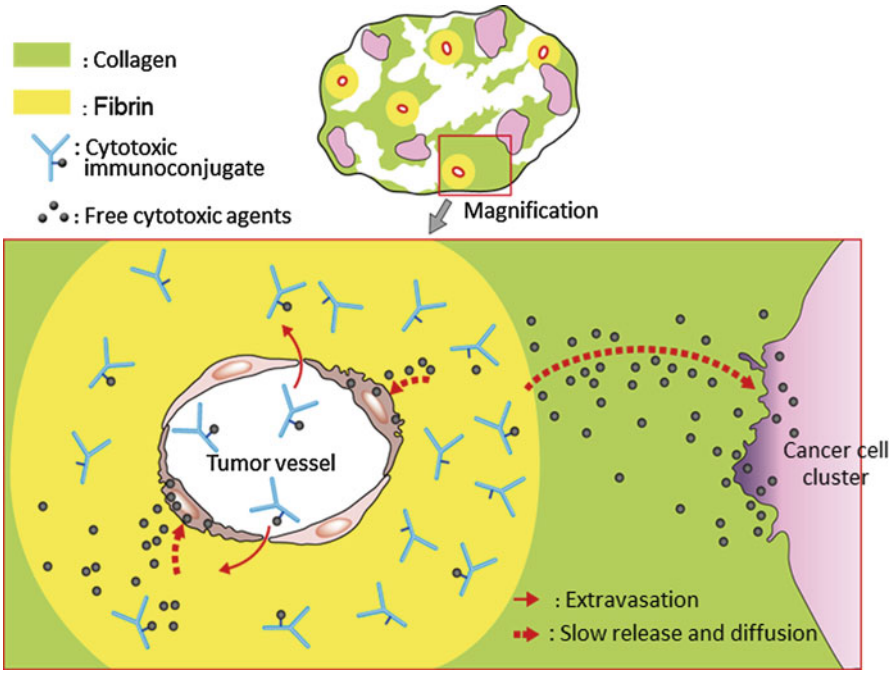


Fig. 6.10 Diagram of new concept of drug delivery using tumor stroma as a ligand. Newly developed anti-fibrin mAb conjugated with SN-38 extravasate selectively from leaky tumor vessels, bind specifically to the fibrin network around the tumor vessels to create a scaffold, and then allow the effective sustained release of SN-38, a time-dependent anti-cancer agent, from the scaffold. Since this released anti-cancer agent is LMW, it is subsequently distributed over the entire tumor-stroma barrier and induces damage not only to tumor cells but also to tumor vessels

Tailored ADC Depending on Quantity of Tumor Stroma

Difference of Tumor Stromal Component Between Malignant Lymphoma and Pancreatic Cancer

Anti-collagen 4 mAb was prepared to evaluate the stromal component. Human malignant lymphoma, RL tumor, consisted of CD20-positive tumor cells and collagen-4-positive blood vessels, which was stained fine linearly but not interspersed fibrously like the intercellular stroma. On the other hand, human pancreatic tumor, SUIT2 tumor reported as the histopathology relatively resembling original human pancreatic cancer [29, 46], consisted of EpCAM-positive cancer cells and collagen-4-positive extracellular component, the latter was composed of both CD31-positive blood vessel wall and high amount of CD31-negative stroma.

Preparation and Characterization of Cell-Targeting or Stroma-Targeting Immunoconjugate-PEG-SN-38 via a Carbamate Bond or Ester Bond

To specify the appropriate immunoconjugate therapy against malignant lymphoma or pancreatic cancer, we prepared two types of the conjugates, one being mAb-PEG-SN-38 via a carbamate bond [47] (Fig. 6.11a) and another being mAb-PEG-SN-38 via an ester bond [29, 30] (Fig. 6.11b). Consequently, six types of immunoconjugates, anti-CD20, anti-EpCAM, anti-collagen 4, or mAb-SN-38 via a carbamate bond or an ester bond, were obtained. The average number of conjugated SN-38 per one mAb (drugs/mAb), the range from 7 to 8.5, was shown in Fig. 6.11c. There was no clear loss of antigen-binding activity of each mAb after the conjugation (Fig. 6.11d). In in vitro release experiment, both bonds can be cut by a carboxylesterase localized in the cytoplasm to release SN-38 inside various cells (Fig. 6.11e). However, in physiological condition (non-enzymatically hydrolysis), the immunoconjugate prepared via an ester bond can release SN-38 gradually and effectively. In contrast, the immunoconjugate via a carbamate bond cannot release SN-38 effectively in the conditions outside the cells (Fig. 6.11e). We then evaluated the release profiles of SN-38 from both type of immunoconjugate in mouse blood, which contained high amounts of carboxylesterase [48]. In in vivo analysis of the mouse plasma, the concentration of unbound SN-38 or bound and unbound of SN-38 from the immunoconjugate via an ester bond or a carbamate bond at 72 h after the mice tail vein injection were shown. Most of the immunoconjugates in the mouse blood were protected from the enzymatic cleavage (Fig. 6.11f). Next, we examined the difference between carbamate bond and ester bond in the combination with cell-targeting or stromal-targeting antibody by the cytotoxicity assay. In RL cells, anti-CD20 immunoconjugate via carbamate bond showed strong cytotoxicity compared to anti-CD20 immunoconjugate via ester bond significantly (anti-CD20 mAb is known to possess high internalization ability). In SUIT2 cells, although no significant difference, anti-EpCAM immunoconjugate via carbamate bond had a lower tendency in the cytotoxic effect compared to anti-EpCAM immunoconjugate via ester bond (anti-EPCAM mAb is known to possess low internalization ability). Anti-collagen 4 immunoconjugate via ester bond showed higher cytotoxic activity than anti-collagen 4 immunoconjugate via carbamate bond in both cells significantly (Table 6.1). These results indicated that a carbamate bond was useful for the immunoconjugate linker to work inside of the cells and an ester bond to work outside the cells.

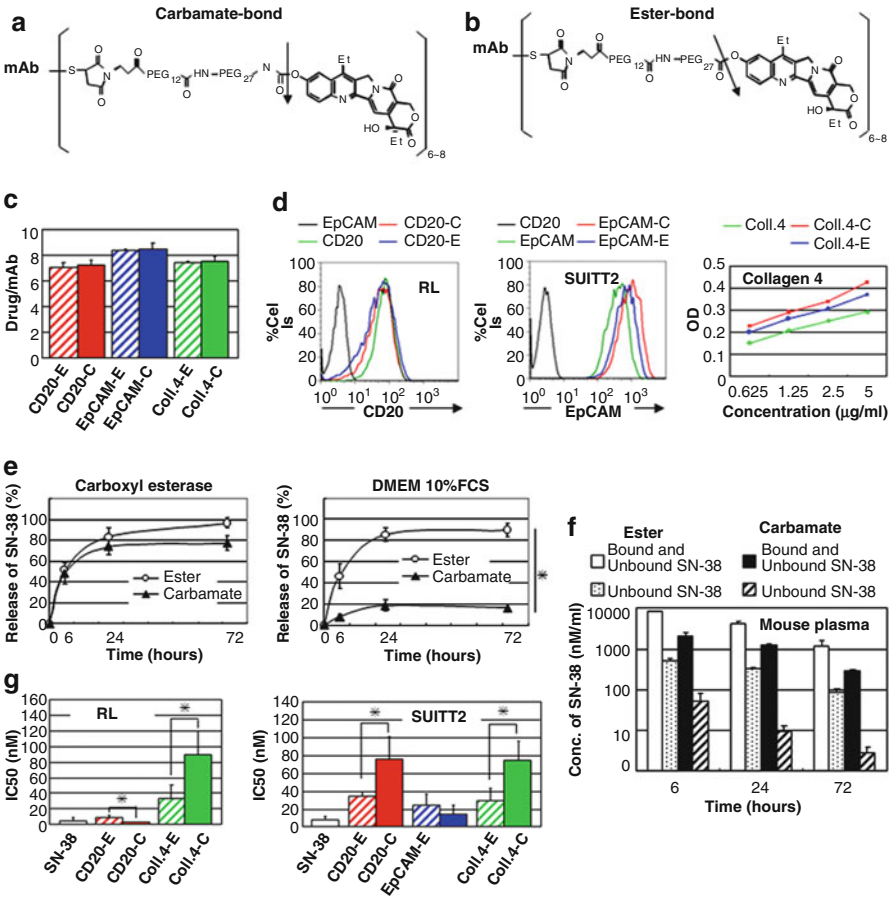


Fig. 6.11 Preparation and characterization of 2 types of immunoconjugqates-PEG-SN-38 via carbamate-bond and eser-bond. **(a) (b)** Drug design of 2 types of immunoconjugates; mAb-PEG-SN-38 via carbamate-bond **(a)** and mAb-PEG-SN-38 via ester-bond **(b)**. One antibody bears 6-8 molecules of SN-38. The arrow indicates the cleavage site for releasing free active SN-38. **(c)** The average number of conjugated SN-38 per one mAb was shown (n=3). Bar=SD. **(d)** Antigen-binding activity of the mAb before and after the conjugation was shown. Anti-CD 20 and EpCAM mAb were examined by FACS analysis using RL cells and SUIT2 cells respectively. Anti-collagen 4 mAb was examined by ELISA using purified protein. **(e)** In vitro release of SN-38 from two types of immunoconjugates in carboxylesterase-contained solution (left) and DMEM 10%FCS (right) (n=3). Bar=SD, *P<0.05. (f) Concentration of bound and unbound form of SN-38, and unbound form of SN-38 from two types of immunoconjugates in the moue plasma at 6, 24, 72 h after the mice tail vein injection, were shown (n=3). Concentrations of SN-38 were determined by HPLC. Bar=SD. **(g)** In vitro cytotoxicity with immunoconugates in RL cells (left) or SUIT2 cells (right) was shown (n=3). Bar=SD, *P<0.05

Table 6.1 IC50 of free SN-38 and SN-38 conjugated to mAb (immunoconjugate) for malignant lymphoma and pancreatic cancer cell line

		SN-38 conjugated to mAb					
Malignant lymphoma cell lines	Free SN-38	CD20				Collagen 4	
		Ester	Carbamate			Ester	Carbamate
RL	4.6±3.7	8.7±2.9 vs. 2.1±1.0*				34±17 vs. 90±30*	
		SN-38 conjugated to mAb					
Pancreatic cancer cell lines	Free SN38	CD20		EpCAM		Collagen 4	
		Ester	Carbamate	Ester	Carbamate	Ester	Carbamate
SUIT2	7.8±3.6	35±5 vs. 77±25*		24±13 vs. 15±9		29±15 vs. 75±22*	

IC50 (50% cell survival) (nM), Mean±standard deviation (n=3), *P<0.05

Cell-Targeting or Stroma-Targeting Immunoconjugate-PEG-SN-38 via Carbamate Bond or Ester Bond Differs Drastically in Their Antitumor Effects Depending on Tumor Stromal Component in Mice

Three mAbs conjugated with SN-38 via carbamate bond or ester bond (administered once, at an equivalent SN-38 dose of 3 mg/kg) were evaluated in order to know their antitumor effects in RL (CD20-positive stroma-poor human malignant lymphoma), SUIT2 (EpCAM-positive stroma-rich human pancreatic tumor). In RL lymphomas, cell-targeting anti-CD20 mAb-SN-38 via carbamate bond showed superior antitumor activity compared to anti-CD20 mAb-SN-38 via ester bond after the treatment (Fig. 6.12a). Stroma-targeting anti-collagen 4 mAb-SN-38 via ester bond showed significant superior antitumor activity as compared to saline as control, but inferior to anti-CD20 mAb-SN-38 via carbamate bond (Fig. 6.12a). On the contrary to RL tumor, in SUIT2 tumor, the most potent antitumor activity was obtained by the stroma-targeting anti-collagen 4 mAb-SN-38 via ester bond (Fig. 6.12b). However, there was no significant difference of antitumor activity between anti-EpCAM mAb-SN-38 via carbamate bond and via ester bond, whereas the antitumor activity of anti-collagen 4 mAb-SN-38 via carbamate bond was inferior to that of anti-collagen 4 mAb-SN-38 via ester bond (Fig. 6.12b). These results clearly indicated that in stroma-poor solid tumors like malignant lymphoma, cytotoxic immunoconjugate should target to the tumor cell surface and ACA should be conjugated to mAb through carbamate bond which can be specifically cut by a carboxylesterase inside the tumor cell after the internalization. On the other hand, in stroma-rich tumors, the immunoconjugate should target to the stroma within tumor tissue and ACA should be attached to the mAb via ester bond which can be cut gradually outside the tumor cell following the accumulation of the cytotoxic immunoconjugate in

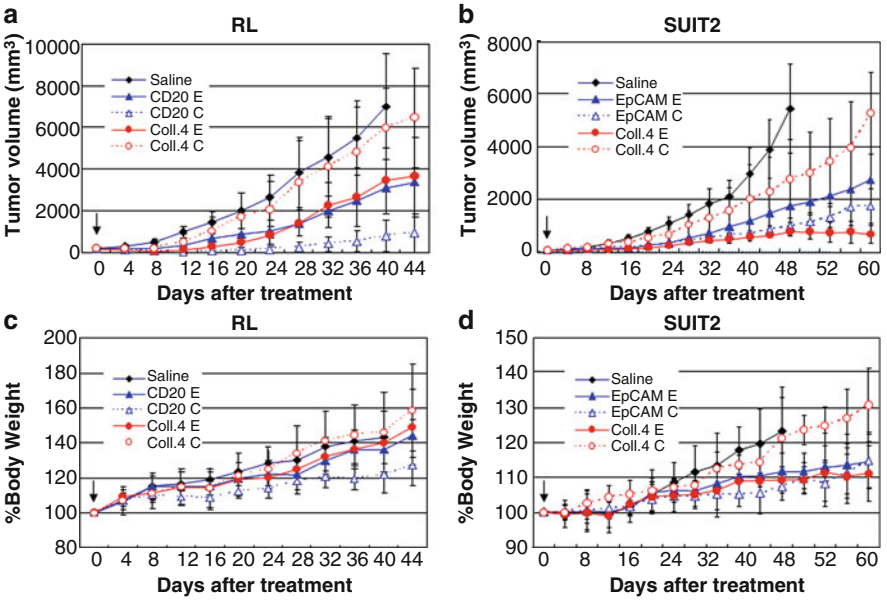


Fig. 6.12 Antitumor effects of immunoconjugates-PEG-SN-38 in the combinations of anti-cell or anti-stroma targeting, carbonate-bond or ester-bond. **(a) (b)** Anti-tumor activities and **(c) (d)** percent changes of body weight were examined. In animal models of RL (A)(C) and SUIT2 (B)(D), the 6 types of immunoconjugates (combined anti-CD20 mAb=CD20, anti-EpCAM mAb=EpCAM or anti-collagen 4 mAb=Coll.4 and ester-bond=E or carbamate-bond=C), or saline as control, were administered once at an equivalent SN-38 dose of 3 mg/kg to separate groups of mice (n=5) by intravenous bolus injection to the mice on day 0. Arrows indicate day of administration and the curves illustrate the effect of treatment on tumor size. $P < 0.0001$ (Saline vs. CD20-E or CD20-C, CD20-C vs. CD20-E, Coll.4-E or Coll.4-C in RL tumor; saline vs EpCAM-E, EpCAM-C or Coll.4-E, Coll.4-E vs. EpCAM-C or Coll.4-C, EpCAM-C vs Coll.4-C in SUIT2 tumor), $P < 0.001$ (Saline vs. Coll.4-E in RL tumor; saline vs. Coll.4-C, Coll.4-E vs. EpCAM-E in SUIT2 tumor). Bar=SD

the tumor stroma. It is remarkable that the feature of tumor stromal component influence the outcome of the two types of immunoconjugation drugs, cell-targeting mAb-PEG-SN-38 via carbamate bond, or stroma-targeting mAb-PEG-SN-38 via ester bond.

Regarding normal tissue distribution and elimination of antibodies and SN-38, there was no difference among immunoconjugates on day 7 after the administration. The dose in this study did not cause significant toxicity as shown by the change of mouse body weight (Fig. 6.12c, d). Moreover, there was no hepatotoxicity, nephrotoxicity, or bone marrow toxicity in mice treated with all three immunoconjugates as compared to controls (Fig. 6.12e). In addition, no autoimmune disease-like adverse effects such as arthritis and nephritis were observed in the administration of anti-collagen 4 mAb, whereas anti-collagen 2 mAb combined with lipopolysaccharide caused severe arthritis [49](Fig. 6.12f).

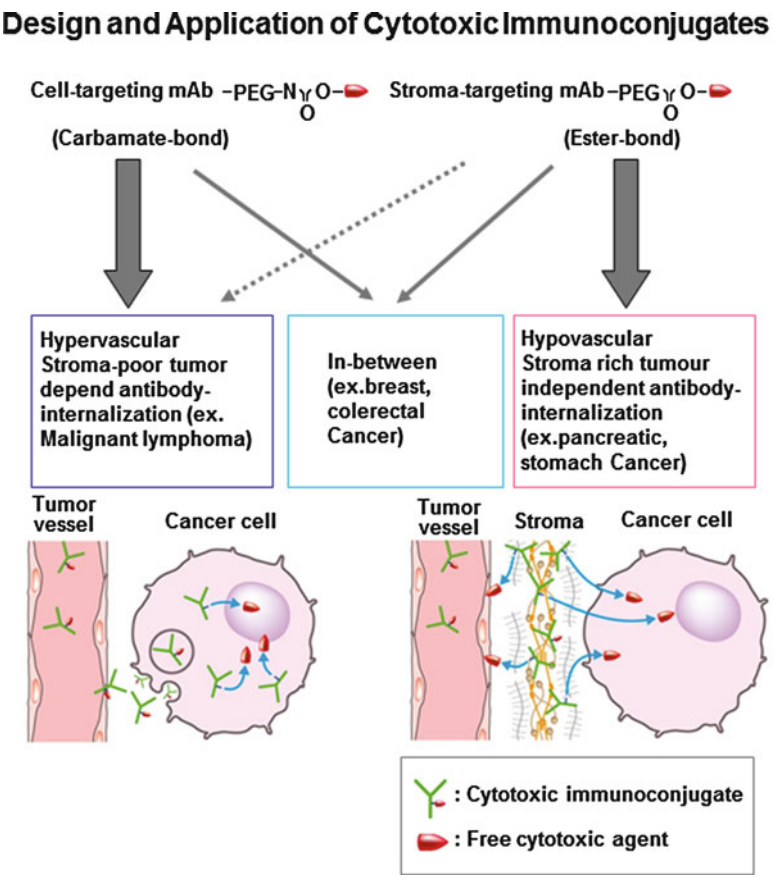


Fig. 6.13 Diagram of Immunoconjugate strategy to tumor tissue component and characteristic of cancer-cells. Design and application of cytotoxic immunoconjugates. SN-38 conjugated cell-targeting monoclonal antibody (mAb) via carbamate-bond is suitable for hypervascular, stroma-poor tumor dependent antibody-internalization. SN-38 conjugated stroma-targeting mAb via ester-bond is suitable for hypovascular, stroma-rich tumor independent antibody-internalization

In general, human cancer is classified into three types according to the tissue component. One is hypervascular stroma-poor tumor such as malignant lymphoma, the second is hypovascular stroma-rich tumor such as pancreatic cancer and stomach cancer, and the third is intermediated tumor between the two types such as breast cancer and colorectal cancer. We thus propose the new therapeutic strategy of immunoconjugates to the feature of individual tumor as tissue stromal component: (1) cell-targeting mAb conjugated with ACAs via carbamate bond for hypervascular and stroma-poor tumor and (2) stroma-targeting mAb conjugated with ACAs via ester bond for hypovascular and stroma-rich tumor, both cell-targeting immunoconjugate via carbamate bond and stroma targeting via ester bond for intermediated type of tumor [49] (Fig. 6.13).

Conclusion

Although there have been numerous reports of genetic and phenotype changes in tumors, a large body of pathological and clinical evidence indicates that there are no pivotal changes in tumor cells that distinguish them from normal dividing cells. Unlike in the case of using antibiotics against bacterial infection, therefore, ACAs need to be delivered selectively to tumor tissues and should be kept there long enough to reproduce the concentrations they reach in the Petri dish, which is a closed space where the cytocidal effects of any ACAs including molecular targeting agents are very strong. In the body, however, administered ACAs are cleared with the passage of time. Furthermore, as described in the main part of this topic, most human cancers possess abundant stroma that hinders the penetration of DDS including ACA-conjugated antibodies specific to surface antigens on cancer cells. We are now concerning that current studies mainly based on molecular and cellular biology while ignoring pathophysiology and pharmacology may be leading the development of antitumor drugs in the wrong direction. The present discovery by a hybrid of stromal biology with organic chemistry may open a new field of science and produce many and useful treatment modalities in the area of oncology, cardiovascular diseases, and inflammation.

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Chapter 7
Cancer Cell Respiration: Hypoxia and pH in Solid Tumors

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Abstract Tumor cells require large amounts of oxygen and nutrients for rapid proliferation. Blood vessel growth is not fast enough to meet these requirements, however, resulting in low oxygen and glucose concentrations in tumor tissues. Tumor hypoxia is closely related to increased levels of glycolysis and in turn accumulation of lactate in the tissue. Therefore, tumor tissue pH decreases in the hypoxic tumors. Gene expression profiles in tumor tissues change in hypoxic environments in order to adapt to the acidic conditions. These genes are involved in angiogenesis, cell growth, apoptosis, and glycolysis. Gene induction is primarily controlled at the level of transcription by hypoxia-inducible factor-1 (HIF-1). HIF-1α is stabilized under hypoxic conditions and forms a heterodimer with HIF-1β. HIF-1 then binds to a hypoxia response element (HRE) within a hypoxia-specific promoter for gene expression. Tumor-specific drug and gene delivery systems have been developed using hypoxia-inducible regulation systems. In this chapter, strategies for pH-specific drug delivery and gene therapy in hypoxic tumors are described.

Introduction

The term "tumor" comes from the Latin word for "swelling," describing a mass in the body caused by uncontrolled, progressive multiplication of cells that have no physiological function. The medical term for tumor is "neoplasm," which indicates a new, abnormal tissue growth in animals or plants. Tumor cells are derived from normal cells that have undergone changes in their DNA sequences that render them

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unresponsive to normal biologic controls for growth limitation. These cells continue to grow faster than the surrounding tissues, resulting in formation of a tumor. In general, neoplasms can be classified as benign or malignant. Tumors that grow slowly and do not infiltrate into the surrounding tissues or metastasize are benign neoplasms. In contrast, rapidly growing cells which infiltrate or metastasize are malignant neoplasms, or cancer.

Approximately 90 % of all known cancers are solid tumors [1]. In general, a single mutation does not lead to significant morbidity and mortality. Rather, accumulated genetic changes can transform a normal cell into a tumor cell. These transformed cells then proliferate continuously to form a mass (solid tumor) within organs such as the lungs, stomach or liver. As the tumor rapidly grows, the microenvironment of the tumor cells changes due to consumption of large amounts of oxygen, resulting in hypoxia [2, 3]. As a result, a solid tumor that is larger than 1 mm in diameter requires its own blood supply due to the diffusion limits of oxygen, nutrients, and growth factors [4].

Avascular tumors tend to have necrotic regions resulting from severe oxygen deprivation [5]. Due to the diffusion limits of the normal blood supply, rapidly growing tumor cells in particular suffer from a lack of oxygen and nutrients since these components only diffuse over a distance of approximately 150 μm after they are released from the blood vessel [6]. Therefore, tumor cells beyond 150 μm from the surface of the tumor could undergo hypoxia-related cellular responses such as metabolic switch, vasodilation, migration to avoid cell death, and induction of angiogenesis [6]. Among these, angiogenesis is the most effective response for overcoming hypoxic stress [7]. As a result, hypoxia has emerged as a major physiologic regulator of neovascularization [8].

There are two types of vessels that reside near tumors: the existing vessels within normal tissues into which the tumor has invaded and tumor microvessels arising from increased expression of angiogenic factors produced by hypoxic tumor cells. These two types of vessels develop structural and physiologic abnormalities that are characteristic of tumor microvasculature [9], making them an attractive target for exploitation by vascular-targeted anticancer agents [10].

Hypoxia has various effects on tumor cells and is often involved in drug resistance. Drug-resistant genes such as the multidrug resistance-1 (MDR-1) gene are induced in hypoxic tumors [11]. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of hypoxia-induced gene expression [12, 13] and is involved in transcriptional regulation of various genes including vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) [14]. Glucose deprivation also induces expression of specific genes such as GRP78 and GRP94, which are glucose-regulated proteins (GRPs) [15]. GRP78 and GRP94 are induced when glucose concentrations are low, increasing cell viability in tumor tissues that have undergone oxygen and glucose deprivation (OGD) [15–17]. The expression of GRP78 and GRP94 is regulated primarily at the transcriptional level. Typically, the promoters of GRPs contain hypoxia response elements (HREs), suggesting that the induction of GRPs is due to hypoxia in addition to low glucose concentrations [16, 17].

Another important characteristic of tumor tissue is low pH. The pH around solid tumors is lower than that of normal tissue [18, 19]. In the setting of hypoxia, tumor cells produce adenosine triphosphate (ATP) primarily via glycolysis rather than the tricarboxylic acid (TCA) cycle. Pyruvate from glycolysis is then converted into lactate by fermentation. During this process, a large number of H^+ ions are produced and transported out of the cells. In normal tissues, these H^+ ions are easily washed away by blood flow; however, limited blood flow in the tumor environment results in a decreased pH. This characteristic may be useful for tumor-specific drug delivery when using pH responsive materials.

Hypoxia and pH are useful conditions for targeted drug therapy. In this chapter, the characteristics of tumor physiology such as tumor hypoxia and pH are discussed and targeted drug delivery methods are introduced.

Cellular Response to Tumor Hypoxia and pH

Mechanism of Cellular Response to Tumor Hypoxia

Tumor growth is characterized by rapid cell proliferation and metabolism, which requires increased levels of oxygen and nutrients. However, this growth occurs at a faster rate than blood vessel formation. The oxygen partial pressure at the core of a tumor is typically less than 2.5 mmHg, indicating severely hypoxic conditions [20]. Hypoxia induces transcription of a series of genes, most of which are angiogenic and antiapoptotic [13, 19]. HIF-1 begins to accumulate at an oxygen concentration of 50 mmHg, which in turn promotes gene transcription [21].

Low oxygen concentrations have a profound effect on the respiratory chain in the mitochondria, resulting in the formation of reactive oxygen species (ROS) [22]. ROS damage cellular proteins and cause cell death in tumor tissues. In addition, ROS may be required for stabilization of HIF-1α under hypoxic conditions, since antioxidants tend to destabilize HIF-1α [23, 24].

HIF-1, a heterodimer of HIF-1α and HIF-1β [25], is a key transcription factor for hypoxia-specific gene expression [12]. HIF-1β is also known as aryl hydrocarbon nuclear translocator (ARNT) [25]. It is a constituent subunit of HIF-1 and its levels are not significantly changed under hypoxic conditions. However, HIF-1α has variable stability depending on the oxygen concentration. Therefore, hypoxia-specific gene expression is primarily controlled by HIF-1α. As a heterodimer, HIF-1 binds to hypoxia response elements (HREs) on the promoters of target genes, thereby facilitating the initiation of transcription [26]. HIF-2 and HIF-3 have also been identified as homologs of HIF-1 [26]. While the expression of HIF-2 and HIF-3 are tissue specific, HIF-1 is ubiquitously expressed. Thus, gene expression in cases of tumor hypoxia is likely primarily mediated by HIF-1.

HREs are present in the promoter regions of genes responsible for angiogenic growth, glucose metabolism, cell proliferation, and apoptosis. For example, VEGF

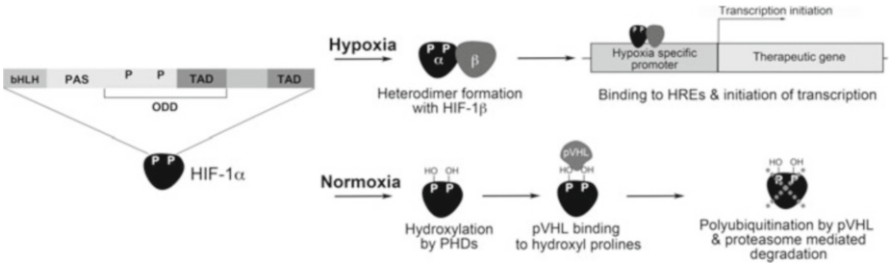

Fig. 7.1 Mechanism of HIF-1 stabilization under hypoxic conditions. *bHLH* basic helix-loop-helix, *PHDs* prolyl hydroxylases, *HIF-1α* hypoxia-inducible factor-1α, *HIF-1β* hypoxia-inducible factor-1β

is a key growth factor for angiogenesis. The VEGF promoter contains HREs and its expression is induced by HIF-1 in hypoxic cells [27]. The insulin-like growth factor-2 (IGF-2) and transforming growth factor-α (TGF-α) genes are also induced by HIF-1, increasing the survival rates of cells under hypoxic conditions [28–30]. Tumor hypoxia facilitates both the adaptation of cells to low oxygen conditions and apoptosis of cells. Some proapoptotic genes are induced by HIF-1, such as the Bcl-2/adenovirus EIB 19-kDa interacting protein 3 (BNip3) gene, which also contains HREs in its promoter region [31]. In cases of tumor hypoxia, ATP production occurs primarily via glycolysis rather than the TCA cycle. Glycolysis of a glucose molecule produces 2 ATPs, while the TCA cycle and oxidative phosphorylation produces 32 ATPs. To compensate for this low efficiency, glucose uptake and glycolysis must be upregulated. Thus, glycolytic enzymes and glucose transporter genes should be induced under hypoxic conditions [32, 33].

The stability of HIF-1 is regulated by prolyl hydroxylases (PHDs) [34–36] (Fig. 7.1). To date, three PHD isoforms have been reported including PHD1, PHD2, and PHD3 [37]. The targets of hydroxylation by PHDs are specific proline residues in the oxygen-dependent degradation domain (ODDD) of HIF-1α [38]. These residues include proline 402 and 564 (Pro402 and Pro564) [35, 39] and are located in a specific consensus sequence (LXXLAP). This sequence is conserved in HIF-2 and HIF-3. The hydroxylation reaction is performed when PHDs split oxygen molecules, and one oxygen atom is subsequently used for the hydroxylation of proline. Therefore, PHDs are more efficient in hydroxylating the ODDD under normoxic than hypoxic conditions. The hydroxylated ODDD is then recognized by von Hippel-Lindau protein (pVHL) [40]. pVHL is an ubiquitin ligase that serves to multi-ubiquitinate the ODDD. Multi-ubiquitinated HIF-1α is then degraded by a proteasome-mediated pathway. Under hypoxic conditions, PHDs are less active and the degradation of HIF-1α is inhibited, thereby increasing the half-life of HIF-1α.

Hypoxic conditions have also been shown to contribute to posttranscriptional regulation, as the stability of mRNA after transcription may vary depending on the oxygen concentration. It was previously reported that the VEGF mRNA had a longer half-life under hypoxic conditions compared with normoxic conditions [41].

The mechanism by which this occurs has been partially characterized in that greater stability of the VEGF mRNA requires both a 5′-untranslated region (UTR) and a 3′-UTR [42]. It was previously shown that the same protein binds to both the VEGF 3′-UTR and the erythropoietin (Epo) 3′-UTR [43, 44]. This Epo RNA-binding protein (ERBP) binds to the Epo 3′-UTR and stabilizes the mRNA under hypoxic conditions. This suggests that a common mechanism is involved in Epo and VEGF mRNA stabilization.

Similar hypoxia-mediated RNA stabilization has been identified for the HIF-1α mRNA and the tyrosine hydroxylase (TH) mRNA [44, 45]. The 3′-UTRs of genes responsible for iron uptake such as the transferrin receptor or ferritin genes have been reported to stabilize these mRNAs under hypoxic conditions [46]. The transferrin receptor and ferritin 3′-UTRs contain iron response elements (IREs) that may be responsible for this hypoxia-specific mRNA stabilization. Interestingly, when the UTRs were separated and linked to other mRNAs such as reporter gene mRNAs, the UTRs retained their stabilizing effects on the newly linked mRNAs under hypoxic conditions [44, 47]. These data suggest that these UTRs may be useful for the stabilization of therapeutic mRNA under hypoxic conditions in tumors.

Recently, RNA interference (RNAi) has been identified as an important mechanism for gene regulation. Tumor hypoxia induces specific microRNAs (miRNAs) and regulates gene expression. miRNAs bind to the 3′-UTR of the target mRNAs and facilitate their degradation, which is mediated by an RNA-induced silencing complex (RISC). An increasing number of hypoxia-specific miRNAs have been identified [48], some of which were shown to be directly involved in tumor growth. The most well-characterized miRNAs are miR-21 and miR-210 [49–53]. miR-21 is overexpressed in various cancers including glioblastoma, hepatoma, and breast cancer [49, 54, 55]. The target genes of miR-21 include programmed cell death protein 4 (PDCD4) and phosphatase and tensin homolog (PTEN) [54, 56]. PDCD4 is a proapoptotic protein, and inhibition of its expression by miR-21 increases tumor growth and invasiveness. PTEN is a tumor suppressor whose expression is decreased in various types of cancers. miR-21 is induced under hypoxic conditions, likely via HIF-1 binding sites in the promoter region [57]. However, it has also been suggested that miR-21 may be induced by other transcription factors such as activating protein-1 (AP-1) in a HIF-1-independent manner [58]. miR-210 is also induced by HIF-1 in cases of tumor hypoxia [59], and its expression is thought to promote angiogenesis and cell survival. An increasing number of miRNAs have been identified as requirements for angiogenesis in tumors, thus rendering them important targets for therapeutic treatment.

Mechanism of Acidification of Tumor Tissue

HIF-1 overexpresses glycolytic or tumor metabolic phenotypes, H^+/lactate monocarboxylate transporters (MCTs), carbonic anhydrase (CA) eco-enzyme, and Na^+/H^+ exchanger 1 (NHE1) molecules, which results in the acidification of the tumor

extracellular environment. First, HIF-1 upregulates the expression of glycolytic enzymes and glucose transporter proteins [60]. As a result, a larger number of glucose molecules are metabolized via glycolysis to produce pyruvate molecules, which is an important alteration necessary for cancer cell survival and proliferation under conditions with limited oxygen and nutrient supply. Under conditions amenable to aerobic metabolism, the resulting pyruvates enter the TCA cycle to produce ATP. However, under hypoxic conditions (anaerobic metabolism), pyruvate is converted into lactic acid directly through the lactate fermentation pathway. About 90 % of pyruvate is converted to lactic acid, which is eventually transported outside of the cell membrane via MCTs [61, 62]. In some tumors, glycolysis and lactate fermentation are increased regardless of oxygen concentration [63]. This is due to the mutations in the genes, which are responsible for oxidative phosphorylation. This defective respiration in mitochondria induces lactate fermentation under normal oxygen concentration, and this effect is referred to as the "Warburg Effect" (aerobic glycolysis). The mutations in succinate dehydrogenases, fumarate hydratase, and isocitrate dehydrogenase reduce the rate of TCA cycle in mitochondria. In addition, these mutations facilitate the accumulation of succinate and fumarate. Succinate and fumarate inhibit PHDs, which are responsible for degradation of HIF-1α under normoxia. Due to low activity of PHDs, HIF-1α is stabilized even under normoxia condition and induces expression of the glycolytic enzymes. In addition, the mutation of the p53 tumor suppressor gene is a hallmark of tumor progress in many cancers. In normal cells, wild-type p53 increases oxidative phosphorylation by inducing cytochrome c oxidase. However, the mutation of the p53 gene increases glycolysis and decreases oxidative phosphorylation [64]. Therefore, glycolysis is induced even in the presence of adequate oxygen in tumors.

During the conversion of pyruvate to lactic acid, a large number of H^+ ions are produced and transported outside of the cell membrane, eventually resulting in acidification of the tumor interstitial space. While the transported H^+ ions would normally be washed away by relatively rapid blood flow, resulting in preservation of the normal interstitial pH [65], the blood flow rate in tumor tissue is often decreased due to abnormal proliferation of tumor cells with limited vascularization. As a result, the transported H^+ ions accrue within the tumor interstitial space [66]. Additionally, HIF also induces eco-enzyme CA, which converts CO_2 into carbonic acid. Consequently, H^+ ions remain in the tumor extracellular fluid when HCO_3^- is taken up via AE molecules on the cell membrane [67]. Finally, hypoxia enhances the expression and activity of NHE1 molecules on the cell membrane. NHE1 can exchange one extracellular Na^+ for one intracellular H^+ ion. Figure 7.2 shows the influence of hypoxia on acidification of the tumor extracellular environment. To confirm that the pH of the tumor tissue is more acidic than normal tissue, pH was measured using a pH electrode [68]. Mean pH values in human subcutaneous tissue and muscle tissue in dogs and rats were 7.52, 7.32, and 7.43, respectively. On the other hand, there was a wider variety of more acidic pH values in malignant tissues, ranging from a pH of 5.8–7.6 in both human and rodent tumors [69]. These findings strongly support the idea that lower tumor extracellular pH (pH_e) can be utilized as a tumor-specific prognostic factor and therapeutic target. Analysis of the relationship between tumor pH_e and tumor volume showed a weak but significant

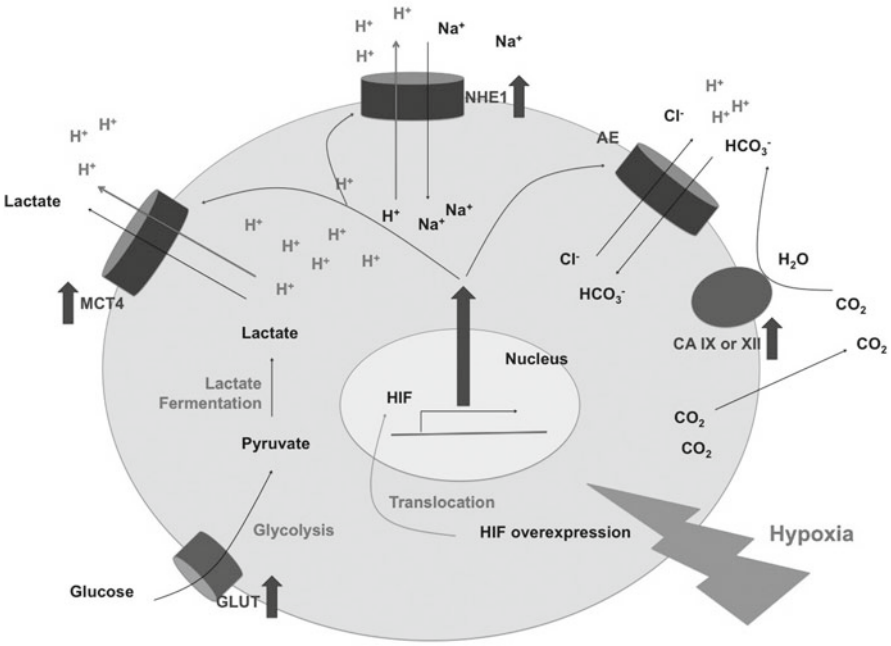

Fig. 7.2 Relationship between hypoxia and acidification of the tumor interstitial space. Under hypoxic conditions, HIF-1 maintains the pH by regulation of NHE1 (Na^+/H^+ exchanger 1), MCT4 (monocarboxylate transporters), and CA (carbonic anhydrase) IX or XII

correlation between the two factors [70]. Tumor pH_e increased as a function of the natural logarithm of tumor volume at a rate of 0.07 ± 0.02 units/ln cm^3 ($p = 0.006$, $r = 0.34$) [71]. However, there was no apparent relationship between tumor histology and pH_e.

These findings demonstrate that not all tumor types have a sufficient pH_e value for effective tumor-specific targeting. Therefore, it is necessary to manipulate the pH_e value in tumor tissues. To this end, glucose administration can decrease the average tumor pH_e from 6.84 to 6.43, with a range of 6.12–6.78 [72]. In addition, inorganic phosphates and m-iodobenzylguanidine (MIBG) can decrease pH_e in cases of glucose-induced hyperglycemia [73]. When glucose is orally administrated to fasting cancer patients in a clinical setting, over half (56 %) of the patients achieved the benefit in terms of pH_e drop [74], demonstrating that pH_e manipulation using glucose administration may be a better strategy for clinical pH-sensitive tumor targeting.

There are both pros and cons to the hypoxia-induced acidic environment of tumor tissues. Acidification of the tumor interstitial space can cause problems with drug permeability and facilitate tumor invasion in some cases. However, it also presents an opportunity for the development of anticancer nanocarriers with pH-sensitive targeting, thereby attenuating the adverse effects of anticancer agents. As a result, several kinds of cancer nanomedicines have been designed.

Hypoxia and pH as Targets for Tumor-Specific Drug Delivery

Most chemotherapy and anticancer agents are not as effective as anticipated because of poor solubility, nonspecific toxicity, lack of tumor selectivity, and multidrug resistance. To overcome these limitations, several nanocarriers have been developed including liposome systems, polymeric micelle systems, and nanoscaled complex systems. Currently, the release kinetics from nanocarriers of anticancer drugs are being concerned. Both fast and slow release kinetics are related to the efficacy of anticancer drugs and drug resistance in cancer cells. Cellular interactions of nanocarriers are also an important concern, given that they should attenuate interactions with normal tissues and increase the amount of time spent in circulation. When nanocarriers approach their target sites, they should interact with and be internalized into cancer cells. Therefore, nanocarrier design demands the presence of a switching mechanism for release kinetics and cellular interactions when the target site is reached. Currently, this property is steering the development of stimuli-responsive or stimuli-sensitive nanocarriers. Stimuli-sensitive nanocarriers are able to respond to small changes in environmental conditions via pathophysiologic signals such as pH, thereby providing them with the desired switching property.

Angiogenic Endothelial Cell-Targeted Drug Delivery

Tumor hypoxia is an excellent target for drug delivery to cancer cells. Specific ligands may be used as targeting moieties, including membrane receptors that are closely related to angiogenesis. Integrin αvβ3, for example, is overexpressed in angiogenic endothelial cells [75]. Proteins that bind to integrin αvβ3 have a consensus sequence of RGD [76] that has been investigated as a target ligand for angiogenic endothelial cells in hypoxic tumors. RGD peptides were directly conjugated to drugs such as paclitaxel or carriers such as PLGA nanoparticles [77]. As a result, systemic administration of RGD conjugated drugs and nanocarriers increased the accumulation of therapeutic agents in tumor tissues [77, 78]. The RGD peptide has since been used for tumor-targeted delivery of anticancer drugs, peptides, nucleic acids, and imaging reagents.

Another example of a hypoxia-inducible membrane receptor is the VEGF receptor. VEGF and its receptors are induced in hypoxic cells [79], and it has been suggested that VEGF produced in these cells may have physiologic effects on hypoxic tissues but not normal tissues [80]. Expression of the VEGF receptor was particularly abundant in endothelial cells undergoing angiogenesis under hypoxic conditions. Therefore, ligands for the VEGF receptor were used to target hypoxic tumors. The VEGF receptor-binding peptides (VRBPs) have been screened using the phage-display library method [81]. Since the identified VRBPs bind to the VEGF receptor directly, they reduce the interaction between VEGF and the VEGF receptor. Thus, VRBPs themselves are antiangiogenic peptides [81]. In addition to this therapeutic effect,

VRBPs were also used as target ligands for hypoxic endothelial cells. The VRBPs were directly conjugated to gene carriers, and subsequent in vitro transfection assays showed that the VRBP-conjugated carriers increased gene delivery efficiency to hypoxic endothelial cells [82].

It should be noted, however, that integrins and VEGF receptors are induced in hypoxic endothelial cells, not in tumor cells. While the use of RGD and VRBP peptides may be useful for antiangiogenic therapy, they cannot eliminate cancer cells directly. Rather, these peptides may facilitate cell death at the core of tumors by inducing hypoxic conditions. Therefore, the combination of antiangiogenic therapy with conventional tumor therapy may offer improved outcomes.

A pH-Sensitive Drug Delivery System for Anticancer Therapy

Two strategies are bringing hope for improved anticancer therapies using precise and timely delivery of potent drugs to the site of action while maintaining therapeutic concentrations over long periods of time. One of these strategies uses tumor-specific molecular targeting [83], which has achieved limited clinical success due to significant heterogeneity in tumor types and cell surface markers [84–86]. Also, these genomic and proteomic targets are occasionally transient and dynamic [84, 87, 88]. Therefore, heterogeneity may explain the unexpected results of this targeting strategy [89]. On the other hand, innovative drug delivery systems (DDSs) are being used to more precisely guide potent drugs to tumor cells, which appear to be a promising and reliable approach [90, 91]. This has resulted in successful anticancer therapy with attenuated toxicity and improved efficacy in preclinical and clinical studies [92]. In DDSs, the mechanism for tumor-specific delivery is exploitation of characteristic tumor microenvironments such as leaky blood vessels, premature lymphatic drainage, heat, and pH.

In vivo stimuli for DDSs include enzymes, oxygen, and protons, with pH being the most attractive target. Compared to the physiologic conditions observed at a pH of 7.4, tumor interstitial and intracellular compartments such as late endosomes and lysosomes are known to be acidic (pH = 6.8–7.2 and pH = 4–6, respectively). Due to sharp pH-dependent structural disruption, pH-sensitive DDS nanocarriers can rapidly release anticancer drugs at the target tumor tissue. For sharp pH-supersensitive DDSs, histidine and sulfonamide have been selected as "platform" polymers due to their multifunctionality and pH sensitivity [93], biodegradability, and fusogenic activity [94–96]. Polyhistidine is a promising pH-sensitive polymer because its imidazole ring has a lone electron pair on the unsaturated nitrogen ($pK_b = 6.5$), thereby possessing pH-dependent amphoteric properties via protonation-deprotonation. These polymers are chemically conjugated with hydrophilic polymers to form multi-block copolymers such as polyhistidine-*b*-poly(ethylene glycol) (polyHis-*b*-PEG) and poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-polyhistidine (PLLA-*b*-PEG-*b*-polyHis), thereby generating hydrophobic anticancer drug-loaded micelle nanocarriers. Furthermore, these block

copolymers are occasionally blended with other copolymers to make mixed micelle systems with improved stability, as "platform" polymer-based nanocarriers are relatively unstable at a pH of 7.4 [97].

Polymeric micelles were first fabricated using polyhistidine (M_n 5 K)-*b*-PEG (M_n 2 K). The micelles were spherical, 114 nm in diameter, and exhibited a unimodal distribution [98]. The critical micelle concentration (CMC) at a pH of 8.0 was 2.3 mg/l, which increased markedly with acidic pH indicating that this micelle nanocarrier is pH sensitive. pH-sensitive polymeric micelles are attractive because of structural destabilization that occurs at an acidic pH, resulting in rapid drug release from the micelles. Conventional pH-sensitive liposome DDSs have been developed that cannot distinguish differences in pH that are less than 1 unit [99]. Therefore, this superior pH-sensitive polymeric micelle system can recognize pH differences between blood (pH = 7.4) and tumor tissues (pH_e = 6.8) after accumulation in the tumor sites via the enhanced permeability and retention (EPR) phenomenon. These findings strongly suggest that this may be a more effective mode of anticancer chemotherapy by providing higher local concentrations of the drug at tumor sites and minimal release of the drug in circulation.

DDSs may also facilitate ligand-targeting systems. For example, trans-activating transcriptional activator (TAT), a cationic cell-penetrating peptide, is able to enter a broad spectrum of cells including both normal cells and cancer cells [100, 101]. In this situation, pH-sensitive micelles can exhibit tumor-specific active targeting through the shield/deshielding mechanism of positive charges on the TAT micelle surface that are controlled by the pH difference between blood and tumor tissue [102]. The nanocarrier consists of two components: a PLLA-*b*-PEG-TAT and a pH-sensitive diblock PSD-*b*-PEG(poly(methacryloyl sulfadimethoxine)-*b*-PEG). At normal blood pH, the sulfonamide is negatively charged. When mixed with the TAT micelle, this sulfonamide shields the TAT by electrostatic interaction. Only PEG is exposed to the outside, which allows the carrier to circulate for a longer period of time. When the system experiences a decrease in the acidic tumor pH, sulfonamide loses its charge and detaches, thus allowing TAT to interact with tumor cells. Another example is pH-induced ligand repositioning on the surface of the micelle (Fig. 7.3). In this case, polyhistidine (M_n 5 K)-*b*-PEG (M_n 2 K) and PLLA (M_n 3 K)-*b*-PEG (M_n 2 K)-*b*-polyhistidine (M_n 1 K)-biotin were used to fabricate a nanocarrier [83]. By decreasing the pH to less than 7.2, the degree of ionization of polyhistidine increased. The interfacial short polyhistidine then became ionized, and at a critical degree of ionization, its hydrophobic interaction with the core phase was weakened. As a result, the biotin moiety was exposed outside of the hydrophilic PEG shell. The exposed biotin could then bind to the biotin receptor, a vitamin B complex, which facilitated biotin receptor-mediated endocytosis. This nanocarrier was further destabilized in the endosome due to the low pH of 6.5, resulting in disruption of the endosomal membrane and release of the anticancer drugs into the cytosol.

Recently, virus-mimetic nanocarriers with pH sensitivity were designed using a hydrophobic polymer core and two layers of hydrophilic shell (Fig. 7.4) [103]. The hydrophobic core was made of poly(histidine-*co*-phenylalanine), i.e.,

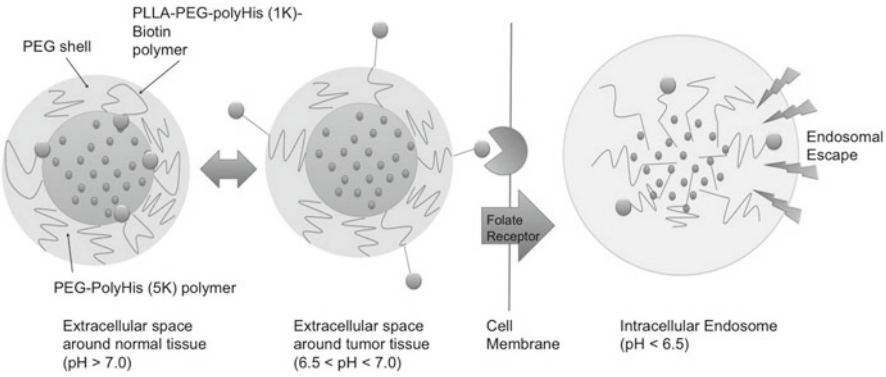

Fig. 7.3 Schematic diagram depicting pH-induced biotin repositioning on the micelle

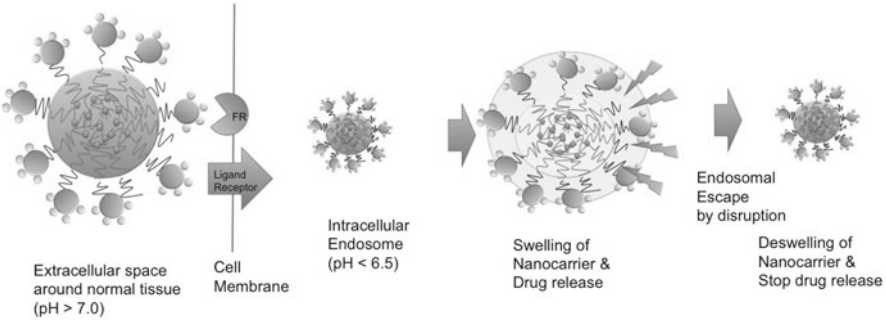

Fig. 7.4 Schematic representation of the virus-like nanogel

poly(His_{32}-co-Phe6), loaded with a model anticancer drug (doxorubicin; DOX) and covered with the first hydrophilic PEG layer as the inner shell. The structures of the core and inner shell were constructed using an oil-in-water emulsion method. The hydrophilic outer shell was made of bovine serum albumin (BSA), which formed a capsid-like structure. Finally, the BSA outer shell was linked with folic acid (F) for specific interaction with the folate receptor (FR), which is overexpressed in many cancer cells [104]. One advantage of this nanocarrier system is the reversible size change (swelling and de-swelling process) that is controlled by repeated pH fluctuations between 7.4 and 6.4. As a result, the release rate of entrapped DOX drugs was accelerated at an endosomal pH (6.4) and slowed at an extracellular and intracellular pH of 7.4–6.8. This manipulation was mediated by the ionization of polyhistidine in the core of the nanocarrier. Another advantage of these nanocarriers is their ability to swell within the endosome after FR-mediated endocytosis, thereby facilitating endosomal escape together with release of DOX drugs via disruption of the endosomal membrane. When the nanocarriers are released into the cytosol, they

rapidly shrink to their initial size, reducing the release of DOX. These nanocarriers are later released from the DOX-induced apoptotic cancer cells and are then able to act on neighboring cells. Therefore, the working role of the nanocarriers is similar to the cycle of parasitic viral infection.

Tumor Hypoxia-Specific Gene Therapy

Gene regulation is an important strategy for tumor hypoxia-specific gene therapy and can be achieved at three levels: transcription, posttranscription, and posttranslation (Fig. 7.5). There are several notable advantages of using hypoxia-specific gene expression systems. First, unlike hypoxic endothelial targeting ligands such as RGD and VRBP, gene regulation can target all types of cells under hypoxic conditions [12]. Additionally, regulation of transcription, translation, and posttranslation is independent of one another, suggesting that the combination of these regulatory strategies may achieve a high level of tumor hypoxia-specific gene expression [105]. Finally, dual-targeted gene regulation is made possible by the combination of hypoxia-specific regulation and tissue-specific regulation. For example, hepatocyte-specific promoters such as the α-fetoprotein promoter can be combined with HREs for specifically targeting hepatomas [106].

Transcriptional Regulation of Therapeutic Gene Expression

Transcriptional regulation is achieved using hypoxia-specific promoters or enhancers. Typical hypoxia-inducible promoters consist of multiple copies of HREs and a basal promoter. For example, the Epo enhancer containing Epo HREs was combined with the simian virus 40 (SV40) promoter for hypoxia-inducible gene expression [107]. Similarly, the HREs from the phosphoglycerate kinase-1 (PGK-1) promoter were combined with the SV40 promoter [108]. This combination was referred to as Oxford Biomedica HRE (OBHRE). OBHRE was used for tumor hypoxia-targeted expression of the herpes simplex virus thymidine kinase (HSVtk) and cytosine deaminase (CD) genes [109, 110]. The efficiency of OBHRE was confirmed in stromal and breast cancers [109, 110].

In addition to the HREs of PGK-1, other HREs of the VEGF, Epo, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes have been evaluated for transcriptional regulation of therapeutic genes [111–114]. The HREs from the VEGF gene were employed for targeting oncolytic viruses to hypoxic conditions within tumor tissue [111, 112]. The genes that are required for viral replication such as ribonucleotide reductase and E1A were regulated by the VEGF HREs. Therefore, under hypoxic conditions, the oncolytic virus was able to replicate and induce cell death. Additionally, Epo HREs and GAPDH HREs were used for HSVtk or CAT gene expression in hypoxic tumor tissues [113, 114].

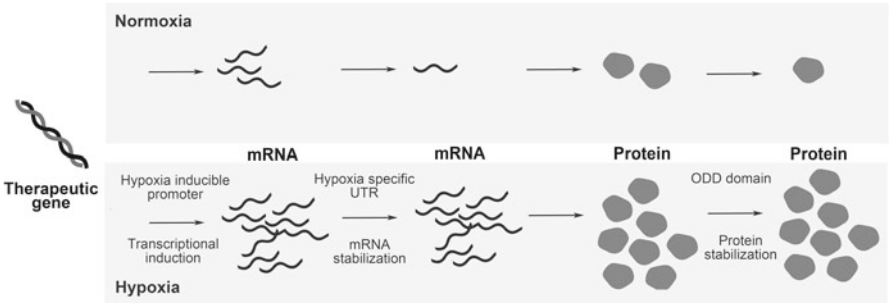

Fig. 7.5 Strategies for hypoxia-inducible gene regulation

Tumor specificity of hypoxia-specific promoters can be further improved by combining with tissue-specific promoters. In breast cancer-specific gene therapy, for example, the estrogen response element (ERE) has been combined with PGK-1 HREs [115]. As a result, EREs and HREs induced death of breast cancer cells in an estrogen- and hypoxia-dependent manner.

It should be noted, however, that hypoxia- and tissue-specific promoters typically have weaker promoter activity than conventional viral promoters such as SV40 and cytomegalovirus (CMV) promoters. As a result, transcription amplification systems have been developed to overcome this problem. Two-step transcription amplification (TSTA) systems are the most widely used for this purpose [116], in which the first unit expresses strong transcriptional activators under the control of hypoxia-specific promoters (Fig. 7.6). These strong transcriptional activators subsequently facilitate the expression of therapeutic genes. The amplification efficiency of the TSTA system is different depending on the promoters that are used. In cases of the HRE and SV40 promoters, the TSTA system amplified gene expression by more than 400 times that of the simple expression system [117].

Posttranscriptional Regulation of Therapeutic mRNA Stability

Gene expression can be regulated at the posttranscriptional level by controlling the stability of mRNA. The VEGF, Epo, and HIF-1α mRNA are stabilized and have longer half-lives under hypoxic conditions [41, 45, 118]. It has been shown that ERBP binds to the Epo 3′-UTR, thereby stabilizing the mRNA [43]. As a result, the steady-state levels of the target mRNA increase, and more proteins are produced. All cellular processes including translation are slowed down under hypoxic conditions in order to preserve cellular resources [45]. In this case, the increased level of mRNA overcomes the slowed translation process and produces more protein. The VEGF, HIF-1α, and tyrosine hydroxylase (TH) 3′-UTRs have been suggested to stabilize their target mRNAs. To date, however, only the Epo 3′-UTR has been

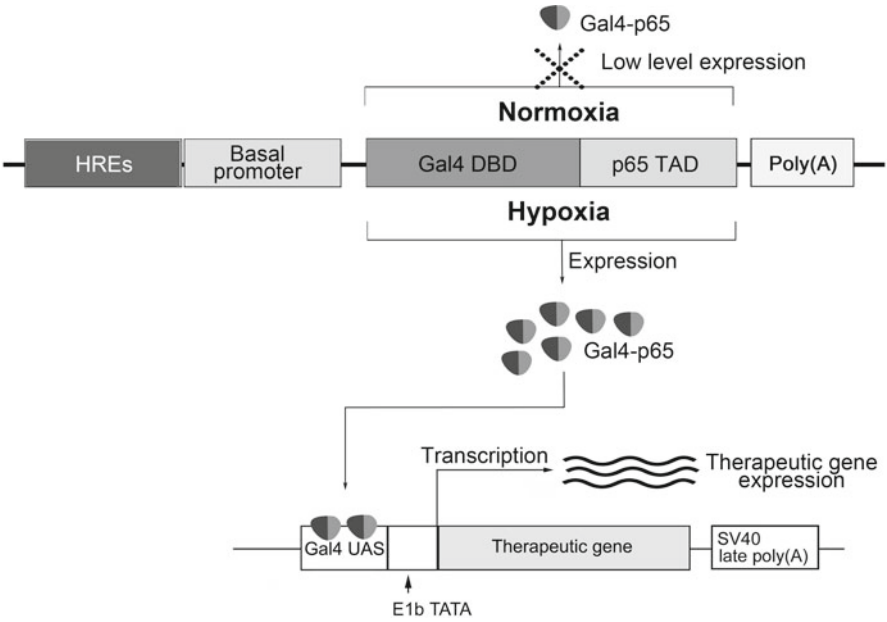

Fig. 7.6 Two-step transcription amplification system

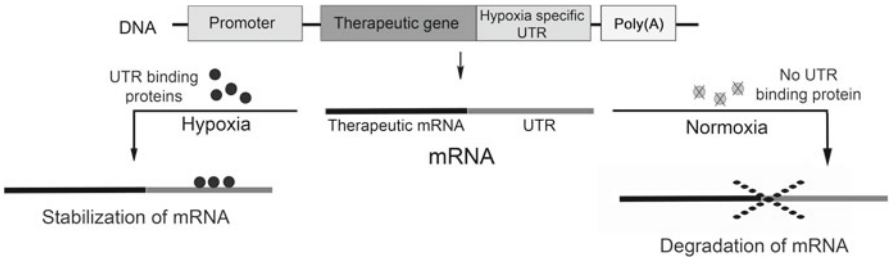

Fig. 7.7 Hypoxia-specific UTR-mediated gene regulation. *DBD* DNA binding domain, *TAD* transactivation domain, *HREs* hypoxia response elements

evaluated for hypoxia-specific gene therapy [119]. The cDNA for the Epo 3′-UTR was combined with the reporter cDNA (Fig. 9), thus the reporter mRNA and Epo 3′-UTR were produced as a mRNA after transcription. Under hypoxic conditions, the Epo 3′-UTR stabilized the reporter mRNA and produced more reporter protein (Fig. 7.7). The application of the hypoxia-specific 3′-UTRs has not yet been reported in tumor gene therapy, but has strong potential for use in tumor hypoxia-specific gene therapy, particularly in combination with tissue-specific promoters as a dual regulation system.

Posttranslational Regulation of Therapeutic Protein Stability

The ODDD plays a key role in HIF-1α stability under hypoxic conditions. Interestingly, when the HIF-1α ODDD was separated from HIF-1α and combined with a reporter protein, the reporter protein became stable under hypoxic conditions [120]. For example, HIF-1α ODDD was used for toxin gene expression in hypoxic tumor cells [121]. The HIF-1α ODDD cDNA was combined with diphtheria toxin cDNA to produce a fusion protein, ODDD-diphtheria toxin A. In a lung cancer model, ODDD-diphtheria toxin A was expressed in a hypoxia-specific manner. Recently, the ATF-4 ODDD was also suggested as a hypoxia-stabilizing ODDD [122, 123]. Interestingly, the ATF-4 ODDD was reported as a target of PHD3, though it is not recognized by pVHL, suggesting that the stabilizing mechanism of ATF-4 may be different from that of HIF-1α [122].

The conjugation of the ODDD leads to modification of therapeutic proteins, in turn limiting their ability to function normally. Indeed, we found that the ODDD in the VEGF-ODDD fusion protein interfered with the normal secretion of VEGF [124]. To overcome this problem, the furin recognition site was integrated between the ODDD and VEGF [125]. Furin contained in the Golgi complex works by recognizing and separating the site between ODDD and VEGF, which facilitates secretion. Therefore, it is important that the ODDD fusion protein is designed in such a way to not disturb the normal functions of therapeutic proteins.

RNAi Against Hypoxia-Inducible Genes in Tumors

HIF-1α is an important target for tumor therapy, and RNAi against HIF-1α has been studied extensively. When HIF-1α was inhibited by short hairpin RNA (shRNA) expressed via the delivery of plasmid DNA [126], tumor growth was reduced. The effectiveness of HIF-1α RNAi may be different depending on cancer type, as tumors have different levels of expression and dependency on HIF-1α. RNAi against HIF-1α showed positive effects in pancreatic cancer, ovarian cancer, oral cancer, and prostate cancer [127–130]. In addition, the inhibition of HIF-1α increased the cellular response to chemotherapeutic drugs by modifying stimulating protein 1 (SP1)-mediated transcription [131]. Similarly, inhibition of VEGF by RNAi has been widely studied for use in antiangiogenic therapy [132–134].

Recently, hypoxia-inducible miRNAs have been developed for hypoxia-specific RNAi [135]. It was previously reported that exogenous miRNA and shRNA expression occupied RNAi machinery in cells and interfered with endogenous miRNA expression [136]. This resulted in severe cytotoxicity and death in experimental animals. Therefore, nonspecific expression of miRNA and shRNA should be avoided, and hypoxia-specific expression systems may be useful for this purpose. The production of hypoxia-inducible miRNAs was accomplished using an miRNA backbone. The specific miRNAs for target genes can be produced under the control

of a hypoxia-inducible promoter. After transcription, pri-miRNAs are cleaved by Drosha, thereby eliminating their caps and polyadenylated tails. In our previous report, miRNA against Src homology phosphatase-1 (SHP-1) was developed using the backbone of miR-30 [135]. The core sequence of miR-30 was replaced with the SHP-1 siRNA sequence. The expression of the SHP1 miRNA was controlled by the Epo enhancer-SV40 promoter for hypoxia-specific expression. In vitro transfection assays showed that the SHP1 miRNA was produced in response to low oxygen concentration.

Antagomir Therapy Against Hypoxia-Inducible miRNA in Tumors

Tumor hypoxia-specific miRNAs are important in hypoxia-targeted therapy. As described above, tumor hypoxia-specific miRNAs inhibit the expression of tumor suppressors and apoptotic proteins [48]. Therefore, inhibition of such miRNAs may be a useful strategy for cancer treatment. To inhibit the action of miRNAs, antisense oligonucleotides against miRNAs (antagomirs) have been studied [137]. For strong hybridization between miRNAs and antagomirs, RNA antagomirs have been used because the RNA-RNA hybrid is stronger than the DNA-RNA hybrid. Locked nucleic acid (LNA) has also been used as an antagomir, since LNA is highly stable and has a strong affinity for miRNA [138–140]. miR-21 was found to be expressed in various hypoxic cancer cells, decreasing the expression of both tumor suppressor genes and proapoptotic genes [48, 141–143]. As a result, miR-21 has been evaluated as a target for anticancer treatment. Interestingly, LNA antagomir against miR-21 has been found to reduce tumor growth in an animal model of glioblastoma [56, 138]. The number of hypoxia-inducible miRNAs continues to increase and their functions are currently under investigation, which may offer future opportunities for novel cancer treatments.

Conclusions

Tumor hypoxia is an excellent target for tissue-specific drug and gene delivery. Hypoxic conditions are known to alter RNA and protein metabolism by slowing down synthesis. However, some genes are rapidly transcribed and translated into proteins as cells adapt to hypoxic conditions. This regulation of gene expression has provided the necessary tools for tumor hypoxia-specific gene therapy. Various regulatory systems have been evaluated and applied to therapeutic gene expression not only for tumor gene therapy but also for ischemic disease gene therapy. Various animal studies have shown that the concept of hypoxia-specific gene regulation is effective for tissue-specific therapy, as well as both knockout and knock-in gene

therapy. Tumor-specific drug delivery systems have also been developed for synthetic macromolecular therapeutics and therapeutic nucleic acids. These targeted drug delivery systems are based on a combination of tumor hypoxia receptors and pH responsiveness, thereby improving tissue specificity and efficiency. Recently, tumor-specific RNAi can also be developed using these hypoxia-specific gene regulation systems, which present a novel method of cancer treatment. Some miRNAs are induced in hypoxic tissues and are directly related to tumor growth. Therefore, tumor hypoxia-inducible miRNAs may be excellent targets for tumor-specific therapy. An increasing number of tumor hypoxia-inducible miRNAs have been studied recently, suggesting that additional targets may be available in the near future. Taken together, recent progress in understanding the mechanisms of cellular response to low oxygen and pH will provide new opportunities for cancer treatments.

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Chapter 8
Tumor Vasculature, EPR Effect, and Anticancer Nanomedicine: Connecting the Dots

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Abstract The progression of a tumor cell mass beyond 2 mm is critically dependent on neoangiogenesis. Angiogenic factors secreted by tumor cells, infiltrating macrophages, and stromal cells aggressively promote proliferation and migration of endothelial cells. The nascent primitive vasculatures are usually morphologically and functionally abnormal due to several features such as the lack of a vascular smooth muscle cell layer, abrupt change of the blood vessel diameter, tortuosity, and leakiness. Those characteristics which alter the blood flow and the transport of molecules in tumors led to the discovery of the enhanced permeability and retention (EPR) of nanosize molecules in tumor tissues. Following its discovery, various anticancer nanoconstructs have been developed with the EPR effect as a central mechanism for tumor targeting. However, the development of these nanodrugs has been hampered by a slow progress towards the clinic. Only nine nanomedicines have been approved for anticancer treatment for the last 26 years. In this chapter, we discuss various aspects that may explain the limited transition for an efficient anticancer nanomedicine. The specificity of the tumor vasculature, the discrepancy in tumor biology, the role of animal tumor models, and the physicochemical characteristics of nanoconstructs are closely examined. This chapter provides new considerations for successful development of EPR-based anticancer nanomedicine.

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Abbreviations

EPR	Enhanced permeability and retention
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
bFGF	Basic fibroblast growth factor
TGF	Tumor growth factor
MMP	Matrix metalloproteinases
NO	Nitric oxide
EBD	Evans blue dye
SMANCS	Styrene co-maleic acid conjugated neocarzinostatin
TDT	Tumor doubling time
HPMA	*N*-(2-hydroxypropyl)methacrylamide
RES	Reticuloendothelial system

Introduction

Angiogenesis is fundamental for many biological processes such as development, reproduction, and wound healing and has been implicated in the progression of a variety of diseases including diabetic retinopathy, rheumatoid arthritis, age-related macular degeneration (AMD), psoriasis, and tumor progression [1–4]. The early stages of the tumor development are characterized by the aberrant activation of oncogenes, inhibition of tumors suppressor genes, and modifications of genes that directly and indirectly control cell proliferation, all as a result of the accumulation of discrete genetic changes and epigenetics alterations [5]. Once the tumor has reached a certain size, the tumor propagation and progression will be dependent on the immediate environment. In 1889, Stephen Paget proposed the "seed and soil" hypothesis based on the concept that the microenvironment of a developing tumor is a crucial regulator of its growth and expansion [6]. The capacity of transplanted tumor cells to promote blood vessel formation was demonstrated by Greenblatt and Shubik [7] and Ehrmann and Knoth [8] who demonstrated that a diffusible factor produced by tumor cells can induce neovascularization. In the early 1970s, Folkman proposed that the tumor growth is essentially dependent on the establishment of its own vascular supply [9, 10]. Independent of the cellular origin of the cancer, angiogenesis is the critical step for the growth of tumours beyond 2 mm as well as the development of metastasis. The activation of tumor angiogenesis relies essentially on the balance between the pro-angiogenic factors and the anti-angiogenic factors.

The induction of the tumor vasculature growth is termed the "angiogenic switch" [11, 12] and is dependent on the increased expression of the pro-angiogenic genes and/or a decreased expression of anti-angiogenic factors. Many potential regulators of angiogenesis have been identified including acidic fibroblast growth factor

(aFGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), angiogenin, interleukin (IL)-8, angiopoietins, angiotensin (ANG)-II, bradykinin, and prostaglandins [1, 13–17]. Negative regulators of angiogenesis were also identified and included factors such as thrombospondin [18], the 16 kDa fragment of prolactin [19], angiostatin [20], endosatin [21], and vasostatin [22].

The increased expression of these angiogenic factors has been demonstrated in several types of cancer and has been associated with increased permeability of the tumor vasculature compared to normal blood vessels [23–25]. Further, angiogenic factors have been associated with structural aberrations of the tumor blood vessels (for review see [26]). The higher permeability of the tumor blood vessels favors the accumulation of macromolecules and lipids in the interstitium of the tumor for extended periods of time. This feature of the tumor vasculature led to the characterization of the enhanced permeability and retention (EPR) effect of macromolecular drugs in solid tumors [27]. The EPR effect is the result of the distinctive vascular permeability of solid tumors [28] and inflammatory tissues [29]. The characterization of this phenomenon allowed the development of the first anticancer nanomedicine by Maeda: styrene co-maleic acid conjugated neocarzinostatin (SMANCS) for the treatment of hepatocellular carcinoma [30]. Following this discovery, several laboratories have developed EPR-based nanomedicine. The main advantage of the EPR-based anticancer nanomedicines is their altered pharmacokinetics caused by their hydrodynamic diameter as it exceeds 7 nm, a size sufficient to escape kidney filtration and urinary excretion [31, 32]. These nanoconstructs can exhibit prolonged circulatory half-life, high area under the concentration/time curve (AUC), and higher partition into tumor tissues [33–36]. Since the first nanomedicine was developed in 1986, the Food and Drug Administration (FDA) and several agencies worldwide have approved over 30 nano-therapeutics for clinical use, 11 of which are for the detection and treatment of various cancers. Despite the improvement in the design and targeting efficiency of these nanomedicines to the tumor site, the transition from the bench to the clinic is particularly slow. In this chapter, we will present an overview of the mechanisms involved in neovascularization, as well as the specific characteristics of the tumor vasculature. We will also discuss the critical considerations that might influence nanomedicine targeting efficiency to solid tumors utilizing tumor vasculature permeability.

Mechanism of Tumor Angiogenesis

The origin of the blood vessel formation is different depending on the biological process which it serves. During embryogenesis, the de novo formation of blood vessel originates from the differentiation of angioblasts into mature endothelial cells and their subsequent assembly into tubes, a process called vasculogenesis [37]. Several angiogenic factors such as VEGF, VEGFR-2, bFGF, and TGF-β

influence angioblast differentiation into mature endothelial cells [38–40]. Further development of these native vessels is the result of angiogenesis, a process in which new capillaries emerge by sprouting from existing ones [37]. Distinctive signaling mechanisms will promote either venous or arterial differentiation [41]. The recruitment of periendothelial cells such as vascular smooth muscle cells or pericytes is essential for the maturation of the blood vessel by inhibiting the endothelial cell proliferation and promoting the formation of extracellular matrix [42]. The periendothelial cells can also assist the endothelial cells to acquire specialized functions in different vascular beds [42]. In contrast, neovascularization taking place at postembryonic stage involves essentially angiogenesis as a result of the proliferation and migration of differentiated endothelial cells [13]. With the exception of few physiological processes such as wound healing and the female reproductive cycle where endothelial cells are transiently activated and proliferating, the endothelial cells are largely quiescent in mature vessels. The percentage of endothelial cells entering the cell cycle is only 0.45 % for arteries and arterioles and 0.11 % for capillaries [43].

Tumor expansion is marked by a constitutive activation of the "angiogenic switch" in most cases [11]. The newly formed blood vessels will emerge by sprouting from existing ones and sustain tumor growth [11]. However, recent studies have challenged these conceptions and identified several concomitant mechanisms contributing to the neovascularization of tumors. These mechanisms have been mainly characterized in the tumor vasculature but their contribution remains poorly understood. New blood vessels can emerge from vasculogenic mimicry where tumor cells can line and form a vessel-like structure [44] or putative cancer stem cells can differentiate into an endothelial cell lineage and contribute to angiogenesis [45]. Other studies have demonstrated the capacity of tumor cells to hijack an existing blood vessel, a process known as vessel co-option [46]. In other cases, new blood vessels can arise through intussusceptive angiogenesis where one existing vessel splits into two new vessels [47]. Several studies have also demonstrated the involvement of bone marrow-derived cells for the repair of adult vessels and the expansion of tumor ones. Endothelial cell progenitors can be mobilized from the bone marrow and transported through the blood circulation to become incorporated into the vascular walls of the growing blood vessels [48].

Tumor Vasculature as a Target for Selective Delivery of Nanomedicine

Normal vasculature networks consist of arterioles, capillaries, and venules and form a well-organized network with dichotomous branching and hierarchic order [49]. Newly formed tumor vessels are usually abnormal in form and architecture with narrowed, tortuous, and fragmented blood vessels. In addition, tumor vessels usually lack a smooth muscle layer and innervation, with defective endothelial linings and basement membranes [50]. Some structures are dilated, saccular, poorly aligned,

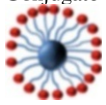
and heterogeneous [51]. Many vascular mediators such as bradykinin [52], prostaglandin [53], nitric oxide (NO) [54], peroxynitrite ($ONOO^-$) [29], matrix metalloproteinases (MMP) [29], and VEGF [55] have been shown to play an important role in these alterations.

As a consequence of these defects, tumor vessels usually harbor wide fenestrations [56]. The blood flow is often irregular with vessels having different diameters and abnormal branching patterns. These tumor vessels are leaky and show increased permeability to large circulating molecules with fenestration sizes ranging from 300 nm to 4,700 nm [57–59]. Furthermore, lymphatic drainage of tumor tissues is generally deficient and limits the clearance of macromolecules [9, 27, 60–62]. Evidence for increased endothelial permeability of tumor vessels to large molecules was clearly demonstrated by Maeda, 26 years ago, using Evans blue dye (EBD) injected intravenously into rodents. After injection the dye bound to albumin in the bloodstream and the complex selectively concentrated into tumor tissue [28]. Additional studies using soluble tracers further demonstrated the extravasation of large molecules from the tumor vessel [63, 64]. EBD extravasation and accumulation in the tumor was the first demonstration the EPR effect concept [28]. The accumulation of nanosize drugs in the tumor tissue is time dependent ranging from several hours to several days [60, 65]. Maeda's work demonstrated that the rate of accumulation of macromolecules and lipids in the tumor was inversely proportional to their clearance rate. Following SMANCS, the first nanomedicine approved and used for the treatment of hepatocellular carcinoma in Japan (see Table 8.1), several laboratories have developed nanosize drug carriers. But, for the past 20 years, few nanomedicines were approved for the chemotherapeutic treatments of various cancers as well as for their detection (see Table 8.1). Among these formulations, liposome nanocarriers achieved significant success such as Doxil, DaunoXome, Depocyt, and Myocet (Table 8.1). Second- and third-generation types of micellar or polymeric drug carriers are currently being developed or evaluated in clinical trials (phase I–III).

To be efficient for cancer treatment, the size and shape of nanoparticles are critical for their accumulation at the tumor site [66]. Several studies have demonstrated that both criteria are essential for the longevity of the nanomedicine in the circulation, their distribution to different organs [66], as well as their recognition and elimination through the reticuloendothelial cells system (RES) [67]. The RES is composed of macrophages present in the liver, spleen, and bone marrow [67]. Generally, particles larger than 100 nm are rapidly eliminated from the circulation by the RES [68, 69]. To decrease their recognition by macrophages, several strategies have been developed, for instance, the addition of synthetic polymers such as polyethylene glycol (PEG) on the surface to sterically hinder interaction with plasma proteins [70] and reduce opsonization [71].

Overall, nanomedicine advantages over conventional drugs rely on the EPR effect and their improved pharmacokinetics that lower their systemic cytotoxicity. A schematic representation of this phenomenon is illustrated in Fig. 8.1. Furthermore drug delivery nanotechnology allows the controlled release of anticancer drugs and might partly circumvent multidrug resistance mechanisms that involve cell-surface protein pumps [72].

Table 8.1 Clinically approved nanomedicines for cancer treatment or imaging

	Name	Therapeutic agent	Approval year	Treatment	References
Conjugate 	SMANCS	Neocarzinostatin	Japan (1993)	Hepatocellular carcinoma	[213]
Liposomes 	Doxil/Caelyx	Doxorubicin	FDA (1995)	Late-stage ovarian cancer Advanced HIV-Associated Kaposi's sarcoma	[214] [215]
	DaunoXome	Doxorubicin	FDA (1996)	Advanced HIV-associated Kaposi's sarcoma	[216]
	Depocyt	Cytarabine	FDA (1999)	Malignant lymphomatous meningitis	[217]
	Myocet	Doxorubicin	Europe (2000) Canada (2001)	Metastatic breast cancer in combination with cyclophosphamide	[218]
	MEPACT	Muramyl tripeptide phosphatidylethanolamine	Europe (2004)	Osteosarcoma	[219]
Polymeric micelles 	Oncaspar	PEG-L-asparaginase	FDA (2006)	Acute lymphoblastic leukemia	[220]
	Genexol-PM	Paclitaxel	South Korea (2006)	Metastatic breast cancer	[221]
Albumin	Abraxane	Paclitaxel	FDA (2005)	Metastatic breast cancer	[222]
Metal 	Feridex	Superparamagnetic iron oxide	FDA (1996)	MRI contrast agent	[223]
	GastroMARK	Superparamagnetic iron oxide	FDA (1996)	MRI contrast agent	[224]

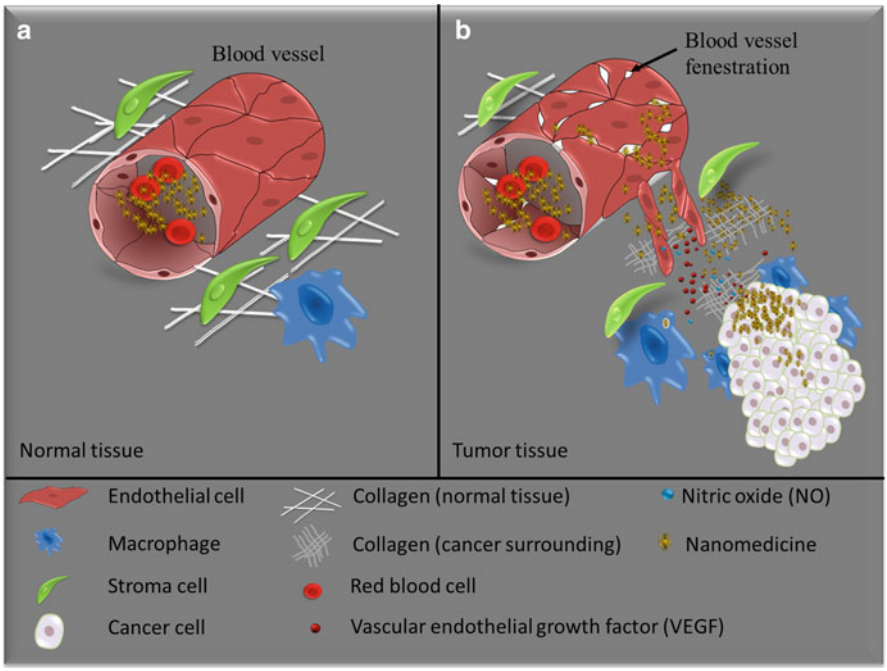


Fig. 8.1 Differences between normal and tumor tissue in relation to the targeting of nanomedicines by the enhanced permeability and retention (EPR) effect. (**a**) Normal tissue contains tightly connected endothelial cells which prevents the diffusion of the nanomedicine outside the blood vessel. (**b**) Tumor tissue contains large fenestrates between the endothelial cells allowing the nanomedicines to reach the matrix and the tumor cells by the EPR effect. VEGF and NO secreted by tumor cells, stromal cells, and macrophages increase permeability and stimulate angiogenesis and the migration of endothelial cells towards the tumor. A considerable proportion of the nanomedicine never reaches the tumor either due to entrapment or nonspecific interaction with collagen composing the matrix, or removal through macrophage endocytosis. Nanomedicines tend to concentrate at the periphery of the tumor, only a small proportion will diffuse to the center of the tumor

Factors Contributing to the EPR Effect

Several studies have demonstrated that the EPR effect is dependent on angiogenic factors produced from the tumor cells, stromal cells, or other cell types such as VEGF, bradykinin, nitric oxide, peroxynitrite, and other cytokines [73–75]. All these factors increase blood flow and promote diffusion and retention of nanomedicines inside tumors.

VEGF

The vascular permeability factor or vascular endothelial growth factor (VPF/VEGF) was originally characterized from guinea pig ascites as a secreted protein

inducing vascular permeability [76] and was later found in various human tumor cell lines [77]. The same protein was also later identified as a specific and potent vascular endothelial cell mitogen [78]. VEGF is highly expressed in most tumors (2–30-fold higher than normal tissue) and was shown to contribute to the tumor blood vessel structural abnormality [79]. The contribution of VEGF to the EPR effect was demonstrated by Claffey et al. who showed a greater extravasation of large molecules in tumors overexpressing VEGF [80].

VEGF is a homodimeric glycoprotein comprised of two identical subunits [78]. VEGF expression is regulated at the level of transcription by alternative splicing of the VEGF gene and the $VEGF_{165}$ isoform is the most abundant and assimilated as the native soluble heparin-binding endothelial mitogen activator [78]. Other VEGF isoforms have been identified and arise from different VEGF splicing such as $VEGF_{121}$, $VEGF_{189}$ and $VEGF_{206}$. The $VEGF_{121}$ isoform is secreted and fully soluble but lacks the heparin binding site, while $VEGF_{189}$ and $VEGF_{206}$ are largely sequestered at the cell surface and extracellular matrix and bind avidly to heparin and heparin-like moieties [78, 81]. In addition to the alternative splicing, a proteolytic activation of VEGF has been demonstrated for $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ following plasmin [82, 83] and matrix metalloproteinase (MMP) activations [84]. These patterns of activation regulate bioavailability and bioactivity and also determine receptor specificities. VEGF acts mainly in a paracrine fashion binding to receptors expressed at the surface of endothelial cells. $VEGF_{165}$ binds to two receptor tyrosine kinases, VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1), as well as Neuropilin (NRP)-1 and NRP-2, transmembrane glycoproteins [85, 86]. In addition, several VEGF-related genes have been identified including VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) [87]. These members of the VEGF gene cluster undergo alternative splicing with the exception of VEGF-C [87]. VEGF-C and VEGF-D were shown to bind to VEGFR-3 (Flt-4) and promote lymphangiogenesis [88].

The expression of VEGF is upregulated by multiple factors including hypoxia. Under hypoxia, hypoxia-inducible factor (HIF)-1α dimerizes with the constitutive HIF-1β to bind to the hypoxic response element (HRE) present in the promoter of the VEGF gene and stimulate its expression [89]. HIF-1α is involved in the activation of transcription of many genes involved in the activation of angiogenesis and other physiologic processes (for review, see [89]).

Bradykinin

Bradykinin (kinin) is a peptide that causes vasodilatation and increased vascular permeability. Bradykinin is generated from its precursor kininogen by limited proteolysis by various serine proteases such as kallikrein, cathepsins, and collagenases [90]. Kininogens are multifunctional glycoproteins mainly synthesized in the liver but also in the kidneys, salivary glands, and endothelial cells [91] and circulate in human plasma in low (50–68 kDa) and high (80–120 kDa) molecular weight forms

[92]. Bradykinins' half-life is a few seconds in the plasma and is rapidly degraded by proteases such as kininase and angiotensin-converting enzyme (ACE) [92]. A greater synthesis of bradykinin has been reported in several types of cancer [52, 93] as well as the expression of bradykinin receptor B2 [94]. The effect of bradykinin appears to be direct as the release of bradykinin triggers vasodilatation and increased vascular permeability as well as indirect as mediated by the production of nitric oxide through the stimulation of the nitric oxide synthase [95], prostaglandins [96], and various cytokines such as interleukin-1, interleukin-6, and interleukin-8 [94]. The permeabilizing action of bradykinin was found to be similar to VEGF but mediated through a different pathway.

Nitric Oxide (NO)

NO is a signaling messenger and contributes to several pathways and biological processes. NO is produced from L-arginine by nitric oxide synthase (NOS) in the presence of oxygen. In pathological conditions such as cancer and inflammatory tissue, NO production is largely increased and plays an important role in the extravasation of large molecules and thus contributes to the EPR effect [54, 60]. Increased NO production was also associated with the overexpression of the inducible form of NOS (iNOS) in the tumor tissues due to the infiltration of leukocytes [97].

Moreover, NO to the same extent as oxidized NO products such as peroxynitrite contributes to the vascular permeability of solid tumor [54]. Peroxynitrite ($ONOO^-$) is a reaction product of NO and anion superoxide $O2^-$ [98]. The increased production of peroxynitrite triggers the maturation of pro-matrix metalloproteinases (pro-MMP) into MMPs, which promotes remodeling of the extracellular matrix and contributes to the vascular permeability [29].

The identification of the factors contributing to the EPR effect has resulted in the development of anticancer nanomedicine. However, with the exception of a few clinically approved nanomedicines (see Table 8.1), many nanoconstructs failed to achieve a significant outcome in the clinic. The lack of complete understanding of the EPR effect and its specific biological implications has so far impaired EPR effect-based therapy as a paradigm for cancer treatment. The following describes some of the factors that could account for the slow transition of the EPR-based nanomedicine to an effective cancer treatment.

Animal Models for the EPR Effect

The EPR effect has been repeatedly proven in animal models through the use of large molecules such as the EBD. EBD binds instantly to plasma albumin which results in a large molecular weight complex of about 7 nm diameter that can simulate the effect of a nanomedicine. A diameter larger than 7 nm will escape renal

filtration and urinary excretion [31], due to the slit diaphragms at the level of the podocyte foot of the glomerulus which prevent the filtration of globular plasma proteins above this size [32]. Therefore, large particles can exhibit prolonged circulatory half-life, high area under concentration/time curve (AUC), and higher partitioning into tumor tissues [33]. After 6 h, there is usually a distinct accumulation in tumor lesions compared to surrounding tissues. Many nanomedicines have been observed to accumulate in tumor tissue from 2-fold and up to 27-fold more than free drugs depending on the nanocarrier, the drug encapsulated, and the xenograft tumor model used (Fig. 8.2a).

The question of whether the results of EPR-based drug targeting in animal models can be faithfully translated to the clinic remains unanswered. Macrophage infiltration has been demonstrated in a large cohort of cancers. The production of VEGF and NO by tumor-associated macrophages (TAM) and their role in cancer development is also well documented [99–101]. To determine the anticancer properties of a given nanomedicine against a specific human cancer, it is necessary to utilize immunocompromised mice to enable the use of human tumor xenografts. However human cancer patients are rarely immunocompromised. A change in macrophage activity in immunocompromised mice [102] can result in less VEGF and NO leading to a tumor with reduced vascular density, which in turn limits the access of the nanoconstructs to the tumor. Furthermore, the results obtained from immunocompromised models differ from results obtained in immunocompetent mice. In various drug delivery systems (conjugates, liposomes, and micelles), the tumor accumulation is a 2-fold higher in immunocompetent mice relative to immunocompromised ones (see Fig. 8.2b). Moreover, immunocompetent mice bear murine tumors and not human cancer cell lines which further complicate interpretation of in vivo animal data and jeopardize its value in predicting the performance of new drugs in clinical trials.

The expression of VEGF and its receptors between commonly used human tumor cell lines and their clinically isolated variants differs. It is clearly evident that tumor cell lines have pronounced expression of VEGF and its receptors with far less variability in comparison to clinical tumors. For example, human breast cancer cell lines MCF-7 and MDA-MB-231 expressed VEGFR-1, VEGFR-2, and VEGFR-3 as well as the ligands VEGF-A, VEGF-C, and VEGF-D with VEGF-B being found only in the MCF-7 cells [103–108]. In contrast, the expression pattern in breast cancer tumors collected from patients is more limited to one specific type of receptor and/or ligand and more importantly not all tumors tested within this cancer type expressed VEGFR and/or its ligand [109–111]. A similar observation was made with prostate cancer and lung cancer.

Relevant to this is the design of nanomedicine targeted to tumors which relies on the conjugation of target ligands that bind strongly to tumor cell-surface receptors to increase cell recognition, cell specificity, and cellular uptake. Galactosamine [112], transferrin [113], and folate [114] have been incorporated in nanomedicine based on the preferential expression of these molecules by cancer cells. Despite promising in vitro studies, these targeted nanomedicines failed to demonstrate significant benefit at the preclinical or clinical level [115]. The discrepancy between

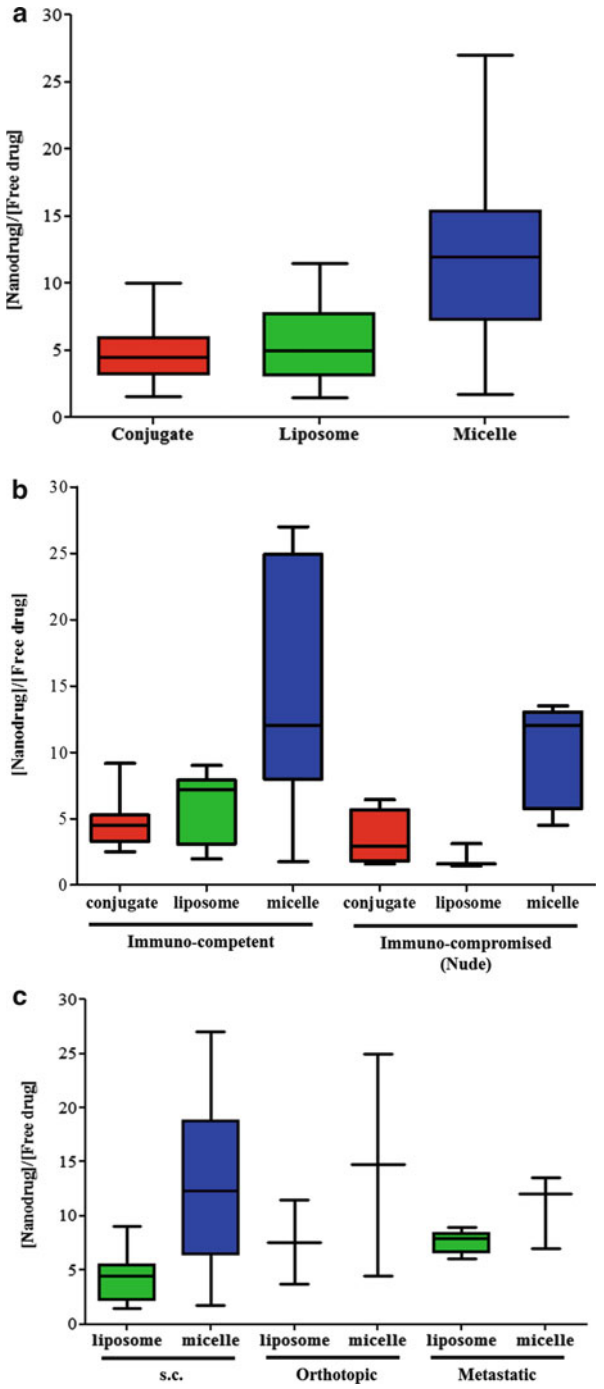


Fig. 8.2 Variability of the different nanocarriers based on their accumulation profile in the tumor, animal models used, and the site of tumor implantation and metastasis in animal model. (**a**) Comparison of the proportion of the different nanocarriers accumulation in the tumor [184–196]. (**b**) Comparison of the tumoral accumulation of different nanocarriers based on the animal model used [184, 189, 194, 197–201]. (**c**) Comparison of accumulation of different nanocarriers based on the site of the tumor implantation either subcutaneous (s.c.), orthotopic, or metastatic [184–197, 202, 203]

the results obtained from testing specific tumor cell lines in tumor models and the clinical trials points further to the sampling errors in generalizing the results of from specific cell line to that of relevant tumors [116].

A substantial difference between tumor models in animals and those of human patients is the progression rate. Animals usually develop a large, clinically relevant tumor (>5 mm) 1 week following subcutaneous (SC) tumor cell inoculation, while such a tumor volume can take years to develop in a human (Table 8.2). This rapid progression rate in animal models results in the overestimation of the targeting role of the EPR effect. Animal tumors developing quickly presumably produce a large quantity of VEGF and vascular mediators to support their rapid growth. In addition a 1 gram tumor mass in a 30 gram mouse is about 3 % of its total weight. In humans, a comparable tumor would weigh 2–5 kg, which is an advanced tumor stage that is not an ideal for utilization of anticancer nanomedicine. Finally, tumors are usually implanted SC in animal models, which allow the developing tumors to take advantage of the extensive cutaneous vascular network for extending their blood supply, a condition that is rarely encountered in human malignancy.

Data collected from available literature to date are plotted in Fig. 8.2c. Although there is a trend towards higher concentration of nanoconstructs in SC models, the results are not conclusive given the limited number of studies. Whether site of tumor development can influence the efficacy of the EPR effect remains an unanswered question.

Tumor Biology Diversity

Tumor Doubling Time (TDT)

Tumor doubling time (TDT) is an important factor to consider when designing EPR-based anticancer nanomedicine. Most cytotoxic drugs selectively target cancers by exploiting differential tumor characteristics such as high proliferation rates, hypoxia, and genome instability. The TDTs provide a selection trait that is exploited by chemotherapeutics that target DNA synthesis and cytoskeleton remodeling. Many chemotherapeutic agents fail to cope with rapidly dividing tumors as the amount of drug necessary to kill a given number of cells will double with each tumor doubling. However, the dose that will elicit dose-limiting toxicity will remain the same. A short TDT is well known to be associated with an unfavorable survival prognosis [117–122]. TDT is a highly heterogeneous, both within and between different tumor types, stages, and grades. There is a large degree of variation of TDT between tumors of different tissue origins. Pituitary adenoma, for example, has an extremely long TDT of 506–5,378 days and within the tumor type the TDT varies by ten times [123], while in meningiomas and neurinomas the TDTs are 6.5 days and 7.67 days, respectively [124]. Some tumor types have a high variation of the TDT, for instance, lung adenocarcinoma has an extremely high variation in TDT of

Table 8.2 Tumor doubling time (TDT) and microvascular density (MVD) in various cancers

	Grade	Tumor doubling time (TDT)	Patient number	References	Gleason's grade	MVD (mean)	Patient number	Marker	References
Prostate cancer		42–1,346 days	34	[225]	3–	60	155	CD34	[226]
					7	79	333	CD34	[226]
					8–10	90	84	CD34	[226]
Hepatocellular carcinoma	Edmonson grade				Edmonson grade				
	I–II	118.2 (37–356)	23	[227]	I–II	254	52	CD34	[228]
	III–IV	70.1 (13–239)	39	[227]	III–IV	216	48	CD34	[228]
Brain cancer	WHO grade				WHO grade				
	I–II	14	9	[229]	I–II	15	30	CD31	[230]
	III	140	7	[229]	III	39	25	CD31	[230]
	IV	69.7	9	[229]	IV	50	22	CD31	[230]
Renal clear cell carcinoma	Fuhrman grade				Fuhrman grade				
	I				I	78.8	16	CD31	[231]
	II	486.4 (159–1,193)	17	[232]	II	56.4	69	CD31	[231]
	III	558.2 (253–987)	7	[232]	III	41.4	20	CD31	[231]
	IV	321	1	[232]	IV	36.8	4	CD31	[231]
Breast cancer					TNM stage				
		11–1,293	118	[126]	I–II	104	74	CD34	[233]
					III	111.1	33	CD34	[233]
					IV	131.9	13	CD34	[233]

964-fold [125] followed by breast cancer with a variation of 117.5-fold [126] (see Table 8.2). TDT can also differ according to the specific cellular origin within a given tissue. Bronchoalveolar cancer, for example, has an extremely varied TDT of 36–1,092 days, a variation of 30.3-fold [125], while small cell lung cancer has a TDT of 61.9–120.4, a mere 1.9-fold difference [127]. In addition, TDT can range depending on tumor grade (see Table 8.2). Poorly differentiated hepatocellular carcinomas corresponding to the Edmonson grade III or IV are highly invasive and have a DT of 13–239 days [128], while well-differentiated tumors corresponding to Edmondson grade I or I–II has a significantly extended TDT of 54.7–1,508.3 days [128] (see Table 8.2). Interestingly, hepatocellular carcinomas are highly vascularized and the microvessel density (MVD) is not affected by the tumor grade (see Table 8.2). Astrocytoma also follows this trend with the TDT of grade IV astrocytoma, according to the WHO grading system, varying between 1.4 and 319 days, the TDT of grade III 30–472 days and the TDT of grade I–II tumors 138–1,045 [129, 130]. The tumor grade also correlated with the MVD, with higher grade having a higher MVD (see Table 8.2). The same trend was observed with prostate cancer and breast cancer where a high grade correlates with a lower doubling time and a higher MVD (Table 8.2).

The primary or metastatic status of a tumor can also cause large fluctuations. For example, primary melanoma may have a DT of 50–377 days [131], while metastatic melanoma may have a DT of 8–212 days [132].

EPR-based anticancer nanomedicine should consider doubling time variation when planning the release mechanism of active chemotherapeutic agents from its nanocarrier, as well as the internalization rate of macromolecular complexes into tumor cells. For example, a slow-releasing amide bond between the polymer backbone and the drug, or slowly internalized liposome, could both be a good choice for tumors with a slow DT. In contrast a fast-releasing micelle or an ester bond linkage can be a better fit for rapidly dividing tumors. Generally, EPR-based nanomedicine has a wider therapeutic window [133], an advantage that can be exploited to shape dose regimens based on individual patient conditions. A tumor's inherent sensitivity to specific chemotherapeutic agents as well as TDT is of the upmost importance in designing EPR-based anticancer nanomedicine.

Microvascular Density (MVD)

The EPR effect is strictly dependent on the vasculature of the tumor with theoretical assumption that all tumors independently of their origin, stage, and organs will behave identically. However, this concept is drastically challenged by a number of reports that show a high diversity in angiogenesis behavior [12, 51, 134, 135]. Nagy et al. have identified six structurally and functionally distinct types of blood vessels in human cancers [134]. Vascular density can provide, in most tumors, a

prognostic indication of tumor progression. As shown in Table 8.2, vascular density is largely dependent on the type of cancer and varies largely within each tumor type. For instance, renal cell carcinoma is highly vascularized [136], while the density of microvessels appears low in head and neck squamous cell carcinoma [137] or in ovarian carcinoma [138]. In addition, higher stages of cancer are well correlated with higher microvascular density as observed in astrocytoma and prostate cancer (Table 8.2), while in other types of tumors such as renal cell carcinoma, no direct correlation can be established between tumor stage and vascular density (Table 8.2). Furthermore, metastatic tumors tend to possess higher vascular density compared to non-metastatic tumors [139–143]. Another element regarding the EPR effect is the secretion of angiogenic factors such as VEGF by the tumor. Vascular permeability can be altered by VEGF as well as a wide array of inflammatory mediators [144], which can affect the extent of nanomedicine accumulation driven by the EPR effect and the penetration of the nanoconstruct into the tumor. As mentioned previously, there is a large heterogeneity in the expression of VEGF between different types of cancers. When designing a nanocarrier, the properties of the targeted tumor tissue such as the cancer type, the microvascular density, and the secretion of permeability factors such as VEGF should therefore be taken into account in order to take full advantage of the EPR phenomenon.

Optimization of Drug Nanocarriers for the EPR Effect

To optimize the engineering of nanoparticles for specific delivery, careful consideration should be undertaken regarding the biology of the tissue being targeted. In many instances, the nature of the nanomedicine itself has been a limiting factor that negatively impacted its chance of clinical success. The loading of active drug into a delivery system can be insufficient due to the physical or chemical limitations to achieve the critical dose needed to treat the tumor. For example, the HPMA copolymer-paclitaxel conjugate showed insufficient drug loading ($\leq$10 %) with a particle size in the range of 12–15 nm [145] and lacked stability due to the use of an ester linker [146]. Consequently insufficient tumor tissue accumulation of the drug was evident in phase I clinical trials [147]. Another factor limiting the efficacy of nanomedicines is the fast release rate of drug in the circulation. For instance, low molecular weight HPMA copolymer-camptothecin conjugate showed a rapid release of drug and quick renal filtration and consequent bladder toxicity in phase I clinical trials [148]. The nanomedicine was designed with a labile ester linker, decreasing its stability and therefore its tumor accumulation [149]. In this example, a low molecular weight (below the renal excretory threshold of 7 nm) coupled to the toxicity associated with a fast release rate resulted in the drug failing to achieve EPR-based pharmacokinetics.

Following are a few considerations inherent to the design of nanomedicine that may significantly influence the outcome of EPR-based drug targeting (see Fig. 8.3).

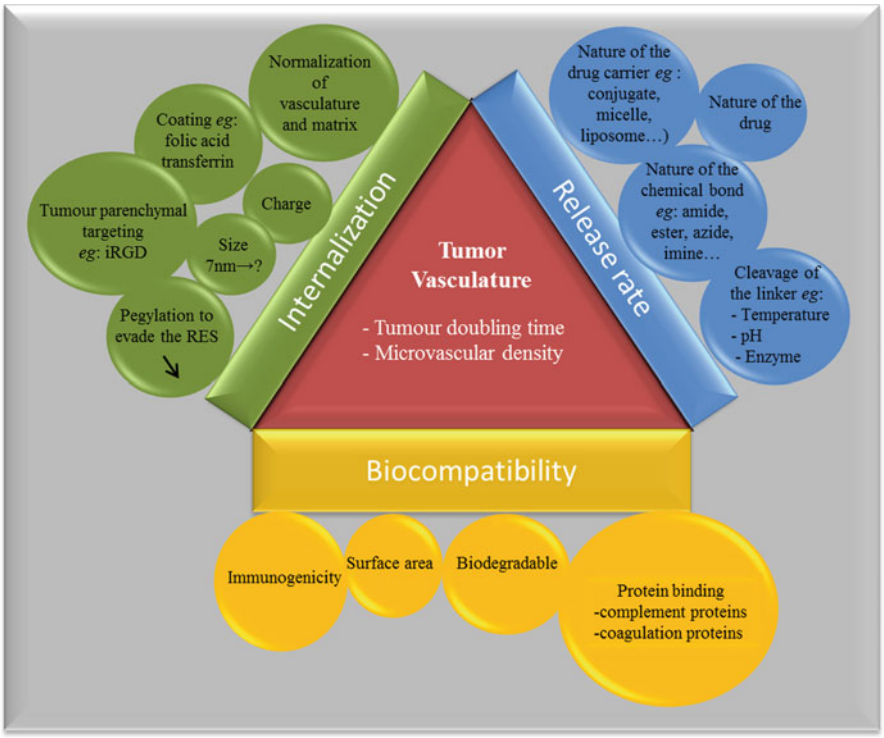


Fig. 8.3 Schematic representation of the variables influencing the clinical application of a nanomedicine. The biocompatibility, internalization, and release should be carefully considered when designing a nanomedicine utilizing tumor vascular abnormalities for targeted cancer treatment

Internalization of the Nanocarrier

The concentration of drug inside the tumor resulting from the EPR effect in a subset of highly vascularized tumors does not guarantee the efficient internalization of the drug within the tumor cells. Multiple factors can influence the cellular internalization process of the nanomedicine. Usually, nanoparticles and polymer-based drug delivery systems are internalized by endocytosis, a multistep process that culminates in the formation of a late endosome which finally fuses with a lysosome [150]. Malignant cells have an accelerated metabolism, a high glucose requirement, and an increased glucose uptake characterized by the elevated expression of glucose transporter proteins (GLUT) [151]. However, recent studies have shown that many cancer cell lines exhibit limited capacity for endocytosis compared to normal cells [152, 153].

Compared to tumor cells, macrophages usually exhibit a higher uptake of nanosized molecules [66, 154] as they can recognize nanomedicine either through

their Toll-like receptor 4 (TLR-4) [155] or through scavenger receptors [156]. Much work has therefore been devoted to the development of nanoparticles which can evade macrophage recognition, resulting in longer circulatory time and increased interaction with target tissue. On this basis, polyethylene glycol (PEG) is the polymer most commonly used to enhance in vivo circulatory half-life [157, 158]. Coating nanoparticles with PEG results in the formation of a polymeric layer which sterically hinders the interaction of nanoparticles with plasma proteins and cell membranes [159] preventing opsonization and phagocytosis by components of the RES [160, 161]. PEG-liposome-incorporated doxorubicin (Doxil®) is approved by the FDA for the treatment of ovarian cancer (see Table 8.1). Additional polymers such as N-(2-hydroxypropyl) methylacrylamide (HPMA), polyacrylamide, or poly(vinyl pyrrolidone) have also been used to improve the circulation time and steric hindrance of nanomedicines [162, 163]. The main disadvantage of this strategy is that it limits the interaction of (stealth) nanoconstructs with the tumor cell membrane and subsequently reduced internalization and uptake by tumor cells. To improve specific uptake by endocytosis, several nanoparticles have been coated with receptor ligands such as folate [164] or transferrin [165] to induce receptor-mediated endocytosis. These coatings increased the accumulation of drug inside tumor cells. However, the practical advantages in the management of human tumors in the clinic remain to be proven. Following intracellular internalization, active drug should be liberated from the lysosomal compartment to reach its cellular target. Mechanisms to escape the lysosomal compartment and improve intracellular targeted delivery have been described by Breunig et al. [166]. Another consideration relevant to relatively large sized macromolecular nanomedicine is their nonspecific interactions with the extracellular matrix; to reach tumor cells, nanoconstructs must move through the matrix, a highly interconnected network of collagen fibers that intermingle with proteins such as proteoglycans and glycosaminoglycans. This semisolid barrier could significantly reduce the amount of nanomedicine reaching tumor cells, either through nonspecific interaction (Fig. 8.1) or simply by impeding convection movements of relatively large sized nanoconstructs [167]. This could lead to nanomedicine being locally concentrated in proximity to the capillary that it leaked from without reaching the target tumor cells.

Recently, several studies have developed methods to circumvent these limitations such as using of the tumor penetrating peptide, iRGD, which has been shown to increase the delivery of nanomedicines in solid tumors by improving its interstitial transport [168]. Additional therapeutic strategies aiming to normalize the tumor vasculature and extracellular matrix in order to improve tumoral penetration of the drug have been described (for review [51, 169]).

Release Rate

Conjugates can be synthesized through covalent linking of drugs to polymeric carriers such as SMANCS (Table 8.1) [30]. In comparison, entrapment of drug inside a micellar structure requires either covalent or non-covalent bonds (ionic, hydrogen

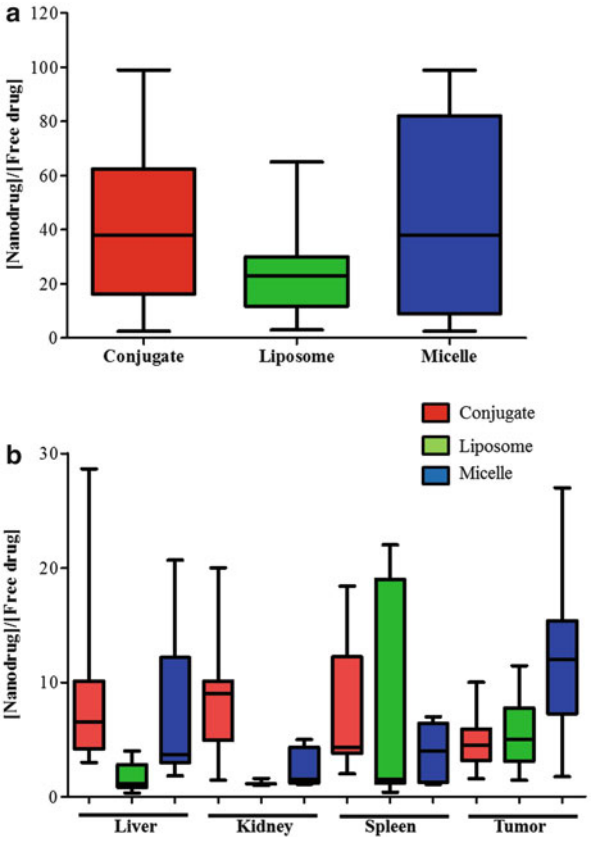


Fig. 8.4 Variation of nanocarrier release rate and accumulation profile. (**a**) Determination of the release rate over a 24 h period of different nanocarriers across several studies [162, 204–212]. (**b**) Comparison of the proportion of the different nanocarriers accumulation in the tumor and various organs [184–197, 202, 203]

bonds, or hydrophobic) and involves a block polymer or copolymer. Various chemical bonds such as amide, ester, azide, imine, hydrazone, thioether, and urethane are currently used to prepare nanomedicines [29, 170]. Based on the nature of these chemical bonds, the release of drug from its carrier can depend on either pH, usually acidic pH of the lysosome [170], temperature [70], or on enzymatic cleavage [171]. Furthermore, the nature of this bond will determine the release rate; for example, an ester bond ensures a rapid release of drug due to an abundance of esterases in plasma, whereas an amide bond will show a slower release profile [29, 148]. The comparison of release rates between polymer conjugates, liposomes, and micelles nanomedicines after 24 h incubation (Fig. 8.4a) in different studies showed a distinctive profile. Overall, polymer conjugates and micelles have a comparable release rate which is higher than that of liposomes (Fig. 8.4a). The release profile of liposomes appears relatively homogeneous with a mean value of 24 % (3–39 %), while the release profile of conjugates and micelles appears heterogeneous across several studies with a mean value of 39 % (2.5–100 %) and 41 % (2.5–100 %), respectively.

In order for a nanocarrier to provide tumor targeting, the carrier should have a stable chemical bond with the cargo drug while in circulation. This prevents the rapid release of free drug and permits a therapeutic effect at the site of action. A rapid drug release from its delivery system in plasma can result in a biodistribution and toxicity profile comparable to its related free drug. In contrast, engineering a stable linkage between the drug and its carrier can result in a slow release rate at the target site and inability to reach the critical therapeutic concentration. The release rate of nanoconstructs needs to be tailored for the treatment of a specified tumor doubling time (see Table 8.2). Thus, the choice of a specific linker is critical for a favorable anticancer outcome of EPR-targeted nanosystems.

Biocompatibility

The EPR-based accumulation of active drug inside the tumor rarely exceeds 5 % of the total dose of nanomedicine administrated by i.v. injection. The majority of the injected dose accumulates in various organs such as liver and spleen and to a minor extent kidneys and lungs [172]. As nanomedicines reach sizes of 7 nm, classical pharmacokinetics cannot be accurately applied due to two drastic changes. Firstly, nanosized drugs cannot be eliminated by renal glomeruli as they exceed the renal threshold of excretion dictated by the pore size in the glomeruli [32, 173]. Secondly, their organ distribution is limited to tissues that have capillaries with large enough endothelial fenestrations to allow macromolecular drugs to pass through [32, 33]. The EPR effect utilizes the unique characteristic of large gaps between endothelial cells that makes up tumor vessels. Usually these gaps can vary from few nanometers to up to 1200 nm in size [174, 175]. At this large size, nanomedicines can preferentially accumulate in tumor tissues. However, tumors are not the only organs with such large fenestrae as the spleen and liver show similar characteristics. Liver sinusoid can have fenestrae of around 100 nm in humans [176], whereas the spleen has large sinusoid lumina of ~ 5 μm that can support extravasation of aged red blood cells [32]. With such a large fenestration size, great amounts of nanosized drugs accumulate in these organs. As shown in Fig. 8.4b meta-analysis of 73 studies over the last 10 years revealed that with all the EPR effect-based nanoconstructs that were used, the liver and spleen were the two major organs competing with tumor for the nanoconstructs (conjugate, micelles, and liposomes). While spleen function can be compensated for by other lymphatic organs, liver damage due to the concentration of cytotoxic nanomedicine remains a challenge to successful anticancer drug targeting. For example, nanoconstructs of cis-platinum have reduced toxicity in the kidney compared to the free drug but result in a dose-limiting liver toxicity [177].

Surface modification of the nanomedicine, such as PEGylation, may increase their retention in the systemic circulation and favor tumor accumulation. However, more than 90 % of PEGylated nanoparticles will still be removed through liver clearance within several hours of administration. Studies have demonstrated that as

little as ~2 % of the total i.v. administered dose was found in the tumor after 4 h [172]. Thus there is a legitimate safety concern regarding the off target accumulation of the drug delivery system. Ideally, the drug carrier should be eliminated after drug release. But, unless the nanocarrier is biodegradable, it will remain in the body and be dealt with as a foreign body. The innate elements of the immune system could be stimulated nonspecifically by these foreign bodies through TLR-4 [155]. Activated macrophages will phagocytose and attempt to degrade the nanocarrier in its lysosomal compartment. Failure to do so may lead to the formation of foreign body giant cells caused by fusion of multiple macrophages or monocytes [178] and ultimately to the formation of lesions resembling granulomas [179]. This can potentially result in the pathological formation of a dense fibrous capsule replacing the original functional tissue. Another concern in relation to the accumulation of nondegradable materials is the induction of malignancy resulting from frustrated phagocytosis and prolonged inflammation [180].

To address these issues, recent work has focused on the development of biodegradable and nonimmunologic drug delivery systems containing either enzymatically or reductively degradable spacers such as poly(-D,L-lactide-co-glycoside) (PLGA) [181] or the star HPMA polymer carrier which enable a controlled degradation of the drug carrier [182, 183]. Some of these carriers demonstrate prolonged blood circulation and tumor drug accumulation but difficulties in the reproducibility of their synthesis could hamper further clinical development [182].

To summarize, EPR-related parameters of a nanoparticle delivery platform such as long circulatory half-life, reduced elimination, and altered distribution could be a double-edged sword. Careful consideration of these parameters is essential for effective, safe, and more personalized cancer treatments.

Conclusion

As a general concept of tumor vasculatures, vascular permeability has allowed the development of a variety of anticancer nanomedicines. In theory, the nanomedicine should decrease systemic toxicity and improve the delivery to the tumor site. However, despite high expectations for this targeting strategy, over the last 26 years, only a few nanomedicines have successfully exploited this concept and made the transition to the clinic. Possible reasons for this slow transition are the lack of control of essential parameters for a good delivery such as release rate, internalization, and biocompatibility. Moreover, the variability of tumors biology such as doubling time and microvascular density can influence the targeting potential of EPR-based nanomedicine. Consideration of these variables as well as the development of modular delivery systems of macromolecules can significantly hasten the transition of anticancer nanomedicine towards clinical application.

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Chapter 9
Pressure Gradients in Solid Tumors

Shawn Stapleton and Michael F. Milosevic

Abstract In order to reach target cells, nanoparticles must transport through the systemic circulation, extravasate from blood vessels, and penetrate the interstitial tissue. These transport processes occur by a combination of convection and diffusion. Tissue pressure gradients play an integral role in directly and indirectly mediating the convective and diffusive transport of nanoparticles in solid tumors. Specifically, poorly regulated tissue pressure gradients reduce the micro-regional delivery, extravasation, and penetration of nanoparticles. In this chapter we discuss the pathogenesis of abnormal tissue pressure gradients in solid tumors, describe their influence on the enhanced permeability and retention (EPR) effect, nanoparticle drug delivery, and review methods to modulate tissue pressure gradients to improve the transport of nanoparticles in solid tumors.

Introduction

Pressure gradients play an active role in maintaining tissue homeostasis by promoting interstitial fluid flow, which transports nutrients and waste products between blood vessels and cells, and macromolecules, such as antigens and cytokines, to

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local draining lymph nodes. Pressure gradients also play an important role in regulating blood flow [1] and modulating important biological processes that contribute to morphogenesis, remodeling of the extracellular matrix (ECM), cell migration, and cell–cell signaling [2–4]. In healthy tissue, the relationship between tissue pressure and structural, physiologic, and molecular factors allows cells to thrive, tissues to grow, and organs to function. In cancer, tissue pressure becomes deregulated, which leads to abnormal pressure gradients and consequently reduced nutrient delivery and waste removal, as well as an altered immune microenvironment. The abnormal pressure gradients are also associated with more aggressive tumor behavior such as increased metastatic potential and altered cellular function [4, 5]. The culmination of direct and indirect effects of abnormal pressure gradients in solid tumors contributes to inter- and intra-tumoral heterogeneity in the EPR effect, manifests as a barrier to drug delivery [6, 7], and contributes to impaired response to radiation therapy [1, 8, 9].

In this chapter, the pathophysiology of abnormal pressure gradients in solid tumors is reviewed with focus placed on the effect of tissue pressure on nanoparticle delivery. Ways of measuring tissue pressure are presented, including recent developments of noninvasively imaging techniques that may be useful in the clinical environment. Finally, methods to modulate tissue pressure are presented along with their impact on improving drug delivery.

Pathophysiology of Tissue Pressure in Solid Tumors

Pressure Gradients in Healthy Tissue

The total pressure experienced by tissue is the combination of solid tissue pressure and interstitial fluid pressure [10]. Solid tissue pressure (STP) is the result of direct contact between solid elements such as cells (epithelial, fibroblasts, and immune cells), ECM proteins (collagen, elastin, and fibronectin), and interstitial gel-like material composed primarily of hyaluronic acid. STP arises from tissue growth (cell division and vascular remodeling), tissue deformation (breathing), and cellular contraction (heart beating). Interstitial fluid pressure (IFP) is the hydrostatic force exerted on the interstitium. IFP arises from convection-driven fluid flow out of capillaries, through the interstitium, and into lymphatic vessels or back into postcapillary venules. Due to the poro-elastic nature of the interstitium, STP and IFP are intrinsically coupled.

In normal tissue, pressure gradients are actively regulated. STP gradients are regulated by (1) cell contraction, which in turn applies mechanical stress on the ECM; (2) realignment of fibroblast cells to locally shield from stress; and (3) ECM remodeling to modulate the tension between cells and other solid components [2, 11]. IFP is regulated by the relationships among key determinants of fluid flow into, through, and out of the tumor interstitium: trans-capillary fluid flow, interstitial fluid flow, and trans-lymphatic fluid flow. The trans-capillary fluid flow into the interstitium is typically described using Starlings law (9.1), which relates the

transmembrane plasma flux to hydrostatic and colloidal osmotic pressure difference between the capillary and interstitium. Mathematically Starling's law is given by

$$J_v = L_p S \times \left[\left(P_c - P_i \right) - \sigma \times \left(\Pi_c - \Pi_i \right) \right], \tag{9.1}$$

where J_v is plasma flux (cm^3/s), L_p is the hydraulic conductivity coefficient (permeability) of the capillary wall (cm/mmHg/s), S is the surface area of the vessel (cm^2), P_c and P_i are the capillary and interstitial hydrostatic pressures, respectively (mmHg), σ is the osmotic reflection coefficient (a dimensionless number between 0 and 1), and Π_c and Π_i are the capillary and interstitial colloid osmotic pressures, respectively (mmHg). Trans-capillary pressure gradients between 10 and 40 mmHg have been measured in different normal tissues [10, 12, 13]. Interstitial fluid flow is driven by IFP gradients and influenced by the hydraulic conductivity of the tissue. It can be described Darcy's law for fluid flow through a porous medium

$$v_i = -K \times \nabla P_i, \tag{9.2}$$

where v_i is the interstitial fluid velocity (cm/s), K is the hydraulic conductivity coefficient (permeability) of the interstitium (cm^2/mmHg/s), and ∇P_i is the spatial IFP gradient. The parameter K is an effective property that represents the combination of the permeability (also referred to as the porosity) of the interstitial space and the viscosity of the interstitial fluid. A few studies have measured interstitial pressure in animal models and reported gradients between 0.08 and 2.74 mmHg/mm [2, 14–16].

The mechanisms driving trans-lymphatic fluid flow are still an active area of research, but it is generally accepted that IFP and interstitial fluid volume are primarily responsible. As interstitial fluid volume increases due to capillary filtration, strain is placed on the ECM that in turn places strain on lymphatic endothelial cells through anchoring filaments [17, 18]. The strain opens gaps between lymphatic endothelial cells, allowing pressure-driven trans-lymphatic flow of interstitial fluid. Mathematical modeling of trans-lymphatic transport is still in its infancy, and attempts to describe lymphatic transport using Starling's law have proven insufficient [17, 19]. While several mathematical theories of trans-lymphatic transport have been proposed [19], it is sufficient to understand the genesis of IFP gradients by assuming a trans-lymphatic fluid flow rate J_L (cm^3/s). Due to conservation of mass, the interstitial fluid velocity (IFV) is related to the trans-capillary fluid flux (9.1) minus the trans-lymphatic fluid flux. That is,

$$\nabla \cdot v_i = \frac{J_v}{V} - \frac{J_L}{V} \tag{9.3}$$

Combining (9.2) and (9.3) reveals that steady-state IFP gradients are driven by the steady-state capillary and lymphatic hydrostatic pressures, as well as capillary osmotic pressure, and are regulated by the hydraulic conductivities of the capillary wall, the interstitium, and lymphatic wall. All of the properties work in unison to regulate IFP in healthy tissue, maintaining it close to, or slightly less than, atmospheric pressure.

Pressure Gradients in Solid Tumors

In solid tumors the tissue pressure gradients become unregulated and are typically characterized by elevated STP and IFP. Structurally, tumors are less compliant than comparable normal tissues due to a higher density of malignant and nonmalignant (stromal) cells, the higher elastic modulus of tumor cells, as well as higher concentrations of ECM proteins such as collagen and fibronectin [20, 21]. Rapid tumor cell proliferation and growth in a confined space combine to cause elevated STP. Although difficult to measure, STP is believed to be elevated in the range of 28–120 mmHg in solid tumors [22, 23]. IFP is elevated in the range of 10–100 mmHg in virtually all solid malignant tumors in both animal models and man [24–28] and is a consequence of abnormal vasculature and lack of functional lymphatic vessels within the tumor tissue.

Elevated STP in solid tumors is a consequence of growth-induced solid stress and compressive solid stress caused by confined growth [29]. Growth-induced solid stress, or residual stress, is caused by proliferating cancer cells that exert force on surrounding cells and ECM structures causing them to deform. The force is transmitted through direct contact or through compliant interstitial components including fibroblasts, other stromal cells, collagen, fibronectin, and hyaluronan [30]. Additionally, the ECM is extensively cross-linked and bound to cellular structures such that contraction of ECM components contributes to increased solid stress [21]. STP and IFP are intrinsically coupled and changes in STP, for example, due to tissue deformation or cell contraction, will lead to equivalent changes in IFP. Alternatively, changes in IFP, for example, caused by increased trans-vascular fluid filtration, cause changes in STP as a result of tensile forces on the ECM and shear stress on cellular and ECM components.

Elevated IFP in solid tumors is a consequence of morphologically and functionally abnormal blood vessels and a lack of functional lymphatic vessel [28]. Unregulated angiogenesis results in tumor blood vessels that are spatially disorganized, tortuous, lack structural hierarchy, are often dilated, and contain excessive branching and shunts [31, 32]. These properties contribute to elevated viscous and geometric resistance to tumor blood flow and may result in elevated capillary pressure—P_c [33, 34]. Unregulated angiogenesis also results in hyper-permeable tumor blood vessels, which have wide inter-endothelial junctions, large fenestrae, and large trans-endothelial channels [35]. The increased permeability of tumor vessels results in a decreased trans-capillary osmotic pressure gradient [36] and increased vascular hydraulic conductivity—L_p. Some studies reported that L_pS is 10–1,000 times higher in tumors compared to normal tissue [37, 38] and can result in as much as a 15-fold increase in trans-capillary fluid flux in solid tumors compared to healthy tissue [28]. Lymphatic vessels are present within the tumor but are generally nonfunctional [39, 40]. Instead, functional lymphatics are located along the tumor margins in the peri-tumoral tissue. The excess fluid leakage in combination with the lack of functional lymphatic vessels causes the central IFP to rise until it nearly equals the average capillary pressure.

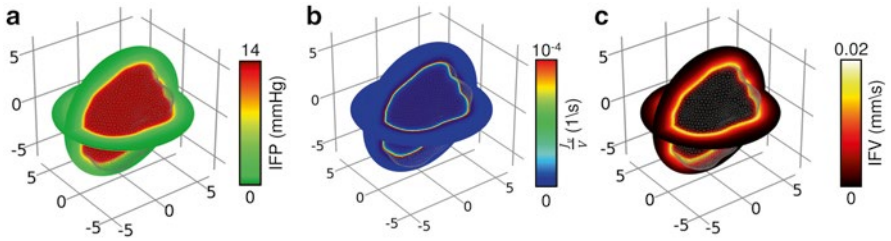

Fig. 9.1 Mathematical modeling demonstrates that steady-state IFP is elevated and uniform in the central tumor volume and drops precipitously at the tumor periphery (**a**). Based on the state of IFP, the predicted rate of trans-vascular convection (J_v/V) is zero within the tumor volume, and trans-vascular fluid flow occurs predominantly along the peri-tumoral regions (**b**). The corresponding interstitial fluid velocity is also zero in the central tumor region and directed outwards at the periphery of the tumor (**c**). This simulation demonstrates that convection is negligible in the central tumor regions and limited to the peri-tumoral regions. The *mesh* denotes the boundary between healthy (*outside*) and tumor (*inside*) tissue and was obtained by a computed tomography scan of an orthotopic cervix tumor xenograft mouse model. The simulation does not consider lymphatic transport and the scale is in mm

Biophysical modeling of convection-driven fluid transport using (9.2) and (9.3) has suggested that steady-state IFP is uniformly elevated in the central tumor volume and falls precipitously to zero near the interface between tumor and surrounding normal tissue [7]. The model predicts that the trans-vascular pressure gradient is largely abolished in the center of tumors and that trans-vascular fluid flux only occurs at the periphery in an outward direction towards the surrounding normal tissue (Fig. 9.1). The predicted radial IFP profiles and microvascular pressure (MVP) have been confirmed using an invasive point-based mapping method in animal tumors [27, 33]. However, significant spatial variation in IFP has been observed in preclinical studies of human malignant melanomas, breast carcinomas, and colorectal carcinomas [25, 41]. In 77 human cervix cancer patients, it was demonstrated that IFP can vary by up to 15-fold (median 2.4-fold) in an individual tumor [42]. Temporal variations in IFP may also be present. IFP may fluctuate with changes in blood flow that occur on a time scale of second to hours [43–49] and changes in the vascular and interstitial hydraulic conductivities over time scales of minutes to days [50–52]. In addition, IFP may increase over longer times due to matrix remodeling [53] and tumor growth [27, 54].

The tumor interstitium is heterogeneous in terms of both morphology and interstitial fluid flow. There are regions with high concentrations of collagen, elastin, and other ECM components that hinder interstitial fluid flow. There also are regions of necrosis where interstitial fluid flow is enhanced. There is constant remodeling of the stromal and ECM architecture in response to cytokine signaling and tissue stress induced by interstitial flow [3, 4]. The spatiotemporal heterogeneity in interstitial architecture leads to variations in the interstitial hydraulic conductivity—K. Furthermore, the interstitial hydraulic conductivity varies nonlinearly with extracellular fluid volume and IFP [55], and tissue with elevated IFP can have up to 100,000-fold higher K compared to normal tissue [3].

It is clear that tissue pressure gradients play an important role in tumor progression, disease dissemination, and treatment response; the latter by acting as a barrier to drug delivery and contributing to radiation resistance [2, 6, 26, 56]. Therefore, techniques for measuring pressure gradients in the clinical environment are required to support future studies of therapeutic intervention designed to modulate pressure gradients, improve drug delivery, and ultimately improve patient outcome.

Measuring Solid Tissue Pressure

The earliest form of measuring STP is palpation, where the stiffness of malignant tumors is determined subjectively by manual manipulation of the tissue. Some quantitative estimates of tumor STP have arisen based on mathematical models describing the accumulated stress in a growing spheroid volume and on experimental tumor spheroid systems [22, 23, 57]. It was proposed by Skalak et al. [29] that residual solid stress can be estimated by making cuts in the tissue and observing resulting shape changes. Using this technique Stylianopoulos et al. [30] showed that preclinical and clinical tumors exhibit significant shape change compared to normal tissue, indicating an elevated amount of residual stress. They demonstrated that residual stress was reduced by depleting collagen, fibroblasts, hyaluronan, and cancer cells, highlighting the importance of these structures in mediating STP. Furthermore, they found that tumor blood flow increased when residual stress was decreased, adding support to the concept that STP can cause vascular constriction and collapse.

Recently, elastography imaging using either ultrasound or magnetic resonance imaging (MRI) has emerged as a potentially viable tool for the assessment of tissue stiffness or elasticity in the clinical setting. Elastography works by applying a stress on the tissue and measuring the resulting deformation (strain). The ratio of these two quantities gives a parameter termed Young's modulus, which represents the stiffness of the tissue. A detailed review of the fundamentals of ultrasound elastography (USE) is given in [58] and magnetic resonance elastography (MRE) is given in [59]. USE measurements have confirmed that tumor tissues have higher STP relative to normal tissue. For instance, Young's modulus was found to be 100 kPa (750 mmHg) in prostate carcinoma compared to 62–69 kPa (465–518 mmHg) in healthy prostate [60]. However, large variations in stiffness for different and similar tissues have been reported, which may be related to the nonlinear nature of the stress–strain relationship and the technical challenges in performing controlled ultrasound elastography measurements [58]. MRE has the benefit of being operator independent and can measure tumor stiffness in 3D dimensions. MRE of breast tumors were found to have a higher shear stiffness (25 kPa or 187 mmHg) compared to breast fibroglandular tissue (7.5 kPa or 56 mmHg) and breast adipose tissue (3.3 kPa or 25 mmHg) [61]. Both USE and MRE can provide spatial maps of tumor stiffness at resolutions on the order of 1 mm or less and therefore may provide probe tissue properties related to STP within solid tumors.

Measuring Interstitial Fluid Pressure

Measuring interstitial fluid pressure has seen only modest developments over the past four decades. All past and currently accepted techniques are based on invasive methods, including (1) micropipettes [62], (2) needle technique [63], (3) wick catheter technique [64, 65], (4) wick-in-needle [66], (5) micropipette/servo-null [67], and (6) fiberoptic transducers [68]. These techniques have proved suitable for use in preclinical and clinical applications where it is feasible to make point-based measurements; however, all of these methods are invasive, are limited to superficial tumors, and have limited ability to spatially map IFP throughout the tumor volume. Therefore noninvasive imaging methods have been sought out to provide spatio-temporal mapping of IFP in a clinical setting. The imaging methods are almost exclusively based on magnetic resonance imaging (MRI).

Invasive Methods to Measure Interstitial Fluid Pressure

The invasive methods are reviewed briefly here, and a more detailed review including a comparison of invasive techniques can be found elsewhere [69]. The micropipette method was developed to measure MVP in frog mesentery [62]. The technique uses glass micropipettes that are pulled and beveled to achieve a tip diameter of 1–3 μm. The micropipette is filled with saline solution mixed with a blue dye. MVP is determined by using a counterbalancing pressure such that blue dye is just being forced into the vessel. The micropipette is the least-traumatic invasive methods and is able to measure pressure in a small region. However, the method is time consuming due to frequent capillary breakage and the need to manually adjust the pressure. The micropipette technique was modified to use a servo-null method to automatically provide the counterpressure. In this case, the micropipette is filled with an electrolytic fluid that has a low and constant electrical resistance. The electrical resistance increases significantly when interstitial fluid or plasma enters the micropipette. The servo-null system detects the change in resistance and triggers a pump to generate an equal counterpressure that restores the micropipette to its baseline electrical resistance. The main drawback of this method is the inability to measure pressure deeper than 2–3 mm in solid tumors.

The needle technique is a simple method to measure IFP deep in the tissue. The method is similar to the original method of [62], except the micropipette is replaced with a 23–30 G hypodermic needle. The needle is introduced into the tissue and the minimum pressure required to make the fluid flow from the needle to the tissue is recorded. This method suffers from frequent obstructions at the tip and potentially erroneous fluid pressure measurements due to the introduction of fluid into the interstitial space.

The wick catheter technique consists of a saline-filled PE50 tube with a cotton or nylon wick (often made using surgical sutures) protruding from the end. The wick catheter is inserted through a 16 G needle that is used to penetrate the skin and is

subsequently removed once the catheter is in place. The wick and PE50 tubing is in communication with the interstitial fluid and recorded using a pressure transducer. The criticism of the wick catheter method is that the wick may be generating osmotic pressure differences, resulting in erroneous estimates of hydrostatic fluid pressure.

The wick-in-needle (WIN) technique combines the desirable qualities of both the needle and wick catheter methods. A 2–4 mm side hole is ground in a hypodermic needle about 3–5 mm from the tip. A wick is then placed into the needle to maintain fluid continuity between the tumor and the apparatus. Fluid communication occurs through the side hole, and given the rigidity and length of the needle, pressure can be measured deep in the tumor tissue. This is the most commonly used technique and the most applicable in the clinical environment, but it is not ideal in the preclinical setting where tumors are typically 4–10 mm in diameter, making repeat measurements and measurements of the spatial distribution of IFP difficult.

Imaging Interstitial Fluid Pressure

There have been several attempts to estimate tumor IFP noninvasively. Lyng et al. [70] attempted to image IFP using the proton relaxation rates measured with MRI. The rationale for this approach is that tumors with elevated IFP have a higher interstitial fluid volume, which may alter the proton relaxation rate. They did not find a correlation between T1 and T2 relaxation rates with IFP measured using the WIN technique. Hassid et al. [71] measured the slow infusion of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) enhancement using MRI. They demonstrated that the estimated rates of trans-vascular efflux and influx were correlated with the steady-state tumor concentration of Gd-DTPA. They produced a spatial map of IFP by assuming that Gd-DTPA concentration was inversely correlated with IFP (regions with poor Gd-DTPA enhancement were considered to have high IFP). They surmised that their technique mapped tumor IFP based on the fact that the radial distribution of IFP (Gd-DTPA concentration) agreed with the predictions of Baxter and Jain [7]. However, this method is directly affected by tumor necrosis and poor central tumor perfusion that can also produce similar patterns of Gd-DTPA enhancement. Haider et al. [72] found a negative correlation between quantitative measures of tumor microvasculature (area under the curve and relative K_{trans}) and tumor IFP. They speculated that these correlations were due to higher blood flow resistance in tumors with high IFP. Zhao et al. [73] used DCE-MRI to create spatially varying 3D maps of vascular hydraulic conductivity and vascular permeability. As well, they used anatomic MRI to derive the tumor and surrounding healthy tissue geometry of a subcutaneous xenograft tumor in a mouse. The image-derived parameters were used in a convection-driven transport model to predict IFP and interstitial fluid velocity in the tumor and surrounding healthy tissue using finite element analysis. They found that IFP was elevated in the central region of the tumor and interstitial fluid velocity was lower on the skin side of the implanted tumor. Additionally, they showed that the shape of the predicted and measured

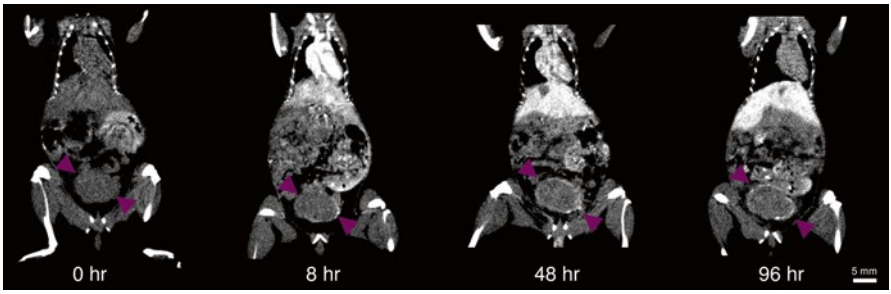

Fig. 9.2 Computed tomography (CT) images of the spatiotemporal accumulation of a CT-liposome contrast agent in an orthotopic cervix cancer mouse model. A predominantly peripheral accumulation of the CT-liposome can be seen at the 48 and 96 h time points in the tumor (*arrows*)

distribution of Gd-DTPA was similar, demonstrating that the transport model predicted the spatial distribution of Gd-DTPA. Gulliksrud et al. [74] also found that K_{trans} was negatively correlated with IFP for non-necrotic A-07 and R-18 melanoma tumor; however, no correlation with IFP was found for necrotic tumors. Kim et al. [75] used diffusion-weighted imaging and the intravoxel incoherent motion (IVIM) analysis technique to estimate blood flow and vascularity. These two properties are reflected by two parameters termed the pseudo-diffusivity (D_p) and perfusion fraction pseudo-diffusivity product (f_pD_p). The authors found a significant negative correlation between IFP and both D_p and f_pD_p. Hompland et al. [76] observed, using DCE-MRI, a high-signal-intensity rim in the tumor periphery that moved outward with time. They assumed that the velocity of the enhanced rim was equal to the interstitial fluid flow velocity at the tumor periphery. They found a significant positive correlation between the enhanced rim velocity and tumor IFP measured using the wick-in-needle technique and observed that cervical cancers with higher rim enhancement velocity also had a higher incidence of lymph node metastasis compared to those with a lower velocity.

All of the current approaches to image elevated IFP rely on the use of low-molecular-weight contrast agents to measure indirect properties associated with elevated IFP, such as interstitial volume, blood flow, vascular morphology, and interstitial fluid velocity. The use of low-molecular-weight contrast agents is likely not ideal due to potentially confounding contributions from diffusive transport. High IFP is a pressure-driven phenomenon and may be more accurately characterized using high-molecular-weight contrast agents that transport predominantly by convection. To date, there have been no published reports demonstrating that the spatiotemporal accumulation of a high-molecular-weight contrast agent is reflective of the underlying IFP. One approach to address this is using liposomal-based contrast agents that encapsulate low-molecular-weight contrast agents such as iohexol or gadolinium. These agents have a high molecular weight and are believed to transport predominantly by convection [77, 78]. We have shown that the spatial distribution of these agents is predominately at the tumor periphery (Fig. 9.2), which is consistent theoretical predictions using a convection-driven transport model [7].

This suggests that the application of a convection-driven mathematical model to the spatiotemporal liposomal distribution could give an accurate assessment of elevated tumor IFP. In general, the challenge inherent in all imaging approaches to estimate elevated IFP is accounting for other properties that might also influence contrast agent transport kinetics, such as diffusion, necrosis, reduced blood flow, and heterogeneous vascular permeability.

Implications for Drug Delivery

The enhanced permeability and retention (EPR) effect is the theoretical foundation for the development and use of macromolecular- and nanoparticle-based medicine [79]. It is an empirical description of the increased accumulation and retention of macromolecular- and nanoparticle-based therapeutics due to the hyper-permeable nature of tumor blood vessels and lack of functional lymphatics [80]. However, the EPR effect is not a consistent phenomenon and can vary considerably between tumors and even within individual tumors. The inter- and intra-tumoral variations in EPR have been highlighted in several studies using imaging technology to track the spatiotemporal distribution of nanoparticles. For example, Gabizon et al. [81] used whole-body gamma camera imaging to monitor the biodistribution and tumor accumulation of ^{111}In-labeled liposomes in patients with solid tumors and found no significant accumulation in all but one tumor. Harrington et al. [82] found considerable heterogeneity in total tumor uptake of ^{111}In DTPA-labeled liposomes between different tumor types and between patients with the same tumor type. More recently, our lab has shown using computed tomography (CT) that the spatiotemporal distribution of iohexol-encapsulated liposomes in rabbit and mouse tumor models is heterogeneous with larger tumors exhibiting predominantly peripheral accumulation [83, 84]. The heterogeneous EPR-mediated delivery of nanoparticles is the result of (1) heterogeneous tumor perfusion [85], (2) heterogeneous extravasation [35, 86], and (3) heterogeneous/limited penetration [6]. Each of these properties can be linked directly or indirectly to the abnormal pressure gradients found in solid tumors (Fig. 9.3). In the following sections, we describe how tissue pressure gradients play a central role in mediating EPR-driven nanoparticle delivery.

Tumor Blood Flow, Pressure Gradients, and Drug Distribution

The chaotic morphology and physiology of tumor blood vessels limit the ability of blood-borne therapeutics to reach the target tissue. Tumor microcirculation is heterogeneous in terms of both morphology and blood flow [44, 85, 87, 88]. Clinical and experimental evidence has shown that vascular density is typically higher along the tumor periphery compared to the central regions [89, 90], and vascular morphogenesis and remodeling occur on a continuous basis [51, 52]. Tumor blood flow (TBF) can be higher or lower than in comparable healthy normal tissues, with

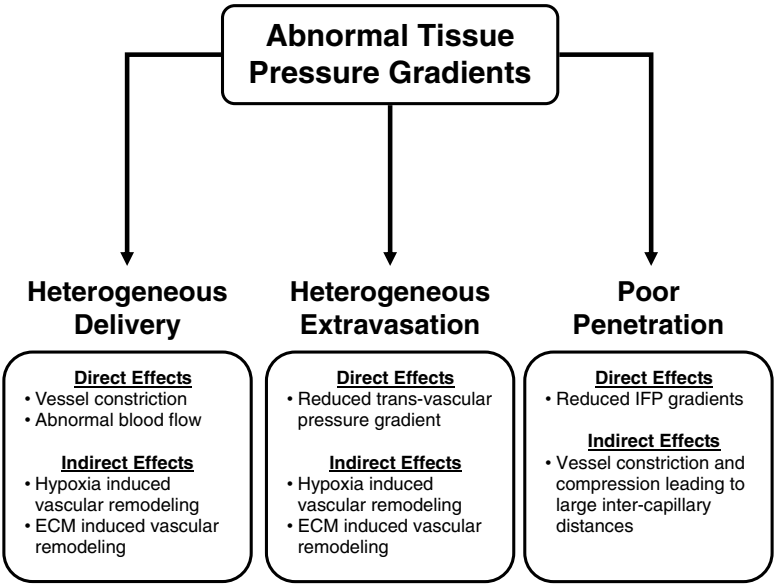

Fig. 9.3 There are several direct and indirect effects that abnormal pressure gradients have on the heterogeneous delivery, heterogeneous extravasation, and poor penetration of nanoparticles in solid tumors

higher blood flow typically observed along the tumor periphery [89]. Vascular constriction and collapse has been observed and is spatially and temporally heterogeneous [91]. These properties contribute to TBF that is intermittent, and exhibits periods of stasis and flow reversal [43–49]. As a result, the extravasation of nanoparticle therapeutics is limited to areas of the tumor with active TBF, and areas deprived of blood flow can only be reached once the agent has extravasated and traveled through the interstitium.

Pressure gradients play an important role in driving the chaotic vascular morphology and blood flow observed in solid tumors. STP contributes directly to heterogeneous drug accumulation through vascular constriction and collapse, leading to increased flow resistance and spatiotemporal heterogeneity of blood flow and thereby limiting the accumulation of nanoparticles to perfused regions of the tumor [1, 30, 57, 92]. Additionally, the higher vascular hydraulic conductivity, L_p, in tumors suggests greater communication between blood flow, trans-vascular fluid exchange, and interstitial fluid flow in solid tumors compared to normal tissue. Mathematical modeling that takes account of the coupling between IFP and tumor perfusion has demonstrated that elevated IFP results in efferent capillary constriction, which in turn leads to flow stasis and redistribution of perfusion from the center to periphery of the tumor [1, 93, 94]. Some experimental data support this hypothesis, where measurements in ovarian cancer demonstrated that blood flow and interstitial fluid flow have a strong positive correlation [28].

STP and IFP may also influence the chaotic morphology and heterogeneous blood flow through pressure-induced modifications of the ECM. STP and interstitial fluid flow actively participate in the remodeling of the ECM, which leads to the release of potent proangiogenic molecules and stimulates tumor angiogenesis [2, 4]. As well, vascular collapse and intermittent TBF driven by elevated IFP and STP may contribute to the development of hypoxia, which in turn stimulates tumor angiogenesis [42]. Therefore, STP and IFP may contribute indirectly to the heterogeneous delivery of nanoparticles by stimulating angiogenesis and further contributing to impaired TBF.

Trans-vascular Transport, Pressure Gradients, and Drug Delivery

The delivery of blood-borne nanoparticle therapeutics is dependent on the ability of the agent to transport out of capillaries and into the interstitium. Vascular permeability is driven by the expression of several factors including bradykinin, nitric oxide (NO), prostaglandins, and vascular endothelial growth factor (VEGF) [79]. The pore size of tumor vessels can be up to 2 μm in diameter, and considerable heterogeneity has been observed within a tumor and between tumors [35, 86]. Vascular permeability is also spatiotemporally heterogeneous as a result of fluctuations in stimulators of angiogenesis, which as previously mentioned are in part driven by abnormal pressure gradients [86, 95, 96]. Furthermore, increased permeability results in the abolishment of osmotic and hydrostatic pressure gradients, specifically in the central tumor region, and hinders trans-vascular convective transport of nanoparticles [7, 33, 36]. Therefore, nanoparticles must rely on the slow process of diffusion to extravasate in regions of elevated IFP. As a result, the trans-vascular transport of nanoparticle therapeutics is spatially and temporally heterogeneous.

As discussed, elevated IFP plays a direct role in limiting the convection-driven trans-vascular transport of nanoparticles in the central region of the tumor. Diffusion is the dominant transport mechanism in central regions that are perfused but lack a trans-vascular pressure gradient. However, observations of intermittent blood flow suggest that transient spatiotemporal fluctuations in MVP and IFP may exist in the central tumoral region and temporarily restore the trans-vascular pressure gradient. Therefore, trans-vascular convection may also contribute to nanoparticle extravasation in the central tumor regions, but this has yet to be experimentally confirmed. The largest effect of abnormal tissue pressure gradients appears to be limiting the extravasation of nanoparticles to the tumor periphery, where significant trans-vascular pressure gradients are present. While diffusive transport contributes to extravasation, theoretical modeling predicts that the rate of trans-vascular diffusion of macromolecules is significantly slower than convection in the presence of modest (~2 mmHg) trans-vascular pressure gradients [97]. IFP and STP may also play an indirect role in the heterogeneous extravasation of nanoparticles by modulating vascular permeability through ECM remodeling and hypoxia-driven angiogenesis.

Interstitial Transport, Pressure Gradients, and Drug Delivery

Experiments have shown that the interstitial penetration of nanoparticles can be severely limited [86]. The penetration of nanoparticles into the tumor interstitium is hindered by several factors including the lack of interstitial fluid pressure gradients and a dense interstitial structure. In tumors with high IFP, interstitial fluid flow is negligible in the central tumor region and the bulk of fluid flow occurs in the peritumoral regions. This has several important implications relating to the poor penetration of nanoparticles. In the absence of interstitial pressure gradients, nanoparticles transport occurs by diffusion. The rate of diffusion depends on several factors including tissue structure, tissue hydration, viscosity of the medium, hydrodynamic radius, and molecular weight of the nanoparticle. Empirical studies have demonstrated an inverse power relationship between the diffusion coefficient and molecular weight, demonstrating that macromolecular agents and nanoparticles diffusion is extremely slow in biological tissue [98]. It has been estimated that it could take a 100 nm liposome over 80 days to diffuse a distance of a 100 μm in tumor tissue, which is likely much longer than the stability of the agent allows for [99]. Along the tumor periphery, outward-directed interstitial fluid flow dominates the inward diffusion of nanoparticles, again limiting the ability of nanoparticles to target cells in the central tumor regions.

Abnormal pressure gradients directly limit the penetration of nanoparticles by reducing interstitial fluid flow, particularly in the central tumor regions. Along the tumor periphery, steep interstitial pressure gradients serve to transport nanoparticles out of the tumor and into the surrounding normal tissue. Interstitial fluid pressure gradients are influenced by the abnormal stromal characteristic of solid tumors leading to hindered interstitial fluid flow and steric resistance to nanoparticles transport [2, 100]. Molecules in the interstitial space such as hyaluronan increase the interstitial fluid viscosity and may impede the convective interstitial transport of high-molecular-weight agents [77]. In addition, abnormal pressure gradients lead to altered blood flow distribution, resulting in an increased intercapillary distances that must be traversed by nanoparticles in order to reach target cells far away from blood vessels.

Methods to Restore Pressure Gradients and Improve Drug Delivery

Many studies have demonstrated that reducing STP and/or IFP can significantly increase the accumulation, improve the distribution, and/or increase the penetration distance of traditional chemotherapeutics and nanoparticle-based therapeutics [101–108]. The strategies employed are based on pharmacological agents, heat, and radiation to reestablish trans-vascular and/or interstitial pressure gradients. As previously discussed, blood flow, vascular permeability, MVP, IFP, and STP are

coupled and thus any strategy to modulate one aspect will influence the others and will result in a positive or potentially negative impact on drug delivery. While these strategies largely have been successful in improving drug delivery, there have been conflicting studies indicating that reducing IFP does not always lead to increased drug accumulation [109, 110]. These conflicting studies highlight that abnormal tissue pressure gradients are not the only barrier to drug delivery and also highlight that many of these agents have complex, interrelated vascular and cellular effects on both the normal and tumor tissues. A summary of the agents that are known to alter IFP and improve accumulation and penetration of nanoparticles in solid tumors is given in Table 9.1.

Improving Blood Flow and Nanoparticle Delivery

Improving blood flow has received the most attention as means of enhancing drug delivery. The aim of these methods is to increase micro-regional blood flow and to reestablish a trans-vascular pressure gradient by increasing MVP and/or decreasing tumor IFP. According to Poiseuille's law, blood flow can be increased by (1) increasing the vessel diameters, (2) increasing the arterial venous pressure gradient, (3) reducing viscous resistance, (4) reducing geometric resistance, and (5) reducing the length of the vessel. Modulating vessel diameter (D) should have the largest impact because blood flow is proportional to D^4. Indeed, several vasoactive agents have demonstrated the ability to modulate IFP, improve TBF, and improve nanoparticle accumulation. There have been a limited number of attempts to modulate viscous resistance, although some strategies have shown promise.

Angiotensin II (AT-II) is a potent vasoconstrictor that has shown potential to improve nanoparticle delivery in solid tumors [111–114]. The mechanism of action for AT-II is complex, but it is generally thought that AT-II causes significant arterial vasoconstriction in healthy tissue and systemic hypertension, leading to a diversion of blood flow to the tumor [89]. Zlotecki et al. [115] found that systemic administration at AT-II results in a 72–82 % increase in mean arterial blood pressure (MABP), a 40–100 % increase in TBF, and a 22–25 % increase in tumor IFP in three different mouse xenograft tumor models. Hori et al. [116] went on to demonstrate that although AT-II increased tumor IFP by roughly 25 %, it increases MVP substantially more (approximately 67 %), thus reestablishing a trans-vascular pressure gradient and promoting improved drug accumulation.

Nitroglycerin (NG) is a vasodilating agent that has been shown to improve the delivery of chemotherapeutics and macromolecular agents [117–119]. NG is metabolized in the mitochondria to produce nitric oxide, which in turn promotes vasodilation and vascular permeability. When administered intravenously, NG has been found to decrease MABP by 39 %, TBF by 32 %, and only transiently decreased tumor IFP by 6 % [120]. When administered topically, NG resulted in a 40–60 % increase in TBF and a two- to threefold increase in accumulation of two macromolecular agents in several different preclinical rat and mouse tumors [119].

Table 9.1 An overview of agents known to lower tissue pressure and promote the accumulation and penetration of nanoparticles

Agent	Possible mechanism of action	Effects on tumor microenvironment	Effects on tumor pressure	Effects on EPR	References
Promoters of tumor blood flow					
Angiotensin II	Vasoconstriction at host arterial level, leading to increased tumor blood flow	↑ MAPB (72–82 %) ↑ MVP (67 %) ↑ TBF (40–100 %)	↑ Tumor IFP (22–55 %)	↑ Accumulation of SMANCS (1.2- to 1.6-fold) ↑ Accumulation and intra-tumoral distribution of liposomes	Zlotecki et al. [115] Hori et al. [116] Li et al. [111] Hattori et al. [112]
Nitroglycerin	Vasodilation and increased vascular permeability	↓ MABP* (39 %) ↓ TBF* (32 %) ↑ TBF** (40–60 %)	↓ Tumor IFP* (6 %)	↑ Accumulation of EB and PZP in several tumor models (two- to threefold)	Zlotecki et al. [115]* Seki et al. [119]**
Bradykinin agonists	Vasodilation and increased vascular permeability	↓ MAPB (2–25 %) ↓ TBF (86 %) ↓ Micro-regional blood flow ↑ Vessel diameter (67–86 %)	↓ Tumor IFP (8–18 %)	↑ Accumulation of 70 kDa dextran and carboplatin in several tumor models (threefold)	Emerich et al. [173] Emerich et al. [121]
Nicotinamide	Vasodilation and reduced spontaneous contractions of vessels	↓ MABP (15 %) ↑ TBF (75 %) ↑ Micro-regional blood flow	↓ Tumor IFP (39–47 %)	Unknown	Chaplin et al. [125] Lee et al. [124] Peters et al. [123]
Pentoxifylline	Reduced blood viscosity	– MAPB ↑ TBF (32–46 %)	↓ Tumor IFP (55 %)	Unknown	Lee et al. [130] Song et al. [131]
Hyperthermia	Increased tumor blood flow and vascular permeability	↑ MAPB (26 %) ↑↓ TBF ↑↓ Vascular permeability	↓ Tumor IFP (78 %; 43 °C for 30 min)	↑ Accumulation of monoclonal antibody fragment in xenografts (two- to threefold) ↑ Accumulation of liposomes in several tumor models (0.4- to 1.6-fold)	Cope et al. [174] Krüger et al. [132] Leunig et al. [133] Kong et al. [175] Song et al. [136]

(continued)

Table 9.1 (continued)

Agent	Possible mechanism of action	Effects on tumor microenvironment	Effects on tumor pressure	Effects on EPR	References
Radiation	Increased tumor blood flow and vascular permeability	↑ TBF (105–122 %, 2–7 days after 10 or 20 Gy) ↑ Vascular permeability (60 %)	↓ STP ↓ Tumor IFP (20–40 % for doses >5 Gy)	↑ Accumulation of Caelyx™ in human sarcoma (1.8- to 4.7-fold, 70 Gy fractionated) ↑ Accumulation of Caelyx™ in osteosarcoma xenograft (two- to fourfold, 8 or 11 Gy) ↑ Accumulation of iron oxide nanoparticles (twofold, 15 Gy) ↑ Accumulation of HPMA copolymer-based drug delivery systems in several tumor models (1.2- to 2-fold, 20 Gy)	Krishnan et al. [138] Tozer et al. [139] Znati et al. [140] Koukourakis [176] Davies et al. [177] Lammers et al. [178] Giustini et al. [144]
Promoters of trans-vascular transport					
Low-dose TNF-α	Unclear. Many vascular effects	↓ MAPB (30 %) ↑ Vessel stability	↓ Tumor IFP (50–70 %)	↑ Accumulation of Doxil and other macromolecules in several tumor models (three- to sixfold) ↑ Micro-regional distribution of Doxil	Kristensen et al. [145] Ten Hagen et al. [148] Brouckaert et al. [147] Seynhaeve et al. [146]

VEGF\VEGFR inhibition	Vascular normalization	– MAPB ↓ Microvessel density (50 %) ↓ Vascular permeability (52 %) ↑ Vessel structure and vascular morphology	↓ Tumor IFP (50–70 %) ↑ Oncotic pressure	↓ Accumulation of 100 nm liposomes (1.5-fold) ↑ Accumulation of 100 nm liposomes in tumor core after low dose of SU5416 ↓ Accumulation of nanoparticles >60 nm ↑ Accumulation of nanoparticles <13 nm	Tong et al. [106] Jain [149] Jain et al. [152] Chauhan et al. [100] Yoshizawa et al. [179]
Promoters of interstitial penetration					
Relaxin (reproductive hormone)	Reorganization and degradation of collagen	↓ Collagen	Unknown	↑ Accumulation of macromolecules in mouse tumor model (two- to threefold)	Brown et al. [180] Perentes et al. [53]
Collagenase	Degradation of collagen	↓ Collagen ↑ *K* ↓ MVP (60 %) ↑ Micro-regional blood flow	↓ Tumor IFP (45 %)	↑ Accumulation and intratumoral distribution of antibodies in mouse tumor model (twofold) – No change is liposome accumulation	Netti et al. [153] Eikenes et al. [107] Erikson et al. [157]
Losartan	Degradation of collagen	↓ Collagen I (19–52 %) ↓ MABP (11 %) – Micro-regional blood flow	Unknown	↑ Accumulation and intratumoral distribution of 100 nm nanoparticles and HSV in several mouse tumor models	Diop-Frimpong et al. [181]
Hyaluronidase	Degradation of hyaluronan	↓ Hyaluronan (19–52 %) ↑ *K* (healthy lung tissue) – MABP ↑ Micro-regional blood flow	↓ Tumor IFP (63–84 %)	↑ Accumulation and intratumoral distribution of antibodies (20 %) ↑ Accumulation and intratumoral distribution of Caelyx™ (fourfold)	Brekken et al. [159] Brekken et al. [160] Brekken et al. [161] Eikenes et al. [101]

(continued)

Table 9.1 (continued)

Agent	Possible mechanism of action	Effects on tumor microenvironment	Effects on tumor pressure	Effects on EPR	References
Paclitaxel\ docetaxel	Tumor cell apoptosis	↓ MVP (33 %) ↑ *K* (109 %) – MABP ↑ TBF (two- to threefold) ↑ Micro-regional blood flow	↓ Tumor IFP (54 %)	↑ Accumulation and intratumoral distribution of 100 nm and 200 nm nanoparticles and liposomal doxorubicin (1.8-fold) ↑ Accumulation and intratumoral distribution of HSV	Griffon-Etienne et al. [163] Lu et al. [162] Nagano et al. [182]
PDGF antagonist	Decreased contraction of stromal fibroblasts and reduced stromal interaction with the ECM	↑ Microvessel density* (67 %) ↓ Microvessel density** (50 %)	↓ Tumor IFP* (44 %)	↑ Accumulation** of liposomal doxorubicin (1.5-fold)	Pietras et al. [165] Pietras et al. [105]* Vlahovic et al. [166]**
Prostaglandin E_1	Decreased fibroblast-mediated collagen contraction	↑ Edema – MABP – TBF – Blood volume – Vascular structure	↓ Tumor IFP (15–30 %)	Unknown	Rubin et al. [168] Salnikov et al. [108]
TGF-β inhibitors	Decreased content of ECM molecules in the interstitium	↓ Hydroxyproline (collagen) ↓ Pericyte coverage – Microvessel density	↓ Tumor IFP (50 %)	↑ Accumulation and penetration of micelle and liposomal doxorubicin (twofold)	Lammerts et al. [170] Salnikov et al. [171]

Note: All values presented as % difference or fold increase relative to control untreated group or pretreatment value
MABP mean arterial blood pressure, *MVP* microvascular pressure, *TBF* tumor blood flow, *IFP* interstitial fluid pressure, *SMANCS* macromolecular neocarzinostatin and poly(styrene-co-maleic acid) polymer drug conjugate, *EB* macromolecular Evans blue dye/albumin complex, *PZP* macromolecular PEG-conjugated zinc protoporphyrin IX drug, *Doxil* liposomal doxorubicin, *K* interstitial hydraulic conductivity, *HSV* herpes simplex virus, *Caelyx*™ liposomal doxorubicin. In the case of conflicting observations, the * and ** denote which study reported the finding

Bradykinin agonists (BA) target the bradykinin B_2 receptor and cause vasodilatation, increased permeability, and improved drug delivery [121]. Intra-femoral infusion of Cereport (a selective B_2 receptor agonist) has been found to produce a transient dose-dependent decrease in MABP (2–25 %) peaking at 1 min post infusion, a decrease in TBF by 86 %, no improvement in micro-regional blood flow, a decrease in tumor IFP of 8–18 %, and a 67–86 % increase in vessel diameter [121]. The larger decrease in MABP compared tumor IFP suggests that BA reestablishes a trans-vascular pressure gradient.

Nicotinamide (NIC) is a vasodilator that has been extensively studied for its radiosensitizing abilities and has been used in combination with carbogen to overcome chronic and acute hypoxia in the clinical setting [122, 123]. Intraperitoneal (IP) administration of NIC has been shown to decrease MBAP by 15 %, increase TBF by 75 %, and decrease tumor IFP by 39–47 % [124]. The significant decrease in tumor IFP compared to the MABP suggests that NIC may reestablish trans-vascular pressure gradients. In another preclinical study, NIC decreased tumor IFP by roughly 40 % within 20 min with recovery to baseline by 1 h [123]. The study also found a significant increase in perfused vessels compared to control tumors, suggesting that NIC improves micro-regional TBF. These results are consistent with several other studies [125–127] and also with previously described theoretical predictions that elevated IFP can lead to flow instability. These studies suggest that NIC improves the trans-vascular pressure gradient by preventing transient fluctuations in blood flow. Furthermore, NIC has had an extensive history of clinical use and the pharmacokinetics, pharmacodynamics, and toxicity profiles in patients are well known [122]. These factors make a potentially strong candidate to test for improved nanoparticle drug delivery.

Pentoxifylline (PTX) is another agent that has been studied due to its potential radiosensitizing abilities [128]. PTX has been found to reduce viscous flow resistance, potentially by altering the biomechanical properties of erythrocytes, and improve blood flow, making it a potentially useful agent to improve drug delivery [129]. In one xenograft study, IP administration of PTX had no effect on MABP but increased TBF by 32 % and decreased tumor IFP by 55 % [130]. Another study showed that PTX increased TBF by 46 % in a syngeneic rat tumor model and roughly 5–30 % in different mouse xenograft tumor models [131]. As of yet, the ability of PTX to improve nanoparticle drug delivery has not been tested. The significant decrease in tumor IFP without change in MABP suggests that PTX will reestablish a trans-vascular pressure gradient and lead to substantially improved convection-driven drug accumulation. Additionally, PTX also has a long history of use both preclinically and clinically, making it a potentially impactful agent.

Hyperthermia (HT) is a mechanical heating technique that has been shown to increase vascular permeability, increase TBF, increase MABP, and decrease tumor IFP [132–134]. While HT can increase TBF and permeability, the effect is not necessarily consistent and depends on many factors including the tumor model, degree of host tissue support, temperature, and the duration of heating [134–136]. In general, the optimal temperatures to increase TBF and permeability appear to be between 41 °C and 42 °C [137]. Leunig et al. [133] found that HT dramatically

reduces tumor IFP by 78 % after 30 min of heating and 94 % after 60 min of heating in amelanotic melanoma subcutaneous tumors implanted in hamsters.

Radiation (RT) has been shown to increase TBF, increase vascular permeability, and reduce IFP [138–142]. One study found that TBF increased by 105–122 % and became more uniform after 10 and 20 Gy single-fraction doses of RT [139]. They found that the improved TBF correlated with reduced RT-induced apoptosis and a reduction in cell density and STP, which was speculated to lead to an opening of previously compressed blood vessels. It also has been reported that radiation doses greater than 5 Gy when delivered in either a fractionated schedule (5 Gy/day) or as single fractions for total doses of 10, 20, or 30 Gy can reduce IFP by 20–35 % [140]. In a follow-up study, these authors found that 10 or 30 Gy RT caused a 12-fold decrease in the interstitial hydraulic conductivity (K) after 5 days, which appeared to be driven by a significant increase (almost threefold) in collagen I levels [143]. As a result RT may reduce interstitial penetration of nanoparticles. Giustini et al. [144] found that 15 Gy decreased IFP by 40 % and increased vascular permeability by 60 % in a syngeneic mouse breast cancer model. These studies suggest that there is a time-dependent nature of transport changes during and after RT, which may depend on dose and fractionation schedule. It is likely that vascular effects dominate during early time points and promote trans-vascular transport, while during late time points, interstitial effects dominate which may hinder interstitial penetration.

Improving Extravasation of Nanoparticles

The hyper-permeable nature of tumor blood vessels reduces the steric hindrance of trans-vascular transport of nanoparticles, leading to greater accumulation. However, hyper-permeable blood vessels also contribute to elevated IFP, impaired trans-vascular pressure gradients, and reduced of convection-driven nanoparticle transport in the central tumor regions. Vascular permeability is also linked to TBF and agents that decrease vascular permeability have been shown to improve TBF and improve micro-regional of some low-molecular-weight and macromolecular agents. However, studies have also shown that reducing vascular permeability may lead to greater steric hindrance to nanoparticle transport potentially limiting any advantage conferred by reducing IFP and improving TBF. Therefore, care must be used when choosing a particular therapeutic strategy.

TNF-α (TNF) has been shown to reduce IFP and improve nanoparticle accumulation [145–148]. While the mechanism of action of TNF remains unclear, one study has shown that TNF reduced MABP by 30 % and reduced tumor IFP by 50–70 % in three different murine xenograft tumor models [145]. The effect was seen at 5 h postinjection, and both MABP and tumor IFP returned to pre-injection values after 24 h. These results suggest that TNF may transiently reestablish a trans-vascular pressure gradient, based on the significant reduction in tumor IFP compared to MABP. Several studies have demonstrated that low-TNF treatment can result in a three- to sixfold increase in accumulation and improved micro-regional distribution of Doxil [146–148].

Vascular "normalization" using antiangiogenic agents has been shown to improve vascular morphology and function [149]. Vascular normalization also decreases tumor IFP and reestablishes a trans-vascular pressure gradient in solid tumors [106, 150]. One of the first studies found that a single injection of DC101 (a VEGFR-2 antibody) caused significant decreases in vascular length (29 %), vascular diameter (37 %), and vascular permeability to albumin (52 %) and a more normalized vascular architecture in several window chamber xenograft mouse models [106]. Furthermore, DC101 caused a sustained 50–60 % decrease in tumor IFP (with no change in MVP) and restored the oncotic pressure gradient as a result of decreased vascular permeability. Additionally, clinical evidence has shown that vascular normalization can decrease tumor blood flow by 30–50 % and tumor IFP by up to 70 % in a limited number of rectal carcinoma patients [151]. There are many more studies of vascular normalization and tumor IFP and these have been reviewed elsewhere [149, 152]. A study by Chauhan et al. [100] demonstrated that different doses of DC101 resulted in increased trans-vascular flux of 12 nm nanoparticles, but no effect on the trans-vascular flux of 60 or 125 nm particles. Additionally, they found that DC101 resulted in zero or nearly zero penetration of 60 or 125 nm nanoparticles in several tumors. This study highlights that while vascular normalization can improve TBF and reestablish a trans-vascular pressure gradient, the benefit may be mitigated by a reduced vascular permeability and increased steric hindrance to nanoparticle extravasation.

Improving Penetration of Nanoparticles

The penetration of nanoparticles by convection depends on the interstitial hydraulic conductivity and interstitial fluid pressure gradients. Additionally, the abnormal structural components of the interstitial space, such as increased collagen and glycosaminoglycans (GAG), present a physical barrier to nanoparticle transport and accumulation. Several strategies have been employed to reduce IFP and STP and improve the penetration of nanoparticles. These strategies involve agents that act to reorganize or degrade the ECM, promote tumor cell apoptosis, or decrease the contraction of fibroblasts and the forces that they exert on other stromal elements. Reorganizing the ECM, degrading the ECM, and promoting tumor cell apoptosis are strategies that reduce intra-tumoral mass effects and increase interstitial hydraulic conductivity [153, 154]. The increased interstitial hydraulic conductivity in turn promotes interstitial fluid flow out of the tumor, lowering IFP and further reducing mass effects and STP. Strategies that cause decreased contraction of stromal fibroblasts lead to a reduction of STP, which in turn reduces tumor IFP [3]. However, increasing interstitial fluid flow may have the undesirable effect of carrying cytokines and tumor cells into the surrounding normal tissue, which may promote further invasion and metastasis [150, 155].

Collagenase (CG) has been shown to degrade fibrillar collagen and reduce IFP. CG was found to improve micro-regional perfusion in mouse xenograft tumor models [156]. A more comprehensive study found that CG can decrease MVP by 60 % and

IFP by 45 %, leading to a reestablished trans-vascular pressure gradient [107]. However, one experiment found that CG did not improve the accumulation or distribution of liposomal doxorubicin [157]. Experiments in multicellular spheroids, where convection is not present, have demonstrated that CG improves the penetration of nanoparticles up to 100 nm in diameter [158]. This illustrates that CG can also improve nanoparticle transport by diffusion.

Hyaluronidase (HLD) is an enzyme that degrades hyaluronan resulting in an increased interstitial hydraulic conductivity, increased interstitial fluid flow, and a reduction in IFP [2, 3, 77]. HLD has been found to increase interstitial fluid flow and interstitial transport of albumin and 440 kDa dextran in the lungs of New Zealand white rabbits [154]. HLD was shown to transiently decrease IFP by 63–84 % in a dose-dependent manner with negligible changes to MABP [159–161]. Eikenes et al. [101] found that HLD decreased IFP by up to 50 % without any change in MVP, which in turn reestablished a trans-vascular pressure gradient. They also found that HLD increased the accumulation of liposomal doxorubicin by fourfold.

Paclitaxel\docetaxel (TXL) causes tumor cell apoptosis leading to improve TBF and reduced IFP [162]. TXL has been reported to induce apoptosis and reduce cellular density in a murine mammary carcinoma (MCa-IV) and a human soft tissue carcinoma (HSTS-26T), leading to decompression of blood vessels 48–96 h after administration [163]. The decompression of tumor blood vessels was associated with a two- to threefold increase in TBF, an increase in K (109 %), a decrease in MVP (33 %), and a decrease in tumor IFP (54 %).

Platelet-derived growth factor (PDGF) antagonists have been found to mediate the contraction of stromal fibroblasts and stromal interactions with the ECM via binding integrins [164]. Furthermore, inhibition of PDGF receptor β1 (PDGF-β1) can reduce IFP [165]. It was found that treatment with a PDGF-β1 antagonists resulted in a significant increase in vessel density (67 %) and reduced tumor IFP by 44 %. One study found that imatinib (Glivec) improved the accumulation of liposomal doxorubicin by 1.5-fold in mice bearing non-small-cell lung carcinoma xenografts [166].

Prostaglandin E_1 (PGE_1) has been found to reduce tumor IFP. The mechanism of action is believed to be due to inhibition of fibroblast-mediated collagen contraction. However, PGE_1 also causes vasodilation, which may contribute to improved trans-vascular transport [167]. Local administration of PGE_1 has been shown to transiently reduce tumor IFP by 15–30 % in two rat tumor models [168]. The reduction in tumor IFP was accompanied by a 40–86 % increase in trans-vascular transport of the low-molecular-weight agent ^{51}Cr-EDTA. Salnikov et al. [108] also reported that PGE_1 reduced IFP in the same experimental system.

TGF-β inhibitors (TGF) target types I and II TGF-β receptors that are involved in ECM regulation [169]. TGF has been shown to decrease tumor IFP by 50 % in a KAT-4 thyroid carcinoma xenograft mouse model [170]. The effect became apparent 10 days after administration, suggesting that the IFP reduction was due to long-term reorganization of the ECM. TGF also reduced the hydroxyproline (a marker for collagen) content of these tumors, providing further evidence to support an effect on the ECM. Salnikov et al. [171] demonstrated that TGF does not necessarily affect

microvessel density. Kano et al. [172] found that TGF can decrease the pericyte coverage of tumor blood vessels. These results suggest that TGF also plays a role in modulating vascular permeability, which may in turn modulate tumor IFP.

Summary

Tissue pressure gradients are important in the development, maintenance, and function of healthy tissue. Both STP and IFP play a crucial role in tumor biology as regulators of angiogenesis, ECM remodeling, cell signaling, cellular adaptation to the microenvironment, and more. As described in this chapter, tissue pressure gradients become unregulated during the switch to a malignant phenotype and play an active and crucial role in the pathogenesis and treatment of cancer. Our current understanding of the dynamics of abnormal tissue pressure gradients in both cancer biology and nanoparticle drug delivery has been driven primarily by the use of mathematical modeling and biophysical reasoning based on experimental observations, typically using in vitro systems. These studies demonstrate that tissue pressure gradients contribute both directly and indirectly to EPR-mediated nanoparticle accumulation. They also demonstrate the importance of developing measurement techniques that relate pressure gradients to specific biological processes and drug delivery in vivo. Exciting studies are emerging, demonstrating how agents that modify pressure can improve drug delivery. The success of these studies will be drastically improved by the development of novel, noninvasive methods for imaging STP and IFP in a spatiotemporal manner. This would facilitate a detailed understanding of the relationship between pressure gradients in tumors and nanoparticle transport and would help guide the optimal use of modifiers to promote nanoparticle uptake.

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Chapter 10
The ADAMs: New Therapeutic Targets for Cancer?

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Abstract The ADAMs are transmembrane proteins implicated in a variety of biological processes including proteolysis, cell signalling, angiogenesis, cell migration, and cell adhesion. Of the 21 ADAMs believed to be functional in humans, approximately one half have been shown to possess protease activity. As proteases, the main ADAM substrates are the ectodomains of transmembrane proteins, especially growth factor precursors, growth factor receptors, and adhesion proteins. Recently, several different ADAMs have been shown to play a role in cancer formation and progression. These include ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, and ADAM28. Consistent with a causative role in cancer, several ADAMs, especially ADAMs 10 and 17, are emerging as potential therapeutic targets for cancer treatment. Indeed, targeting these ADAMs with either low molecular weight inhibitors or monoclonal antibodies has been shown to have anticancer activity in multiple preclinical systems. Although early phase clinical trials have shown no serious side effects with a dual ADAM10/17 low molecular weight inhibitor, the consequences of long-term treatment with these agents are unknown.

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Introduction

It is now widely accepted that we have reached the limit with respect to chemotherapy for cancer treatment. Indeed, in recent years, the main focus in the development of new cancer treatments has shifted from cytotoxics to targeted therapies. Amongst the most successfully targeted molecules are members of the HER family of proteins, especially EGFR and HER2 (Table 10.1). Activation of the HER family of proteins is generally mediated by the release of ligands from their inactive precursor forms. These ligands include TGF-alpha, amphiregulin, HB-EGF, and heregulin. This release is catalyzed by specific ADAMs, including ADAM10 and ADAM17. ADAM10 and ADAM17 thus play a pivotal role in EGFR/HER signalling and consequently in controlling cell growth, cell death, and migration. Consistent with their role in modulating cell proliferation, cell death, and migration, a number of ADAMs have been implicated in the formation and progression of cancer.

The aim of this chapter is to review the current status of targeting ADAMs for the treatment of cancer. Firstly, however, we provide a brief overview of the structure and biological functions of the ADAMs.

Structure and Function of ADAM Proteins

The ADAMs are a family of transmembrane and secreted proteins, possessing a multidomain structure (Fig. 10.1). The transmembrane forms may contain up to eight separate domains. Starting from the N-terminal end, these are a prodomain, a metalloproteinase domain, an integrin-binding domain, a cysteine-rich region, an EGF (epidermal growth factor)-like domain, a transmembrane sequence, and an intracellular C-terminal region [1–3]. Some of the ADAMs, such as ADAM10 and 12, have also been shown to undergo glycosylation [4–6]. This glycosylation appears to be necessary for processing and localization of these ADAMs to the cell membrane.

Table 10.1 Anti-EGFR/HER2 agents in clinical use or undergoing clinical trials

Antibodies	Target	Cancer
Trastuzumab (Herceptin)	HER2	Breast, gastric
Pertuzumab	HER2	Breast
Cetuximab	EGFR	Colorectal
Panitumumab	EGFR	Colorectal
Tyrosine kinase inhibitors		
Gefitinib	EGFR	Lung (non-small cell)
Erlotinib	EGFR	Lung (non-small cell)
Lapatinib	EGFR/HER2	Breast
Neratinib	EGFR/HER2/HER4	Breast, lung
Afatinib	EGFR/HER2/HER4	Breast, lung

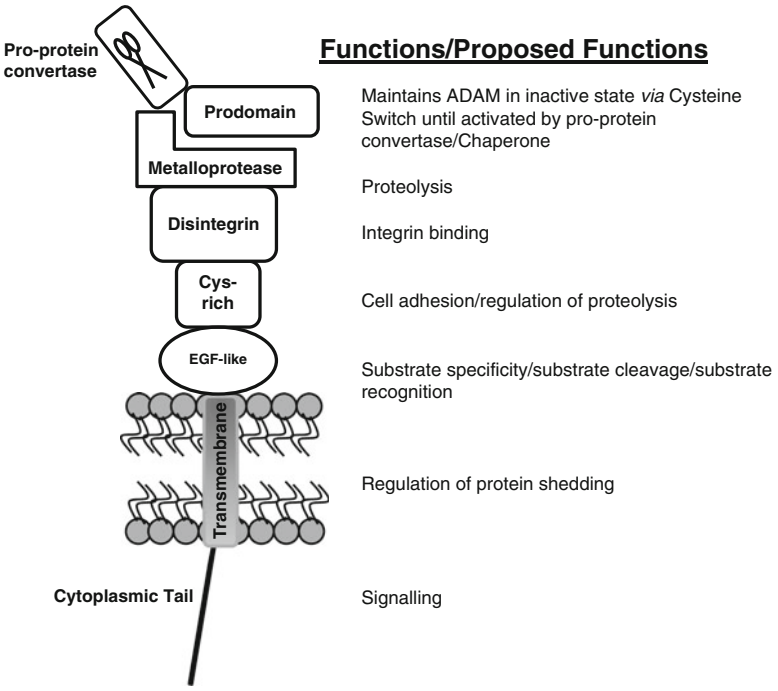

Fig. 10.1 Prototypical structure of a membrane-tethered ADAM protease in the inactive state, i.e., prior to removal of the prodomain by a proprotein convertase

As well as transmembrane forms, several ADAMs can also exist as soluble forms. These soluble forms may result from differentially spliced mRNAs or from proteolysis of the parental protein. Thus, differential mRNA splicing results in both membrane and soluble forms of ADAM9, ADAM11, ADAM12, ADAM15, and ADAM28 [1]. At least four different forms of ADAM15, arising from alternative splicing, have been described [7]. As well as differential splicing, different forms of specific ADAMs may be generated by proteolysis. For example, ADAM10 has been shown to be processed into several different forms by ADAM9, ADAM15, and gamma-secretase [8].

The ADAMs have been implicated in several different but related biological functions including proteolysis, cell signalling, cell adhesion, cell migration, proliferation, and vasculogenesis. Of these, the best documented role is proteolysis. Indeed, many of the ADAM-mediated activities are likely to result from their protease action. ADAMs shown to possess protease activity include ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM28, and ADAM33 [1].

Unlike the classical MMPs which mediate degradation of the extracellular matrix proteins, the main substrates for the ADAM proteases are the external regions of transmembrane proteins [3]. Cleavage of these ectodomains generally occurs within the first 20 amino acids external to the cell membrane. Little information is

Table 10.2 Some of the best characterized substrates for ADAM proteases

ADAM	Substrates
8	L-selectin, CD23
9	HB-EGF
10	Notch, EGF, betacellulin, cadherin E, CD44, L1, HER2
12	HB-EGF
15	E-cadherin
17	TNF-alpha, TGF-alpha, HB-EGF, amphiregulin, epiregulin, heregulin, Il-6R, L-selectin, Notch
19	Heregulin
28	von Willebrand factor (VWF)

available regarding consensus cleavage sites, although ADAM17 has a preference for alanine and valine at positions P1 and P1′, respectively; leucine/glutamate and valine at positions P2 and P2′, respectively; alanine and serine at P3 and P3′, respectively; and leucine and serine at P4 and P4′, respectively [9].

As well as the amino acid sequences, glycosylation at or close to the scissile bond of the substrate may also modulate substrate recognition and specificity [10]. Thus, with a TNF-based peptide substrate, glycosylation was found to enhance ADAM8 and ADAM17 activities but decreased ADAM10 activity. In contrast to the ADAM protease activities, glycosylation did not affect MMP activity. These findings suggest that an exosite on the ADAMs interacts with a glycan moiety on the substrate [10].

Table 10.2 lists some of the best characterized substrates for the different ADAM proteases. It is clear from this table that most ADAM proteases can potentially cleave several different membrane substrates. Indeed, the specific substrate cleaved depends on the specific cell context. Thus, Notch is a substrate for both ADAM10 and 17, with ADAM10 acting on ligand-bound Notch1 and ADAM17 on the non-liganded form [11]. Similarly, although ADAM17 is the principle sheddase for TGF-alpha, TNF-alpha, and L-selectin when both ADAM10 and ADAM17 are present, in a situation of ADAM17 deficiency, ADAM10 can cleave these substrates [12].

Role of ADAMs in Cancer Formation and Progression

Since specific ADAMs promote cell proliferation and migration, it is not surprising that they have been shown to be involved in cancer formation, invasion, and metastasis [13–15]. Currently, the best evidence of a role for ADAMs in cancer exists for ADAM17, although ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, and ADAM28 have also been implicated. Most of the data implicating ADAMs in cancer is based on preclinical studies involving cell line and animal model systems [13–15]. However, the available indirect data from human cancers are also consistent with preclinical findings. For example, several different ADAMs have been shown to be increased in different cancers and to correlate with histopathological

indices of aggressive disease as well as with poor patient outcome [13–15]. Thus, in breast cancer, ADAM17 has been shown to be associated with ER-negativity, high tumor grade, triple-negative status, and poor outcome [16–18].

The specific mechanism(s) by which ADAMs play a role in cancer is unclear and indeed is likely to vary from ADAM to ADAM. Thus, ADAM10 may participate either by activating Notch signalling, releasing EGF and betacellulin, and thereby activating EGFR signalling or shedding CD44 and thereby promoting metastasis. On the other hand, ADAM17 is likely to act by releasing growth factors such as TGF-alpha or heregulin and as a result activating HER protein signalling. Alternatively, ADAM17 may participate by releasing TNF-alpha or modulating Il-6 receptor signalling [13, 15]. Recently, ADAM28 was shown to promote lung cancer metastasis by cleaving and inactivating the proapoptotic protein, von Willebrand factor (VWF) [19]. Inactivation of VWF appeared to prolong cancer cell survival within the blood vessels, thus increasing the probability of metastasis. A further mechanism by which ADAMs may promote cancer is by modulating adhesion and cell migration [11, 12].

Briefly, the evidence implicating ADAMs in malignancy is as follows [11, 12]:

- Increased levels of several ADAMs in model systems enhanced in vitro invasion, proliferation, and promoted tumor formation in vivo, while decreased expression reduced these processes.
- In human tumors, high levels of specific ADAMs were found to correlate with indices of aggressive disease such as large tumor size, high tumor grade, metastasis to local lymph nodes, and poor outcome for patients.
- Selective inhibitors against certain ADAMs reduced or blocked tumor cell growth in model systems (see below).

ADAMs as Therapeutic Targets for the Treatment of Cancer

Since considerable evidence from model systems suggests that specific ADAMs are causally involved in cancer formation and progression, it might be expected that inhibition of these proteases could be used to treat cancer. At least four potential approaches exist to block ADAM protease activity [15]. These include the use of low molecular weight synthetic inhibitors, monoclonal antibodies, purified or synthetic forms of ADAM prodomains, and modified forms of the naturally occurring endogenous inhibitors, TIMPs. Of these potential approaches, the most widely investigated involves the use of low molecular weight synthetic inhibitors and monoclonal antibodies.

Low Molecular Weight Synthetic Inhibitors

Several low molecular weight synthetic inhibitors against different ADAMs, especially ADAM10 and ADAM17, have been described in recent years (Table 10.3,

Table 10.3 Low molecular weight ADAM inhibitors that have undergone preclinical or clinical investigation as potential cancer agents (see also Fig. 10.2)

Inhibitor	Primary target(s)	Company	References
INCB3619	ADAM10/17	Incyte	[20, 21]
INCB7839	ADAM10/17	Incyte	[22, 23]
PF-5480090 (TMI-002,WAY18022)	ADAM17	Pfizer	[18, 24, 25]
GI254023X	ADAM10	GSK	[26, 27]
GW280264X	ADAM10/17	GSK	[28]
KB7785	ADAM12	Nippon Organon	[29]

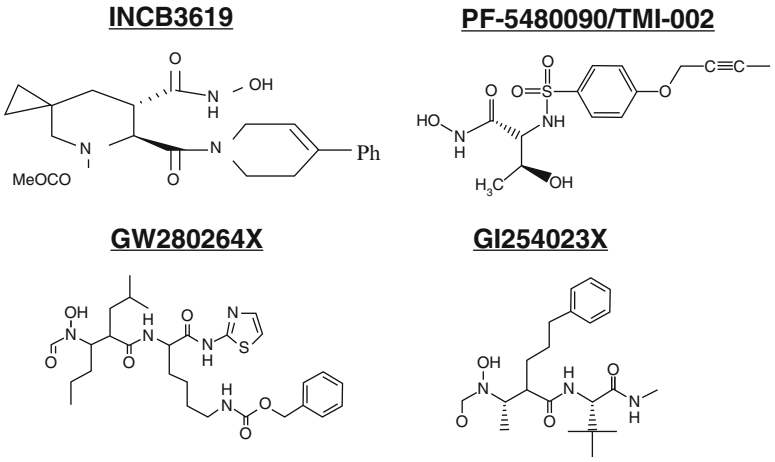

Fig. 10.2 Chemical structures of hydroxamic acid-derived ADAM inhibitors currently in preclinical evaluation in cancer models. INCB3619, developed by Incyte Biotechnology (Wilmington, Delaware), is a dual ADAM10/17 inhibitor [21]. PF-5480090 (formerly WAY-18022/TMI-002; Pfizer) is a specific ADAM-17 inhibitor [24]. GW280264X is also a dual ADAM10/17 inhibitor and GI254023X is ADAM-10 specific (both from GlaxoSmithKline) [26]

Fig. 10.2). Most of these are hydroxamate based and bind to the MMP-like catalytic site of the ADAMs where they chelate zinc. A number of these are selective for specific ADAMs and have undergone preclinical investigations as potential anticancer agents. These include INCB3619 (Incyte), PF-5480090 (previously known as TMI-002 or WAY-18022; Pfizer), GI254023X, and GW280264X (GSK).

Of these inhibitors, one of the most-detailed investigated is INCB3619. INCB3619 is an orally active compound that selectively inhibits ADAM10 and ADAM17 with IC_{50} values of 22 and 14 nmol/L, respectively [20, 21]. Although having little inhibitory activity against ADAM9 or ADAM33, INCB3619 was found to block MMP2 proteolytic activity (IC_{50}, 35 nM) and MMP12 (IC_{50}, 17 nM). In contrast, it had little specificity for MMP1, 3, 7, 9, or 14 [20, 21].

In an early study, Zhou et al. [20] found that INCB3619 inhibited the release of several EGFR/HER ligands, including heregulin, TGF-alpha, HB-EGF,

amphiregulin, and EGF. In addition, it sensitized small-cell lung cancer (NSCLC) cells in culture to the EGFR tyrosine kinase inhibitor, gefitinib. Also, using NSCLC cell lines in vitro, INCB3619 was found to increase apoptosis and reduce the apoptotic threshold for paclitaxel. Consistent with this finding, INCB3619 decreased tumor growth and enhanced the therapeutic benefit of paclitaxel in a xenograft model of these cells.

In other animal models, INCB3619 was shown to synergize with cisplatin in reducing growth of head and neck cancers and with paclitaxel in inhibiting growth of breast cancers [21]. A clinically relevant and important finding with the animal models investigated was that administration of INCB3619, in contrast to previous studies with broad-spectrum MMP inhibitors [30, 31], did not appear to induce musculoskeletal side effects [21].

In contrast to INCB3619, PF-5480090 is a selective inhibitor of ADAM17, being greater than 250-fold more selective vis-à-vis ADAM10, MMP1, MMP7, MMP9, and MMP14 [24]. Its selectivity for MMP8 and 13, however, is only 17- and 48-fold more specific than for ADAM17. As with INCB3619, early studies in an animal model showed no evidence of fibroplasia following administration of PF-5480090 [24]. In contrast to PF-5480090, a broad-spectrum MMP inhibitor induced fibroplasia in the same animal model [24].

Using a broad panel of breast cancer cell lines in culture, McGowan et al. [18] reported that PF-5480090 blocked release of the EGFR ligand, TGF-alpha, decreased levels of phosphorylated EGFR, and inhibited cell proliferation in a cell type-dependent manner. Interestingly, in this study, PF-5480090 decreased growth of the cell lines irrespective of their molecular subtype, i.e., whether cells were ER-positive, HER2-positive, or triple negative. A potentially important finding using the cell lines investigated was that ADAM17 catalytic activity levels correlated with sensitivity to PF-5480090. This finding suggests that if PF-5480090 were to enter clinical use, a predictive marker might be available for identifying potentially responsive patients.

In addition to the antigrowth activity found in breast cancer cells following treatment with PF-5480090 alone, preincubation with the drug enhanced response to several different specific cytotoxic and anti-HER agents (neratinib and afatinib) [18]. The extent of enhancement, however, was both cell line and drug dependent (Fig. 10.3). As well as decreasing cell proliferation, inhibition of ADAM17 has been shown to block cell invasion (McGowan, Mullooly and Duffy, unpublished observation) (Fig. 10.4). Furthermore, this decrease in invasion was significantly enhanced by combination with the pan-HER inhibitor, neratinib (Fig. 10.4).

The growth-inhibitory effects of PF-5480090 are not limited to breast cancer, as this agent has also been shown to decrease the growth of colorectal cancer cells (CRC) in culture [25]. Furthermore, combinations of suboptimal concentrations of PF-5480090 with an anti-EGFR antibody or a selective EGFR kinase inhibitor resulted in cooperative growth inhibition in the CRC cell lines investigated [25]. PF-5480090 might thus be of value, if combined with existing anti-EGFR-directed therapies in the treatment of colorectal cancer or other EGFR-dependent cancers such as NSCLC.

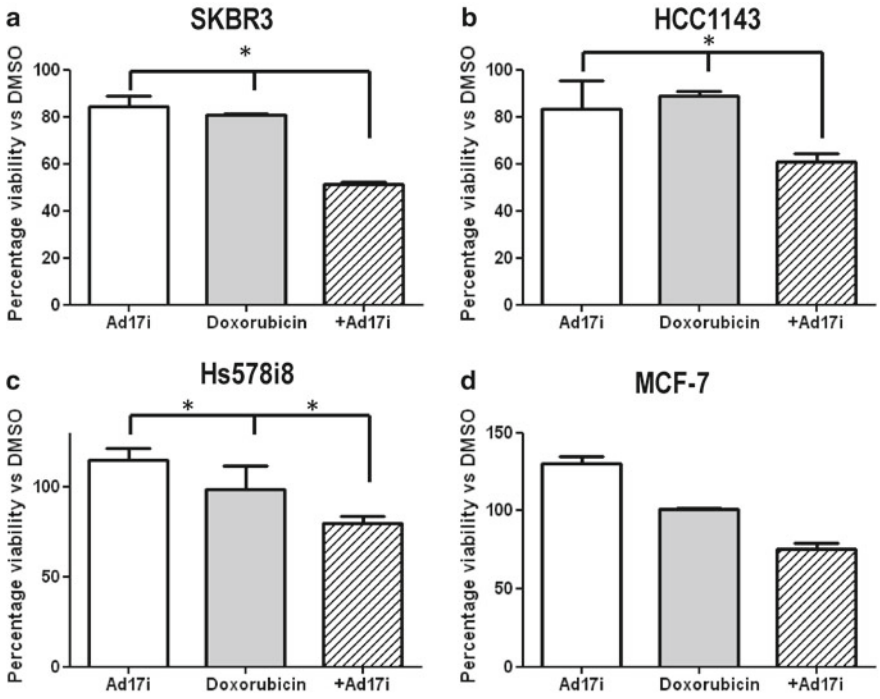

Fig. 10.3 Pretreatment of SKBR3 (**a**), HCC1143 (**b**), Hs578i8 (**c**), and MCF-7 (**d**) breast cancer cell lines with an ADAM-17 selective inhibitor (Ad17i) (1 μM) resulted in a significant enhancement of the growth-inhibitory effects of the cytotoxic agent doxorubicin (0.05 μM). Cellular viability by measured by the MTT assay. *$p<0.05$ versus ADAM17 inhibitor or doxorubicin alone. Data was analyzed using the Student's paired *t*-test

GI254023X (GSK) is a selective inhibitor of ADAM10, possessing >100-fold selectivity for this ADAM vis-à-vis ADAM17. In cell-based assays, GI254023X blocked release of Il-6 receptor, CX3CL1, and CXCL16 [26, 28]. As with PF5480090 and INCB3619, GI254023X has been shown to inhibit the growth and invasion of breast cancer cell lines in culture [27].

To our knowledge, the only low molecular weight ADAM10/17 inhibitor that has undergone clinical trials as an anticancer agent is the dual ADAM10/17 inhibitor INCB7839 (Incyte) [22, 23]. Preliminary results suggest that this drug is generally well tolerated with no significant musculoskeletal side effects or anti-EGFR-related side effects such as skin rash. Furthermore, there were no reports of drug-induced increases in liver enzymes, bone marrow toxicity, or increase in cardiomyopathy [23]. Evidence of target inhibition was the finding that administration of INCB7839 decreased shedding of different HER ligands and the extracellular domain of HER2 [22].

Most of the work to date on ADAM inhibitors as potential anticancer agents has focused on ADAM10 and 17. However, a putative ADAM12 inhibitor, known as KB7785 has been shown to inhibit the shedding of HB-EGF and suppress

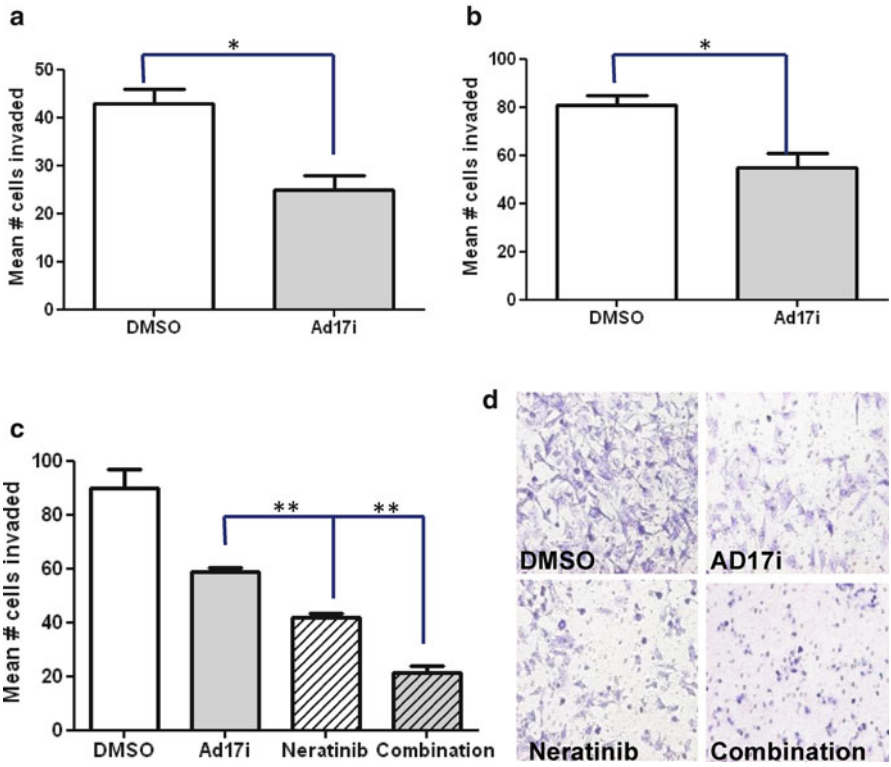

Fig. 10.4 Treatment of MDA-MB-231 (**a**), Hs578i8 (**b**), and Hs578t (**c**) breast cancer cells with an ADAM17 selective inhibitor (Ad17i) (5 μM) resulted in a significant reduction in cellular invasion through a Matrigel™ layer compared to vehicle controls (*$p < 0.05$ compared to vehicle control). In addition, treatment of Hs578t cells with a combination of an ADAM17 specific inhibitor plus the pan-HER inhibitor, neratinib (0.1 μM), was more effective at inhibiting invasion (**c**, **d**). **$p < 0.01$ compared to either alone. Data was analyzed using Student's *t*-test

translocation of its C-terminal fragment into the nucleus [29]. KB7785 was found to block the in vitro growth of gastric cells in a dose-dependent manner and induce apoptosis. Furthermore, KB7785 induced cell cycle arrest and suppressed expression of cyclin A and c-myc. These activities were enhanced by cetuximab, a monoclonal antibody that binds to EGFR. The target specificity of KB7785, however, was not determined.

TIMPs

For most if not all mammalian proteases, endogenous or naturally occurring inhibitors have been identified. For the MMPs, four such inhibitors have been identified,

i.e., TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [32]. The inhibitors have two main structural domains, an N-terminal domain which binds to the active site of the MMPs, thereby blocking their activity, and a C-terminal domain that contains six conserved cysteine residues [32]. As with the low molecular weight synthetic inhibitors discussed above, the TIMPs also act by chelating the active site zinc.

The current thinking is that TIMPs show little selectivity for the different MMPs, although it should be stated that this has not been investigated in depth. In addition to their ability to inhibit MMP activity, TIMP-1 and TIMP-2 have also been shown to stimulate cell proliferation and participate in cell signalling [32]. It is unclear as to whether these growth-promoting effects are independent or dependent on protease inhibitory activity.

As well as inhibiting MMPs, certain TIMPs, especially TIMP-3, have been shown to block the protease activity of specific ADAMs [1–3]. Thus, TIMP-3 has been shown to inhibit ADAM10, ADAM12, ADAM17, and ADAM33 [1–3]. In one report, ADAM17 was found to exist as a dimer in association with TIMP-3 on the cell membrane [33]. Following activation of the ERK or p38 MAPK pathway, the ADAM17 dimeric configuration was transformed into a monomeric state. This resulted in the increased cell membrane presence of ADAM17 and disassociation of TIMP-3. Removal of TIMP-3 from ADAM17 led to increased activation of TGF-alpha [33].

Because of their pleiotropic functions and broad specificity (potentially for multiple MMPs and ADAMs), intact TIMP proteins are unlikely to be used to treat cancer or indeed any disease. Although attempts have been made to alter or engineer TIMPs in order to enhance their protease inhibitory ability and increase specificity [34, 35], these modified TIMPs do not appear to have undergone evaluation as potential anticancer agents.

ADAM Prodomains

Another potential approach for blocking ADAM protease activity is with the isolated prodomain peptide of the relevant ADAM. ADAMs are initially synthesized as inactive precursor proteases with the N-terminal prodomain blocking catalytic activity. The prodomain maintains inactivity until it is removed by a furin-type proprotein convertase or by autocatalysis. Theoretically, therefore, isolated prodomains are potential specific inhibitors of the corresponding ADAM.

In one of the few studies to have investigated a potential protease inhibitory role for ADAM prodomains, Moss et al. [36] reported that a recombinant form of the mouse ADAM10 prodomain, comprising amino acid residues 23–213, inhibited human ADAM10 catalytic activity. The inhibitory ability of this peptide appeared to be selective for ADAM10, as little effect was found against ADAM8, ADAM9, or ADAM17 or several MMPs (MMP1, 2, 3, 9, 13). In vivo studies with this peptide do not appear to have been reported.

Monoclonal Antibodies

Several monoclonal antibodies are now in use to treat different cancers. Indeed, of all the newly available biological or targeted therapies, specific monoclonal antibodies are amongst the most efficacious. Lendeckel et al. [37] were one of the first to investigate a potential cancer therapeutic role for antibodies against specific ADAMs. These authors showed that anti-ADAM15 and anti-ADAM17 antibodies inhibited the proliferation of both MCF-7 and MDA-MB-453 breast cancer cell lines in vitro. In contrast, the growth of MCF-7 cells appeared to be stimulated following treatment with an anti-ADAM12 antibody.

More recently, Tape et al. [38] developed a monoclonal antibody against ADAM17, known as D1(A12) that targets both its catalytic and disintegrin domains. In vitro, this antibody inhibited the release of several ADAM17 substrates including TNF-alpha, TNFR1-alpha, TGF-alpha, HB-EGF, amphiregulin, and interleukin-6 receptor-alpha, while in an animal model it blocked the release of TGF-alpha, amphiregulin, and TNFR-alpha [38, 39]. Using an animal model of ovarian cancer, D1(A12) was found to significantly reduce tumor growth [39].

Rather than using a native monoclonal antibody, Yamamoto et al. [40, 41] generated an ADAM17-specific scFv (single-chain variable fragment) which was fused to a CD3-specific scFv to generate a bispecific T-cell engager antibody [A300E-BiTE (bispecific T-cell engager antibody)]. The fusion product bound to ADAM17 and CD3 on the membrane of tumor cells and T cells, respectively. In the presence of primary human peripheral blood mononuclear cells or human T cells, addition of A300E-BiTE resulted in ADAM17-specific killing of prostate tumor cells [40].

A further study with this antibody showed that it could be conjugated to the cytotoxic agent doxorubicin or to the Pseudomonas exotoxin A. Furthermore, these conjugates were found to kill ADAM17-expressing cells in vitro. This cytotoxic effect was dependent on the presence of ADAM17 on the membrane of target cells. Animal studies with these conjugates are eagerly awaited.

Potential advantages of therapeutic monoclonal antibodies over low molecular weight compounds for the treatment of cancer include better target specificity, potential to be conjugated with other anticancer agents such as cytotoxic drugs, radioisotopes, and immuno-targeting molecules; and potential to induce antibody-dependent cellular cytotoxicity (ADCC). Disadvantages include cost of production and inability to administer orally.

Potential Side Effects from Anti-ADAM Treatments

Overall, few major toxicities have been reported in the limited animal model studies performed to date with ADAM inhibitors. However, based on ADAM-deficiency experiments carried out in these models, some side effects, especially relating to skin disorders and defects in immunity and/or inflammation, might be expected. Franzke et al. [42] showed that mice lacking ADAM17 in keratinocytes developed

defects in the epidermis which progressed to chronic dermatitis at the adult stage. These abnormalities were due to failure to produce TGF-alpha and EGFR signalling in the presence of ADAM17 deficiency.

In a further study, p53-induced ADAM17 resulted in keratinocyte differentiation and suppression of squamous cell carcinoma formation [43]. In this model, ADAM17 appeared to promote keratinocyte differentiation by activating Notch1 rather than EGFR. Although ADAM10, like ADAM17, can also activate Notch1 (see above), ADAM10 did not appear to be involved in enhancing keratinocyte differentiation in this study.

Further possible side effects of ADAM inhibition, especially inhibition of either ADAM10 or ADAM17, are depressed inflammation and immunity. This is likely as several of the ligands activated by ADAM17 are proinflammatory, as they stimulate both the acquired and innate immune system [9]. These proinflammatory molecules include TNF-alpha, Il-6R, and L-selectin. Indeed, cell type-specific deletion or hypomorphic knock-in of ADAM17 in animal models clearly shows that this ADAM is involved in inflammation, especially intestinal inflammation [9, 44]. Thus, Chalaris et al. [44] showed that mice deficient in ADAM17 were highly susceptible to dextran sulfate-induced colitis. This resulted from impaired release of EGFR ligands resulting in the failure to phosphorylate the downstream protein STAT3. This in turn led to defective regeneration of epithelial cells and degradation of the intestinal barrier [44].

Consistent with animal model studies, immune defects were observed in two human subjects with a hereditary deficiency in ADAM17 catalytic activity [45]. The female subject died at 12 years due to a parvovirus B19-associated myocarditis. The male, however, led a relatively normal life despite multiple skin infections. The depressed immunity in these two subjects appeared to relate to a failure to produce TNF-alpha.

As well as ADAM17, ADAM10 also appears to play a role in the immune system. Using B lymphocyte-specific ADAM10-deficient mice, Chaimowitz et al. [46] detected decreased numbers of follicular Th cells which resulted in decreased amounts of T helper cells and impaired germinal center formation. Thus, in this animal model, ADAM10 appeared to be essential for optimal maintenance of lymphoid structures following antigen challenge.

Based on the above findings, it might be expected that the use of ADAM10/17 inhibitors in humans would result in increased skin lesions and depressed immunity. Currently, however, little information is available regarding possible side effects of ADAM inhibition in humans. As mentioned above, the only ADAM10/17 inhibitor investigated in clinical trials for cancer treatment is the dual ADAM10/17 inhibitor INCB7839 (Incyte) [22, 23, 47]. Phase I/II clinical trials with this agent reported no major toxicity. Consistent with the animal model studies (see above), it was particularly encouraging to see a lack of musculoskeletal side effects [22], which was a major problem with the early metalloproteinase inhibitors investigated. Continued caution however, with respect to long-term toxicity, will be necessary, especially as ADAM10 and ADAM17 shed a wide variety of biologically important membrane proteins [1–3].

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Chapter 11
Role of the Extracellular Matrix: Enzyme Activities and Metastasis

Il-Kyu Choi and Chae-Ok Yun

Abstract The local milieu of malignant tumor cells has key roles in cancer progression. A major component of the niche is the extracellular matrix (ECM), a complex interdigitating meshwork of macromolecules with multiple biophysical and biochemical characteristics. Although tightly controlled during normal tissue development and homeostasis, the ECM is mostly deregulated and becomes disorganized in cancer. Abnormal ECM has an impact on cancer progression by promoting tumor malignancy and metastatic dissemination. Importantly, the altered ECM in tumor is associated with deregulated ECM-regulating enzymes (matrix metalloproteinases, lysyl oxidase, urokinase plasminogen activator, and cysteine cathepsin). Excess expression of ECM-regulating enzymes alters behavior of cancer cells in the tumor niche, and its sustained upregulation results in the progressive breakdown of normal ECM which is replaced by tumor-derived ECM, thereby allowing tumor malignancy and cancer cell dissemination. Thus, ECM-regulating enzymes act as essential mediators of deregulating and disorganizing ECM. In this chapter, we will review and discuss how ECM-regulating enzymes generate disruption of ECM homeostasis and contribute to cancer progression, especially cancer metastasis.

Introduction

Cancer is the primary cause of disease-related mortality and morbidity. Even though therapeutics for a variety of localized cancers has advanced significantly, metastatic cancers still remain poorly responsive to conventional therapies such as surgery,

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radiation therapy, and chemotherapy. Hence, metastatic cancers are implicated in an extremely poor clinical outcomes [1, 2]. Once cancer cells have disseminated to distant organs, there is no definitive therapeutic strategies available for effective treatment. Thus, the highly disseminated properties of cancers represent the major obstacles for effective eradication of the diseases, and there is still a therapeutic challenge to cure disseminated metastatic diseases. One of the major causes of this treatment failure has been the lack of understanding of the processes that govern how malignant tumors progress and metastasize to distant organs. Accordingly, it is critical to understand the underlying mechanisms of metastatic spread of cancer cells for uncovering novel therapeutic targets and treatment strategies.

Basic cancer research has mainly focused on determining how cellular components of tumor microenvironment initiate and promote cancer progression [3, 4]. However, recent progress has also highlighted the importance of non-cellular components of the local microenvironment, especially the tumor-derived extracellular matrix (ECM) during cancer progression, because ECMs are an important regulator which plays roles in regulating diverse essential cellular processes in the niche [5–9]. This tumor-derived ECM is characteristically different from normal ECM. The normal ECMs are highly governed by various regulatory mechanisms [10], whereas tumor ECMs are typically deregulated and become disorganized by abnormal control of the regulatory mechanisms [11, 12]. The disruption of ECM dynamics is especially attributed with deregulated ECM-regulating enzymes. The aberrant ECM-regulating enzymes result in the destruction of normal ECM which is then replaced by tumor-derived ECM in the microenvironment and consequently modify physiological cues that function as important mediators for cancer progression. Thus, excessive and uncontrolled ECM remodeling through deregulated ECM-regulating enzymes affects cancer progression by enhancing tumor malignancy and metastatic dissemination. In this chapter, we will review and discuss not only the diverse roles of the ECM that are needed for normal tissue homeostasis but also how ECM-regulating enzymes create abnormal ECM during malignant transformation and contribute to cancer progression.

Functions and Characteristics of ECM

The ECM is the non-cellular component observed within all animal solid tissues and a complex interdigitating meshwork of multiple proteins and polysaccharides in the extracellular interstitial space within the tissues. This matrix provides a vital physical scaffolding as well as a source of various biochemical reactions essential for maintaining tissue homeostasis and regulating tissue function [13]. For instance, ECM physically generates stiffness, porosity, insolubility, spatial arrangement, and topography of tissues, thereby creating unique mechanical strength, cushioning, and elasticity of a given tissue [12, 14]. The matrix also has an important role in protecting cells via a buffering capacity capable of sustaining its fluid retention [15].

In addition to biophysical properties, the ECMs also bind to a variety of extracellular signaling molecules (growth factors, hormones, and cytokines), so that the ECM-bound signaling molecules elicit structural organization of vital tissues and physiological response by inducing intracellular signaling pathways and modulating gene expression [9]. These biochemical functions of the ECM need transmembrane adhesion receptors (ECM receptors) such as integrins, discoidin domain receptors, and syndecans that interact directly with the different ECM components and that also bind, via intracellular adaptor proteins, to the cytoskeleton [16–20]. Additionally, the ECM induces cytoskeletal rearrangement of cells through cell-ECM interaction and in turn results in cell movements [20]. Thus, ECMs enable cells to interact and sense with their environment through diverse signal transduction, leading to regulation of cellular behavior.

Importantly, these physical, biochemical, and physiological characteristics of the ECM in each tissue can be significantly different between tissues (e.g., breast vs. muscle) and even within one tissue (e.g., skin epidermis vs. skin dermis) as well as from one physiological condition to another (e.g., normal tissue vs. neoplastic tissue), because physical, topological, and biochemical composition of ECMs is not only tissue specific but also tremendously heterogeneous. Similarly, the relative ratio among cells and ECMs varies markedly between tissues or organs. For example, the connective tissues mainly consist of ECMs, whereas the epithelial tissues mostly consist of densely packed cells with relatively little ECM. Taken together, ECMs create functionally discrete physical, biochemical, and physiological features of the given tissue dependent on specific compositions and concentration of ECM components, thereby organizing cells and serving unique functions of the tissue as well as allowing cells and tissues to promptly adapt to the external niches.

Component Parts and Structures of ECM

The ECM consists of a large collection of diverse molecules including collagens, non-collagenous proteins, and proteoglycans with various physical and biochemical properties [21, 22]. Collagen is the most abundant fibrous structural proteins and the main structural element of the ECM in a multicellular animal. This fibrous protein confers upon stiffness, strength, and flexibility, thus restricting the distensibility of tissues [23]. Collagens also mediate cell adhesion, chemotaxis, migration, and tissue development [23].

The major structural elements of non-collagenous proteins are elastins, fibronectins, and laminins [15]. Elastins are responsible for the unique elastic properties of tissues [24, 25], enabling tissues to maintain their shape following stretching or contracting as well as skin to return to its original position after being poked or pinched. Fibronectin is a cell surface-associated glycoprotein that regulates diverse cellular interactions with the ECM components such as integrins, collagens, fibrins, and heparins [26]. This protein plays an important role in inducing cell attachment, migration, and proliferation. Therefore, fibronectins are crucial for cell motility

during development and have also been associated with cancer invasion and metastasis [23, 27]. Laminins are a family of large extracellular glycoprotein that is a vital component of the structural scaffolding in almost every tissue of an organism, because it is the major component in the basement membranes. Accordingly, laminin is an integral part for both differentiation, migration, and adhesion of cells and the maintenance and survival of tissues.

Proteoglycans (PG) are highly glycosylated proteins that have covalently attached anionic glycosaminoglycan (GAG). The GAGs are polyanionic molecules, and the net negative charges attract cations (sodium, potassium, and calcium) which stick water molecules after GAG interacts with the cations [23]. Thus, PG generates gel-like hydrated networks that fill the majority of the extracellular spaces between cells, and the unique physical and hydrodynamic characteristics regulate the movement of fluid and solute, resistance to compressive forces of tissues, and the diffusion of secreted growth factors [15, 28, 29]. In addition, GAG-mediated hydration facilitates cell migration and invasion through increase in porosity [30, 31].

Structurally, the interstitial matrix, which is observed in most tissues and packs the extracellular interstitial space within the tissues, is a major category of ECM. It makes up cells distributed in meshwork of collagens (type I, II, and III collagens), proteoglycans, and different glycoproteins including elastin and fibronectin. The collagens together with fibronectin provide the structural and mechanical integrity of the tissue [32, 33]. The proteoglycans also regulate the movement of fluid and solute, growth factor- and cytokine-binding functions, and resistance against the stress placed on the matrix [28, 34]. Thus, the interstitial matrix not only confers upon tensile strength and architecture of the tissue but also mediates cellular signaling indirectly by binding growth factors and cytokines.

Basement membrane of epithelial cells, which is a specialized form of sheetlike ECM, is the other major category of ECM. The basement membrane is more compact and less porous than interstitial matrix and has a characteristic composition containing type IV collagen, laminins, fibronectin, other glycoproteins, and linker proteins such as nidogen/entactin, which link type IV collagens to other ECM proteins [35]. The primary function of the basement membrane is to anchor down the epithelium to its loose connective tissue underneath, to provide mechanical support, and to separate tissue parenchyma (epithelial cells) from the underlying stroma (connective tissues). It also regulates cell polarity, proliferation, differentiation, and gene expression [36, 37]. Additionally, basement membrane functions as a physical barrier, preventing malignant tumor cells from invading the deeper tissues [38]. Early steps of malignant progression are thus restricted to the epithelial layer by the extracellular mechanical barrier (basement membrane).

Deregulated ECM in Cancer

As discussed above, the ECM is a complex network of macromolecules with multiple physical, biochemical, and biomechanical properties which plays vital roles in regulating behaviors of cells residing in the local microenvironment. Due to this

importance of the matrixces on cell and tissue homeostasis, the ECMs are tightly governed through diverse regulatory mechanisms [10]. Importantly, aberrant control of the regulatory mechanisms may cause dysregulation and disorganization of ECMs, resulting in abnormal cellular behavior and ultimately loss of tissue homeostasis and functional role. Thus, it has been proposed that alterations in ECM homeostasis should lead to diseases. Indeed, disruption of ECM dynamics is implicated in disease progression and is well documented in clinical data of various diseases, especially cancer [11, 12].

ECMs generally become deregulated and disorganized in malignant tumors, so that tumor ECM is distinct from its properties compared with normal ECM [39]. For instance, the cancer tissue is commonly much stiffer than normal tissue (400 Pa vs. 150 Pa); even breast cancer tissue can be ten times stiffer than normal breast tissue (1.5 kPa vs. 150 Pa) [8, 40]. The elevation in tissue stiffness alters ECM properties and triggers migration of cancer cells [8, 40]. In addition, dynamics of ECM remodeling is excessive or uncontrolled in cancer, whereas it is well-controlled in normal tissues. This aberrant ECM remodeling elicits the degradation of normal ECM and its replacement with tumor-derived ECM in the niche and finally alters physiological cues that function as essential inducers for cancer progression. Thus, the disruption of ECM dynamics influences cancer progression by enhancing tumor malignancy and metastatic dissemination. This abnormal ECM is involved in aberrant expression and activities of ECM-regulating enzymes which act as main contributors of the processes, deregulating and disorganizing ECM. Therefore, it is important to understand how ECM-regulating enzymes may lead to abnormal ECM and contribute to tumor malignancy and metastatic progression. In the following sections, we will review and discuss how deregulated ECM-regulating enzymes such as matrix metalloproteinases (MMPs), lysyl oxidase (LOX), urokinase plasminogen activator (u-PA), and cysteine cathepsins generate aberrant composition and structural organization of the ECM and potentiate cancer progression at different steps of cancer development, especially cancer cell dissemination.

Deregulated MMPs in Cancer

MMPs are zinc-dependent endopeptidases that mediate various cellular behaviors by degrading and remodeling ECMs. Expression and activity of these enzymes is greatly elevated in almost every type of human tumors [11, 41–43]. This high level of MMPs changes behavior of cancer cells in the tumor microenvironment, and their retained expression results in the active destruction of normal ECM and its replacement with tumor ECM, thereby promoting tumor progression and correlating with poor clinical outcome [44, 45]. These clinical observations have been supported by different reports that diverse MMPs were first identified and have been repeatedly cloned as cancer-associated genes or metastasis-specific genes from neoplastic cells [5, 41, 42]. The strong causal relationships between MMP overexpression and tumor malignancy have been further supported by recent reports that

transcriptome studies in both murine and human tumors demonstrate the essential roles of MMPs in metastatic disease, especially in highly aggressive late-stage tumors with poor clinical prognosis [46–49]. Thus, MMPs were suggested to be important in cancer invasion and metastasis. Furthermore, recent reports indicate that MMPs are associated with several steps of cancer development.

The Roles of MMPs in Cancer Invasion and Metastasis

For cancer metastasis to distant sites, cancer cells have to cross several ECM barriers. First, they traverse the epithelial basement membrane and invade the interstitial stroma, and then they enter into (intravasation), survival in, and exit from (extravasation) the lymphatic or blood vessels, thereby establishing new proliferating colonies (Fig. 11.1). Hence, tumor cells upregulate MMPs and/or enhance expression of MMPs in neighboring stromal cells in order to degrade the basement membrane and invade the surrounding tissue. This proteolytic activity is also required for a cancer cell to invade a nearby blood vessel, extravasate at a distant location, and then invade the distant tissue to seed a new metastatic site.

Tumor cell migration is one of the first events that occur during tumor invasion and metastasis. Degradation of type IV collagen, laminin-5 through MMP-2 and membrane-type 1 matrix metalloproteinase (MT1-MMP) generates a cryptic peptide that promotes migration of tumor cell [50, 51]. MT1-MMP also sheds CD44, the main receptor for hyaluronan, from tumor cell surface, and MT1-MMP-mediated shedding of CD44 stimulates motility of tumor cells, however the exact mechanism by which shedding of CD44 leads to increased tumor cell migration remains unknown [52]. In addition to interact with the ECM, the localization of MMPs to specialized surface protrusions, known as invadopodia, on the cancer cell membrane is integral to their capacity to facilitate cancer cell invasion [53]. Active MMP-2 interacts with αvβ3 integrin on the surface of cancer cells, and αvβ3 integrin-associated cell surface MMP-2 promotes cancer cell-mediated collagen breakdown and invasive behavior of cancer cells [54]. Similarly, MMP-9 binds to CD44 on the cancer cell surface and this surface presentation of active MMP-9 stimulates cell-mediated type IV collagen degradation and tumor cell invasion [55]. MMPs not only induce the degradation of ECM but also activate intracellular signaling for tumor cell migration and invasion. For example, MMP-1 induces PAR1 (protease-activated receptor 1)-dependent signaling by cleaving at the proper site for receptor activation, resulting in tumor cell migration and invasion [56]. Pro-MMP-9 also exerts a potent effect on cancer cell migration by activating intracellular signaling pathway (MAPK and PI3K pathways), irrespective of the degradation of ECMs [57]. Epithelial to mesenchymal transition (EMT) is a key hallmark of cancer progression to metastasis. During EMT, cancer cells actively induce downregulation of cell-cell adhesion molecules, disruption of cancer cell polarity, and acquisition of a mesenchymal-like phenotype, resulting in promoted migratory

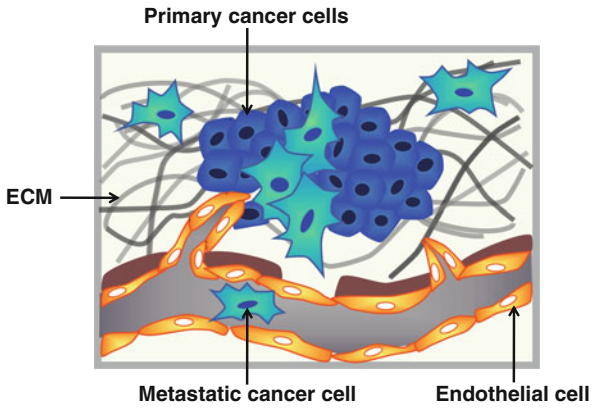

Fig. 11.1 Degradation of ECM and enhanced migration and invasion of cancer cells

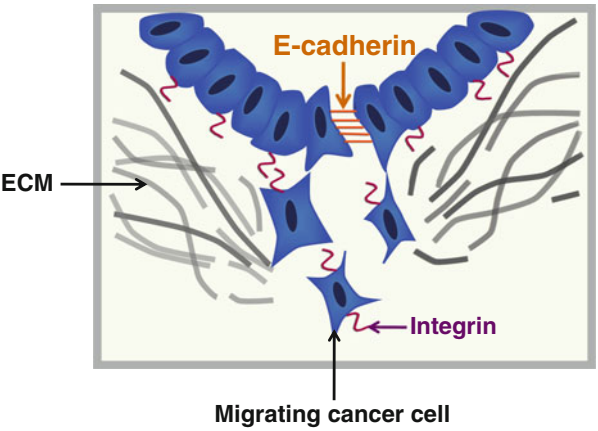

Fig. 11.2 Downregulation of cell-cell adhesion and induction of EMT by truncation of E-cadherin

and invasive ability of cancer cells and consequently leading to accelerated metastatic potential of cancer cells [58]. This EMT can be induced by MMPs. For example, E-cadherin is an intercellular adhesion molecule that has a pivotal role in maintenance of epithelial cell polarity and architecture and is deregulated during EMT of cancer cells [59]. The E-cadherin is truncated by MMP-1, MMP-3, MMP-7, MMP-9, and MT1-MMP, thereby triggering the release of E-cadherin fragment, the loss of cell-cell adhesion systems, and in turn the facilitation of EMT, followed by facilitated migration and invasion of cancer cells [60–63] (Fig. 11.2). Transforming growth factor-β (TGF-β) is also known to initiate and maintain EMT through various mechanisms [64–67]. In a recent study, Illman et al. reported that MMP-28 elicits proteolytic activation of latent TGF-β and TGF-β-dependent EMT [68].

MMPs have also been involved in the late steps in the metastatic cascade, when the cancer cells intravasate into, survive in, and extravaste from the lymphatic or blood circulatory system. Expression of MMP-9 in cancer cells is correlated highly with their intravasative capacity [69]. Cancer cells expressing MT1-MMP acquire increased survival capacity when cancer cells are injected intravenously [70]. Likewise, there is a significant positive correlation between high MMP-2 and/or MMP-9 activity and the survival rate of cancer cells in systemic circulation following intravasation [71]. In addition, endothelial cells trigger extravasation of cancer cell through MMP-9 production [72]. This MMP-9 might contribute to the extravasation steps catalytically but its precise mechanisms are not yet clear.

The Roles of MMPs in Cancer-Associated Immune Suppression

The immune system can seek out and destroy neoplastic cells, but tumor cells employ a variety of mechanisms to avoid host immune response. In particular, this tumor immune evasion may be essential for metastatic dissemination of cancer cells, since intravasated cancer cells are directly exposed to the host immune system in the lymphatic or blood circulatory system. MMPs can mediate the cancer-associated immune suppression. For example, the proliferation and development of T lymphocytes are mediated by interleukin-2 (IL-2) signaling complex. MMP-9 interrupts IL-2 signaling by cleaving interleukin-2 receptor-α (IL-2Rα), inhibiting the proliferative capability of the T lymphocytes, and finally attenuating a T cell-mediated antitumor immunity [73]. MMP-9 also liberates active TGF-β (an important immunosuppressive cytokine observed in most human tumors) localized in the tumor stroma, thereby eliciting suppression of a T cell-mediated immune response capable of eradicating tumors [74, 75]. Additionally, a cleavage fragment of α1-proteinase inhibitor is induced by MMP-11 which diminishes the sensitivity of cancer cells to natural killer cells, leading to the promotion of tumor growth and invasiveness [76, 77].

The Roles of MMPs in Cancer Cell Proliferation

MMPs have an impact on cancer cell proliferation through induction of growth-promoting signals. To this end, MMPs process cell membrane-associated growth factors or growth factors sequestered by ECM components, followed by releasing active growth factors and in turn stimulating tumor growth (Fig. 11.3). Epidermal growth factor receptor (EGFR) is upregulated in more than one third of all patients with solid tumors and involved in cancer cell proliferation and progression (2009, 16, 3797-3804). MMP-3 and MMP-7 drive elevated shedding of the membrane-bound ligands of EGFR or heparin-binding EGF (HB-EGF), leading to excessive

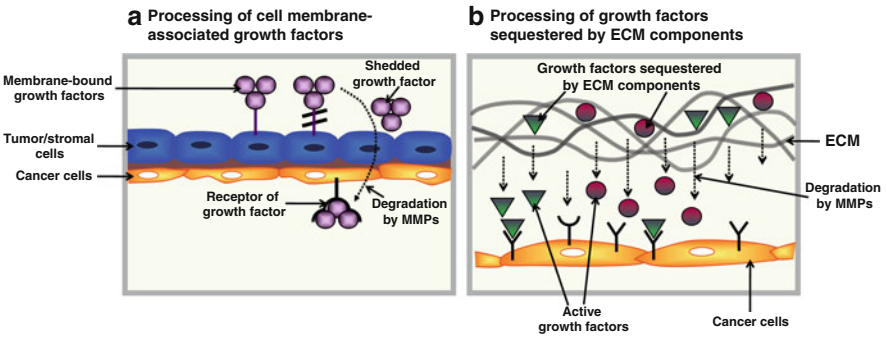

Fig. 11.3 Processing of cell membrane-associated growth factors (**a**) or growth factors sequestered by ECM components (**b**), leading to the stimulation of tumor growth

EGFR signaling and cancer cell growth [78, 79]. MMP-1, MMP-2, MMP-3, MMP-9, and MMP-11 also cleave insulin-like growth factor-binding protein (IGFBP), release insulin-like growth factors (IGF), and transactivate the insulin-like growth factors receptor (IGFR), resulting in uncontrolled proliferation of cancer cells [80–83]. In addition, MMPs can indirectly modulate proliferative signals, since the shedding of E-cadherin by MMPs (MMP-1, MMP-3, MMP-7, MMP-9, and MT1-MMP) regulates the β-catenin downstream signaling, thereby inducing expression of the β-catenin downstream gene cyclin D1 and promoting cell proliferation [84].

The Roles of MMPs in Cancer-Associated Angiogenesis

Tumor angiogenesis is the process of forming new blood vessels from a preexisting vasculature and is pivotal for cancer progression, because the new tumor vasculature provides oxygen and nutrients essential for cancers to grow as well as even the main route for cancer metastasis [85, 86]. The initial step in this process needs to degrade physical barriers (the vascular basement membrane) and in turn to generate angiogenic factors for proliferation and invasion of endothelial cells into the tumor stroma. MMPs participate in these events and thus contribute to cancer-associated angiogenesis.

MT1-MMP is present at the leading edge of endothelial cells and enables them to migrate and invade along tumor stroma through surface-bound proteolytic degradation, thus allowing enhanced endothelial cell migration and capillary tube formation [87, 88]. Similarly, active MMP-2 interacts with $\alpha v\beta 3$ integrin on the surface of angiogenic blood vessels, and $\alpha v\beta 3$ integrin-bound cell surface MMP-2 facilitates type IV collagen cleavage, endothelial migration, and eventually angiogenesis [54]. In addition, MMPs have been implicated in tumor-induced angiogenesis by liberating angiogenic mitogens, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), localized in the tumor stroma, followed by

potentiated cancer-associated angiogenesis [89–91]. For example, MMP-9 promotes the release of ECM-bound VEGF [90]. Likewise, the process of ECM components by MMP-1 and MMP-3 liberates active bFGF [91].

Deregulated LOX and Its Roles in Cancer

LOX is a copper-dependent extracellular enzyme that mediates the cross-linking of collagens or elastins in the extracellular space. This LOX-induced cross-linking plays a vital role in maintaining tensile strength and structural integrity of normal tissues, which is essential for normal connective tissue function and remodeling. Conversely, abnormal expression and/or enzymatic activity of the LOX may affect disease development and progression. Indeed, excess expression of LOX has been observed in cancer patients and patients with high LOX-expressing tumors have a poor prognosis [8, 92, 93]. During cancer progression, collagens of the various types such as type I, II, III, V, and IX collagens exhibit increased deposition and then LOXs are upregulated in response to elevated collagen deposition [94–96]. Consequently, overexpression of LOX results in high ECM stiffness, leading to facilitated cancer cell migration that promotes invasion and metastasis of cancer cells [14, 97]. The increased tumor matrix stiffness also elicits enhanced integrin clustering, mechanotransduction, and eventually promoted cancer cell migration [98, 99]. Additionally, LOXs drive cancer metastasis by activating cell signaling and transcriptional gene regulation, as evidenced by the observation that exogenous expression of LOX in cancer cells induces activation of the focal adhesion kinase (FAK)-Src signaling complex that mediates changes in actin filament polymerization, resulting in promoted motility and metastasis of cancer cell [7].

Deregulated u-PA and Its Roles in Cancer

u-PA is a serine protease associated with the fibrinolytic system activating plasminogen to plasmin, a broad-spectrum ECM- and fibrin-degrading serine protease [100]. Alterations in host expression of this u-PA are also causally involved in cancer progression, especially cancer invasion and metastasis [101]. Various studies have shown an upregulation of the u-PA in different human malignant tumors (breast, prostate, lung, bladder, colon, liver, ovary, gastric, cervix, kidney, and brain) in contrast to the corresponding normal tissue, and high level of the u-PA correlates with the metastatic potential of cancer cells as well as inversely correlates with the overall survival rate among cancer-bearing patients [102–111]. The effects of u-PA in cancer metastasis can be mediated by degradation of the fibronectin causing both cancer cell motility and activation/release of metastasis factors such as FGF-2, hepatocyte growth factor/scatter factor (HGF/SF), and TGF-β localized in the tumor milieu [101, 112, 113]. Additionally, u-PA-activated plasmin can contribute to

cancer invasion and metastasis. Firstly, it degrades several ECM components such as type IV collagen, laminin, fibronectin, proteoglycan, and fibrin [111, 114, 115], an important step for cancer invasion and metastasis. Secondly, it activates the MMPs such as MMP-1, MMP-3, MMP-9, MMP-12, and MMM-13 and thus facilitates cancer cell invasion and dissemination [116–120]. Thirdly, it liberates metastasis factors including FGF-2 and TGF-β localized in the tumor stroma. Finally, plasmin catalyzes the conversion of pro-u-PA to active u-PA which in turn elicits further plasmin generation.

Deregulated Cysteine Cathepsins and Its Roles in Cancer

The human cysteine cathepsin family makes up 11 members (cathepsin B, C, F, H, K, L, O, S, V, W, and X/Z), which have conserved active site cysteine and histidine residues [121, 122]. These intracellular proteases play crucial roles in terminal protein degradation in the acidic milieu such as cellular endosomes/lysosomes [123]. The cysteine cathepsins also have different physiological roles including antigen processing and presentation in the immune system, collagen turnover in bone and cartilage, and neuropeptide and hormone processing [124–128]. In addition to the normal physiological functions, the cysteine cathepsins have been known to stimulate cancer invasion and metastasis [129, 130]. In accordance with these reports, they are upregulated in patients with various types of cancer and their high levels are correlated with malignant progression and poor prognosis [131]. During neoplastic progression, these proteases are translocated to the cell surface of cancer cells or secreted into the extracellular microenvironment of tumor in which cysteine cathepsins require acidic condition for optimal activity, followed by facilitation of cancer invasion and metastasis [130, 132, 133]. For instance, at the cancer cell surface or the extracellular space, the cathepsins directly cleave ECM components such as laminin, fibronectin, tenascin-C, and type IV collagen, thereby affecting invasion and metastasis of cancer cells [134–138]. Recently, E-cadherin is also identified as a novel cathepsin substrate. The cathepsin-mediated degradation of E-cadherin elicits its loss of adhesive properties of cancer cells and increased migration potential of cancer cells, thus indicating another mechanism by which cancer cell invasiveness could be achieved [139]. In addition, pericellular cathepsins can activate other proteases such as pro-MMPs and pro-u-PA, which in turn can degrade the ECM components (collagens, laminin, fibronectin, gelatin, and tenascin), thus driving the invasion and metastasis of tumor cells [140–143].

Conclusion

ECM is one of the most important key regulators of cellular behaviors and tissue functions. Accordingly, highly controlled ECM homeostasis is essential for regulating diverse cellular processes, allowing for correct normal tissue development and

homeostasis. When this normal ECM homeostasis is perturbed by deregulated ECM-regulating enzymes (MMPs, LOXs, serine proteases, and cysteine proteases), ECM becomes abnormal and the disruption of ECM homeostasis contributes to life-threatening pathological conditions. The expression and the activity of ECM-regulating enzymes are excessive and/or uncontrolled in cancer. Aberrant expression and activities of ECM-regulating enzymes alter behaviors of cancer cell in the tumor niche, and its sustained upregulation elicits the progressive breakdown of normal ECM and its replacement with tumor-derived ECM, thereby facilitating malignant progression and cancer cell dissemination as well as correlating with poor clinical prognosis. The cancer progression and metastasis caused by deregulated ECM-regulating enzymes are responsible for millions of deaths worldwide and indicate a challenging obstacle with respect to clinical treatment. This increased understanding of how ECM-regulating enzymes generate abnormal composition and structural organization of the ECM which lead to cancer progression and metastasis will help guide better inhibitors and protease-based drugs. Ultimately, further research should focus on tracing the processes underlying cancer-associated deregulation of ECM-regulating enzymes at the molecular level in order to achieve the goal of effective targeting of deregulated ECM-regulating enzymes in malignant tumors.

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Chapter 12
The Role of Non-cancerous Cells in Cancer: Pancreatic Ductal Adenocarcinoma as a Model to Understand the Impact of Tumor Microenvironment on Epithelial Carcinogenesis

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Abstract Pancreatic ductal adenocarcinoma is a deadly tumor. Despite several advances in conventional and targeted therapies over decades, its mortality remains slightly less than its frequency. One of its main features is its compact stroma which is formed by pancreatic stellate cells. In the last decade, it became evident that the stromal component of the tumor is not a passive scaffold, but an active player in carcinogenesis. This component is mostly missing in our experimental settings. Even in genetically engineered mouse models where a fibrotic stroma is visible, tumor responses are different than in humans. Our inability to recreate the tumor microenvironment often leads to optimistic results in the therapy of pancreatic cancer. This temporary optimism is often lost after first clinical trials. Here we would summarize various approaches to treat pancreatic cancer and scrutinize their pros and cons from a biologic point of view.

In this chapter the data on pancreatic cancer, possible mechanisms of its therapy resistance, and especially the role of tumor stroma will be scrutinized.

Incidence and Clinicopathological Features of Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is a disease with a dismal prognosis in which the incidence almost equals mortality. Relative 5-year survival rate ranges from practically null in metastatic disease to 9 % in regional (cancer has spread to lymph nodes near the pancreas) to 22 % in localized stages. Despite many advances

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in diagnostic modalities and treatment options, the overall 5-year survival rates for all stages of pancreatic cancer only slightly increased from 2 to 6 % monitored over three observation periods from 1975 to 2007 [1]. There is no difference in estimated affection between men and women [1]. In a study assessing 213 patients with PDAC, Freeny and colleagues reported that tumors are located in the pancreatic head in 64 %, in the body in 22 %, and in the tail in 10 % of the cases [2]. Symptoms of pancreatic cancer are mostly subtle and detected, in almost all cases, at advanced stages. Thus, pancreatic cancer often develops unperceived. Typical symptoms are abdominal pain and jaundice, due to tumor infiltration of nearby structures such as nerves and the bile duct.

As of today, surgical resection of an early stage tumor is the only curative option. Unfortunately, non-resectable disease is seen in more than three-quarters of patients as a consequence of contiguous organ or vascular invasion and/or distant metastases [2, 3]. Adding to these dismal numbers are the missing screening strategies and the inability to recognize early cancer or precursor lesions, since they are usually below the detection threshold of contemporary diagnostic tools [4]. As environmental risk factors, obesity, diabetes, and smoking have been identified to be associated with pancreatic cancer [5, 6]. Genetic predisposition, though rare, through oncogenic mutations—or as a consequence of chronic inflammation in familial chronic pancreatitis—has a strong association with pancreatic cancer [7–9].

Origin of Pancreatic Ductal Adenocarcinoma

Ductal Origin

Due to the duct-like morphology of the cancer, three preneoplastic lesions of ductal origin are thought to be the precursor lesions, namely, pancreatic intraepithelial neoplasia (PanINs), intraductal papillary mucinous neoplasms (IPMNs), and mucinous cystic neoplasms. The last two are also the most commonly encountered cystic tumors of the pancreas.

Pancreatic Intraepithelial Neoplasia

Pancreatic intraepithelial neoplasia, which arises within intralobular ducts, is perhaps the best-analyzed precursor lesion of ductal adenocarcinoma of the pancreas. PanINs occur frequently and are histologically well defined by tissue architecture and cytological atypia [10–12]. The progression of PanIN to invasive cancer has been intensively studied on the molecular level [13, 14], and recently genetically engineered mouse models (GEMM) have been generated that fully recapitulate the entire spectrum of lesions from precursor to invasive pancreatic cancer [15, 16].

Investigations for genetic changes in PanINs revealed versatile results, ranging from bi-allelic inactivation (*MAD4*/*DPC4, TP53,* and *p16*/*CDKN2A* genes) to activating point mutations (K-*ras2* gene). Furthermore, telomere shortening was

identified to be a universal feature of PanINs, even in the earliest putative precursor lesion PanIN-1 [17]. These genetic changes follow a timely order; therefore, there is an accumulation of mutations as the lesion advances from PanIN-1 to PanIN-3 [18–20]. Thus, PanINs can be categorized by morphological appearance as well as by genetic expression analyses. Importantly, the grade of dysplasia of the PanIN lesion was reported to correlate with a stepwise increase of K-*ras* mutations [21]. As K-*ras* mutations are present in almost all pancreatic ductal adenocarcinoma cases [22], it is likely that PanINs are precursor lesions to ductal adenocarcinoma of the pancreas. Importantly, there is a consistent stromal activation and deposition of extracellular matrix around these preneoplastic lesions [23].

Cystic Tumors

Although more than 20 different cystic lesions of the pancreas are described, approximately 90 % of the lesions are intraductal papillary mucinous neoplasms (IPMN), mucinous cystic neoplasms (MCN), solid-pseudopapillary neoplasms (SPN), or serous cystic neoplasms (SCN) [24]. As of today, with the exception of SCN, of which the majority is benign, most cystic neoplasms are considered precursor lesions with malignant potential and deserve surgical resection when certain criteria are fulfilled (see below).

SCN are mostly asymptomatic tumors with a mean diameter of 5 cm, which grow smaller in size in females [25] and which occur mainly in the tail of the pancreas [26]. Their epithelium is lined with inconspicuous single layer of cuboidal or flattened, glycogen-rich cells with clear or eosinophilic cytoplasm [27]. The nuclei are usually centrally located, small, and hyperchromatic. Mitoses are absent. The central star-shaped scar and the stroma separating the cysts are composed of acellular collagenous connective tissue [28]. The treatment of choice is conservative in most of the cases. Surgery is indicated rarely due to symptoms and large size.

MCN are cystic epithelial neoplasms, typically affecting perimenopausal female patients and arising mostly in the body or tail of the pancreas [29]. Macroscopically, they are composed of thick-walled multilocular cysts that can become very large [30]. Microscopically, the cysts are lined with a tall, columnar, mucin-producing epithelium that resembles endocervical epithelium [31]. Due to their malignant potential, surgical resection is the therapy of choice.

IPMN are characterized by intraductal proliferation of neoplastic mucinous cells and, by definition, larger than 1 cm in size. They are predominantly seen in the elderly (mean age, 65 years) with slight male predominance [32]. 70 % of IPMN occur in the head of the pancreas, where they form radiologically detectable masses. Separation from PanIN is primarily based on their larger size. The epithelium of IPMN comprises mucin-producing cells and often exhibits papillary architecture. The mucosa is further subclassified into gastric type (best prognosis), oncocytic type, intestinal type, or pancreatobiliary type (worst prognosis) [33]. Depending on their localization, they can be main-duct type, branch-duct type, or mixed type. The main-duct type is considered malignant and should always be resected if the diameter of the pancreatic duct is more than 5 mm. The branch-duct type IPMN

harbor less risk of malignancy; therefore, surgical resection is offered selectively when particular criteria are fulfilled (see for a detailed review: [34]).

SPN typically afflict women in their late twenties. The lesions are relatively evenly distributed throughout the pancreas. Size is often large [35]. Sections display various degrees of hemorrhage and necrosis. A distinctive tumor tissue pattern is created by focal dyscohesion of cells away from the vasculature. The nuclei are uniformly round to oval with occasional grooves; the cytoplasm is eosinophilic or clear. Due to their malignant potential, surgical resection is recommended.

Acinar Origin: Atypical Flat Lesions

Alternative to ductal origin, acinar and centroacinar cells have been proposed as the originating cells in PDAC since 1970s [36]. Stanger et al. provided evidence that ductal metaplasia resulted from the expansion of centroacinar cells as neoductules rather than transdifferentiation of acinar cells in a fraction of *Pten*-deficient mice [37]. Knockout of *Pten* caused an islet phenotype and early, highly proliferative expansion of the ductal lineage in these rodents.

Aichler and colleagues recently analyzed atypical flat lesions (AFLs) of the pancreas in detail [38]. They observed AFL in areas of acinar-ductal metaplasia (ADM) and described them to consist of tubular structures lined by cuboidal cells with enlarged nuclei and prominent nucleoli, a high nuclear-cytoplasmic ratio, and the presence of mitoses. Presence of AFL in tissue sections could be easily identified at low magnification because of peculiar appearance of its loose but highly cellular stroma surrounding the lesions. Although originating in ADM areas, both murine and human AFLs displayed a ductal phenotype. A comparative immunohistochemical profile of human and mouse AFL revealed a nearly identical expression pattern without quantification for trypsin/amylase, Muc1, Mib1, Smad4, Pdx1 (cytoplasmic positivity in human tissue, loss of expression in mouse specimens), CK5, and α-SMA. Striking difference was the predominant negativity of mouse tissue for p53 expression, whereas human tissue revealed presence of p53 [38].

Tumor Stem Cells

Although several tumor or cancer stem cell (CSC) populations have been identified in pancreatic adenocarcinoma (PDAC), it has not been elucidated yet whether these populations fit the cancer stem hierarchy or the classical stochastic clonal evolution model of tumor growth. Despite these uncertainties, another model still has its attraction: the “seed and soil” theory, which describes cancer development as a process involving cell-autonomous changes (the seed) and the local microenvironment of the tumor (the soil) [39]. Transferred to the situation in PDAC, its stroma represents the soil, whereas the CSCs would provide the seed. The conceptual idea was raised that

pancreatic CSCs reside in a niche, providing space for cell-cell interactions, sheltering them from stressors and genotoxic insults under a regulatory network comprised by the extracellular matrix, stromal cells, and soluble factors released by the latter ones [40]. However, the debate on the role of pancreatic CSC remains controversial [15].

The marker expression profile of pancreatic CSCs is similar to other entities as they exhibit specific membranous proteins such as CD24, CD44, ESA (EpCAM, epithelial-specific antigen) in a triple positivity [41], CD133 and CXCR4 [42, 43], c-Met [44], ABCG2 (ATP-binding cassette subfamily G member 2) [45], and cytosolic enzyme ALDH-1 [46]. In xenograft mouse models, sorted cells from low-passage PDAC, which were positive for the individual markers or combinations of these, recapitulated the histology and cellular heterogeneity of the ongoing pancreatic tumorigenesis. For nestin and other known stem cell markers such as SOX2 and Oct-4, detection by immunohistochemical staining on pancreatic tissue was also reported [47]. Oct-4- and SOX2-positive cells were found in small duct walls. Double positivity was also detected, but no additional co-expression of nestin, CD34, or CK19, the most relevant marker for ductal cell differentiation. Interestingly, CXCR4 expression was detectable as of early stages of pancreatic carcinogenesis and maintained during progression to invasive cancer [23], whereas SOX2 seemed to be involved only at later stages [48]. CSCs expressed increasing levels of Nestin under hypoxic conditions and demonstrated higher proliferation rates and self-renewal capacity [49].

The interactions of putative CSCs and the predominant stromal cells in PDAC are manifold. For instance, CD133-positive pancreatic cancer cells displayed increased cellular migration and invasion potential after co-culture with stromal cells [50]. That silencing *CXCR4* in CD133+ cells could attenuate these effects suggests that stromal cells could be responsive to SDF-1, the chemokine receptor ligand, and thus functionally support CSCs. As CD133 expression levels in cancer are regulated by HIF1α, the hypoxic condition in pancreatic cancer tissue could contribute to the propagation of CSC phenotype displaying cancer cells, triggering cellular adaptation, and maintaining a non-differentiation environment opposed to normoxic condition [51].

Endocrine Origin

Although once a popular concept, endocrine cells of the pancreas are now thought less likely to be involved in the carcinogenesis of PDAC [52].

Role of Tumor Microenvironment in Pancreatic Carcinogenesis

On the morphological level, pancreatic ductal adenocarcinoma is characterized by a prominent, dense fibrotic cancer stroma, called desmoplasia. Desmoplastic tissue is primarily comprised of fibrillary collagens (type I and III), fibronectin and

proteoglycans, and pancreatic stellate cells (PSC) have been identified to be the major source of excessive deposition of these extracellular matrix proteins [53, 54]. PDAC stroma is a complex structure containing PSC, proliferating myofibroblasts, inflammatory cells, macrophages, pericytes, endothelial cells, and pathologically enlarged nerve fibers [55–62]. Though the fibrotic matrix produced by normal cells likely functions as a physical barrier, evidence was found that it could also modulate and even initiate carcinogenesis by providing a physical scaffold and a pool for cytokines and growth factors [63–66].

The desmoplastic stroma of the PDAC impacts on its aggressiveness [67, 68]. Continuous PSC activation in periacinar spaces at the invasive front of the activated stroma also leads to the commonly encountered chronic pancreatitis-like changes around the tumor leading to the replacement of the normal parenchyma by fibrosis [69]. It is not clear whether this stromal activation is a part of carcinogenesis or an effort by the host tissue to confine the preneoplastic lesions. Whatever the reason may be, the fibrotic stroma causes hypoxia due to distortion and compression of tissue vasculature and damages in fine innervation, likely contributing to the chemoresistance of pancreatic cancer [63, 70–72]. We have previously argued that selective pressure applied by the desmoplastic stroma leads to the evolution of pancreatic cancer cells. Consequently, somatic evolution (from PanIN or AFL) of invasive pancreatic cancer could be viewed as a sequence of phenotypical adaptations to this fibrotic barrier, highlighting the importance of the barren tumor microenvironment in the behavior of pancreatic cancer [73, 74].

Microenvironment of Pancreatic Cancer Cells

Pancreatic Stellate Cells

In the normal pancreas, quiescent pancreatic stellate cells are located in the periacinar spaces in close vicinity to the basal aspect of acinar cells, capillaries, and terminal nerve fibers. This cell population can be identified by expression of desmin and GFAP. Moreover, a very typical feature of quiescent PSC is the numerous retinoid-containing fat droplets in their cytoplasmic compartment, which display strong autofluorescence [75]. PSC extend long cytoplasmic projections along the base of adjacent acinar cells, similar to that of pericytes in the mammary gland. Four to seven percent of all parenchymal cells in the normal pancreas represent quiescent PSC [54], whereas in tumor sections, PSC can outnumber the cancer cells [76].

During pancreatic injury, like in acute or chronic pancreatitis (CP), resident PSC transform into an activated, myofibroblast-like phenotype, in which they express α-smooth muscle actin (SMA) filaments (Fig. 12.1) and secrete excessive amounts of extracellular matrix proteins, which comprise fibrous tissue (desmoplasia) [77]. Through secretion of growth factors and cytokines, pancreatic cancer cells can

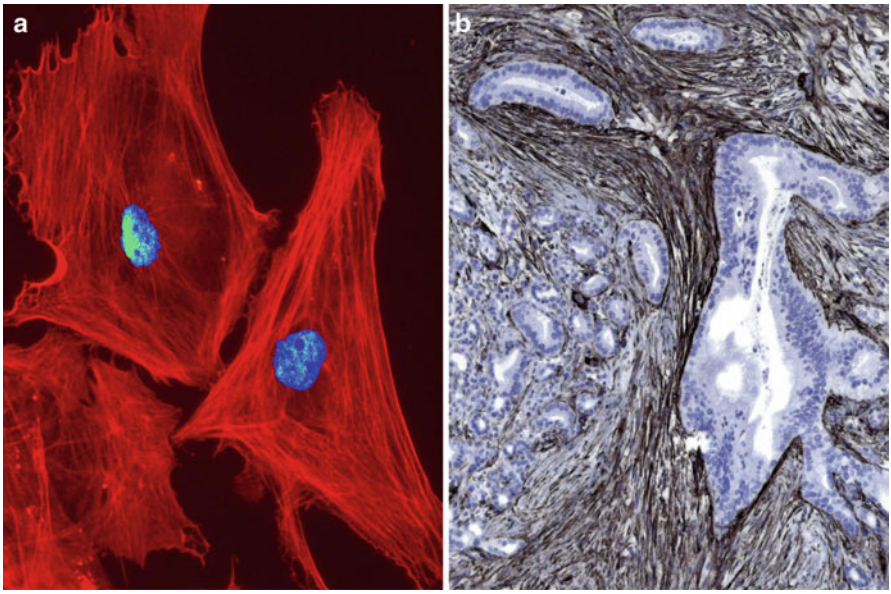

Fig. 12.1 Pancreatic stellate cells: Pancreatic stellate cells are the producers of extracellular matrix proteins in the diseased pancreas. Alpha smooth muscle actin expression is a typical feature of pancreatic stellate cells showing their transdifferentiation into a myofibroblast-like phenotype from their quiescent form. (**a**) Immunofluorescence analysis of cultivated primary pancreatic stellate cells. Alpha smooth muscle actin (*red*) and DAPI (*blue*) staining marks the cell cytoskeleton and the nucleus, respectively. (**b**) Immunohistochemistry analysis of PDAC tissues. Alpha smooth muscle actin (*brown*) expression marks the activated PSC around PanIN lesions, tubular complexes, and cancer structures. Hematoxylin + eosin staining, original magnification: 100×

activate the PSC within their immediate vicinity. Once activated, PSC can perpetuate their own activity by forming autonomous feedback loops. In the pancreas, stellate cells are the only source of some extracellular matrix proteins such as periostin, over which such an autonomous feedback loop runs [63, 69].

Efforts to return PSCs to a relatively quiescent phenotype have led to the identification of mediators of the reconstitution of such inactive state, which primarily target the MAPK pathway (p38, JNK, or ERK) in response to retinol and retinoic acid [78]. Evidence exists that forced expression of PPAR-γ, C/EBP-a, or albumin might also inactivate PSC [79]. PSC synthesize and secrete fibrillary collagens, fibronectin, and laminin, all of which have been reported to increase cancer cell growth and promote resistance to chemotherapeutic agents in vitro [77, 80–85]. Not surprisingly, subcutaneous tumors induced in nude mice reached a larger volume when pancreatic cancer and stellate cells were co-injected. On the other hand, PSC not only synthesize extracellular matrix proteins but also regulate turnover of the extracellular matrix through their ability to produce various matrix metalloproteinases (MMPs) and their inhibitors [86]. It was shown that PSC predominantly

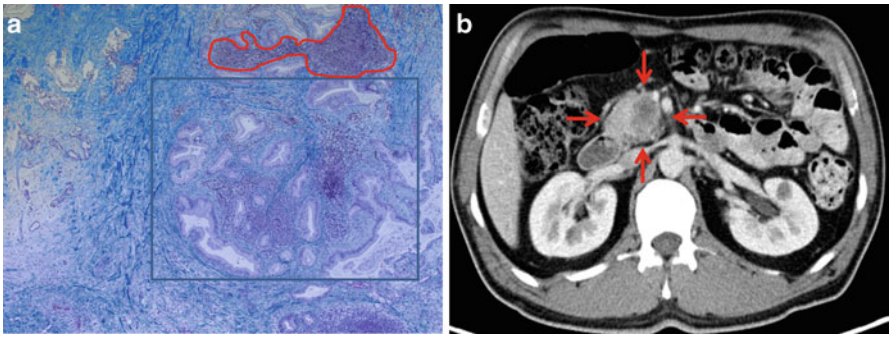

Fig. 12.2 Pancreatic ductal adenocarcinoma. (**a**) Mason's trichrome staining of pancreatic ductal adenocarcinoma. *Blue* (aniline) stains the fibrillary collagen-rich stroma. Several ductal cancer structures are marked in the *rectangular area*. Notice the dense infiltration by inflammatory cells (*encircled with red*). (**b**) Contrast-enhanced computed tomography image of a pancreatic ductal adenocarcinoma located in the pancreatic head (*arrows*), appearing as a hypodense (less contrasted, *darker*) lesion in the normal contrasted pancreatic parenchyma

secrete MMP-2, MMP-9 (both gelatinases involved in the degradation of basement membrane collagen type IV), and MMP-13 (a collagenase). MMPs and their tissue inhibitors are associated with inflammation, fibrosis, angiogenesis, and cancer invasion [87–93].

Extracellular Matrix

The extracellular matrix (ECM) comprises the interstitial substance and the basement membrane. The ECM of the pancreas is composed mostly of glycoproteins, collagens, growth factors, and proteases (Fig. 12.2). Activation of PSC during pancreatic injury leads to remodeling of the ECM with deposition in ECM proteins in CP and PDAC [76, 94]. Matrix metalloproteinase-2 and matrix metalloproteinase-9, as well as EMMPRIN, mostly produced by PSC, were found in PDAC patient samples in their active forms, indicating steadily ongoing remodeling of the ECM in the context of pancreatic neoplasia [95–97].

In PDAC, there are several reports on the tumor supportive effects of various ECM components. In general, ECM influences growth, differentiation, survival, motility, and cancer cell invasion of both by providing a physical scaffold and by acting as a reservoir for soluble mitogens. Several ECM components are reported to contribute to the aggressive phenotype of PDAC [98] and its motility [63, 99, 100]. Fibronectin and collagens type I and IV have been also identified to promote the migration of pancreatic tumor cells in an integrin-dependent manner [101, 102]. Recently, it was shown that stromal tenascin-C, rich in pancreatic stroma, enhances pancreatic cancer cell growth and motility [103]. Basement membrane laminin

expression predicts the outcome of curative resection and adds to gemcitabine resistance of pancreatic cancer cell lines [104, 105]. Furthermore, Kanemaru and colleagues reported that vitronectin mediates integrin β1-dependent pancreatic cancer cell adhesion through thrombin stimulation and Aprile et al. showed that biglycan expression in the ECM correlated with worse prognosis [106, 107].

These manifold examples demonstrate and underline the pro-malignant role of ECM components in pancreatic tumor biology. However, contradictory reports also exist. For example, the amount of osteopontin found in the pancreatic cancer ECM correlates positively with the survival of patients with PDAC [108]. Despite abovementioned in vitro data, abundant collagen in the stroma of PDAC hints a dormant type of tumor stroma and correlates significantly with a favorable prognosis [64].

Angiogenesis

According to classical reasoning, tumors cannot grow more than some millimeters without accompanying neoangiogenesis. However, most of the evidence for this type of reasoning is coming from in vitro experiments or from suboptimal setups where three-dimensional tumor architecture with its stroma is not recreated (i.e., injection of tumor cells subcutaneously into nude mice or chorioallantoic membrane assay).

There is an in vitro data showing that almost all components of pancreatic cancer microenvironment (inflammatory cells, tumor cells, cancer cells) producing pro-angiogenic substances, contradicting at the same time the clinical situation in humans where PDAC is known to be hypovascular and hypoxic [49, 69]. Due to the hypovascularity, contrast-enhanced computed tomography scanning of pancreatic cancer patients results in images with hypodense appearance of the tumor (Fig. 12.2) [44, 109]. When quantitatively analyzed, there is a significant reduction of microvascular density in CP and PDAC compared to that of the normal pancreas [23]. This is mostly due to the combined antiangiogenic effects of pancreatic cancer and stellate cells [23]. Despite these facts, several lines of evidence also indicate that neoangiogenesis is a vital process in the development of pancreatic cancer [110, 111]. Although PDAC is not a grossly vascular tumor, it often exhibits enhanced foci of endothelial cell proliferation. VEGF-A is believed to be a critical factor for tumor angiogenesis [111]. Several studies have reported positive correlation between blood vessel density, tumor VEGF-A levels, and disease progression in PDAC [56]. In vitro, PSC exhibit pro-angiogenic properties and produce significant amounts of VEGF. For example, conditioned media of PSC induce angiogenesis both in vitro and in vivo through the production of vascular endothelial growth factor and nonvascular endothelial growth factor family members such as FGF-2, IL8, and periostin. VEGF is induced by many mechanisms including mutant K-*ras* and p53 expression (commonly present in pancreatic cancer) or by growth factors such as FGF-2 and TGF-β and through transcription factors such as hypoxia-inducible factor 1 alpha and SP1 induced by hypoxia [112–115]. It is likely that PSC may exert different effects on angiogenesis depending on the site (invading front vs. dense fibrotic areas) and disease stage (early vs. advanced) [75].

Inflammatory Cells

The microenvironment of pancreatic cancer comprises ECM proteins, activated PSC, endothelial cells/capillaries, and immune cells. As inflammation is contributing to several other malignancies, it is also a key player in the development of pancreatic cancer by release of cytokines through immune cells and upregulation of pro-inflammatory pathways [116]. On the other hand, tumor cells themselves produce cytokines and growth factors, which may have chemotactic effects on inflammatory cells, perpetuating the cycle of cancer-inflammatory cell interactions [117].

The composition of immune cells found in the peritumoral connective/parenchymal tissue of pancreatic cancer was shown to be predominated by macrophages and mast cells, which exert a more detrimental than protective role and therefore may support its metastatic potential. Macrophages and mast cells express pro-angiogenic factors bFGF, VEGF-A, and VEGF-C, and tumors with high number of infiltrating macrophages/mast cells have a worse prognosis due to high intra-tumor microvessel density [56]. It is likely that such (relatively) vascular tumors might benefit from anti-angiogenic therapy. The assumption that a subgroup of pancreatic cancer tissues are better vascularized than the rest, however, still needs to be proven [56]. In a later study, elevated levels of cytotoxic T cells and DCs were observed in addition to infiltrating macrophages [118]. The same study demonstrated presence of multiple inflammatory mediators including TGF-β1, IL-1, IL-6, IDO, COX-2, CCL2, CCL20, and CXCL8 in the tumor milieu which are also known to exert tumorigenic properties.

There is also a cross talk between the inflammatory cells and the PSC. For example, TGF-β production by infiltrating granulocytes at the invasive front of pancreatic cancer correlates with the expression of collagen mRNA, promoting the stromal reaction by sustaining PSC activity [119]. Taken together, it is generally believed that chronic inflammation acts more pro-tumorigenic than tumoricidal in the development and progression of pancreatic cancer.

Neural Cells

Extra-pancreatic neural invasion by cancer cells precludes curative resection of the tumor [120–123]. Pathologically enlarged nerves are found in pancreatic cancer as well as in chronic pancreatitis. Several lines of evidence suggest that there is mutual tropism between nerves (including perineural cells as well as the neuronal axons) and cancer cells [124–130]. In the diseases of pancreas, as the number of pathologically enlarged nerves increases, normal terminal innervation (found in the periacinar spaces) is reduced [131]. The absence of cancer cells in chronic pancreatitis hints that several neuropathic changes are environment related. The possible factors inducing such changes are PSC, hypoxic stroma, as well as inflammatory cells [75].

For example, PSCs display intrinsic neurotrophic features and induce neurite outgrowth of isolated neurons in an in vitro neuroplasticity model where both cells are co-cultured [132]. Haas et al. reported that PSC produce NGF as a consequence of TGF-β-mediated activation of the ALK5 pathway [133].

Targeted Therapies

Gemcitabine is now accepted as the standard chemotherapeutic agent used to treat PDAC in palliative, neoadjuvant, and adjuvant settings. It provides survival advantage of approximately 6 weeks in the case of advanced pancreatic cancer. Numerous phase III trials evaluating gemcitabine in combination with other cytotoxic drugs have failed to demonstrate any significant overall survival advantage over gemcitabine alone [134, 135], which lead to the development of novel therapies targeted against molecules known to be crucial in pancreatic carcinogenesis (e.g., K-*ras* and EGF signaling). Below, we will summarize four different approaches. The common denominator of all these approaches is that, despite their success in the preclinical setting, they have largely failed in the clinical setting, hinting at our inability to recreate the tumor microenvironment in various experimental setups.

Anti-angiogenic Therapies

VEGF-Trap, a modified soluble VEGF receptor that consists of the second immunoglobulin-like domain of VEGF-R1 and the third immunoglobulin-like domain VEGF-R3, suppressed the growth of four pancreatic cancer cell lines as reported by Fukasawa and colleagues [136]. Therapy with TKI258, a tyrosine kinase inhibitor to FGFR, PDGFR, and VEGFR, led to significant growth delay and improved survival in established tumors, demonstrated in subcutaneous and clinically relevant orthotopic models [59]. Also assessed in an orthotopic animal model of pancreatic cancer, oral application of anti-angiogenic VEGFR inhibitor ZK261991 revealed significant survival benefits after curative tumor resection [137]. In a further study by Wicki and colleagues, anti-VEGF-R2 antibodies, which were covalently bound to pegylated liposomal doxorubicin (PLD), selectively depleted VEGF-R2-expressing tumor vasculature with high efficacy [138]. These promising results using anti-angiogenic therapies have paved the way for clinical trials.

However, there is a great discrepancy between such experimental results and the clinical reality. In a double-blind, placebo-controlled, randomized phase III trial of gemcitabine (standard chemotherapy) and bevacizumab (a humanized monoclonal antibody that recognizes and blocks vascular endothelial growth factor A) versus gemcitabine and placebo in 602 advanced pancreatic cancer patients, Kindler et al. could not show any benefit of the addition of this anti-angiogenic agent to the standard chemotherapy [139]. Possible reasons of this discrepancy will be discussed below.

EGF Inhibition

Erlotinib, a tyrosine kinase inhibitor of human epidermal growth factor receptor type I (HER/EGFR), is the only targeted drug that is approved by the FDA as it prolongs survival in a subgroup of PDAC patients that develop a skin rash. However, despite its high costs, the overall survival advantage is minimal; it only increases median survival from 5.91 months (gemcitabine plus placebo) to 6.24 months (gemcitabine plus erlotinib)—approximately 10 days—in patients with advanced PDAC [140].

Herceptin (trastuzumab) is a monoclonal antibody that interferes with the HER2/neu receptor (human epidermal growth factor receptor 2). Its binding action promotes anticancer benefits in approximately 20 % of patients with pancreatic cancer, who overexpress HER2/neu [141, 142]. However, treatment with this targeted drug holds the potential for development of resistance against trastuzumab therapy. Mechanistically, Herceptin can act in two ways, either by preventing growth factors to bind to HER2 and thereby blocking their stimulating effects on cancer cells or by stimulating the immune system to attack and kill the cancer cells, to which Herceptin is bound. Treatment of patients diagnosed with metastatic pancreatic cancer overexpressing HER2/neu with gemcitabine plus Herceptin displayed only modest benefit regarding 7-month median survival over gemcitabine alone in a cohort of 34 patients [143]. A phase III study with 745 patients that included comparing gemcitabine plus cetuximab, an anti-EGFR monoclonal antibody, versus the monotherapy in patients with advanced pancreatic adenocarcinoma demonstrated no improvement in outcome [144]. Recently published data from a phase II trial, in which the combination of gemcitabine, oxaliplatin, and cetuximab (GOC/GEMOXCET) was analyzed for extension of progression-free survival, presented similarly disappointing lack of significant improvement, emphasizing the need for further development or other approaches [145].

K-ras *Inhibition*

Pancreatic cancer samples display high frequency (more than 90 %) of mutations in the K-*ras* proto-oncogene [146], which results in constitutively active Ras protein. The cells carrying the mutation contain an enzyme known as farnesyl transferase. Its activity is required by the mutated cells, which are undergoing cell division. K-*ras*-mutated cells proliferate continuously. Specific drugs that inhibit farnesyl transferase to induce cell cycle arrest have been developed and being evaluated in clinical trials with cautious future perspectives [147]. In fact, some of these drugs enhanced radiosensitivity [148] but failed as effective monotherapy to prolong median survival in surgically incurable, locally advanced, or metastatic pancreatic cancer [149]. In a study comparing metastatic pancreatic cancer patients harboring the point mutation in codon 12 of the K-*ras* gene with wild-type individuals,

no significant relationship between K-*ras* mutation and response or overall survival was found after (GOC/GEMOXCET) first-line therapy [150]. Hence, neither monotherapy nor combined approaches have shown any clinical benefit for patients.

MMP Inhibition

Matrix metalloproteinases (MMPs) are key players in pancreatic tissue maintenance [151], inflammation [152], in the turnover of the desmoplastic reaction [153], and during tumor invasion and metastasis [154, 155]. Regulation of MMPs is tightly orchestrated by TGF-β1, through its ability to cross talk with various other cytokines [88]. Moreover, direct correlation between MMPs and mutated K-*ras* has been reported to promote pancreatic fibrosis, a critical step in pancreatic carcinogenesis (see above) [156].

As a consequence of the overall impact of MMPs in pancreatic cancer, novel MMP inhibitors without intrinsic cytotoxic activity have emerged. Recently, a triazine derivative was synthesized and presented as a highly potent MMP inhibitor [157]. Ukrain (or NSC-631570), a semisynthetic proprietary product containing alkaloids and Thio-TEPA, downregulated MMP-2 and MMP-9 gene expression levels in three pancreatic cancer cell lines and decreased cell invasion [158]. Furthermore, it showed no adverse effects in combination with gemcitabine on patients receiving adjuvant therapy following surgical resection for pancreatic cancer. This combined treatment was regarded as safe and seemed to extend overall survival time, although high recurrence rates were observed (80 %) [159]. However, this approach has also largely failed in the clinical setting [160].

Although multi-MMP inhibitors did not help improving survival in PDAC in clinical trials, there is still hope for more specific inhibitors. Previous broad-spectrum MMP inhibitors were generally plagued by a lack of efficacy, and the majority of drug makers have since invested in other targets. However, since the end of the previous trials, much has been learned about MMPs, notably the need for drug specificity, as some MMPs are regarded as being protective and others not. Thus, these newer inhibitors are being designed with specificity in mind [161, 162].

Stroma as a Hurdle in the Therapy of Pancreatic Cancer

Pancreatic ductal adenocarcinoma is one of the six most lethal malignancies in the world. Survival has not improved substantially in the past 30 years despite advances in conventional therapies (chemotherapy and radiotherapy) as well as targeted therapies against epithelial tumor cells [163]. These disappointing results are also surprising, as major discrepancies exist between optimistic experimental results and the clinical reality [67]. Even with highly toxic combination chemotherapy regimens, the median survival for patients with advanced pancreatic cancer is less than a year

[164]. Molecular targeted agents—as exemplified above—have shown good results against pancreatic cancer cells in vitro and in animal experiments. However, the results of several clinical trials have not shown any clinical benefit [131, 139, 140, 160, 165–167]. We have previously argued that the discrepancy between experimental results and the clinical reality might in part result from the inefficiency of our current experimental setups and animal models in recreating the tumor microenvironment and the fibrotic stroma of PDAC [73].

It is now well accepted that the fibrotic stroma of pancreatic ductal adenocarcinoma is not a passive scaffold for the malignant cells but an active player in carcinogenesis as well as in therapy resistance. Recent compelling data show that the microenvironment (activated stroma/pancreatic stellate cells in particular) plays an important role in pancreatic cancer progression, metastasis, and therapy resistance (see above) [73]. Assuming that the fibrotic stroma in PDAC forms a barrier for the delivery of therapeutic agents, targeting the tumor stroma (in combination with chemotherapy) is thought to be a promising novel treatment option. Here we would summarize various approaches and scrutinize their pros and cons of such therapies from a biologic point of view.

Elimination of PSC

Pancreatic stellate cells and the desmoplastic stroma of pancreatic cancer impact on tissue perfusion significantly. Continuous cellular proliferation (cancer as well as PSC) within the confines of a stiff extracellular matrix results in increased interstitial pressure, which compresses the capillaries, venules, and lymphatics. The obstruction of the outflow further increases the interstitial pressure perpetuating the tissue edema as arteries keep on pumping blood into the tumor where outflow is obstructed [47, 168].

The discovery of hypovascularity and impairment of tissue perfusion seen in pancreatic cancer contradicts the general assumption of tumors inducing neoangiogenesis and points to a critical role for PSCs in defining therapeutic strategies [169, 170]. We have previously shown that, although PSC are more potent than pancreatic cancer cells in terms of VEGF production, they paradoxically inhibit tumor angiogenesis and reduce tumor perfusion [69]. Stellate cells inhibit tumor-induced angiogenesis by two major mechanisms. On one hand, PSC cleave various collagen molecules (i.e., collagen XVIII) to create potent anti-angiogenic substances like endostatin [130]. On the other hand, through deposition of ECM proteins in the periacinar spaces, they mechanically compress capillaries and form a barrier for tissue perfusion [130]. The anti-angiogenic effects of the fibrotic stroma have also been shown to occur in genetically engineered mouse models [72, 171, 172]. In these models, the fibrotic stroma is not only hypovascular but many of the existing capillaries are nonfunctional [72]. It is likely that the fibrotic and already hypovascular microenvironment of pancreatic cancer is one of the reasons for failure of anti-angiogenic therapies in pancreatic cancer in the clinical setting [139].

Recent studies in a genetically engineered mouse model with poor tumor perfusion and gemcitabine resistance showed that administration of IPI-926, a drug that

depletes tumor-associated stromal tissue by inhibition of the Hedgehog signaling pathway, could transiently increase intra-tumoral vascular density and gemcitabine delivery [72]. The authors concluded that hypovascularity and inefficient drug delivery were important contributors to chemoresistance in pancreatic cancer [72]. The results of this study formed a cornerstone for the usage of anti-fibrotic therapies in pancreatic cancer, and many other groups showed similar results in various animal models. The rationale behind these studies can be summarized by the idea that chemotherapy resistance of pancreatic cancer is due to inefficient drug delivery, which is a consequence of the fibrotic stroma. Hence, when one removes this fibrotic wall, chemotherapeutic drugs can better penetrate the tumor, killing cancer cells more effectively, hence prolonging survival.

It is possible that fibrosis forms a barrier around the tumor cells, preventing effective delivery of chemotherapy in the many cases. However, this should not automatically lead to the reasoning that PSC produce the ECM to form a barrier to prevent chemotherapy penetrance. Could this really be their sole purpose in pancreatic cancer? If this argument was true, how could we explain, despite the promising results of the abovementioned study in mice, why inhibition of the sonic Hedgehog signaling in humans in a phase II clinical study (IPI-926-03 trial) increased mortality? In fact after an interim analysis, this study was stopped at the beginning of 2012 because of increased mortality in the treatment arm. As of 2012, as seen on the National Institutes of Health (NIH) web page (http://www.clinicaltrials.gov/), there are ten registered studies using various Hedgehog inhibitors in PDAC. The unexpected results of the abovementioned study will certainly increase awareness of adverse effects, but results of the clinical trials are yet to be seen [173].

We have previously argued that anti-fibrotic therapies at advanced stages could be a double-edged sword [68]. In our opinion, it is not certain whether fibrosis acts only as a barrier for chemotherapy or also as a defense against tumor spread [173]. Both in humans and in genetically engineered mouse models, the earliest PSC activation and ECM deposition take place around precursor lesions like PanIN or atypical flat lesions known to harbor genetic defects like K-*ras* mutations. It is likely that premalignant cells are kept dormant by environmental factors (here the stroma). However, when this suppressive environmental control is lost (e.g., through aging), genetically malignant, but dormant lesions may progress into invasive cancer [173].

Elimination of Extracellular Matrix

In 2012, two different research groups used another approach to loosen the compact ECM in PDAC by targeting hyaluronan—a major component in the ECM—by PEGylated human recombinant PH20 hyaluronidase (PEGPH20) [171, 172]. In the genetically engineered mouse model, the enzymatic digestion of hyaluronan led to a reduction in interstitial fluid pressures, enabling better drug delivery and thereby prolonging survival of the mice [171, 172]. Nonetheless, all mice died from their tumors, hinting that the failure of gemcitabine to provide a cure is due not only to

inefficient drug delivery but also to drug resistance. These promising results in the animal setting have already paved the way for a clinical trial using PEGPH20 (NCT01453153). Like the inhibition of Hedgehog signaling study (IPI-926-03 trial), the effect of this therapy on humans also remains to be seen.

However before pursuing this approach, we should first understand why PSC are depositing ECM around pancreatic cancer cells. In the body, stromal cells deposit ECM as an innate defensive reaction to form a barrier between what is harmful and the rest of the body [173]. Fibrosis happens in the form of callosity in hands and feet as a protection against mechanical trauma (i.e., if one wears a shoe that does not properly fit or works in the garden on a weekend). A fibrotic capsule is also found around parasitic cysts [174]. For example, pericyst is a thick fibrotic layer around hydatid liver cysts caused by *Echinococcus granulosus*, which is completely formed by the host stromal cells to prevent parasitic penetration. In tumor biology, fibrotic capsule formation is a defensive reaction coming from the stroma around the tumor, and tumors with a capsule have better prognosis than infiltrative tumors without a capsule [66, 68]. In line with these arguments, collagen deposition has a favorable impact on patient survival in PDAC [64]. All these facts point that the fibrotic stroma around cancer cells may also have a protective function. Therefore, nonselective targeting of the stroma may as well be counterproductive.

Selection of Aggressive Cancer Clones

The desmoplastic reaction observed in PDAC is characterized by a compact stroma, which is known to be hypovascular and hypoxic. In this hostile microenvironment, to overcome nutrition and oxygen deprivation, cancer cells are forced to undergo a selection process that leads to the evolution of resistant clones. Indeed, it has been shown that as hypoxia deepens, susceptible clones become eradicated, leaving behind negatively selected hypoxia-resistant ones. This negative selection occurs partially through suppression of pro-apoptotic pathways, which creates a cross-resistance to chemotherapy-induced apoptosis [175]. In 2000, Hanahan and Weinberg distilled properties of cancers into six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [23, 176]. This review was updated in 2011 and a significantly greater focus on the importance of the tumor microenvironment was added [177].

According to the prevailing model of tumor progression summarized by Hanahan and Weinberg, human tumors develop through a succession of genetic and epigenetic changes that confer increasingly malignant characteristics on cells. This multistep process has been likened to Darwinian evolution within the microcosm of living tissues, in which the units of selection are individual cells [178]. On the other hand, as proposed by Gatenby and Gillies, this malignant evolution cannot take place without interactions with the tumor microenvironment. According to this theory, carcinogenesis requires tumor populations to surmount distinct microenvironmental

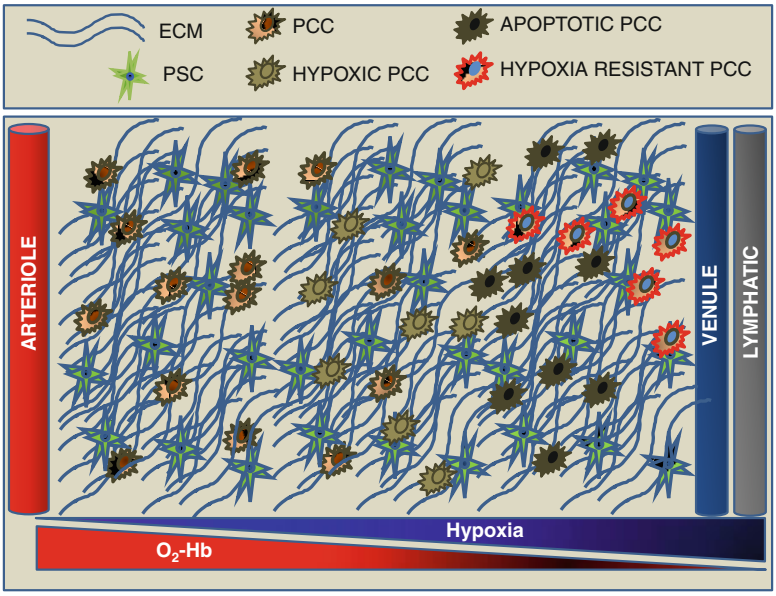

Fig. 12.3 Schematic description of the evolution of malignant cancer cells under selection pressure applied by the fibrotic and hypoxic microenvironment. In order to survive in an ever-changing habitat, tumor cells evolve and adapt to the microenvironmental proliferation barriers that arise during the process of carcinogenesis. This clonal selection eventually leads to the rise of resistant tumor populations that can survive in a hostile microenvironment. As tumors outgrow their vascular supply, malignant cells adapt to survive in a hypoxic milieu. Hypoxia and its consequences like acidosis and reactive oxygen species are not only highly selective but are also able to induce genetic instability in the tumor cells perpetuating the malignant evolution of cancer cells

proliferation barriers that arise in the adaptive landscapes of normal and premalignant populations growing from epithelial surfaces [74]. Therefore, somatic evolution of invasive cancer can be viewed as a sequence of phenotypical adaptations to these barriers [64]. These microenvironmental barriers—specifically hypoxia, acidosis, and reactive oxygen species—are not only highly selective but are also able to induce genetic instability in the epithelial cell tumor [175, 179]. As a result, malignant cancers are dynamically evolving clades of cells living in distinct microhabitats that almost certainly ensure the emergence of therapy-resistant populations [179] (Fig. 12.3).

Conclusions

Since conventional and targeted therapies aiming at cancer cells have largely failed to prolong survival in pancreatic cancer, targeting the infrastructure of the tumor, hence its stroma, is a novel strategy [173]. It is believed that fibrotic and hypovascular stroma forms a barrier around cancer cells, hindering effective delivery of chemotherapy. Theoretically, anti-fibrotic therapy should reduce the compactness of

the stroma and reduce the interstitial pressure, allowing better delivery of chemotherapy. This approach has worked successfully in a genetically engineered mouse model but failed in humans, paradoxically increasing mortality in the treatment arm. This discrepancy between experimental data and clinical reality results mostly from the inefficiency of our current experimental setups in recreating the tumor microenvironment. Despite the significant amount of in vitro data suggesting the pro-tumorigenic roles of activated stellate cells, it is likely that the initial activation of PSC around preneoplastic lesions is a defensive reaction. However, due to the robustness of cancer as a system, which can evolve in order to adapt and survive in this hostile microenvironment, the (inadequate) tumor-suppressive pressure created by the microenvironment eventually leads to the selection of aggressive cancer clones, indirectly contributing to the aggressiveness of the tumor. However, after the selection of aggressive clones has already taken place, applying anti-fibrotic therapy can be a double-edged sword [173].

Since our chemotherapeutic agents are not powerful enough to eradicate all cancer cells (i.e., tumor-promoting cells) in the tumor, breaking down the stromal wall may also lead to the increased dissemination of cancer cells. Here, an analogy can be drawn with destroying the walls of a prison to get inside after the inmates have barred the doors [173]. We are better able to get in, but probably they are also better able to get out. It is possible that fibrosis forms a barrier around the tumor cells, preventing effective delivery of chemotherapy in the many cases. However, until we have the right tools to fight cancer effectively or diagnose it in an early stage (i.e., before the selection of aggressive clones), it is not wise to apply anti-fibrotic therapy non-selectively to all, which may facilitate tumor spread.

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Part IV
The Dynamic Problem of Tumor Heterogeneity

Chapter 13
Heterogeneity of Cancers and Its Implication for Targeted Drug Delivery

Tracy A. Denison and You Han Bae

Abstract In most cases, cancer has eluded significant gains in treatment and cure despite the continuous effort to develop novel, potent therapies. The diversity between cancers is now more recognized, such as with breast cancers, which are currently stratified into distinct subtypes based on certain receptor expression profiles and optimal treatments. However, it is also becoming clear that significant cellular heterogeneity exists even within a single primary or metastatic tumor of a patient. In many ways, therapy development has lagged in trying to accommodate the challenge of intratumoral heterogeneity. This chapter reviews some of the sources of tumor heterogeneity such as the diversity of the malignant cell population, considering both the cancer stem cell (hierarchical) and stochastic progression models. Heterogeneous aspects of the tumor microenvironment are also discussed such as associated stromal cells or hypoxia. Finally, the expression distribution of some biomarker drug targets in clinical tumor specimens is also reported to exemplify the challenging reality within the clinical setting when designing drug delivery systems targeting a single marker.

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Introduction

The etiology and progression of cancer both stem from a complex array of contributing biological and physiological factors. The heterogeneity of tumors between patients has led to the realization that cancers even of the same tissue can be categorized as very different diseases with diverse drug responses and progression patterns. This awareness has prompted the clinical approach to cancer therapy in many cases to be based first on a characterization of what type of biomarkers are present, and this plays a strong role in determining the current standard treatment regime. Neoplastic cells are triggered by complex and multifaceted genetic and epigenetic drivers of malignancy, and it is now more apparent that even within the same tumor, subpopulations of cancerous and neighboring cells are contributing unique and separate roles in the progression of the disease. While diversity between cancers of the same tissue has stratified treatment applications in the clinic, currently most standard and developing therapy approaches are designed on a premise that all cells within the cancer are homogeneous and should respond equally to the treatment, despite growing evidence that multiple contributions from heterogeneous cells are involved. The heterogenic differences within the tumor of a cancer patient may be based on the progressive mutation of clonal subpopulations, altered differentiation hierarchy of cancer stem-like cells, alterations of stromal or other cells in the microenvironment of the tumor, varied conditions across the tumor such as hypoxic gradients, and ramped up metabolic activity or hormonal sensitivity which might be indicated by an expression profile of specific receptors or antigens.

While drug delivery research has continued to develop novel and clever approaches to more specifically deliver a drug to the appropriate location or to have enhanced interactions specific to malignant cells, most proposals still fail to address the rampant cellular heterogeneity found within clinical tumors [1]. Some approaches may indicate overoptimistic preliminary data when tested in relatively homogeneous experimental animal tumors, but ultimately fail in clinical phases in part for not addressing the level of cellular heterogeneity in clinical tumors (Fig. 13.1). Tumor cell heterogeneity is, admittedly, a very challenging problem, but it is also one of the major impediments to producing real breakthroughs in cancer treatment. Some of the best advances in cancer therapy are arguably found in certain cancers where the majority of malignant cells are persistently sensitive to a single treatment. Perhaps the most notable success story thus far is imatinib, which appears to induce sustained remission for a majority of chronic myeloid leukemia patients, although this drug does indicate some inherent or eventual acquired resistance in a subset of patients [2]. However, most cancers thus far have yet to exhibit any singular Achilles heel that will be easily targeted for successful and permanent remediation, which is likely due in large part to the heterogeneity of the malignant cell population. Significant advances in cancer therapy will undoubtedly have to address the challenges of cellular heterogeneity. This chapter presents some of the traits influencing heterogeneity among tumor cells and conditions across their microenvironment. Evidence of heterogeneity within clinical tumor samples of some identified cell biomarkers considered as potential cancer drug targets is also presented.

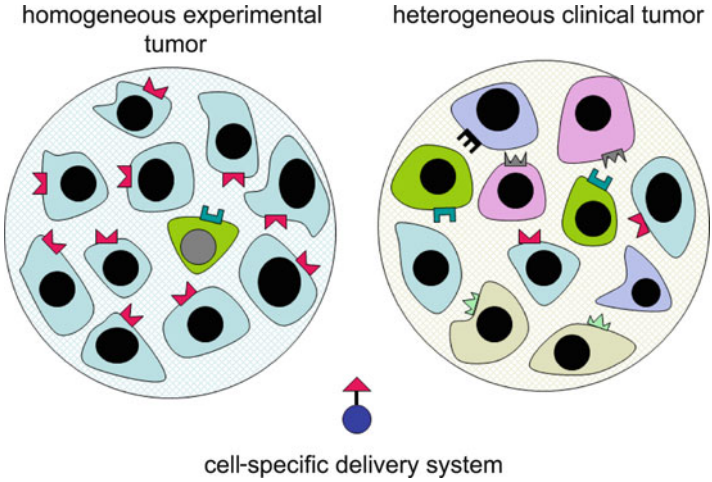

Fig. 13.1 Cartoon schematic of the relative cellular homogeneity of experimental animal tumors compared to the cellular heterogeneity of human clinical tumors. Many cell-specific drug delivery systems are designed following the assumption that essentially all tumor cells exhibit the selected target. Image adapted with permission from the graphical abstract from [1], © 2012 Elsevier

Heterogeneity of Malignant Cell Populations

Intertumoral heterogeneity between patients can be very apparent, particularly when neoplastic diseases occurring within the same tissue or organ are very different in terms of morphology, progression, and drug sensitivity. This is exemplified by the multiple clinical classifications for breast cancer. Currently, breast cancer is categorized in part by the presence of certain receptors for estrogen, progesterone, or epidermal growth factor, resulting in at least five possible subtype diagnoses: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive, Claudin-low, or basal-like breast cancer [3]. Each of these may warrant a different therapeutic regime, but it is becoming clear that further stratification may be necessary for improved treatment success [4]. Trastuzumab, an antibody drug developed for HER2 positive breast cancer, is highly effective for some patients but not for others in this group. Variations in this success may depend on correct identification of other chromosomal alterations that may exist within this cancer type [5].

Cancer cells within the same tumor are also heterogeneous in many aspects including phenotypic expression, level of drug resistance, and capacity for initiating new tumor growth. Tumor cells can engage in complex interactions between populations of cells and the signals that influence their gene expression. In this regard, a tumor is somewhat comparable to a new organ acting within the host [6]. Organs have populations of cells at organized stages of differentiation as well as stromal cells to support their structure and function. Organs can have complex spatial organizations that house niches where individual cells maintain specialized functions. Evidence now suggests

that similar complexity exists for interactions of individual tumor cells among themselves, associated stromal cells, and even with the system of the host [7–10].

However, unlike organs, tumors deviate in other ways from the integrated cooperation with the rest of the body. Clearly, tumor cells override signals that restrict unbridled cell proliferation. Tumor cells evade signals promoting apoptosis and immune signals that would flag malignant cells for removal. Yet cancer cells also at times exploit legitimate existing signaling pathways that can aid them in their survival and expansion. These may include innate differentiation and proliferation hierarchies, paracrine signaling relationships critical during embryonic development, or inflammatory signaling normally helpful in wound healing [9]. The chaotic and rogue behavior of cancer cells results in the partitioning of differing subpopulations that can be diverse in their malignancy and contribution to progression of the disease.

There are various types of evidence for cancer cell diversity within a single patient. Human ovarian cancer cells can exhibit heterogeneity in their cellular drug resistance and the expression of stem-like phenotypes according to their spatial location within the tumor [11]. Liu et al. report the use of antibody-conjugated quantum dots to simultaneously visualize the expression of four different cellular markers on fixed biopsies of human prostate tumors. The multiplexed resolution was able to discern a heterogeneous distribution of both premalignant and malignant cells located within glands that would likely appear to be benign under traditional histological evaluation [12]. Heterogeneity is also observed between metastatic growths and original tumor within the same patient. Ding et al. published an account of metastasized basal-like breast cancer from a single patient. They collected samples from the primary tumor, peripheral blood, a cerebellar metastasis, and a xenograft culture of the primary tumor and then proceeded to sequence the genomes of each sample to find any differences [13]. A small set of mutations were noted between the different samples, although in this case most mutations observed were also associated with the primary tumor. However, the tissues in the xenograft and metastasis were highly enriched for certain mutant allele frequencies, suggesting certain subpopulations were more selected in the new environments. These findings suggest that the patterns of heterogeneity within the primary tumor may not match those found in metastatic growths [13]. In another analysis of samples from a single patient, differences among separate metastatic foci of the same melanoma patient were shown to exhibit heterogeneous morphology and surface antigen expression, suggesting new metastatic regions are also heterogeneous from each other [14].

While cell heterogeneity is frequently observed in tumors, there is a debate as to the source of this variability. Evidence has suggested such heterogeneity may be a product of either hierarchical or stochastic models. Perspectives related to these theories are discussed below.

Cancer Stem Cell Theory and Heterogeneity

While it still remains controversial, increasing evidence for the cancer stem cell hypothesis has arisen in recent years [15]. This theory asserts that tumors are driven

by stem cells that give rise to proliferative and multi-differentiated progenies that are still genetically identical to their progenitors. Meanwhile, a smaller population of stem-like cancer cells is also maintained that are relatively more quiescent. These so-called tumor-initiating cells are also known as cancer stem cells. Many blood cancers and some solid tumors display the evidence of subpopulations of cells that can initiate tumor growth and give rise to cell progeny of more differentiated phenotypes [10, 16]. These side populations appear to be enriched for tumor-initiating cells, but not necessarily fully characterized as being all equivalent in their tumor-initiating capacity [17]. Cancer stem cells have been identified by surface markers as well as other means, such as by dye exclusion or ALDEFLOUR assays [18]. While cancer stem cells might result from a stem cell dysfunction, it is not certain that tumor-initiating cells must derive from normal stem cells. However, once transformed into a cancer stem cell, this model suggests that tumor-initiating cells will drive tumor progression and cell differentiation in a hierarchical manner, with most of the progeny becoming highly proliferative and ultimately differentiated and no longer possessing stem-like capacity [15].

The cancer stem cell theory would require a new paradigm for drug therapy. Most traditional drugs have been designed to disrupt the mechanisms of highly active proliferation of the bulk tumor cells, but it is thought that the less proliferative cancer stem cells are more drug resistant due in part to their more quiescent nature and also perhaps to enhanced mechanisms of drug exclusion [19, 20]. In order to prevent cancer recurrence, the cancer stem cells would have to be effectively targeted during the first regimen of treatment under this model. However, the potential positive side of this theory is that if cancer stem cells drive tumor growth and malignancy, they present a very specific target for new therapeutics, which if successful could provide more definitive cures for many cancers.

Stochastic Theory and Heterogeneity

The stochastic model of tumor progression is based on continual genetic mutation that supports the emergence of new clonal populations evolutionarily favored to thrive in the existing environmental conditions. Under this theory, tumor heterogeneity is derived from the existence and evolution of multiple clonal subpopulations that are competitively viable in the tumor environment. While it is possible that one dominant clonal population could form the bulk of the tumor for a time, it would be expected that eventually new mutations would introduce clonal variants and more heterogeneity within the tumor.

To effectively treat cancer following the stochastic model, it would be necessary to try to kill all malignant transformed clonal populations. Furthermore, this model supports the evolutionary concept that the introduction of a therapy (or any change of condition) could select for new dominant populations. Thus, it would be necessary to devise a treatment regime capable of adapting to altered population growth rates or to try to exploit predictable vulnerabilities caused by this phenomenon.

This is in significant contrast to the cancer stem cell theory where it is really only crucial to target the cancer stem cells with drugs.

Modified Alternatives Reconciling Both Theories

This chapter does not aim to resolve the debate between the hierarchical and stochastic models of tumor progression, but merely reflects on how the tumor heterogeneity arising from either model might need to be considered for effective drug therapy. It is very possible that evidence for both theories will continue to accumulate. However, both hypotheses suggest that significant heterogeneity of cells exist within a tumor and that a single therapeutic approach may not likely be effective in killing all cancer cells. It is also possible that these seemingly conflicting theories may not be entirely mutually exclusive [15, 21]. With the countless occurrences that might combine to result in neoplasia, it seems plausible that some cancers may develop by overriding natural stem cell differentiation hierarchies, whereas others may develop by self-reinforcing and runaway mutagenesis that allows for stochastic evolution and competition among the most malignant clonal populations. Others have proposed that perhaps the evolution of cancer itself may also allow for a transition between the hierarchical and stochastic patterns. For example, Tian et al. have suggested that as the epigenetic landscape of cancer cells becomes severely destabilized, they may lose their connection to any hierarchical patterns that were previously driving their behavior [22]. If cancer can ascribe to entirely different fundamental driving mechanisms, or perhaps even worse, migrate between them, then development of therapies to tackle the heterogeneity achievable within these scenarios will be even more crucial.

Other reports propose the concept of phenotypic equilibrium occurring in populations of cancer cells between stem and non-stem cancer cells. This hypothesis may also offer an alternative explanation for the confusing array of cancer cell behavioral data. Gupta et al. report data suggesting that breast cancer stem cells, luminal cells, and basal cells (defined by antigen expression profiles) can stochastically transition between these states following a Markov model [23]. This model asserts that cells expressing a certain phenotype will exhibit distinct probabilities of either remaining in that state or transitioning to another state. Not all transition probabilities are equal, but with time and a stable environment, this model predicts a steady equilibrium ratio of each phenotype existing within the population. Moreover, these findings suggest that it is possible for non-stem cells to convert to stem cells, although this phenomenon occurs at a lower frequency. Iliopoulos et al. also report a similar finding that non-stem cells can convert back to cancer stem cells in response to the secreted signal interleukin-6 [24]. These findings were observed in breast and prostate cancer cells, and it is not yet clear if this phenomenon will be observed in most cancers generally.

The equilibrium concept poses other challenges for heterogeneous drug target ing. In this case it would be important to target all cells but, in particular, aim to prevent the conversion of non-stem cells to stem cells since the stem cell population seems to promote tumor recurrence. Gupta et al. also reported that drug sensitivity of the various phenotypes in a population may also follow a Markov model, and thus, this model may also be informative in developing new drug strategies against the heterogeneously shifting population [23].

Heterogeneity of the Tumor Microenvironment

It is becoming clear that transformed cancer cells interact with aspects of their environment in ways that impact the progression of the disease. Evidence is accumulating that some of these other parameters affect the cancer cells in significant and important ways. The effect of neighboring stromal cells and hypoxic conditions within the tumor on cancer cells is discussed in this section.

Stromal Cell Contributions to Tumor Progression

Tumors consist of non-transformed stromal cells, such as fibroblasts, endothelial cells, and immune cells, that are now being shown to have important interactions with cancerous epithelial cells [8, 9]. Fibroblasts associated with tumor cells, sometimes called carcinoma-associated fibroblasts (CAFs), develop a distinct phenotype compared to normal fibroblasts and can maintain this phenotype for several passage doublings even when removed from the presence of carcinoma cells [25]. Unique aspects of this phenotype include an increase in the myofibroblastic marker, alpha-smooth muscle actin (α-SMA) and the capacity to contract collagen gels. These fibroblasts influence the growth of tumor cells by reciprocal paracrine signaling involving stromal cell-derived factor-1 (SDF-1) and transforming growth factor beta 1 (TGF-β1) [26]. Co-implantation xenograft studies of cancer cells with CAFs showed that tumor volume increased more quickly with the CAFs than would occur without them [25]. Tlsty and Coussens showed that tumorigenesis could even occur when non-transformed epithelial cells were coinjected with CAFs [6]. This suggests that CAFs can acquire an independent and persistent phenotype that contributes to tumor progression.

As CAFs clearly contribute to tumor progression, they may offer some targets for tumor therapy [27]. One target would be to inhibit receptors or signaling molecules of the many soluble signals between fibroblasts and epithelial cells [8]. Another work is uncovering some specific cell markers associated with tumor stroma. One example is fibroblast activation protein (FAP), a protease within the dipeptidyl peptidase IV gene family. This protein is not expressed significantly in normal tissue,

but is upregulated in some tissues during tissue remodeling, wound healing, and inflammation. In cancers, it has been observed in the fibroblasts of epithelial tumors and in sarcomas. The proteolytic capacity of this membrane protein could potentially contribute to matrix remodeling, possibly suggesting a role in angiogenesis and metastasis [28]. FAP expression may also help the tumor to evade an antitumor immune response. Some have considered the use of monoclonal antibodies against FAP or the use of inhibitors to FAP protease activity as an approach to tumor therapy [29].

Hypoxia Within the Tumor Microenvironment

The heterogeneity across spatial regions of a tumor can also have a strong influence on the biology of individual tumor cells. Some of these local differences result from the proximity of a cell to gradient concentrations of paracrine factors released from other tumor cells or from stromal cells. It is also known that the extracellular matrix (ECM) can significantly influence cell behavior, and cancers are known to misregulate factors controlling the remodeling of their matrix or integrin receptors which are influenced by the ECM ligands [30]. The organization of the ECM is likely to vary throughout a large, aggressive tumor [31]. Other variations in local environment are caused by accessibility to the content delivered by perfusion of plasma and blood cells within the circulatory system, including oxygen and endocrine signals [9].

As tumor growth is often rapid and unorganized, cells at some locations within a solid tumor will experience hypoxia because uncoordinated capillary formation will leave interior cells severely isolated and lacking access to adequate oxygen. Hypoxia is an important but complex factor affecting cancer cells, and clinical testing shows that up to 50–60 % of locally advanced solid tumors have heterogeneously distributed regions of significant hypoxia [32]. While it would seem intuitive that limited oxygen would be helpful to slow down the growth of cancer cells, it turns out that hypoxia can influence the metastatic and stem-like properties of cells [33, 34]. This may explain the unfortunate finding that hypoxic tumors tend to correlate with a worse prognosis in some cancers [32].

Hypoxia has been shown to correlate with resistance to both radiation and chemotherapy [35], and a number of reasons may support the role in drug resistance. First, hypoxia usually correlates with poor vascularization and diffusion limitations, and thus any drug delivered through the blood stream will also encounter transport limitations to hypoxic regions. Furthermore, cells reduce proliferation rates in low oxygen and thus may evade therapies that target mitotic mechanisms. Also, it appears that cells may also revert towards or maintain any stem cell-like properties during hypoxia, perhaps invoking the attributes of cancer stem cells [34]. Finally, hypoxia appears to promote the evolution of cancer cells with capacity to metastasize as well as withstand nutritive deprivation, both of which might also help facilitate the mechanisms of drug resistance or evasion [33].

Heterogeneity of Biomarker Expression

A number of biomarkers have been discovered to be highly expressed on cancer cells compared to normal cells. It is hoped these biomarkers could be potential drug targets that would enable the specific killing of cancer cells while sparing normal cells throughout the body and reducing the harmful side effects of many chemotherapeutic agents. These markers are attractive target candidates because they are either uniquely or highly overexpressed in some cancers, but are rarely expressed in most normal cells at least in spatial locations easily accessible to drugs passing through the systemic blood flow [36, 37]. Most of these markers participate in some ways to basic metabolic functions and would corroborate with the likelihood that malignant cells have drastically increased their basal metabolic consumption and proliferation rates. These markers can serve as antigens for monoclonal antibody drugs or are natural receptors that might be manipulated to internalize a drug-conjugated ligand into the cell. The following sections report on some of the markers that have received considerable attention as potential cancer targets for their unique overexpression on malignant cells and in some cases for their cellular transport abilities. Clinical evidence suggesting the prevalence and prognostic significance of these markers in cancer patients is discussed.

Transferrin Receptor

Transferrin (Tf) is a glycoprotein that is responsible for iron transport from plasma to cells in virtually every tissue in the body. It is capable of binding two ferric (Fe^{3+}) ions, referenced as holo-Tf in this iron-bound state [36]. Holo-Tf binds to the transmembrane transferrin receptor 1 (TfR), also known as CD71, at the cellular surface. Iron is important for certain standard metabolic reactions found in all cells, and evidence has supported the notion that highly proliferative cells, including cancer cells, express higher levels of TfR [38]. The TfR is a homodimeric receptor that when bound to holo-transferrin is transported together with the transferrin via clathrin-coated pits into the cell. This complex is located into endosomal compartments, where a significant drop in pH to 5.5 results in the release of the iron ions, after which the Tf–TfR complex is relocated back to the cellular membrane and the iron-free transferrin (apo-Tf) is easily dissociated from the receptor at extracellular pH levels to be available for further iron sequestration. The transferrin receptor is an attractive target because it has been shown to be overexpressed in a variety of cancers and has been considered as a target for solid tumors [38], blood cancers [39], and even a possible delivery vehicle for bound toxins through the blood–brain barrier [40]. The following sections review some of the reported clinical data regarding the overall level and heterogeneity of TfR expression in solid tumor tissue.

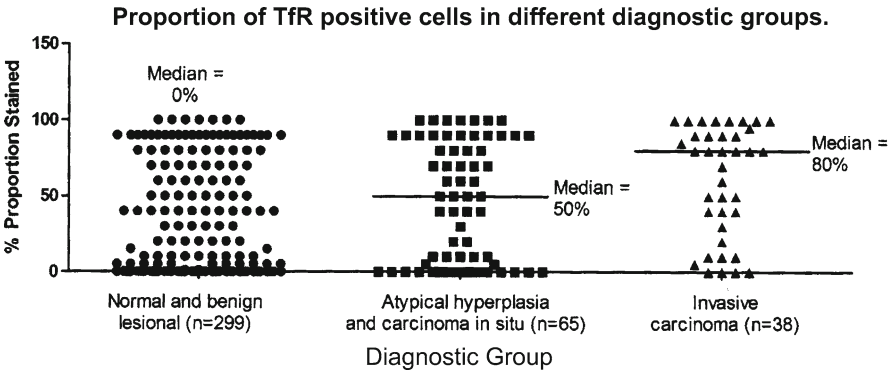

Fig. 13.2 Proportion of transferrin receptor-positive cells in different diagnostic groups. Image reprinted with permission from [41], © 2011 Wolters Kluwer Health

Breast Cancer

Transferrin receptor has been shown to be overexpressed in breast cancer. Singh and colleagues present a thorough immunohistological analysis of TfR staining in stratified grades of human breast tissue within three diagnostic groups: (1) normal and benign lesional, (2) atypical hyperplasia and carcinoma in situ, and (3) invasive carcinoma [41]. They report that the median percent proportion staining for each of these groups was 0 %, 50 %, and 80 %, respectively (Fig. 13.2). The intensity of positive staining was evaluated independently by two different expert pathologists based on the following scoring system: 0 (negative), 1 (mild), 2 (moderate), and 3 (strong). The percentage of samples with high staining intensity scores increased for more severe diagnostic groups compared to normal or benign tissue. Collectively these results suggest that TfR overexpression correlates with a worse prognosis but that rarely do all cells in the tumor stain completely positive for TfR expression. Habashy et al. also report that TfR overexpression is correlated with a number of prognostic factors in clinical breast cancer specimens suggesting a possible connection with tumor progression and aggressiveness [42]. These factors include larger tumor size, higher histological grade, poorer Nottingham prognostic index, distant metastases, and higher mitotic counts. It was correlated also with other markers associated with aggressive tumor phenotypes such as EGFR, HER2, and p53 but was inversely correlated with the expression of hormonal receptors for estrogen, progesterone, and androgen. However, despite being inversely correlated with hormonal status, it seems that the subset of hormone receptor-positive breast cancer patients that also expressed TfR were more inclined to develop drug resistance and have an overall worse prognosis. Perhaps the expression of TfR could serve as a secondary marker to stratify the prognosis of hormone receptor-positive patients and potentially offer an alternative target when traditional treatments are not working.

Pancreatic Cancer

Ryschich and colleagues report that the TfR is highly expressed in human pancreatic cancer [43]. Positive staining (considered as over 80 % of cells staining positive) was indicated in 82 % (32/39) of samples, and 11 % (4/39) of samples displayed heterogeneous expression (defined as 25–80 % cells stained). They found that normal pancreatic tissue expressed no staining ($n=8$). They also reported that 75 % (9/12) of metastatic growths from pancreatic cancer also stained positive for TfR. This suggests that TfR expression in pancreatic cancer is common and consistent between primary and metastatic tumor. The small portion of cancer samples indicating negative or heterogeneous staining suggest perhaps that TfR expression is highly correlated with cancer progression in this disease and that expression upregulates quickly in this process rather than being a drawn out progression which would seem more likely for other cancers with more occurrences of moderate expression. However, a larger cohort would confirm this more convincingly, and not as much was reported regarding the prognostic indications of TfR expression for these patients either, leaving partly unaddressed some of the rationale for targeting TfR in this cancer.

Brain Cancer

Recht et el. first report in 1990 that TfR is highly expressed in glioblastoma multiforme (GBM) brain tumor [44], which is the most lethal form of brain cancer. Staining intensity was broken into categories of (1) less than 25 % of cells, (2), 25–75 % of cells, or (3) over 75 % of cells staining positive for TfR. Staining pattern was also noted as diffuse or focal. TfR staining in normal brain tissue was observed primarily in endothelial cells or in some rare glial cells. For 90 % (9/10) GBM human samples, the staining intensity was over 75 % and the pattern was observed as diffuse. While the GBM sample was the largest and most consistent, this group reports that all brain tumor sections displayed more cells positive for TfR immunoreactivity than any of the normal brain tissue sections.

Interestingly, Calzolari et al. report that the less studied transferrin receptor 2 (TfR2) is also highly expressed in GBM [45]. This receptor is expressed in few tissues normally, but was shown to be only expressed by malignant cells in brain tumor. For GBM, 40 % (14/35) of samples expressed a score of 0 (no staining) or 1+ (very weak staining), 28.6 % (10/35) samples received a 2+ score (moderate staining visible at 100–200× field), and 31.4 % (11/35) of samples were classified as 3+, meaning their TfR2 staining was very intense and easily detectable at 20× field such that some cellular details were obscured by the staining. These results suggest that a fairly continuous spectrum of TfR2 expression may be found across a swath of GBM patients. Calzolari et al. also present evidence that TfR2 may be expressed in tumor cells with tumor-initiating capacity (stem-like compartment) and that TfR2 expression indicates a better prognosis for the patient, perhaps explained by higher sensitivity of TfR2-positive cells to the drug temozolomide.

While it is not fully clear how transport mechanisms may differ in the brain for TfR compared to TfR2 for transferrin drug conjugates, it may be worth exploring this related receptor as well to fully consider any and all options for delivering drugs across the blood–brain barrier to treat tumors.

Folate Receptor

Folate (also called folic acid or vitamin B_9) is a necessary vitamin nutrient that is important to all cells for the role it plays in DNA synthesis, repair, and methylation. For most cell types the majority of transport into the cell is handled by proton-coupled transporter or a reduced folate carrier [36]. However, folate receptor-α (FRα) is a glycosylphosphatidyl-anchored glycoprotein receptor that also can transport folate into cells and is considered the most commonly expressed folate receptor (FR) in adult tissues. This receptor is not transmembranous, but instead it is bound to the outer lipid layer of the cell membrane and is thought to commonly locate to lipid rafts [46]. It is weakly expressed in most tissues but has indicated abundant overexpression in numerous cancers. It is especially characterized as being highly expressed (possibly up to 90 %) in a number of gynecological cancers [47]. Evidence also shows that it may be overexpressed in other cancers including breast [48], head and neck [49], lung [50], and colon [51].

Folate has been an attractive candidate for conjugated drug targeting because of evidence that even large molecules bound to folate are carried into the cell via endocytosis together with the folate receptor [52]. This robust transport compatibility of folate conjugates seems to be unique to FRα and not the other more common transport mechanisms, making it a potential specific carrier targeting only cancer cells overexpressing this receptor. While there is some possibility that subsets of epithelial cells normally expressing folate receptor (i.e., kidney, lung) could take up folate-conjugated drugs, in these cases cells are usually only found expressing this receptor on the apical side and thus are less likely to sequester a folate-conjugated drug that was delivered systemically. Furthermore, some reports indicate that FR expression on malignant cells is 10- to 100-fold higher than the normal levels expressed by kidney or lung epithelial cells [53]. The receptor and conjugated folate complex is internalized into the cell via clathrin-independent carriers. Besides the conjugation of toxins or other molecules to folate for drug delivery, the other approach to drug targeting FRα is the use of monoclonal antibodies against this receptor [54].

Parker and colleagues developed a quantitative radioligand binding assay technique to quantitatively compare active FR levels in normal and cancerous human tissues [53]. This protocol isolated soluble cellular membranes with microfiltration based on molecular weight cutoff and then acid-stripped folate receptors of any bound folates to not confound their measurement following an incubation with [^{3}H]-folic acid. A broad array of tissue types were analyzed, although in many cases the sample numbers were fairly limited. They reported certain ovarian tumors to express high levels of FR, indicating 100 % high expression in each of the serous

carcinoma ($n=7$), endometrioid carcinoma ($n=4$), and metastatic tissue groups ($n=4$). Normal ovarian tissue expressed low or negligible levels of FR ($n=12$). The radioligand binding assay offers specificity for active FR and enhanced sensitivity by using radioisotopes. However, it does not offer much information regarding the distribution of FR expression through a population of cells in a tissue section or any information regarding subcellular distribution. Similar limitations are found in using quantitative reverse transcriptase polymerase chain reaction or immunoblotting. While immunohistochemistry (IHC) has other limitations, it does offer more visual information regarding the heterogeneity of FR expression across a tumor section or even regarding its subcellular localization. The following sections report the findings of clinical sample analysis using IHC to analyze FR expression in various types of cancer, as well as any reported correlation of FR to tumor staging or patient prognosis.

Colorectal Cancer

Shia and colleagues present the evaluation of FRα staining of colon tumor tissue samples. Tissue samples evaluated included normal, adenoma, primary cancer, and metastatic growths in the lung and liver [51]. Patient samples were overall scored as 0, 1+, or 2+ based on the systematic evaluation of three core sections each scored as negative, weak, moderate, or strong staining (Fig. 13.3). Normal ($n=152$) and adenoma ($n=42$) samples each indicated 93 % of the samples were negative for any staining of FRα. Primary cancer samples ($n=177$) were significantly higher for FRα staining but still suggested that a large portion of these patients (67 %) exhibited no FRα staining at all and only 9 % scored the highest rating of a 2+, suggesting that most FRα-positive patients have a heterogeneous expression. The metastatic samples were lower in number ($n=26$ for both lung and liver) but also were significantly more stained for FRα than normal tissue. However, lung samples had 62 % score a 1+ and 77 % of the liver samples scored a 0, suggesting perhaps that for most metastatic colorectal tumor growths, FRα is not highly expressed in the majority of cells.

This study also reports a high correlation between FRα expression and negative prognostic factors with poor overall survival. This may suggest that the cells expressing FRα are more destructive than those that are not and perhaps targeting these cells is sufficient. However, this is not proven yet with certainty. Perhaps the cells with no or moderate FRα expression are also contributing in some role to the tumor progression as well and will need to be considered for drug targeting with other methods, assuming FRα targeting is an effective option to kill cells expressing this receptor.

Shia's group followed up this study with another report in 2011 that focused on the expression of FRα in resected colorectal metastatic growths in the liver [55]. This study found that roughly the same ratios of liver metastases expressed FRα as were found in their previous studies for primary colorectal tumors. In this case, 25 % (37/150) expressed FRα staining with 10 % being of strong intensity. However, 75 % (113/150) were negative for any expression. This study could not verify that

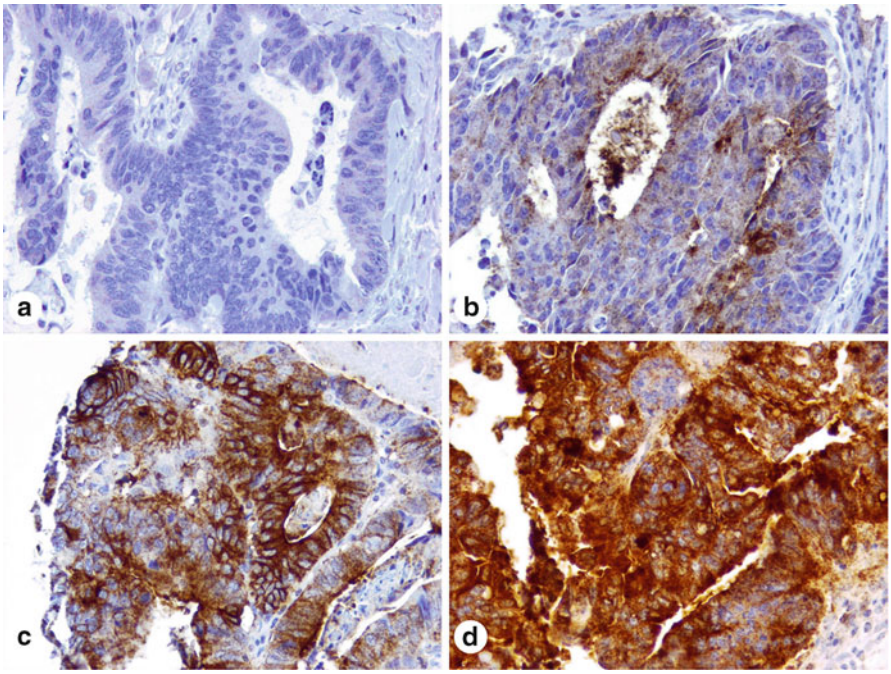

Fig. 13.3 Examples of negative (**a**), weak (**b**), moderate (**c**), and strong (**d**) immunohistochemical staining for FRα in colorectal adenocarcinoma. Image used with permission from [51], © 2008 Elsevier

FRα expression remains consistent between the primary tumor and liver metastases; however, the similar ratios suggest this may be a possibility. The significance of FRα remains unclear as it did not correlate with a number of other clinical or pathological characteristics which indicated a short survival prognosis for the patient. However, FRα expression itself was significantly correlated to being higher in patients with survival times less than 2 years compared to patients with a greater than 10-year survival. Further work would be needed to clarify if FRα-positive colorectal tumors or their metastases warrant a treatment regime targeted to this receptor, but it appears likely that neoplastic growths expressing this marker typically do so with significant heterogeneity.

Head and Neck Cancer

Saba and colleagues examined the expression of the folate receptor in squamous cell carcinoma of the head and neck (SCCHN) [49]. Samples were scored as 0 (negative for any staining), 1+ (under 25 % of cells staining positive), and 2+ (more than 25 % cell staining). Primary tumors were analyzed and when possible, lymph node metastatic samples from the same patient were also analyzed for FR

expression. Expression levels of 1+ or higher were found in 45 % (43/95) of primary tumors and 40 % (19/48) of lymph node metastases. A score of 2+ was found in 25 % (24/95) of primary tumors and 23 % (11/48) of lymph node metastases. Folate receptor expression strongly correlated with a shorter disease-free survival time in general, but the prognosis for overall survival was only significantly lower in patients with FR-positive metastases. This evidence suggests that FR expression correlates with progression of the disease and a worse prognosis at least after the cancer has metastasized. However, it still remains to be elucidated whether FR expression is found almost exclusively on malignant cells and all malignant cells express sufficient folate receptor for targeting. The highest scoring of 2+ in this study is categorized as low as only 25 % cell staining suggesting that certainly the majority of SCCHN cells are highly heterogeneous for actual levels of FR expression.

Li et al. report that FRα was commonly expressed to some level on 62 of 72 patients with poorly differentiated nasopharyngeal carcinoma [56]. No staining for FRα was found on 10 samples of normal nasopharyngeal epithelium from patients who had undergone septoplasty. The samples were scored as 0, 1, 2, or 3 by even quartiles of percent cell staining (0–25 %, 26–50 %, etc.). A significant correlation between FRα expressing scoring and clinical staging of the cancer was found, with higher FRα expression found in later stage cancer. This study also showed that FRα expression correlated with resistance to the drug Taxol for many nasopharyngeal carcinoma cell lines. Further clarification of how biomarker status indicates drug resistance would also be important to more fully characterize when assessing proper treatment of heterogeneous tumors.

Lung Cancer

Folate receptor expression is a normally occurring phenomenon in lung epithelial tissue, thought to help invoke an antibacterial function by keeping folates away from bacteria in the lung [50]. Conversely to the status in other cancers, high FR expression in some lung cancers may indicate a better prognosis. Iwakiri et al. use real-time polymerase chain reaction (PCR) to show that FR transcript levels are reduced in poorly differentiated, advanced p-stage non-small-cell lung cancers which have a worse overall survival and disease-free survival [57]. Surgically removed lung adenocarcinoma tumors with high FR expression also have a more favorable prognosis [50]. The lung may be an exceptional and more complicated tissue to use FR status for directing therapy and prognosis. For squamous cell carcinoma, thought to arise from more central lung cells normally not expressing FR, the lack of FR expression might indicate the cell of origin for this cancer type, although a smaller subset of FR-positive squamous cell carcinomas might derive from a somewhat different cell source. High FR expression is usually, but not always, found on lung adenocarcinoma tumors, which are thought to arise from cells normally expressing FR. Thus, for this lung cancer subtype, FR expression may be a natural occurrence and not correlated to disease progression. The evidence

that FR expression suggests a better prognosis suggests that FR status is indicative of a milder transformation of the neoplastic cells that is not as dangerous in this type of cancer. The fact that FR expression is highly correlated with adenocarcinoma suggests FR status may also be used as a classification and prognostic factor as well as a potential drug target. The study by O'Shanessy et al. indicates that molecular profiling together with careful histological classification may be crucial to understand what drug regime is to be successful [50]. For example, the monoclonal antibody drug bevacizumab improved survival in non-squamous histology (together with standard chemotherapy), but was accompanied with excessive risks in squamous cell carcinoma patients [58, 59]. Thus, tumor heterogeneity is not only important to account for in drug treatment, but careful identification of the cancer origin may suggest that the same target may not be as effective for differently evolved cancers.

Gynecological Cancers

Kalli et al. report the characterization of FRα expression assessed by immunohistochemistry [60]. Scoring was based on assessment of any positive staining for FRα but classification percentages based on quartiles of cells stained were also reported. Overall, 72 % of patients (134/186) indicated some positive staining for FRα. The majority (59.7 %) of these FRα-positive patients were considered to have high staining (>75 % of cells staining positive), and the next largest group (26.9 %) had staining on 51–75 % of the cells. This suggests that for ovarian cancer FRα-positive tumors, a strong majority of the cells will likely express this receptor, although some cells will undoubtedly not express significant amounts. This study analyzed various forms of ovarian cancer, with at least some FRα-positive samples in each type, but overwhelmingly the serous tumor morphology was nearly always positive for FRα staining in 81.7 % of the samples, being statistically more likely than all other morphologies combined to be FRα positive. Chen et al. also report that serous ovarian cancer patients tend to be high in FRα expression [61]. The study by Kalli et al. also analyzed the FRα expression in recurrent tumors and synchronous metastatic tumors for a smaller subset of patients with sufficient data. They found generally that recurrent tumors and metastatic tumors exhibited the same FRα status as the primary tumor. This suggests that a successful FRα therapy may work for metastatic tumors as well as the primary tumor. It also shows that chemotherapy does not appear to likely alter the FRα status of recurrent tumors.

Kalli et al. evaluated the role of FRα status on time to recurrence and found no direct correlation when controlling for other variables, even when only considering the more highly expressing samples (>50 % cell staining). The same pattern was true when considering overall survival. However, the results from Chen et al. are in disagreement with these results because they found that serous ovarian cancer patients with FRα overexpression strongly correlated with chemoresistance (37/37) and had significantly worse disease-free interval and overall survival times. Further work with larger cohorts of patients who are well stratified by cancer type and careful observation of additional factors will help to clarify the real role in FRα as a prognostic indicator for ovarian and other gynecological cancers.

Dainty et al. report that FOLR1 gene (for FRα protein) is upregulated in uterine serous carcinoma (tested by microarray) and that other genes can be proven to correlate with FR expression such as mesothelin and cyclooxygenase [62]. Conversely, caveolin expression has been found to be inversely correlated with FR [63], a cell membrane associated protein whose expression has shown evidence of preventing malignant transformation [64]. As cancer is often hypothesized to develop over the course of multiple hits to the genome via mutation or other causes, it seems plausible that effective therapy may also require consideration of multiple targets to achieve sufficient inhibition of cancer cells [65]. Tumor heterogeneity may require further advances in combined therapy to account for these differences. An array of accompanying targets may need to be characterized for each tumor, and perhaps treatments to reinforce expression of signals bolstering protection (such as caveolin in some cases is reported to do) might also be considered.

Human Epidermal Growth Factor Receptor 2

Human epidermal growth factor receptor 2 (HER2) is a transmembrane receptor that may be overexpressed in approximately 15–30 % of breast cancers to varying degrees [66, 67]. This type of breast cancer is generally aggressive and usually distinct from those types known to overexpress the hormone receptors for estrogen or progesterone. HER2 has evolved from a marker used simply for diagnostic assessment of the presence of cancer to become the target of the monoclonal antibody drug trastuzumab [68]. The effectiveness of trastuzumab for treating HER2-positive breast cancers has made the need for uniform and clarified evaluation of HER2 status paramount for proper diagnosis and treatment in the clinic. Like most cellular markers, HER2 expression occurs with varying levels of heterogeneity, and accounting for this will be important in understanding how to deploy the correct treatment.

The College of American Pathologists (CAP) has issued guidelines for evaluation of HER2 status of invasive breast carcinomas in 2007 [67]. These guidelines indicate how tumor tissue IHC staining results should be stratified to provide a score of 0 or 1+ (negative), 2 (equivocal), or 3+ (positive). The highest score of 3+ indicates that at least 30 % of cells express a uniform and intense staining. Equivocal staining is classified as a score of 2+ and is characterized by circumferential staining in over 10 % of cells but the staining ring is thin. A score of 1+ is for cases where more than 10 % of cells exhibit faint membrane staining but cellular staining is circumferentially incomplete. A score of 0 indicates no staining for HER2 is observable. These classifications have also been explained by others, including Hicks and Kulkarni [68], and an example of their IHC scoring by these guidelines is portrayed in Fig. 13.4.

Fluorescence in situ hybridization (FISH) is another approved method clinically for evaluating the status of HER2. Rather than staining protein by immunohistochemical methods, this approach uses fluorescently labeled nucleic acid probes

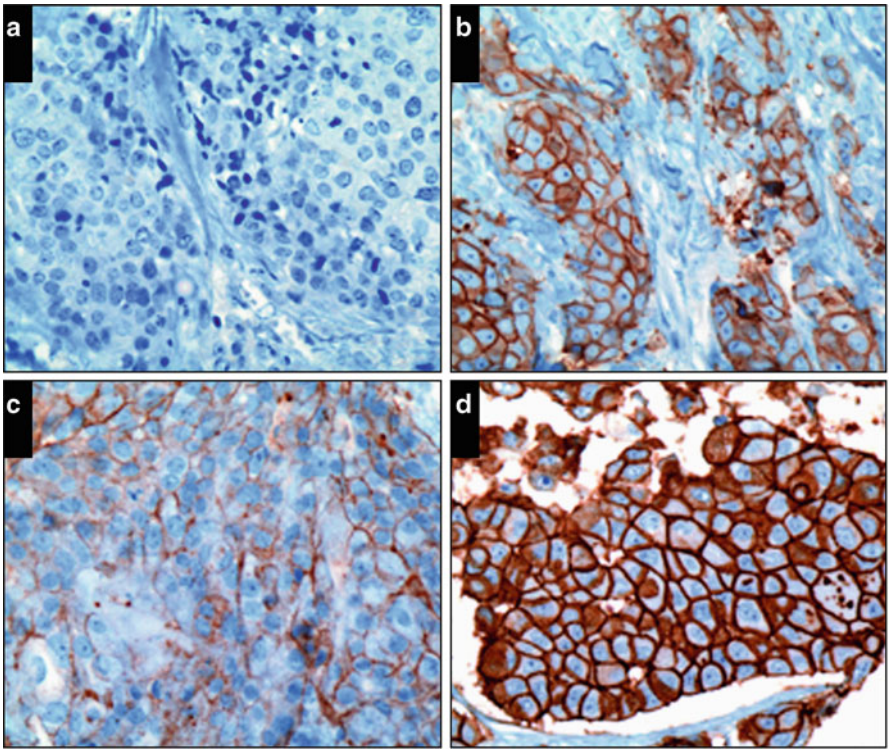

Fig. 13.4 Immunohistochemical (IHC) assessment of the level of HER2 protein expression at the tumor cell membrane using the US Food and Drug Administration-approved Dako HercepTest kit according to the manufacturer's instructions. (**a**) Cases with no membrane staining are scored as IHC 0. (**b**) Cases scored as IHC 2+ demonstrate circumferential membrane staining in more than 10 % of tumor cells, but the staining ring is thin. (**c**) Cases scored as IHC 1+ demonstrate partial weak membrane staining in more than 10 % of tumor cells with no complete circumferential staining. (**d**) Cases scored as IHC 3+ demonstrate circumferential staining in more than 10 % of tumor cells, but the staining ring is thick and has retractile quality (**a**–**d**, 400×). Image used with permission from [68], © 2008 American Society for Clinical Pathology and © 2008 American Journal for Clinical Pathology

which bind to and thus enumerate the average number of HER2 gene copies within the nuclei of tumor cells. It is believed that HER2 overexpression generally correlates with chromosomal instability causing multiple copies of the gene to be replicated within the chromosome 17 where the gene is found. FISH staining utilizes the centromeric enumeration probe to chromosome 17 (CEP17) to provide information regarding the number of chromosome 17 copies present. Normal cells typically have two of each chromosome (diploid), while some cancer cells may have abnormal chromosomal numbers (aneuploid). The FISH method also uses the HER2 probe, which binds directly to the gene on chromosome 17. When both probes are visualized together, cells can be imaged and assessed for a HER2/CEP17 ratio which

indicates if the number of HER2 genes has been multiplied on the chromosome. If the ratio is greater than 2.2, then it is also considered as HER2 positive [67]. The CAP guidelines suggest that IHC staining is sufficiently reliable when samples receive a score of negative (0 or 1+) or as positive (3+). However, samples deemed as equivocal (2+) are recommended to be tested also by FISH to better confirm the HER2 status for determining the best treatment for the patient.

Burrell et al. report on the role that chromosomal instability may play in HER2 expression and its impact on drug resistance [69]. Chromosomal instability is a major contributor of tumor cell heterogeneity, resulting in subpopulations with various levels of copy number of the HER2 gene. HER2 signaling is known to drive chromosomal instability, and treatment with trastuzumab is observed to reduce this instability in tumor cells, likely through its influence on genes involved in the mitotic apparatus and perhaps the regulation of cyclin-dependent kinase activity [69]. Anthracycline drugs have been shown to improve response when combined with trastuzumab [70]. Further clinical studies suggest that associated multiplicity of HER2 in chromosome 17 correlates with improved response to combined anthracycline therapy [71]. Also, some preliminary results suggest that platinum-based chemotherapy agents might also selectively effect cancer cells with chromosomal instability [72, 73], which may be an advantageous alternative as platinum agents are associated with less cardiotoxic effects than anthracyclines. While anthracyclines and platinum agents appear to be more effective on cells with chromosomal instability, cells with this feature do indicate a greater resistance to taxane drugs [73]. When taxanes are combined with trastuzumab, there is an additive clinical benefit, which suggests that perhaps these two drugs are distinctively targeting separate cell populations of low or high chromosomal instability [69]. Overall, these findings taken together suggest that careful combination therapies may be important for treating HER2-positive tumors when substantial heterogeneity of expression is resulting from chromosomal instability.

While it is apparent that evaluating the intratumoral heterogeneity of HER2 status within a tumor can be both challenging and critical for determining the optimal therapy, there is some evidence that intertumoral heterogeneity of HER2 expression may need to also be considered. Arslan et al. reviewed the literature of clinical data pertaining to the consistency of HER2 expression between primary and metastatic tumors [74]. Reports have been varied as to the level of discordance between these types of lesions, probably in part due to variation in the number of patients assessed in each study as well as variation in the sampling, processing, and evaluation of samples resulting from the different personnel involved. Most studies evaluated using immunohistochemistry or sometimes FISH in combination or separately. Reported studies propose discordance may be as high as 33.2 %, although many reported levels are lower. Both possibilities of positive primary tumor with negative metastasis as well as negative primary with positive metastasis have been reported. The cause for the subset of patients with discordant tumors is often ascribed to errors in the acquisition or processing of samples, which can be a significant problem. However, there does seem to be evidence, even for samples twice confirmed for HER2 status with both IHC and FISH, that some small but possibly significant

portion of breast cancers do present with differing HER2 status between primary and metastatic lesions [75]. The underlying biology of such a phenomenon is unclear at this time. Any level of discordance between the primary and metastatic tumors would be important for proper treatment prescription. While appropriate treatment regimes will have to be investigated further for discordant tumors, it seems likely that chemotherapies targeting the metastatic growths might be more critical than targeting the primary tumor when it is difficult to target both equally.

Potts et al. present a modified method to evaluate HER2 status using IHC that may be more informative regarding tumor heterogeneity than currently recommended scoring methods [76]. The need to understand heterogeneity in a clinical sample can be paramount. In particular, tumor status is often evaluated from core needle biopsy that in some cases the relatively small sample of tumor collected may happen to poorly represent the majority of the tumor. This problem is much more likely in a tumor with substantial intratumoral heterogeneity. For example, it has been reported that adjacent sections within a tumor can be so drastically different as to be scored as 3+ positive or as negative when viewed in isolation [77]. This level of proximate contrast may be rare in most patients; nevertheless, it lends credence to the importance of accounting for tumor heterogeneity when attempting to determine the most effective therapy for the individual. The proposal by Potts et al. suggests that scoring be done at various "nests" of cells within the tumor to get a score of cell-level heterogeneity (within the local nest) and also a conglomerate score of tumor-level heterogeneity (across the set of cell nests within the same tumor). They also propose the calculation of a diversity index that would allow pathologists to better understand the regional heterogeneity across a sample. This number would be higher when significant clusters of cells scored greater differences. For example, a tumor section with some areas scoring a 3+ score and others a 0 score would have a higher diversity index than a tumor section with most areas cells scoring as 1+ or 2+, even though both samples might obtain a similar overall score. Trastuzumab is confirmed to be most effective on cells strongly expressing HER2, so an understanding of the diversity of subpopulations across the tumor would assist in formulating the most effective therapy or combination therapy to hopefully target all of the populations.

Conclusion

This chapter has presented some of the evidence for the abundant and diverse contributors of tumor cell heterogeneity, whose underlying mechanisms have clearly not been fully resolved. Cancer cells may act following a hierarchical pattern or clonal evolution. They may have significant interactions with the associated stromal cells and may strongly react to their microenvironmental conditions such as hypoxia. Cancer cells, like normal cells, are influenced by a complex array of input factors that can vary over time, and the level of sensitivity to any one factor might also vary with time. It does seem promising that many cancers appear to express unique

markers or highly overexpress others that might be targeted either by monoclonal antibodies or for their natural transport receptor function. However, even for markers that have been shown to be much more highly expressed in cancer tissue, most of the reported cases indicate that rarely will all tumor cells intensely express these markers. Given that many of these markers correlate with a worse prognosis or tumor grade, it seems reasonable to suspect that the cells expressing these markers may be more active in tumor progression and malignancy.

Nevertheless, further work will need to validate that a potential drug target truly correlates with the malignant cell type, or else other drug targeting approaches will need to be included to kill the non-marker expressing cells. Even if one marker clearly could identify the malignant population, it still may be that a combination of drug treatments with different mechanisms will be needed for effective cytotoxicity of these cells. However, it is very likely that not all tumor subpopulations need to be equally targeted. This idea is exemplified by the cancer stem cell theory. It is also suggested by the current clinical recommendations that breast cancers with as few as 1 % estrogen receptor-positive (ER+) cells are to be classified as positive [78]. This assessment results from the well-documented benefit of endocrine therapy for ER + patients with a relatively moderate risk from this treatment.

Most clinical reports discussed in this chapter indicated that potential drug targets are expressed along a continuous spectrum. It is possible that a relatively smooth spectrum of marker expression suggests that the marker upregulates as the cells progress along a commonly shared pathway to a more malignant phenotype. However, it is also unclear if perhaps for some tumors, variant clonal subpopulations of cells are becoming malignant through differently evolving pathways, some of which result in the expression of the marker where others do not. This might be more likely the case for a cancer following the stochastic model as compared to the cancer stem cell model and could explain the reason why some patient tumors stain with very intense but starkly contrasting regions in close proximity. Certainly, a better understanding of the biology causing the heterogeneity within tumors will need to be better understood to determine the premise for designing the appropriate combinations of drugs or therapies. It will be important to not only diagnose the components of heterogeneity within a tumor but the cellular origin of the cancer and the progressive evolutionary pathway of the cancer to better design drug treatments that stop the real drivers behind each individual cancer.

The increasing development of more powerful and informative technical assay options will allow for better characterization of heterogeneity in clinical tumor samples. Microarray technology will perhaps allow for the discernment of one or more interacting cell signaling pathways that will clarify the rationale for the inhibition of certain targets. This may also better determine the role of increasing mutagenesis of competitive clonal variant or the origin of cancer stem-like cells. Improved and standardized histological and other techniques will reduce human error between labs and help identify equivocal samples where a misdiagnosis could result in a completely ineffective treatment choice. As techniques and information regarding the real level of heterogeneity between patients and within the malignant tissue of a single patient improve, clinicians and medicinal chemists should be able to stratify

unique neoplastic diseases by their most effective drug treatment to cater best to the patient's individual needs. Hopefully, future techniques will also clarify any real but subtle differences between a patient's primary and metastatic tumors. All cancer will need to be treated but organized by priority of which neoplastic growths are the most life-threatening to the patient. Better ways to score and standardize the communication of these evaluations in the clinic will be helpful.

The complexity of tumor heterogeneity is certainly daunting, but should not be seen as insurmountable. The increasing capacity for researchers and clinicians to accumulate, organize, and analyze large quantities of data should make it possible to eventually stratify cancer diseases sufficiently to identify improved treatment for a majority of patients. Already, many cancers of the same tissue have been organized into very different yet recurring disease patterns that can be managed more effectively than a few years ago. These same approaches should now also be implemented in greater efforts at the intratumoral level to reveal similar patterns that emerge and can explain the underlying causes of tumor heterogeneity and how to initially deal with them. The challenge will undoubtedly be ongoing, but the ability to make great strides in this area seems less contingent now on technology and knowledge and more dependent on the collective efforts of clinicians and scientist to maintain the required focus and collaboration.

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Chapter 14
A Study of Cancer Heterogeneity: From Genetic Instability to Epigenetic Diversity in Colorectal Cancer

Maria Giovanna Francipane and Eric Lagasse

Abstract Cancer is the leading cause of death worldwide. Despite improvements in diagnosis and treatment over the past two decades, cancer continues to present a serious challenge to oncologists, especially when the disease has already spread to a distant site at the time of diagnosis. The high degree of variation in gene expression, observed not only in tumors arising from different tissues but also in tumors arising from the same tissue, and sometimes in distinct areas of the same tumor, is likely to be responsible for evolutionary adaptation and consequently tumor survival.

Cellular heterogeneity has historically been viewed solely as the result of genetic instability. However, it has now become increasingly clear that changes in gene expression that occur without altering the DNA sequence—better known as *epigenetic changes*—can likewise contribute to tumorigenesis. Elucidating the mechanisms that account for cancer heterogeneity will be essential to the design of new drugs capable of overcoming the major limitations of current therapies. These limitations include the treatment of cancers able to escape immune surveillance or adapt to chemotherapy regimens as well as invasive and metastatic cancers.

Here, we review recent progress in the understanding of tumor genetics and epigenetics and translate these findings into potential clinical practice.

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Introduction

The word *cancer* comes from the Latin translation of *karkinoma*; the term was derived by Hippocrates (460–370 B.C.) from the Greek word for crab, *karkinos*. *Karkinoma* was used by the Greek physician to describe a malignant growth because veins spreading outward from the tumor mass reminded him of crab claws. Due to these angiogenesis observations, Hippocrates is considered the first person to clearly recognize the difference between malignant and benign tumors. We now know that apart from their histological features, other substantial differences occur between these two groups of tumors, including the presence in malignant tumors and the absence/infrequency in benign tumors of phenotypic instability [1, 2]. Inherent instability of tumor cells is a widespread phenomenon in cancer that drives tumor progression through the generation of more aggressive subtypes undergoing a positive Darwinian selection. Starting from Boveri's suggestions of genetic instability in cancer [3], many groundbreaking discoveries have been made in recent decades in the field of molecular biology, making it increasingly clear that genetic instability is not the only driving force for tumor progression. Epigenetic modification of DNA or of chromatin-associated proteins, a heritable change in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence, can lead to critical changes in gene function and drive tumor progression to an invasive cancer. It has also been proposed that cancer-initiating mutations could even follow an epigenetic disruption of progenitor cells [4]. Thus, epigenetics might play an important role in both cancer pre-initiation and progression.

Understanding *cancer diversity* is crucial to achieve improved diagnosis and patient treatment. Indeed, the elucidation of the mechanisms that allow cancer cells to constantly adapt and evolve during the course of the disease will help prevent cancer growth and progression. Importantly, due to their potential reversible outcome, epigenetic changes are being investigated as potential therapeutic targets, and this has led to the development of new anticancer drugs.

In the first part of this chapter, we will summarize major genetic and epigenetic pathways involved in the pathogenesis of human cancer. In the second part, we will focus on one of the best-defined models for genetic and epigenetic progression, colorectal cancer (CRC). Finally, we will discuss how emerging information about genetic and molecular diversity can be used to assess cancer risk and/or guide therapy.

Genetic Instability

Chromosome instability (CIN) and microsatellite instability (MSI) are the major genetic instability pathways that can lead to cancer pathogenesis. In the following paragraphs, we will consider the most important molecular contributors toward the progressive loss of a stable karyotype thereby initiating and sustaining cancer.

Chromosome Instability

CIN refers to an increased rate of the loss or gain of whole or large sections of chromosomes during cell division. This increased rate of unbalanced chromosomal rearrangement eventually leads to a multistep accumulation of genetic abnormalities, including amplification of proto-oncogenes and inactivation of tumor suppressor genes, which may directly promote tumor cell growth. For instance, loss of tumor suppressor genes often results from the loss of genetic information inherited from one parent, a phenomenon known as the loss of heterozygosity (LOH) [5].

An imbalance in chromosome number is also referred to as *aneuploidy*. Although aneuploidy can be detected at early steps of malignant transformation, and even in certain premalignant lesions, the number of chromosomal aberrations usually increases with tumor progression [6–8]. Whether chromosome abnormalities can be both the cause and the effect of cancer is still under investigation. Similarly, the scientific community is divided over the assignment of the origin of chromosomal abnormalities. Many studies suggest that aneuploidy arises from the inability to faithfully ensure equal segregation of chromosomes during mitosis [9, 10]. This mitotic chromosomal instability has been mainly correlated to numerical and functional abnormalities of centrosomes. Indeed, the presence of multiple centrosomes can lead to multipolar mitosis, enabling the survival of tetraploid cells and the generation of an aneuploid population that evolves to become genetically unstable and tumorigenic [11]. However, it should be considered that centrosome abnormalities effectively destabilize chromosomes only in cells with a compromised spindle checkpoint function. Usually, cell cycle checkpoint activation slows or arrests cell cycle progression, thereby allowing for efficient repair and thus preventing transmission of DNA damage to the progeny [12, 13]. The fate of damaged cells mainly depends on the status of the p53-dependent G1 cell cycle checkpoint pathway [14]. In the presence of p53, mutant cells are rapidly eliminated through cell cycle arrest and/or apoptosis, whereas a defective p53 pathway permits their propagation. Consistent with this, loss of p53 function is associated with increased aneuploidy [15–17], gene amplification [18], point mutation [19], and homologous recombination [20].

Cyclin-dependent kinases (CDKs) are targets of checkpoints that control entry into the next phase of the cell cycle. The activity of CDKs is frequently deregulated in tumor cells due to genetic or epigenetic alterations of CDK–cyclin complexes or to downregulation of several CDK inhibitors including p21CIP/WAF, p27KIP, and p16INK4A [21]. Centrosome amplification can be correlated with multiple genes of the cell cycle engine. For instance, centrosome duplication is controlled by CDK2/cyclin E complex, which is inhibited by p21CIP/WAF [22, 23]. Thus, overexpression of cyclin E or p21CIP/WAF inhibition results in centrosome amplification. Mutational inactivation of p21CIP/WAF is infrequent [24]; however, aberrant p21CIP/WAF promoter gene methylation is common in cancer and results in strikingly reduced expression of its regulated protein [25]. These findings lead to the idea that aneuploidy may not be only genetic in origin.

In addition to defects in either cell cycle machinery or checkpoints as potential causes of CIN, other mechanisms, such as telomere erosion, may be involved in the generation of unstable cells. Telomeres are specialized DNA structures located at the end of chromosomes with an important role in the prevention of chromosome fusion [26]. Normal somatic cells show a progressive loss of telomeres during DNA proliferation due to end replication problems of DNA polymerase, eventually leading to replicative senescence. Telomere erosion has been linked to both tumor suppression and genetic instability. Dysfunctional telomeres activate DNA damage response. In the setting of a competent p53 pathway, this initiates senescence and apoptotic programs to inhibit tumorigenesis, whereas in cells with mutant p53, dysfunctional telomeres promote genome instability and progression to cancer [27, 28]. Telomere-related CIN results from repeated breakage–fusion–bridge cycles (BFBCs), and this is thought to be a key event in tumorigenesis of different tissues, including colon [29], cervix [30], and blood [31].

Like telomere erosion, DNA palindrome formation can lead to genetic instability by initiating BFBCs [32]. However, it is unknown how palindromes form, although they appear early in cancer progression.

Every cell division presents a chance for mutations. Because stem cells have the property of self-renewal, any mutation conferring a selective growth advantage occurring in the stem cell compartment will be perpetuated into its progeny. This genetic lesion, in turn, can lead the daughter cells to acquire new properties through additional cycles of genetic aberrations. This concept has been well demonstrated for chronic myeloid leukemia (CML). Following radiation exposure, the BCR/ABL oncogene is likely to induce genetic instability in CSCs that predisposes the progeny to increased BFBCs [33]. Such important findings can also be applied to chemotherapy and explain why sequential treatment with multiple tyrosine kinase inhibitors still fails to completely eradicate the disease [34].

The Opposing Roles of Aneuploidy

Although the so-called aneuploidy hypothesis postulates that an abnormal chromosome number can drive tumor progression, some researchers have argued that aneuploidy is only a benign side effect of transformation [35]. Indeed, several lines of evidence demonstrate that an altered karyotype can decrease the rate of cell proliferation or even cause cell death. Using centromere-associated protein E (CENPE) heterozygous animals, which develop whole chromosome aneuploidy in the absence of mutations that compromise chromosome segregation fidelity, Weaver et al. have found that aneuploidy promotes tumorigenesis in some contexts and inhibits it in others [36]. Specifically, low rates of CIN promote tumors, whereas high rates of CIN cause cell death. Thus, aneuploidy can act both as a tumor inducer and a tumor suppressor. Such an effect is also analogous to chemotherapy-induced genetic instability, in which high levels of DNA damage lead to cellular death and tumor regression. The most probable explanation for the impairment of cell fitness is the *gene dosage hypothesis* in which gains or losses of whole chromosomes immediately

alter the dosage of hundreds of genes in a cell, leading to imbalances in critical proteins [37]. The possible resulting changes include the alteration of the function of a specific protein, the defect of stoichiometric-sensitive complexes, the favoring of promiscuous molecular interactions, and the accumulation of improperly folded or aggregated proteins negatively affecting cell proliferation. However, aneuploid cells are often able to trigger adaptive dosage compensation responses at the proteome level which may be accelerated by aneuploid-induced genetic instability, suggesting the existence of a functional and destabilizing positive feedback loop of aneuploidy in cancer.

The role of aneuploidy in tumorigenesis remains poorly understood. It is conceivable that cellular outcome is dependent on the extent of aneuploidy induced. This could explain why aneuploidy can be compatible with normal growth and development. Polyploidy is common, for example, in the liver, where frequent multipolar mitosis yield diverse hepatocyte populations, some with aneuploidy [38]. Interestingly, the genetic variation found in hepatocytes is postulated to be an advantage for liver function by allowing the cellular selection of discrete hepatocyte populations to expand and protect the organ from certain injury and poisonous substances [38].

Microsatellite Instability

MSI refers to length alterations of mononucleotide or dinucleotide repeats (e.g., TTTT or CACACA) located mostly in intronic DNA sequences. MSI is mainly due to errors during DNA replication and to a defective post-replicative repair system. Indeed, defects in both DNA mismatch repair (MMR) and base-excision repair (BER) systems have been identified in MSI-positive tumors. The DNA sequences repaired by the MMR system are residual mismatches that have evaded proofreading during replication. Base mispairs, if not corrected by the MMR system, may cause nucleotide transitions or transversions, allowing a novel base to alter the authentic genetic sequence. Importantly, the role of MMR proteins in the repair process can be uncoupled from the MMR-dependent cell-killing response, the latter being based on the ability of MMR proteins to trigger checkpoint activation and apoptosis in response to DNA damage [39, 40].

In late 1993 [41], altered CA repeats in colon cancer were correlated for the first time to a mutation in a gene which codes for a factor essential for replication fidelity or repair. At the same time, Lynch syndrome (also termed hereditary nonpolyposis CRC, HNPCC) was associated with germ-line mutations to one of two MMR genes, human mutL homologue 1 (hMLH1) or human mutS homologue 2 (hMSH2), with mutations of other MMR genes being rare [42–45]. hMLH1 and hMSH2 genes were also reported as inactivated via promoter DNA methylation in a sporadic subset of MSI-positive tumors [46, 47]. In the remaining tumors, no identifiable MMR gene mutations were found, indicating that additional factor(s) could have been responsible for the MSI phenotype [48–52].

Although CIN and MSI can be distinguished from one another by their molecular characteristics, evidence suggests that there might be some degree of overlap. In a study by Goel et al., 3.4 % of the analyzed CRCs showed the coincidence of MSI-high (MSI-H) and LOH events [53], and in the poorly metastatic KM12C cell line, both patterns of genetic instability were found to coexist [54].

Epigenetic Instability

The term epigenetics is defined as the heritable but potentially reversible changes in gene expression that occur without alterations in the DNA sequence [55–58]. Epigenetic modifications include DNA methylation, histone modifications, and microRNAs (miRNAs). Accumulating evidence indicates that these modifications are profoundly altered in human cancers. The key players of such complex processes comprise a long list of enzymes cooperating together and include DNA methyltransferases (DNMTs), methyl-CpG binding proteins, histone modifying enzymes, chromatin remodeling factors, transcription factors, and chromosomal proteins.

DNA Methylation

DNA methylation involves the addition of a methyl group to the 5′ position of the cytosine pyrimidine ring. In mammals, this phenomenon occurs exclusively at a cytosine followed by guanine (CpG). About 70–80 % of CpG sites contain methylated cytosines in somatic cells [59]. Although the CG dinucleotides are present along all chromosomes, the CG density is higher in some areas than others [60]. These so-called CpG islands are present in the promoter and exon regions of approximately 40 % of mammalian genes and regulate gene expression. Several experiments have shown that methylation of promoter CpG islands plays an important role in gene silencing [61], genomic imprinting [62], X-chromosome inactivation [63], the silencing of intragenomic parasites [64], and carcinogenesis [65, 66].

Although the origin of aberrant DNA methylation patterns remains to be established, several studies have suggested that alterations in the DNA methylome could be directly affected by diets that are deficient in folate and methionine; exposure to metals, such as arsenic, lead, and chromium; and inflammation or viral/bacterial infection, i.e., chronic inflammatory bowel disease (IBD) and *Helicobacter pylori* infection of gastric epithelial cells [67].

Epigenetic factors play a critical role in development, dictating the rules that establish and maintain *stem cell identity*. Loss of cellular identity leads to an increased ability to grow and proliferate, ultimately causing the onset of cancer.

Oct4, Nanog, and Sox2 transcription factors are expressed by embryonic stem cells (ESCs) during development, conferring pluripotency [68–71], but are repressed through promoter hypermethylation during adulthood [72, 73]. In the context of cancer, expression of these ESC-associated genes occurs [74] in accordance with the idea that cancer arises through the dedifferentiation of fully committed and specialized cells or from "maturation arrest" of stem cells [75]. Specifically, DNA hypomethylation has been found in a variety of human cancers [76–84] and affects not only Oct4, Nanog, and Sox2 but a long list of genes. The extent of hypomethylation has been correlated with tumor grade and prognosis in liver, breast, and ovarian cancers [85–87], but not in prostate cancer [88]. Thus, the inappropriate epigenetic (re)activation of tissue-specific genes plays a critical role in cancer.

DNA hypomethylation in tumors also occurs at repetitive sequences. Half of the human genome consists of highly repeated, interspersed DNA sequences, and recent studies have highlighted that their hypermethylation represents a mechanism to prevent chromosomal instability, translocation, and gene disruption caused by the reactivation of transposable elements, such as SINE (short interspersed elements), LINE (long interspersed elements), and HERV (human endogenous retroviruses) sequences. Indeed, loss of methylation at these elements contributes to oncogenic transformation or tumor progression [89–91].

Besides DNA hypomethylation, de novo methylation within the promoter region of tumor suppressor genes has also been observed in cancer. The retinoblastoma gene (Rb) was the first classic tumor suppressor gene in which CpG island hypermethylation was detected [92, 93]. Following this discovery, other tumor suppressor proteins including von Hippel–Lindau (VHL), INK4A, E-cadherin, MLH1, and breast cancer 1, early onset (BRCA1) were found to be silenced in cancer through hypermethylation of their promoters [46, 94–100]. The so-called CpG island methylator phenotype (CIMP) was first described by Toyota et al. in 1999 [101]. In their study, two distinct types of hypermethylation were found: one appearing as a result of the aging process and the other, specific for cancer. Age-related methylation was shown to be very frequent in primary CRCs, while cancer-related methylation was relatively infrequent and never observed in normal colon mucosa. Detailed analysis of this latter type of methylation revealed a prominent pattern, suggesting the presence of a hypermethylator phenotype in a subset of CRCs. The authors concluded that through its ability to silence multiple genes simultaneously, CIMP can be considered functionally equivalent to genetic instability, resulting in the rapid accumulation of multiple molecular aberrations with a potential to trigger the neoplastic process. Additional work from other groups has suggested that promoter hypermethylation of tumor suppressor genes can follow the formation of transcriptionally inactive chromatin [102]. From this point of view, hypermethylation could be held responsible for maintaining gene silencing, rather than initiating it. Importantly, hypomethylation or hypermethylation may not result in gross changes in gene expression per se, as cancer appears to be linked to a global epigenetic disequilibrium [103].

DNA Methyltransferase and Polycomb Genes: Key Players in Epigenetic Silencing

The enzymes directly responsible for CpG island hypermethylation of tumor suppressor genes are the DNMTs. Both increased expression and increased activity of DNMTs have been found in human cancers, including colon cancer [104–107]. Polycomb group (PcG) proteins have been suggested to serve as recruitment platforms for DNMTs [108, 109], helping maintain the transcriptional repression of target genes through many cycles of cell division. PcG genes are organized in two multiprotein complexes, Polycomb repressive complex 2 (PRC2) and 1 (PRC1), which have been implicated in silencing initiation and stable maintenance of gene repression, respectively [110].

Among the most studied PRC1 members is B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), which contributes to CSC self-renewal in part by inactivating the INK4A-ARF locus-encoded p16INK4A and p14ARF proteins, thus delaying the onset of senescence [111]. However, Bmi-1 can also act in an INK4A independent manner, for example, modulating Wnt and Notch pathways [112]. Enhancer of zeste homologue 2 (Ezh2), the histone methyltransferase of PRC2, plays a master regulatory role in controlling stem cell differentiation [113], cell proliferation [114], early embryogenesis [115], and X-chromosome inactivation [116]. Moreover, a functional link between dysregulation of Ezh2 and repression of E-cadherin during cancer progression has been reported, suggesting a critical role for this PcG gene in the invasive process [117]. A correlation between the cell cycle machinery and Ezh2-mediated epigenetic gene silencing has also been demonstrated. Specifically, CDKs have been found to phosphorylate Ezh2, maintaining its oncogenic and gene-silencing functions, and ultimately contributing to the aggressive phenotype of tumors [118]. Briefly, many cancer types show an overexpression of Ezh2, predicting poor prognosis, metastasis, and chemoresistance [119–124]. A significant association between polymorphisms of the Ezh2 gene and cancer risk/outcome has been reported for the first time in lung cancer [125] and more recently in CRC patients [126], thus introducing the concept of epigenetic polymorphism testing for cancer therapy. However, our comprehension of the precise role of PcGs in tumorigenesis and mechanisms of their regulation remains incomplete. While there are about 15 unique PcG genes in Drosophila [127], in mammals there are multiple orthologues of many PcGs, making possible hundreds of different combinations to assemble multiprotein complexes. Further studies are needed to complete this puzzle and obtain useful information to develop new ways to treat, cure, or even prevent cancer.

Histone Modifications

The histones constitute a family of small basic proteins that are involved in the packaging of eukaryotic DNA. Histone N-terminal tails may undergo many

enzymatic posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. These modifications provide an important regulatory platform for processes such as gene transcription and DNA damage repair. For instance, acetylation of the lysine residues at the N terminus of histone proteins leads to chromatin relaxation by reducing the affinity between histones and DNA. Decompaction of the chromatin structure allows accessibility of the DNA by RNA polymerase II (Pol II), stimulating gene transcription.

The combination of histone posttranslational modifications is thought to give rise to a *histone code* that is interpreted by an array of diverse proteins. These proteins can be divided into three classes: "readers," "writers," and "erasers." Misreading, miswriting, and mis-erasing of histone methylation marks can be associated with oncogenesis and progression [128]. Mixed lineage leukemia (MLL) is an example of cancer driven by epigenetic alterations involving histone modifications [129]. These leukemias are characterized by translocations of the MLL gene, which normally methylates histone H3 on lysine 4 (H3K4), a mark typically associated with gene activation. MLL translocations encode MLL fusion proteins that have lost H3K4 methyltransferase activity and possess the ability to reprogram differentiated myeloid cells into multipotent CSCs. Changes in global histone modification patterns have also been observed in other cancers, including lymphoma, breast, colon, bone, cervix, lung, testis, neuroblastoma, osteosarcoma, and prostate [130–132]. Particularly, global loss of monoacetylation and trimethylation of histone H4 has been reported as a common hallmark of human tumor cells [130].

miRNAs

miRNAs are short noncoding RNAs that bind to complementary mRNA molecules, promoting their degradation and/or translation into a protein. Studies suggest that the human genome may encode over 1,000 miRNAs, a limited number compared with the number of mRNAs, typically estimated at ~30,000 [133]. However, miRNAs may regulate hundreds of mRNAs, affecting a range of processes, including organismal development and the establishment and maintenance of tissue differentiation [134, 135]. Importantly, an epigenetic crosstalk between miRNAs and DNA methylation has been reported. Specifically, a wide range of tumor suppressor miRNAs has aberrant methylation profiles in human cancers. Mir-127 and mir-124 are examples of the first two miRNAs identified that undergo transcriptional inactivation by CpG island hypermethylation [136, 137]. Epigenetic repression of these molecules leads to changes in histone modifications; thus, epigenetic modifications are profoundly linked to each other. Figure 14.1 shows a summary of both genetic and epigenetic mechanisms that drive cell transformation and promote cancer development and progression.

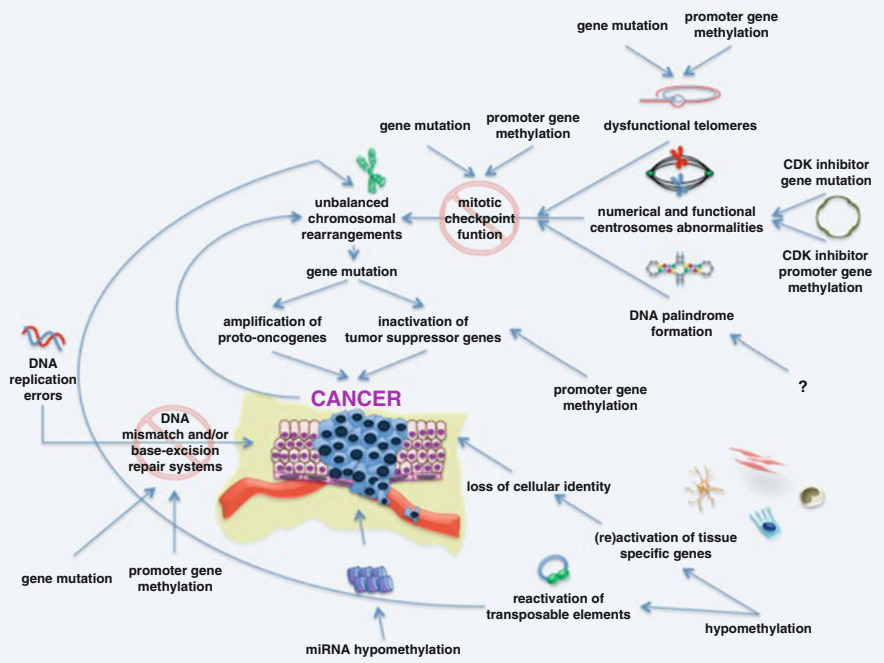

Fig. 14.1 Scheme illustrating the mechanisms that drive cell transformation and promote cancer development and progression. Both genetic and epigenetic mechanisms are depicted

The Genetic and Molecular Diversity of Colorectal Carcinoma

CRC is a leading cause of cancer deaths worldwide. Roughly, three molecular subtypes of CRCs have been described: CIN, MSI, and CIMP. A small subgroup of tumors also exists in which none of these phenotypes have been detected [138].

According to the CIN pathway, the classical multistep pathway of colon carcinogenesis proposed by Vogelstein et al. in 1988, CRC develops as a result of the pathologic transformation of a normal colonic epithelium into a dysplastic epithelium and ultimately into an invasive cancer through an adenomatous polyp. Aberrant crypt foci (ACF), microscopic surface abnormalities first identified in carcinogen-treated rodents [139] and later in human colon [140], are postulated to be a precursor to the adenoma due to the presence of molecular and genetic abnormalities, i.e., MSI [141]. Particularly, ACF formation is initiated by mutations in the adenomatous polyposis coli (APC) tumor suppressor gene [142]. APC is considered a strong negative regulator of the Wnt pathway, being part of the β-catenin destruction complex, which also includes the scaffold proteins axin or conductin/axin2, casein kinase I (protein kinase CKI), and glycogen synthase kinase 3β (GSK3β). In normal cells, this complex phosphorylates β-catenin, leading to its ubiquitination and

destruction by proteasome 26 S [143]. Loss of APC leads to β-catenin accumulation in the cytosol, binding to cytosolic T cell-factor/lymphoid-enhancer-factor (Tcf/Lef) proteins, translocation of the resulting complex to the nucleus, and activation of transcription [144]. Target genes include c-myc and cyclin D1 [145, 146]. Thus, one effect of APC inactivation is proliferation of the affected cells.

The importance of APC dysfunction in colon cancer is well established. Individuals who inherit a defective allele of the APC gene suffer from familial adenomatous polyposis (FAP), an autosomal dominant disease in which thousands of colonic polyps, many of which will progress to cancer if not removed, are developed during childhood and adolescence [147]. Furthermore, somatic mutation of the APC gene is found in the majority of sporadic CRC [148]. APC has usually been implicated in CIN, but this is still a matter of debate. Michor et al. have developed a mathematical approach for the cellular dynamics of colon cancer initiation, showing that genetic instability is an early event and thus a driving force of tumorigenesis, since a small number of CIN genes are sufficient to initiate colorectal tumorigenesis before APC inactivation [149].

ACF are considered microadenomas. In Vogelstein's model, the progression from microadenoma to intermediate adenoma is accompanied by K-ras activation [150]. The K-ras gene encodes a 21-kD protein (p21ras) involved in G protein-mediated signal transduction. Ras mutations usually lead to constitutive activation of the signaling pathways controlling cell proliferation and differentiation [151]. After the formulation of Vogelstein's theory, K-ras mutations were actually reported to occur in every step of colon carcinogenesis. Such an idea was supported by two observations: (1) both small and large adenomas sometimes have the same incidence of K-ras mutations and (2) K-ras mutations can be heterogeneous within the same carcinoma [152–154], suggesting a correlation to late tumorigenesis. By using a different sampling method to collect tumor DNA, Ishii et al. showed that K-ras mutations are instead homogeneous within the same carcinoma, and therefore they do not occur in late carcinogenesis [155].

The transition from an intermediate adenoma to a late adenoma is characterized by the loss of the deleted in colorectal cancer (DCC) tumor suppressor gene. Identified in 1990 by Fearon et al. within a previously described LOH region at 18q, the DCC gene encodes a protein which has been suggested to allow intestinal cell migration from the base to the top of the glandular crypts by reducing cell–matrix contacts and reinforcing cell–cell contacts through association with ezrin/radixin/moesin and merlin (ERM-M) proteins [156, 157]. Mutations of both DCC alleles contribute to tumor development by disrupting such contacts. In addition to DCC, SMAD2 and SMAD4 tumor suppressor genes are the targets of 18q LOH [158, 159]. Whereas mutations of DCC and SMAD2 seem to be very rare in CRC [160, 161], SMAD4 inactivation is likely to be involved in advanced stages such as distant metastasis [162].

Finally, allelic loss of the p53 tumor suppressor gene allows a growing tumor with multiple genetic alterations to evade cell cycle arrest and apoptosis, thus permitting a late adenoma to progress to carcinoma [150].

In summary, Vogelstein's colon carcinogenesis model includes five key steps: (1) APC gene mutation leads to hyper-proliferation and (2), in succession, the formation of a class I adenoma; (3) a class II adenoma forms following K-ras activation; (4) loss of DCC is then responsible for class III adenoma formation; and (5) invasive cancer requires mutation of the p53 gene [150].

Our understanding of the molecular pathogenesis of CRC has advanced significantly since Vogelstein's model was initially proposed, resulting in several reconsiderations of the so-called Vogelgram. We now know that many more genes and steps may be involved. Some of the early evidence that there were multiple molecular pathways to CRC came from identification of different histological and genetic features between CRCs in Lynch syndrome and CRCs developing through the Vogelstein's adenoma–carcinoma sequence. Lynch-associated CRCs are more commonly right sided, often poorly differentiated or mucin-producing, and have a dense lymphocytic infiltrate and a Crohn's-like reaction. Genetically, as we have already discussed, Lynch-associated CRCs are characterized by mutations in the DNA MMR system which are likely responsible for MSI. As shown in 1999 by Salahshor et al., mutations in APC and p53 are not necessary for initiation and progression of such MSI-positive CRC [163]. These types of tumors carry instead a mutation in the type II TGF beta receptor (TGFβR2) resulting in the inhibition of the TGFβ signaling pathway and a low metastatic rate. In accordance, Warusavitarne et al. have demonstrated that restoring TGFβ signaling reduces tumorigenicity and increases invasion and metastasis in MSI-H CRC cell lines [164].

Additional evidence of the existence of multiple adenoma–carcinoma sequences came from the classification of colorectal polyps into two major groups: conventional adenomas and serrated polyps, the latter including hyperplastic polyps (HP), sessile serrated adenoma (SSA), sessile adenomas (SA), and mixed polyps [165]. Serrated polyps are usually found in the left colon, are smaller in size than adenomatous polyps, and have erroneously been considered as benign in nature. However, an equivalent to the adenoma–carcinoma sequence has recently been suggested for adenomas arising from those polyps, which includes an activating mutation in the BRAF gene as the initiating event triggering the malignant transformation of the polyp [166]. Somatic molecular alterations associated with serrated polyps also include K-ras mutations, hMLH1, and MGMT methylation, the prevalence of which varies according to the subtype of serrated polyp [167]. The evidence that serrated polyposis is a genetic predisposition is accumulating. Its genetic basis is yet to be fully determined, though a small number of patients have reported mutations in mutY homolog (*E. coli*) (MUTYH) [168], phosphatase and tensin homolog (PTEN) [169], and ephrinB2 (EPHB2) genes [170]. Figure 14.2 illustrates how different pathogenic pathways can be involved in initiation and progression of right- versus left-sided colon cancers.

One of the intriguing questions is whether the three above-described pathways of colon carcinogenesis initiate in identical cells or whether three different cells are the targets of multiple mutations. Over the last decade, the opinion on cancer biology has drastically changed. Contrary to the longstanding clonal evolution model described by Nowell in the late 1970s [171], the CSC hypothesis has recently proposed that not every cell of the body could be the target of malignant

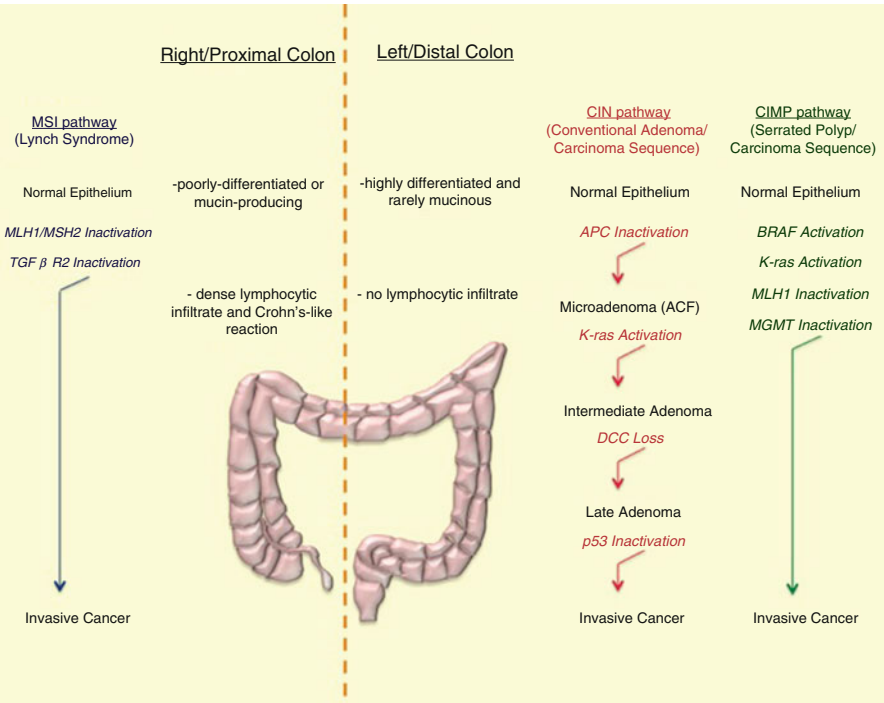

Fig. 14.2 A simplified scheme illustrating how different pathogenic pathways can be involved in initiation and progression of right- versus left-sided colon cancers

transformation. The limited lifespan of a committed cell is likely shorter than the time necessary to accumulate tumor-inducing genetic changes. Therefore, cancer-initiating capability could be a unique feature of the long-lived, self-renewing stem cells [172]. The CSC hypothesis is neither a universal model for all cancers nor for all patients with the same disease. While some cancers have been hypothesized to initiate as a stem cell disease, they may then progress by clonal evolution of their CSCs, as CRC has been suggested to do through CIN [173]. The aforementioned pathways of colon carcinogenesis could be derived from three different CSCs. Importantly, epigenetic modifications are likely to occur in these cells prior to the first gatekeeper mutation. Indeed, five lines of evidence suggest the existence of an epigenetically disrupted progenitor-cell population from which tumors arise: (1) tumor-related properties are stable but reversible; (2) global epigenetic changes must precede the earliest genetic alterations as they are always found, even in benign neoplasms; (3) cloned mouse melanoma nuclei can differentiate into normal mouse cells, indicating tumor properties can be reprogrammed and therefore are epigenetically controlled; (4) neoplastic clones can be maintained solely by a small population of cells with stem cell properties; and (5) the tumor microenvironment can affect the epigenetic state of progenitor cells [4]. Consistently, aberrant promoter

methylation of several genes (p16, MINT31, MINT2, MINT1, MGMT, hMLH1 HLTF, and SLC5A8) has been observed in ACF, thus confirming that epigenetic disruption is a primary rather than a secondary event in colon tumorigenesis [174–176]. From this point of view, tumor heterogeneity and progression could be explained independently of genetic clonal evolution. This means that the ability to metastasize may not require subsequent mutation and clonal selection within a large tumor mass but could be an intrinsic feature of the progenitor cell from which the tumor arises. Unfortunately, no unifying theory has emerged to explain cancer origin and progression. This is an urgent challenge to address in the future in order to achieve targeted cancer therapies.

Cancer Diversity: From Players to Clinical Application

Early FAP and Lynch syndrome diagnoses and appropriate CRC follow-up care can improve survival. Genetic tests for both diseases have been developed. These detect mutations in the APC and MMR genes (MSH2 and MLH1), respectively, and can be used to assess risk and guide treatment decisions. Unfortunately, the accuracy of tests to detect germ-line mutations in candidate genes continues to be challenging [177, 178] and triggers debate over the ability of a proposed test to predict responsiveness to chemotherapy. For instance, a few research groups have recently evaluated classical MMR genes as predictive or prognostic biomarkers for colon cancer, and according to the most recent study, they are independent predictors of disease-free survival (DFS) in patients with stage III colon cancer receiving adjuvant 5-FU–oxaliplatin combination therapy (FOLFOX) [179–183]. Important findings about the utility of knowing the MSI status of non-MMR genes to select patients for chemotherapeutic treatment have recently came from Dorard et al., which have considered in their study a previously unknown mutation in the gene encoding the chaperone heat shock protein (HSP) 110. HSP110 T_{17} intronic DNA microsatellite mutations in MSI CRC result in the loss of HSP110 exon 9 and expression of a truncated protein, HSP110ΔE9, increasing tumor sensitivity to anticancer agents such as oxaliplatin and 5-FU [184].

Throughout this chapter, we have provided evidence to support the epigenetic origin of cancer. Importantly, as we gain insight into the functional significance of global changes in chromatin structure, and as new tools for specific and efficient detection of epigenetic marks become available, there will be an enormous opportunity to develop markers for disease diagnosis and drug response, as well as strategies to prevent further disease progression. In this context, the recent advent of microarray technologies has allowed the identification of epigenetic signatures for different cancers. Each tumor type has been suggested to have a specific “hypermethylome” [185], thus defining CpG hypermethylation maps for a growing list of primary tumors, including glioblastoma [186], acute myeloid leukemia [187], ovarian carcinoma [188], astrocytoma [189], and colon cancer [190]. As the list of tumor

suppressor genes that are silenced through promoter hypermethylation grows, a correlation with response to therapy is investigated. For instance, transcription factor AP-2 epsilon (activating enhancer binding protein 2 epsilon), also known as TFAP2E, has recently been found to be hypermethylated in CRC patients correlating with the overexpression of the Wnt antagonist Dickkopf-related protein 4 (Dkk4) and chemoresistance [191]. Thus, the importance of epigenetic modifications in predicting patient prognosis and response to chemotherapy is increasingly recognized by several studies. Epigenetic markers may be detected easily in circulating DNA (cirDNA) in the plasma or other bodily fluids. For instance, circulating methylated septin (SEPT) 9 DNA in plasma is considered a biomarker for CRC [192]. However, further studies are needed to clearly define specific markers for accurate cancer detection and risk assessment. Consistently, the first epigenome-wide DNA modification profiling of plasma or other bodily fluids from cancer patients has been provided only recently by Cortese et al. in the context of prostate cancer [193].

Importantly, due to their reversibility, epigenetic changes are being investigated as potential therapeutic targets, leading to the development of new anticancer drugs. The first generation of Food and Drug Administration (FDA)-approved epigenetic-based drugs includes two DNA-demethylating agents, 5-azacytidine (AZA) and decitabine (DAC), and two histone deacetylase (HDAC) inhibitors, vorinostat (Vo) and valproic acid (VA). These drugs were developed for the treatment of blood diseases, in particular myelodysplastic syndromes (MDS), against which they were reported to be highly effective, leading to significant improvements in patient quality of life and survival [194]. Although epigenetic drugs in clinical trials for hematological malignancies have been successful, results were much more disappointing for solid tumors, probably because CSCs in solid tumors are confined to a niche that is less reachable by these drugs. Moreover, epigenetic drugs were reported to be toxic, triggering common side effects including nausea, vomiting, diarrhea, and myelosuppression. Nevertheless, the observation that low doses of DNMT and HDAC inhibitors together are able to reverse gene silencing associated with promoter methylation has created much interest. Particularly, the combination of HDAC and DNMT inhibition has been reported to be very effective (and synergistic) in inducing apoptosis, differentiation, and/or cell growth arrest in human lung, breast, thoracic, leukemic, and colon cancer cell lines [195]. Combining current cancer treatments with distinct chromatin remodeling factors may reduce the effective drug concentration and related systemic toxicity; however, other questions remain to be addressed. Specifically, pleiotropic effects and the lack of specificity of epigenetic drugs continue to pose important implications for clinical treatment. Indeed, epigenetic drugs have recently been reported to be able to wake up metastasis-related genes [196]. This finding strongly highlights the need to accurately assess the clinical effectiveness and side effects of putative epigenetic treatments before human testing. This can only be achieved through a full comprehension of cancer dynamics at the cellular and molecular level.

Concluding Remarks

One of the main unresolved problems of current available therapeutic treatments for cancer is the lack of selectivity combined with the lack of efficacy. To design a more successful approach and possibly achieve complete tumor regression, it will be necessary to identify the genetic as well as the epigenetic alterations underlying cancer etiology and progression, not only for each cancer, but probably for each patient. In conclusion, cancer can be viewed as a complex adaptive system [197]. Cancer cells evolve and adapt to resist the death-inducing stimuli they are subject to. As opposed to old-fashioned chemotherapy, emerging and future personalized therapies will help controlling the occurrence of unstable cells with acquired multidrug resistance by targeting only tumor cells while sparing normal cells and tissues.

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Chapter 15
Nanotherapeutics in Multidrug Resistance

Min Han and Jian-Qing Gao

Abstract The development of resistance to a variety of chemotherapeutic agents, also known as multidrug resistance (MDR), is a main impediment to the success of cancer chemotherapy, which refers to many factors such as increased efflux, blocked apoptosis, decreased drug influx, and altered cell cycle regulation. Considerable efforts have been devoted to develop chemosensitizers to conquer drug resistance, while their safety and unwanted pharmacokinetic drug interaction hindered their clinical applicability. Nano-sized drug carriers have great superiority in overcoming drug resistance due to the improved therapeutic index of drugs, enhanced drug targeting in tumor sites, and success in escaping from recognition of ABC transporter-mediated drug efflux. This chapter summarizes the most recent developments in the field of nanotherapeutics toward overcoming drug resistance by drug-targeted delivery, increased intracellular availability, changed subcellular localization, and combination of drug delivery with the agents that regulate intracellular pH, energy delivery, and apoptotic threshold.

Introduction

Cancer is one of the major causes of death worldwide. Multidrug resistance (MDR), which is classically defined as a state of resilience against structurally and/or functionally unrelated drugs, is the main obstacle in cancer therapy. Generally, MDR can be divided into two types: intrinsic MDR and acquired MDR. Intrinsic MDR can be favored by the selection pressures in the tumor microenvironment, whereas acquired MDR can be induced by the traditional chemotherapy in common dose. Many factors can contribute to MDR, such as increased efflux, decreased drug influx, mutated cell cycle regulation, and blocked apoptosis.

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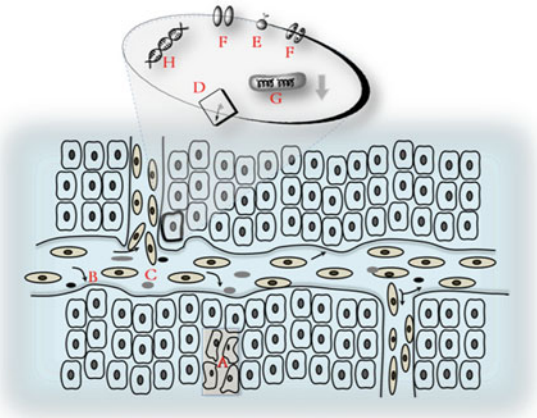
The intracellular concentration of drugs and therapeutic efficiency can be increased using nanovehicles, which can escape from the recognition of efflux pumps and thus be endocytosed by tumor cells. Moreover, drug resistance gene and protein inhibitors can also be loaded in vehicles to modulate cell apoptosis and intensify drug efficacy.

Tumor Microenvironment and Cancer-Initiating (Stem) Cells

The microenvironment of a tumor cell contributes to the development of MDR and determines cell response to chemotherapy. The microenvironmental selection pressures that contribute to the development of MDR also always make the cells hypersensitive to growth stimulation.

Meanwhile, complex phenotype transformations can occur in cancer cells under hypoxic conditions, which are necessary for cell survival under such conditions. This kind of survival is a cascade initiated by the translocation of the alpha subunit of hypoxia-inducible factor (HIF) from the cytoplasm to the nucleus. The translocation is followed by abnormal tumor vasculature, hypoxia, decreased pH, increased interstitial fluid pressure, and alterations in the expression of tumor suppressors and oncogenes. Among them, abnormal (i.e., leaky and unorganized) tumor vasculature and the occurrence of hypoxic regions (transient and/or chronic), which are both common to all solid tumors, have been confirmed to play the most critical roles. Other hallmarks of the tumor microenvironment include the upregulation of oncogenes and DNA repair mechanisms as well as the downregulation of tumor suppressors and cell cycle regulation. Besides, the introduction of growth factor receptors and nutrient importers result in the complexation with the beta subunit of HIF to form an active transcription factor. This HIF complex binds to hypoxia-responsive elements on target genes that are always relevant to invasion, proliferation, metabolism, and drug resistance (Fig. 15.1).

Tumor-initiating cells, commonly called cancer stem cells (CSCs), represent a small proportion of cancer cells possessing the common properties of normal stem cells (SCs). CSCs can initiate new tumors after injection into animal models, which is different in other cancer cells. The small proportion of cancer cells have the function of drug resistance modulating the metastasis of cancer cells, thus resulting in the relapse of cancers by acting as an obstacle in cancer therapy. Tumor drug resistance is reported to be closely associated with CSCs because of their intrinsic or acquired properties, including the following: quiescence, specific morphology, ability to repair DNA, ability to enhance the expression of antiapoptotic proteins and drug efflux transporters, as well as ability to detoxify enzymes. Currently available radio- and chemotherapies can kill the majority of cancer cells but are usually unable to eliminate the initiating CSCs that are protected by specific resistance mechanisms. Surviving CSCs promote the growth of new tumors and metastases, resulting in cancer relapse. The recurrent tumors become even more malignant and spread more quickly. Moreover, they become resistant to previously used radiotherapy and drugs, making them more difficult to treat and leading to increased patient suffering. Different signaling pathways and genes are involved in the



A: Hypoxic regions
B: Leaky vasculature
C: Discontinuous, unorganized vasculature
D: Decreased pH and increased interstitial fluid pressure
E: Upregulation of growth factor receptors
F: Upregulation of ABC transporters
G: Decreased OXPHOS, increased anaerobic metabolism
H: Upregulation of oncogenes and anti-apoptotic factors, down- regulation of tumor suppressors and pro-apoptotic factors

Fig. 15.1 Schematic description of the selection pressures in the tumor microenvironment that leads to the development of multidrug resistance. Selection pressures such as hypoxia (*A*), genetic mutations in regulatory genes, and altered regulation of apoptotic factors (*H*) can lead to cellular adaptation and aggressive MDR characteristics. Such characteristics include increased expression of growth factor receptors (*E*), increased expression of drug efflux pumps (*F*), reversion to anaerobic metabolism (*G*), decreased pH (*D*), and increased interstitial fluid pressure (*D*). The abnormal vasculature in the microenvironment of tumors (*B*) and (*C*) contributes to hypoxia (selection pressure) as well as to invasion and metastasis (from [1])

maintenance of CSCs in the tumor microenvironment, which refers to a range of signaling pathways and genes. Based on the relevant phenotypes, CSCs can be characterized as a small subpopulation of cancer cells, for example, $CD34^{+}/CD38^{-}$ in leukemia cells, $CD44^{+}/CD24^{-}$ in solid tumors, and $CD133^{+}$ in other tumors. Therefore, therapy strategies immediately applied after general cancer therapy is the most promising treatment option to achieve the goal of targeting CSCs [2].

Mechanisms of Drug Resistance in Tumors and Modulation of Drug Resistance

The characteristics of MDR include abnormal vasculature, regions of hypoxia, upregulation of ATP-binding cassette (ABC) transporters, aerobic glycolysis, and an elevated apoptotic threshold. The major course of antitumor drug resistance involves

five stages: (1) decreased drug influx, (2) increased drug efflux predominantly through ATP-driven extrusion pumps frequently of the ABC superfamily, (3) activation of DNA repair, (4) metabolic modification, and (5) detoxification and inactivation of apoptosis pathways with parallel activation of antiapoptotic cellular defense modalities. Members of the ABC superfamily, such as P-glycoprotein (Pgp/ABCB1), multidrug resistance proteins (MRPs/ABCC), and breast cancer resistance protein (BCRP/ABCG2), can act as ATP-driven drug efflux transporters by forming a unique barrier against chemotherapeutics as well as numerous endotoxins and exotoxins (Fig. 15.2).

Although these mechanisms are independent of each other and can work separately, their functions are constantly interconnected and synergistic. Cancer cells that are adapted to the reduction/loss/alteration of specific drug target, enhanced drug metabolism, and enhanced cellular repair are often resistant to a group of drugs that are similar in either structure or function. For cancer cells that are adapted to reduced drug uptake, enhanced drug efflux and drug compartmentalization alter drug accumulation within cancer cells, leading to resistance to a variety of drugs that are structurally and functionally independent [4].

Drug Delivery in Overcoming the Drug Resistance of Tumors

Various nanovehicles have been specifically designed to overcome the drug resistance of cancer cells. The drug cargo is usually released from the nanovehicle either extracellularly in the tumor or in the tumor microenvironment, i.e., the stroma and vasculature supporting the cancer cells, or intracellularly, typically through cellular uptake by receptor-mediated endocytosis [5].

Nanoparticulate systems such as liposomes, polymeric micelles, and polymer–drug conjugates have led to about two dozen clinically approved therapeutic products [6]. Other nanoparticles (NPs) that reportedly deliver therapeutic cargoes in combination include oil nanoemulsions [7], mesoporous silica NPs (MSNPs) [8], and iron oxide NPs [9, 10] (Fig. 15.3 and Table 15.1).

Using nanocarriers for the treatment of MDR is highly advantageous because they can bypass efflux by ABC transporters. Nanocarriers are internalized by nonspecific endocytosis (or facilitated uptake for targeted nanocarriers), which results in higher intracellular accumulation [46]. Nanotechnology-based cancer therapy accomplishes two or more objectives in one therapeutic strategy and can dramatically improve the therapeutic index of an agent. This strategy can enable the reduction of toxicity by increasing the bioavailability, and it also converts an agent with a low therapeutic potential into a drug candidate.

Multifunctional NPs are often engineered to achieve two or more of the following objectives: drug delivery, RNAi/DNA delivery, active targeting, decreased clearance, imaging/tracking, and stimuli-responsive capabilities. To date, NPs combining a cytotoxic drug and an agent for neutralization of a well-defined mechanism of drug resistance have been tested in vivo, but none has reached clinical trials yet.

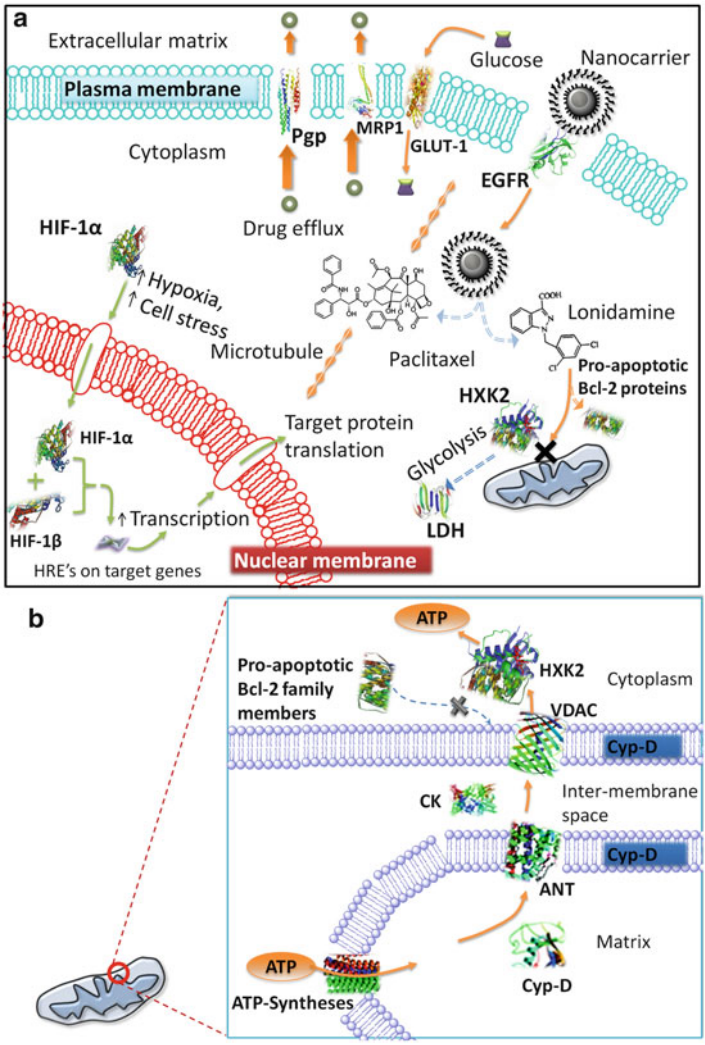


Fig. 15.2 (**a**) MDR characteristics and treatment strategy. HIF-1α is located in the cytoplasm and associated with a complex of regulatory proteins under normoxic conditions. Under hypoxic conditions and cell stress, HIF-1α translocates into the nucleus, complexes with HIF-1β, and then binds to hypoxia-responsive elements on target genes that increase transcription and subsequent translation (e.g., EGFR). Current treatment strategies utilize a nanocarrier modified with EGFR-specific peptides to capitalize the overexpression. This receptor targeting allows facilitated uptake of the formulation, followed by the release of active agents. Receptor targeting of this nanocarrier system to the EGFR receptor should decrease residual toxicity associated with traditional chemotherapy, whereas the combination of paclitaxel with lonidamine offers a unique strategy for terminating the energy supply of MDR cancer and induce apoptosis. (**b**) Hexokinase 2 (HXK2) and lonidamine. This figure depicts the association of HXK2 with the components of the mitochondrial permeability transition pore complex (mtPTP) and coupling of the components to mitochondrial ATP synthase. ATP exits the mitochondria bypassing ATP synthase to the adenine nucleotide translocator (also located in the inner mitochondrial membrane), to the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane. Association of HXK2 with VDAC prevents binding of pro-apoptotic Bcl-2 family member proteins to the mtPTP (from [3])

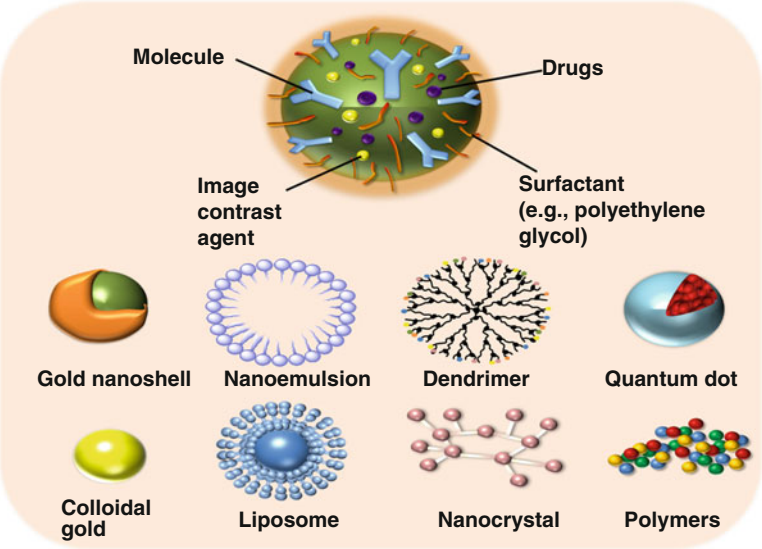


Fig. 15.3 As multicomponent multifunctional systems, nanoparticles can be functionalized with hydrophilic polymers (e.g., PEG), targeting molecules (e.g., antibodies), drugs, and imaging contrast agents. The interior core can be solid (e.g., quantum dots), liquid (e.g., liposomes), or contain an encapsulated drug (from [11])

Table 15.1 Selected examples of formulation-based therapy toward overcoming drug resistance in cancer

	Approach to overcoming the drug resistance of tumors		
Nanocarrier platform	Changing subcellular localization	Increasing intracellular availability	Targeting cancer stem cells
Nanoparticles	[12–15]	[16–19]	
Micelles		[20–24]	
Liposomes		[25, 26]	[27]
Dendrimers	[28]	[29–32]	
Polymer–drug conjugate		[33, 34]	[35]
Mesoporous silica nanoparticles	[36]	[37, 38]	
Iron oxide nanoparticles		[9, 10]	
Oil nanoemulsion		[7]	
Others	[39]	[21, 40–45]	

Nanocarriers for Tumor-Targeted Delivery

Several specific approaches are currently being explored as strategies for future cancer therapy using nanomedicines for the delivery of chemotherapeutic drugs, chemosensitizers targeting drug resistance proteins, or diagnostic aids. Accumulation

of nanocarriers at the tumor site is actually enhanced relative to the normal tissue because of the enhanced permeability and retention (EPR) effect [47]. The EPR effect results in higher accumulation of nanocarriers at the tumor site as compared with the control because of the leaky vasculature that allows passage of nanocarriers into the tumor matrix. Meanwhile, receptor targeting is also being extensively explored in experimental and clinical researches. Receptor targeting is aimed at selectively increasing the accumulation of a nanocarrier system at the tumor site by engaging a biological target that is overexpressed in cancer cells. The surface of the nanocarriers is modified with a ligand or antibody for receptor targeting, antigen targeting, or carbohydrate targeting.

The approaches to targeted nanocarrier for overcoming drug resistance are: (1) targeting the proliferating bulk of tumor cells and their intracellular compartments, (2) addressing the crosstalk between tumor cells and their microenvironment in an attempt to minimize the contribution of the stroma and vasculature to tumor cell survival and proliferation as well as to minimize drug resistance, and (3) targeting CSCs or tumor-initiating cells (TICs) [48].

Targeted delivery to the bulk of tumor cells has been extensively studied, and folate, EGFR-2 (or HER2), and transferrin are some of the most commonly used ligands. Similarly, the attachment of anti-HER2 onto NP surfaces also improves the cellular internalization of gelatin/albumin and gold NPs. Transferrin, an iron-binding glycoprotein, is a well-studied ligand for tumor targeting because of the upregulation of its receptors in numerous types of cancer. Meanwhile, various approaches aimed at targeting the microenvironment of tumor cells or the cross talk between tumor cells and their supporting stroma and/or vasculature are being developed.

Hypoxic conditions in many tumors can be potentially selected for the development of nanocarriers with redox-specific labile bonds, which can selectively target the microenvironment as well as increase drug accumulation and efficacy. Moreover, the depletion of oxygen levels in tissue (i.e., hypoxia) has long been considered as a major feature of the tumor microenvironment, which is a potential contributor to the enhanced tumorigenicity of CSCs. Targeting hypoxic factors with small interfering RNA (siRNA) or topoisomerase inhibitors are reportedly effective in overcoming drug resistance in preclinical studies. Therefore, the development of effective, systemic, and therapeutic approaches specifically focused on the tumor microenvironment is highly desirable. HIF-1α is an attractive therapeutic target because it is a key transcription factor in tumor development and only accumulates in hypoxic tumors. Cationic mixed micellar NPs consisting of amphiphilic block copolymers poly(ε-caprolactone)-block-poly(2-aminoethylethylene phosphate) (PCL29-b-PPEEA21) and poly(ε-caprolactone)-block-poly(ethylene glycol) are suitable carriers for HIF-1α siRNA to treat hypoxic tumors. These NPs are an excellent example of a clinical strategy of specific siRNA therapy for cancer treatment aimed at the hypoxic tumor microenvironment [49].

The concept of CSCs has been explored since the late 1930s, and these concepts have been solidified and received considerable attention in recent years. The two main aspects concerning CSCs are as follows: (1) CSCs are regular SCs that

uncontrollably grown and caused cancer and (2) CSCs arise from a subpopulation of cancer cells. In many situations, both of these concepts are rational and related to the microenvironment of a tumor. The survival and accumulation of drug-resistant CSCs following chemotherapy or radiotherapy are common explanations for the recurrence of increasingly invasive and malignant tumors. Many novel molecular targets are bound to be developed with continued in-depth research on CSCs, although it can be challenging for cancer therapy. As such, inhibiting the SC factor in MDR cells may increase the effectiveness of treatment by reducing the apoptotic threshold of these cells.

To date, the main directions in the treatment of drug-resistant cancer cells and CSC targeting are associated with four main areas. First is the design of novel gene-targeting therapies (e.g., siRNA, miRNA, and antisense oligos) against the proteins responsible for the intrinsic drug resistance and survival of CSCs, such as drug efflux transporters, antiapoptotic proteins, and members of underlying signaling pathways. Second is the development of novel and efficient small drug molecules and inhibitors, as well as polymeric drug conjugates and nanocarriers, which are able to target to the niche of CSCs. Third is the development of sensitive bio-imaging approaches, including theranostics, for the precise location of CSCs. Fourth is the potential application of physical destruction methods, such as thermoablation, photodynamic therapy (PDT), laser therapy, and surgery.

Multiple transporters have been found in CSCs, including Pgp, BCRP, and MRP. The expression of MRP1 (ABCC1) and the activity of the apoptosis inhibitor β-livin cause a high survival rate for glioblastoma CSCs after etoposide treatment. Cell surface markers expressed by CSCs/TICs are generally shared by normal somatic SCs. However, the differences between the subtle surface antigens as well as signaling pathway and metabolic alterations of CSCs/TICs and normal somatic SCs may be exploited for the selection for targeted delivery of NPs in this field. For example, the overexpression of CD44 in cancer cells is strongly linked to therapeutic drug resistance. Another marker, $CD133^{+}$, previously found in abundance in the embryonic epithelium, is also expressed in CSCs of many cancers. Therefore, CSC targeting can be potentially applied using surface carboxylic groups. Many CSC-associated surface biomarkers, such as CD44 and CD133, can be utilized for targeting dot in anticancer therapies by vectorized nanocarriers. Recently, Wang et al. [49] designed anti-CD133 mAb-conjugated single-walled carbon nanotubes. They demonstrated that these nanotubes can selectively target $CD133^{+}$ glioblastoma cells and assist in their photothermal destruction by a NIR laser.

The concept of "a niche" maintenance in CSCs is widely accepted by researchers because of its specific protective microenvironment as one of the intrinsic properties of CSCs. This property potentially allows them to hide in a quiescent state in tissues and avoid the damage of chemotherapy. Various physicochemical methods for the specific destruction of CSCs and the CSC-supporting environment (niche) are currently being investigated. The Notch pathway plays a critical role in the connection between angiogenesis and self-renewal of CSCs and can thus be considered as a potential therapeutic target. The niche is defined as the microenvironment where CSCs are located and where they interact with other types of cells. Evidently, the

CSC niche is a dynamic supportive system that contains a variety of cell types, cytokines, and signaling pathways. Several Notch inhibitors are being developed [50]. Mamaeva et al. [51] recently described the application of another type of nanocarriers, namely, MSNPs, for the targeted delivery of γ-secretase inhibitors of Notch signaling, which are potentially effective against CSCs.

Wnt signaling is another well-known pathway that plays a major role in embryogenesis and cancer development. Similarly, blocking the Wnt pathway in $CD133^+$ colon cancer cells results in the reversal of their resistance to 5-fluorouracil [52].

Increasing Intracellular Availability and Changing Subcellular Localization

Reaching the tumor site as well as their intracellular site of action is important for therapeutics. For this phenomenon to occur, the therapeutics must escape the endosomal pathway and subsequent lysosomal degradation. Many strategies have evolved to ensure endosomal escape. A popular strategy is to modify the particles with cell-penetrating peptides that enable cell entry while evading lysosomal degradation. Another method for endosomal escape is to use pH-sensitive nanocarriers such as poly(ethylene glycol) (PEG)-modified dioleoyl phosphatidylethanolamine pH-sensitive liposomes. The pH of the intra-tumor destabilizes the liposomes, causing them to fuse with the endosomal membrane and subsequently release the cargo into the cytoplasm. Similarly, an optimized, pH-sensitive, mixed micelle system conjugated with folic acid is prepared to challenge MDR in cancers. The micelles are composed of poly(histidine-co-phenylalanine)-b-PEG and poly(L-lactic acid)-b-PEG-folate. Doxorubicin (DOX)-loaded micelles effectively kill both wild-type sensitive (A2780) and DOX-resistant ovarian MDR cancer cell lines (A2780/DOX(R)) through an instantaneous high dose of DOX in the cytosol, which results from active internalization, accelerated DOX release triggered by endosomal pH, and endosomal membrane disruption [53].

For polymer micelles, some polymers have effects on the function or expression of some efflux pump proteins. For example, Pluronic 85 (P85) can prevent the development of MDR1 phenotype in leukemia cells in vitro and in vivo as determined by Pgp expression and functional assays of the selected cells. In addition to mdr1, P85 alters the changes in genes implicated in apoptosis, drug metabolism, stress response, molecular transport, and tumorigenesis [54]. Meanwhile, our previous studies demonstrated that liposomes not only increase DOX levels allocated to nuclei but also extended retention in the nuclei of resistant cells [55]. Many clinical first-line anticancer drugs, such as DOX, camptothecin (CPT), and cisplatin, are DNA toxins that destroy DNA or its associated enzymes. Their cytotoxicity is maximized once they are inside the nucleus probably because of the direct damage to DNA. Thus, similar to therapeutic genes, these drugs have to localize in the nucleus to exert their pharmacological effects. For drug-resistant tumor cells, the drug can

pump out for the existence of Pgp protein. Thus, encapsulating the drug in the nanocarrier is important to overcome the function of Pgp. A polylactide-surfactant block copolymer poly(L-lactide)-vitamin E TPGS (PLA-TPGS) has been synthesized using bidentate sulfonamide zinc ethyl complex as an efficient catalyst, and its self-assembled NPs are used as carriers of DOX. The activity of Pgp in drug-resistant breast cancer MCF-7/ADR cells is found to decrease after incubation with PLA-TPGS NPs. In addition, the nuclear accumulation and cytotoxicity of DOX are significantly increased by encapsulation of the drug into the NPs [56].

Similar results are obtained using a biodegradable polymer coupled to a photosensitizer, and the resulting photosensitizer NPs are loaded with the chemotherapeutic agent DOX. The combination of photosensitizer and chemotherapeutic agent has a synergistic action on a DOX-resistant breast cancer MCF-7 cell line. This combination of photodynamic activity in a powerful nanocarrier loaded with the chemotherapeutic agent DOX can be used to deliver two types of cancer therapy simultaneously, and the addition of TPGS can further enhance the entry of DOX into the nucleus [57].

As reported, the unique and evolutionary highly conserved major vault protein (MVP) is the main component (more than 70 %) of vaults, which are ribonucleoparticles with a hollow barrel-like structure that still contains two additional proteins and vault RNAs (vRNA). Identification of MVP with human lung resistance protein, together with its upregulation in Pgp-negative chemoresistant cancer cell lines, suggests that vaults play a role in cellular detoxification processes and consequently contribute to MDR by drug sequestration or shuttling drugs from the nucleus to cytoplasmic vesicles [58]. Thus, polyamidoamine (PAMAM) dendrimers are functionalized by a polysaccharide hyaluronic acid (HA) to effectively deliver DOX as well as MVP-targeted siRNA to improve DOX chemotherapy in MCF-7/ADR cells by downregulating MVP expression. As a result, co-delivery of siRNA and DOX by PAMAM-HA exhibits satisfactory gene silencing effect as well as enhanced stability and efficient intracellular delivery of siRNA. This phenomenon allows DOX to enter into the nucleus efficiently and induce more subsequent cytotoxicity than when siRNA is absent as a result of MVP knockdown [29].

For the cell interior, the mitochondrion is the major organelle implicated in the cellular bioenergetic and biosynthetic changes accompanying cancer. These bioenergetic modifications contribute to the invasive, metastatic, and adaptive properties typical in most tumors. Moreover, mitochondrial DNA mutations are linked to the bioenergetic changes in cancer. Targeting to tumor cell metabolism or mitochondria has been proposed as a novel strategy for the treatment of tumor. The most important aspect in the physiology of cancer is the role of mitochondria in energy metabolism and cell cycle regulation. Strong evidence supports the rationale for the development of anticancer strategies based on mitochondrial targets. Mitochondria play a key role in the complex apoptotic mechanism and trigger cell death through several mechanisms, such as disrupting electron transport and energy metabolism, releasing or activating proteins that mediate apoptosis, and altering the cellular redox potential.

Nanotechnology, which encompasses materials and methods at the nanoscale, is an attractive approach to designing mitochondrial therapeutics that either target or avoid mitochondria. Nanosystems that target mitochondria can enhance efficacy in treating mitochondrial diseases, whereas those that avoid mitochondria may help reduce mitochondrial toxicity. The surface modification of nanocarriers can also be tailored to achieve subcellular localization such as mitochondrial targeting, which is often achieved using mitochondrial leader sequences or the negative membrane potential of mitochondria through the use of "mitochondriotropics." Mitochondriotropics are molecules that have delocalized positive charges such as triphenyl phosphonium [59].

For mitochondrial targeting, the selective accumulation of Au NPs in the mitochondria of cancer cells has been reported [60]. Their long-term retention decreases the mitochondrial membrane potential and increases the reactive oxygen species level that enhances the likelihood of cell death. Taking advantage of the development of SV30, a new analog of the pro-apoptotic molecule HA14-1, 57 nm organic solvent-free lipid nanocapsules loaded with SV30 (SV30-LNCs) are formulated using an inversion phase process. Encapsulated SV30 is found to improve mitochondrial targeting, which may elicit considerable interest toward the development of mitochondrion-targeted nanomedicines [61]. In addition, the known mitochondriotropic ligand triphenyl phosphonium (TPP) has been conjugated on the surface of a dendrimer. A fraction of the cationic surface charge of G(5)-D is neutralized by partial acetylation of the primary amine groups. The newly developed TPP-anchored dendrimer (G(5)-D-Ac-TPP) is efficiently consumed by the cells and demonstrates good mitochondrial targeting [62].

Combination Therapy Toward Overcoming Drug Resistance

Many combinatorial NP formulations have been successful in reversing MDR in vitro and in vivo of cancer models by co-delivering chemosensitizing agents and chemotherapy agents. Among many cellular mutations that diminish the effectiveness of anticancer drugs, the overexpression of multidrug transporters and altered apoptosis are the two underlying mechanisms by which cancer cells acquire resistance to multiple structurally and mechanistically unrelated drugs. NPs of 10–200 nm in diameter have shown more favorable antitumor pharmacokinetic profiles than small-molecule drugs. These drug-loaded NPs exhibit prolonged systemic circulation lifetime, sustained drug release kinetics, and advanced tumor accumulation [6]. Various NP platforms such as liposomes, polymeric micelles, dendrimers, nanoemulsion, and mesoporous silica particles have been used to carry broad classes of therapeutics, including cytotoxic agents, chemosensitizers, siRNA, and antiangiogenic agents (Fig. 15.4 and Table 15.2).

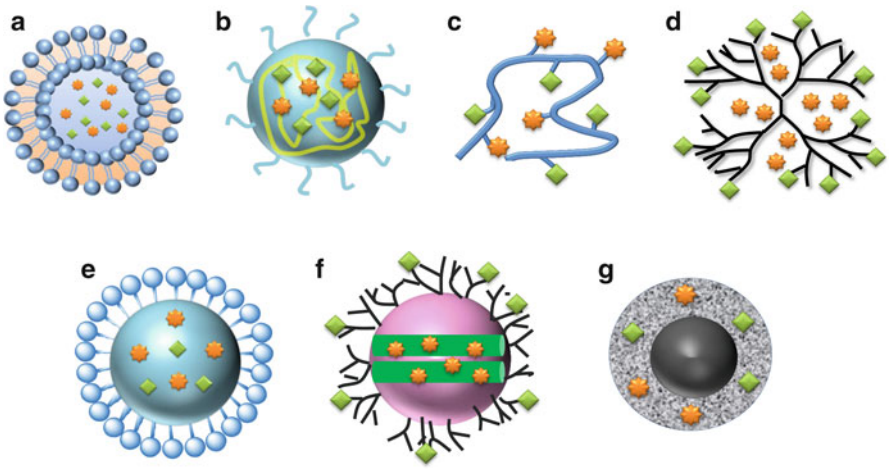


Fig. 15.4 Schematic of nanoscale drug carriers used for combinatorial drug delivery: (**a**) liposome, (**b**) polymeric micelle, (**c**) polymer–drug conjugate, (**d**) dendrimer, (**e**) oil nanoemulsion, (**f**) mesoporous silica nanoparticle, and (**g**) iron oxide nanoparticle (from [63])

Combination of Drug Delivery and Drug Efflux Modulation

Drug resistance is considered to be the main reason for therapeutic failure in advanced cancer treatment. In many cases, drug transporter proteins (e.g., Pgp and MRP) that can pump out the intracellular drug are always overexpressed in most drug-resistant cancer cell lines. These drug transporter proteins are some of the most extensively characterized barriers to chemotherapy. Accordingly, a number of nanocarriers have been designed to sensitize drug-resistance tumor cells because they can aid in drug escape from the transporters, inhibit ATPase activity, or indirectly deplete cellular ATP, thereby leading to enhanced intracellular accumulation of therapeutic agents [68].

During chemotherapy, one of several ABC drug transporters, such as Pgp, MRP1, or ABCG2, becomes upregulated in some cancer cells. This phenomenon causes insensitivity to drugs and, subsequently, drug resistance. To date, the genes for 48 ABC proteins have been identified in the human genome and subdivided into seven families (ABC A–G) based on structural and sequential similarities. The decrease in intracellular drug accumulation is always caused by an undetermined energy-dependent, carrier-mediated mechanism. Not until 1976 was a 170 kDa cell membrane glycoprotein named Pgp discovered, and its link to the MDR phenotype was confirmed by Juliano and Ling [88]. In addition to Pgp (ABCB1), MRP1 (ABCC1), and ABCG2, at least 12 other ABC transporters are currently linked to MDR or can cause reduced intracellular drug accumulation.

Attempts on inhibitor-based chemosensitization and on the identification of new inhibitors of ABC transporters are currently ongoing. A large number of cancer-treating drugs have been identified as substrates of Pgp, including Vinca alkaloids,

Table 15.2 Selected examples of formulation-based combination therapy toward overcoming drug resistance in cancer

Nanocarrier platform	Combined or sequential treatment	Main bioactive or cytotoxic cargo	Tumor type	Approach to overcoming drug resistance of tumor	Ref.
Nanoparticles	Curcumin	Doxorubicin	K562/ADR cells	Modulation of drug efflux and apoptotic threshold	[64]
	Lonidamine	Paclitaxel	SKOV3-TR cells and the MDA-MB-435 cells	Modulation of apoptotic threshold	[3]
	Bcl-2 siRNA	Cisplatin	HEp-2 cells and HeLa cells	RNA interference therapy	[65]
	Photodynamic therapy	Doxorubicin	NCI–ADR–RES cells	Combination drug and energy	[66]
	Tariquidar	Paclitaxel	JC and NCI–ADR–RES cells	Modulation of drug efflux	[67]
	Pgp siRNA	Paclitaxel	JC cells	RNA interference therapy	[68]
	Survivin shRNA	Paclitaxel	A549/ADR cells	RNA interference therapy	[69]
	Curcumin	Nutlin-3a	Y79 cells	Modulation of apoptotic threshold	[70]
	Idarubicin	Doxorubicin	P388/ADR cell lines	Modulation of drug efflux	[71]
	MDR-1 siRNA	Paclitaxel	$SKOV3_{TR}$ cells	Modulation of drug efflux	[72]
	Mitomycin C	Doxorubicin	MDA-MB-435/LCC6/MDR1 cell line	Modulation of drug efflux	[73]
	Fluorodeoxyuridine	Cytarabine	HepG2 cells	Modulation of drug efflux	[74]
	Photosensitizer	Doxorubicin	MCF-7/ADR cell line	Combination drug and energy	[57]
	C6-ceramide	Paclitaxel	SKOV-3 xenografts	Modulation of apoptotic threshold	[75]
	Tamoxifen	Paclitaxel	$SKOV3_{TR}$ cells	Modulation of apoptotic threshold	[76]
	Verapamil	Vincristine	MCF-7/ADR cell line	Modulation of drug efflux	[77]

(continued)

Table 15.2 (continued)

Nanocarrier platform	Combined or sequential treatment	Main bioactive or cytotoxic cargo	Tumor type	Approach to overcoming drug resistance of tumor	Ref.
Liposomes	MRP1 siRNA and BCL-2 mRNA	doxorubicin	H69AR/MDR, MCF-7/ADR and HCT15 cell	RNA interference therapy	[78]
	Amlodipine	Topotecan	HL-60/MDR cell line	Modulation of drug efflux	[79]
	Quinacrine	Vincristine	Drug resistant human leukemia cells	Modulation of drug efflux	[80]
	Tariquidar	Paclitaxel	SKOV-3 cell line	Modulation of drug efflux	[81]
	Verapamil	Doxorubicin	K562 leukemia cells	Modulation of drug efflux	[82]
Micelles	Photosensitizer	Doxorubicin	MCF-7/ADR cell line	Combination drug and energy	[83]
	MDR-1 siRNA	Doxorubicin	MCF-7/ADR cell line	Modulation of drug efflux	[84]
Mesoporous silica nanoparticles	P-Glycoprotein siRNA	Doxorubicin	KB-V1/ADR cells	RNA interference therapy	[85]
	MRP1 and BCL2 mRNA	Doxorubicin and cisplatin	A549 cell line	RNA interference therapy	[86]
	Bcl-2 siRNA	Doxorubicin	A2780/AD cells	Modulation of apoptotic threshold	[8]
Iron oxide nanoparticles	Rapamycin	Paclitaxel	MCF-7/ADR cell line	Modulation of drug efflux	[9]
Dendrimers	MVP siRNA	Doxorubicin	MCF-7/ADR cells	RNA interference therapy	[29]
Nanoemulsion	Curcumin	Paclitaxel	$SKOV3_{TR}$ cells	Modulation of apoptotic threshold	[7]
Gold nanorods	ASCL1 siRNA	Doxorubicin	NE cancer cells	RNA interference therapy	[87]

taxanes, etoposide, teniposide, colchicines, actinomycin D, CPTs, imatinib mesylate, saquinavir, methotrexate, and mitoxantrone. An appealing approach to overcoming MDR is the co-administration of a chemotherapeutic agent and a Pgp inhibitor. Nanocarrier systems containing a combination of cytotoxic drugs and efflux pump inhibitors, such as cyclosporine, verapamil, and tariquidar, have been used to suppress the MDR effect. The first attempt to co-deliver a chemosensitizer with chemotherapeutics in a single nanocarrier was a polyalkylcyanoacrylate NP system loaded with Pgp inhibitor cyclosporin A (CyA) and DOX [89]. Against a DOX-resistant leukemia cell line (P388), the co-encapsulation of CyA and DOX induces nearly a twofold increase in toxicity compared with DOX-only NPs. The enhanced efficacy is not observed when free CyA is applied with the DOX-only NPs. This finding suggests that the NP-coordinated delivery of two bioactive agents is essential for their cooperative activity. Various drug delivery nanovehicles are engineered to evade or overcome drug extrusion by drug efflux transporters, thereby resulting in enhanced chemotherapeutic drug accumulation in the cytosol and/or the nucleus of cancer cells and consequent elimination of tumor cells. These nanovehicles include oil nanoemulsions, polymeric micelles, liposomes, copolymeric NPs conjugated to quantum dots, and metallic NPs.

Permanent elimination or deactivation of any ABC transporter is unrealistic and unreasonable because of their important physiological and pharmacological roles in the human body. However, MDR in cancer caused by the overexpression of ABC drug transporters can be transiently modulated by various means, including direct inhibition, gene silencing, transcriptional regulation, and drug encapsulation. However, no clinically applicable inhibitor of ABC transporters exists to date. The reason for the unsuccessful clinical trials is complex but may be predominantly due to the unfavorable toxicity of inhibitors. MDR in cancer is apparently caused by multiple mechanisms that operate either independently or in unison. Overexpression of drug transporters is just one of the many reasons that cancer cells have adapted to survive the diversity of agents used in cancer chemotherapy. Over 30 years has passed since the discovery of Pgp in 1976, yet no simple and feasible solution to overcoming MDR in cancer has been discovered. The complexity and identification of new MDR-linked ABC transporters produce more challenges. Nevertheless, based on the new discoveries and advancements made on the identification, biological characterization, and structural analysis of MDR-linked ABC transporters over the years, we are one step closer to understanding clinical MDR in cancer.

Combination Drug Delivery and Modulation of Apoptotic Threshold

Cell apoptosis requires a minimum cellular threshold to be overcome. In cancer cells, this threshold is elevated to the extent that extracellular and intracellular insults sufficient in inducing apoptosis in normal cells have no effect. MDR cells have developed various mechanisms for increasing their apoptotic threshold. Decreased ceramide levels and the Warburg effect are the two major mechanisms that MDR cells utilize to increase their apoptotic threshold. The response to MDR

is associated with alterations in the apoptosis pathways. Therapeutic NPs have been developed to co-encapsulate compounds that repair the dysfunctional apoptotic signaling. One example of such pro-apoptotic compound is ceramide, which is produced by cells under environmental stress and serves as a key messenger in programmed cell death.

An increasing number of studies have implicated ceramide, sphingosine-1-phosphate, as well as the genes involved in their biosynthesis, catabolism, and signaling, in various aspects of oncogenesis, cancer progression, as well as anticancer drug resistance and radiation resistance. Based on these findings, several research groups have used the strategy of inducing elevated levels of ceramide to decrease the threshold of apoptotic signaling in MDR cells while simultaneously delivering a cytotoxic drug (e.g., paclitaxel) using polymeric NPs.

A polymeric micelle formulation based on poly(ethylene oxide)-poly(epsilon-caprolactone) (PEO-PCL) for co-delivering exogenous ceramide and paclitaxel to address ceramide metabolism has been developed [90]. Against a paclitaxel-resistant ovarian cancer cell line (SKOV-3TR), the combinatorial formulation is found to increase the paclitaxel sensitivity of MDR cells to the same level as non-MDR cells. Combination with ceramide shows a 100-fold increase in efficacy compared with paclitaxel-only NPs. In another study, polymeric blend NPs have been prepared for the co-encapsulation of paclitaxel and C6-ceramide (CER), a synthetic analog of ceramide [75]. In vivo studies indicate that combination therapy with NPs harboring both paclitaxel and CER can enhance apoptotic signaling and reduce the tumor volume at least twofold compared with traditional standard paclitaxel monotherapy [75]. Yet another approach to increasing intracellular ceramide is the use of siRNA to silence glucosylceramide synthase. This strategy decreases the expression of Pgp in MDR cells, verifying the significance of ceramide in apoptotic modulation [91].

Combination Drug Delivery and Intracellular pH Modulation

The decreased pH associated with MDR cells has been utilized in many strategies for overcoming MDR. Some strategies are aimed at altering intracellular pH; others make use of pH-sensitive constituents to control the release of drugs. Novel pH-responsive polymers such as poly(β-amino ester), soluble below pH 6.5, are incorporated into NP formulations to localize the release of therapeutic agents in the acidic cellular environment of tumors and subcellular endosomal/lysosomal compartments.

Drugs encapsulated in pH-sensitive polymeric micelles have also been developed to target MDR cancer. Zwitterionic oligopeptide liposomes (HHG2C(18)-L) containing a smart lipid (1,5-dioctadecyl-L-glutamyl 2-histidyl-hexahydrobenzoic acid, HHG2C(18)) have been developed to overcome the barriers faced by anticancer drugs on the route from the site of injection into the body to the final antitumor target within transport steps with multiple physiological and biological barriers. HHG2C(18)-L shows a multistage pH response to the tumor cell (the mitochondria

in this case). Their multistage pH response leads to more effective entry of anticancer agents into the tumor cell, improved escape from the endolysosomes, and accumulation in the mitochondria [92].

Nanocarriers for Combination Drug and Energy

Any organ heated to temperatures between 41 and 46 °C is defined as hyperthermia. Hyperthermia leads to reversible cell damage; however, when used as an adjunct treatment, it can help increase the efficacy of chemotherapy and enhance radiation-induced tumor damage. Hyperthermia has been utilized to change the morphology of a tumor to enhance the delivery of polymeric and liposomal NPs by increasing the blood flow to the tumor. It has also been successfully combined with DOX-loaded liposomes that target the folate receptor of tumor cells [93]. These temperature-sensitive systems can be designed to release drug payloads in the presence of specific temperature triggers.

In addition, clinical improvements to ultrasound focusing are being developed to improve the control and precise targeting of ultrasonic waves [94]. Combining localized ultrasound with nanocarrier therapies can exert a dramatic effect on the reduction of the residual toxicity associated with chemotherapy. Meanwhile, PDT is a form of cancer treatment that involves the use of photosensitizers as therapeutic agents. Under light irradiation, photosensitizers enter a triplet state of excitation. This triplet state of energy is easily transferred to oxygen molecules, which are subsequently converted into reactive oxygen species that are capable of damaging cells [95]. This method of treatment has high selectivity because only the cells exposed to both light and photosensitizer are affected.

RNA Interference to Overcome MDR

The clinical applications of small-molecule drugs that inhibit Pgp are not all successful. Hence, therapeutic strategies using RNA interference technology to overcome MDR are actively being explored. siRNA is a short double-stranded RNA that shows specific and effective gene silencing activity by the sequence-specific downregulation of a complementary messenger RNA. Therapeutic applications of siRNA have been limited because of their rapid enzymatic degradation by ribonuclease activity in serum and poor cellular uptake by passive diffusion [96]. The reversibility of the MDR phenotype of human cancer cells through the activation of the RNAi pathway by knocking down the MDR1/Pgp encoding mRNA was first reported in 2003 [97].

Drug efflux transporter genes that are being targeted include ABCB1 (MDR1/Pgp) and ABCC1 (MRP1), and these genes have been studied for decades. Gene silencing may be achieved at the mRNA level using siRNA constructs or antisense oligodeoxynucleotides (asODNs), which results in decreased MDR1 expression. Various drug delivery carriers for the targeted silencing of drug resistance genes

have been described, including liposomes and different polymers, typically of cationic nature such as chitosan and its derivatives. A micellar system consisting of degradable poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) block copolymers with functional groups on both blocks has been prepared. The functional group on the PCL block is used to incorporate short polyamines for complexation with siRNA or to chemically conjugate DOX using a pH-sensitive hydrazone linkage. This system is used to improve the efficacy of DOX in multidrug-resistant MDA-MB-435 human tumor models that overexpress Pgp. The improvement is carried out by the simultaneous intracellular accumulation of DOX and siRNA against Pgp expression [98].

Targeting MDR1 gene transcripts has also been developed by harnessing bacterium-derived minicells encapsulating specific siRNA duplexes and chemotherapeutics [99]. Minicells targeted by specific antibodies to surface receptors of tumor cells are then used to deliver synergistic cargoes to tumor xenografts with high specificity.

Among the mechanisms of drug resistance independent of drug efflux pumps that have been targeted with an NP approach, some modalities are related to the Bcl2 and HIF1α genes. Bcl2 family proteins are regulators of programmed cell death (particularly apoptosis), and the HIF1α gene encodes for a transcription factor that plays a key role in the cellular response to hypoxia. Gene silencing is performed using siRNA or as ODNs. MSNs are utilized for the simultaneous delivery of Dox and Bcl2 siRNA [8]. Dox-loaded MSNs modified with amine-terminated PAMAM dendrimers facilitate conjugation with Bcl2 siRNA. Moreover, the simultaneous delivery of Bcl2 siRNA significantly suppresses Bcl2 mRNA and efficiently overcomes the MDR phenotype presumably using an inhibitory activity that these PAMAM dendrimer-based NPs exert on Pgp-mediated drug efflux [8].

A chemotherapeutic agent (DOX) and Pgp siRNA can be co-encapsulated by MSNPs and transported to a drug-resistant cancer cell line (KB-V1 cells), subsequently accomplishing cell killing in an additive or synergistic fashion [85]. Although a number of research have reported the RNAi modulation of cancer MDR in vivo, the lack of an efficient delivery strategy for administering shRNA to cancer patients is the major drawback. Various strategies have been explored but with no successful results. These studies demonstrate that a more efficient mode of delivery and nanocarriers, which are a promising platform for the efficient delivery of RNAi, is important in the clinical application of RNAi.

Conclusion

The unsatisfactory therapeutic effect of chemotherapy in treating solid tumors is multifactorial, and the occurrence of clinical tumor drug resistance is usually caused by a complex and unknown mechanism. Moreover, solid tumors are heterogeneous, structurally complex, and contain different kinds of cell. To the best of our knowledge,

although various nanocarrier platforms for targeted delivery of anticancer drugs have already undergone in vivo testing in animal models and clinical evaluation in humans, no reports exist on NPs for the delivery of drug combinations aimed at overcoming drug resistance. The development of appropriate combinations of chemotherapies and nanotherapies, including novel gene-silencing, drug efflux-inhibiting, and CSC-targeting strategies, are the most effective methods of treating drug-resistant and aggressive tumors.

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Chapter 16
Stem Cells and Cancer

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Abstract It is widely accepted that cancers arise from cancer stem cells that possess self-renewal capacity and can recapitulate the tumors derived from it. However, the cellular origin of cancers is not clear. Emerging evidence is accumulating showing that cancers of distinct subtypes within an organ may derive from different "cell of origin." Cancer stem cells may originate from long-lived normal stem cells, transit amplifying progenitor cells or more committed differentiated cells through different mechanisms by which cancer cells acquire unlimited self-renewal capacity. The identification of these crucial target cell populations may allow earlier detection for the prevention of malignancies, and it may also provide targets that could be exploited for eventual elimination of cancers with aggressive phenotype.

Introduction

Although the cancer death rate was decreased by 1.8 % per year in men and by 1.6 % per year in women during the recent past 5 years in the USA, a total of 1,638,910 new cancer cases and 577,190 deaths from cancer are predicted to occur in the USA in 2012 [1]. The cause of the cancer death is mainly attributed to tumor relapse and metastases after conventional treatment because of de novo (intrinsic) or acquired (extrinsic) drug-resistant of cancer cells. Mounting evidence has demonstrated that cancers could arise from a rare population of cells that possess self-renewal capacity, and can recapitulate the tumors that derived from it [2, 3]. These cells are named as cancer stem cells (CSCs). Considerable evidence has suggested

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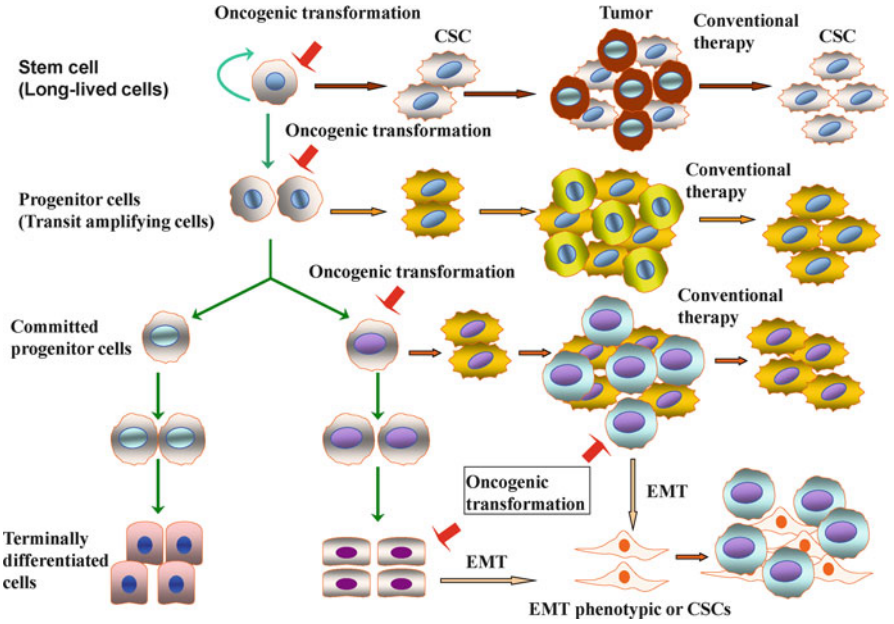

Fig. 16.1 The cellular origin of cancer and cancer cell drug resistance. The cancer cells could be originated from quiescent long-lived normal stem cells, transit amplifying and cycling progenitor cells or committed differentiated cell through oncogenic transformation. Cancer cells also could be generated through the acquisition of epithelial-to-mesenchymal transition (EMT) phenotype that is reminiscent of cancer stem cells (CSCs). Tumor cells are composed of non-tumor initiating cells and tumor initiating CSCs that are responsible for tumor recurrence after conventional treatment, and the CSCs are responsible for the development of tumors, tumor drug resistance, tumor progression, and metastasis

that CSCs are responsible for drug resistance after therapy [4–6]. Therefore, identification of the origin of CSCs is one of many prerequisites for targeted therapeutic strategy to eliminate CSCs, and thereby preventing cancer development, recurrence and progression. However, tumors exhibit significant heterogeneity in their cellular morphology, proliferative capacity, genetic lesions, gene expression profiling, and therapeutic response even in the same tissue of origin. To date, the cellular origin of cancers is still elusive. This chapter focuses on reviewing the evidence for cellular origin of cancers in various tissues, and also discusses the source of CSCs that are generated from the acquisition of epithelial-to-mesenchymal transition (EMT) phenotype that resembles CSCs so that strategies could be developed for the elimination of the root of the cancer such as CSCs. The concept of EMT and CSCs are presented in a hypothetical diagram (Fig 16.1).

Normal Adult Stem Cells

Normal adult stem cells are cells retaining the extensive self-renewal and differentiation capacities, and as such constitute a population of long-lived cells that are not only responsible for daily turnover in maintaining the tissue homeostasis but also underlie

the regenerative response upon tissue injury. Emerging evidence indicates that there is dual activity of stem cells in the same tissues: cycling or transit amplifying stem cells (transit amplifying progenitor) and quiescent stem cells or long-lived stem cells [7]. The cycling stem cells fuel the turnover to maintain the tissue homeostasis by dividing into determined differentiated cell types, and the quiescent stem cells are responsible for regenerative response upon tissue injury by dividing asymmetrically into two daughter cells: one remaining to continue the process of cell renewal, and the other daughter cell starting the process of differentiation [7].

Identification and isolation of normal stem cells is necessary for the elucidation of the cellular and molecular mechanisms that maintain tissue integrity and for designing more effective treatments for cancer that is believed to be initiated from rare population of CSCs, although it is important that such treatment strategy must identify distinction between normal stem cells and CSCs. Analysis of the properties and regulatory mechanisms of stem cells, however, has been limited by the lack of defined markers for their prospective isolation. Shackleton and coworkers have isolated mammary stem cells (MaSCs) with a $Lin^{-}CD29^{hi}CD24^{+}$ phenotype from mouse mammary cells. They found that a single cell could reconstitute a complete mammary gland in vivo, which is consistent with self-renewal capacity, and predetermined differentiating ability of cells [8]. Stingl and colleagues have purified a rare subset of adult mouse mammary cells that were able individually to regenerate an entire mammary gland within 6 weeks in vivo. Moreover, they found that these mammary stem cells were a rapidly cycling population in the normal adult [9].

Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49) was used to identify stem cells in multiple adult tissues and cancers, especially in small intestine and colon [10–13]. Barker and colleagues conducted lineage-tracing experiments in adult mice using an inducible Cre knock-in allele and the Rosa26-lacZ reporter strain. They found that Lgr5-positive crypt base columnar cells are the stem cells of the small intestine and colon because these cells can generate all epithelial lineages over a 60-day period [14]. Lgr5 is the targets of Wnt signaling pathway that is critical regulator for normal adult stem cells. Aldehyde dehydrogenase (ALDH), a detoxifying enzyme that confers resistance to alkylating agents such as cyclophosphamide was also used as stem cell marker for isolation of normal stem cells [15], especially hematopoietic stem cells (HSCs) because of high expression of ALDH in hematopoietic stem cells [16]. An alternative method has been applied to isolate a subset of population of stem-like cell based on side population (SP) technique [17]. This method was created based on most common defense mechanism adopted by stem cells because of their high expression of members of the ABC super-family of membrane transporters [18, 19]. Mounting evidence has shown the identification of markers that are used to identify normal stem cells, which in fact could be applied for the isolation and purification of CSCs because similar gene expression profiles have been shown to exist between normal stem cells and CSCs. Thus, the question rises whether normal stem cells are the cell of origin of cancer cells or CSCs or not, which is further discussed in the following sections.

Cancer Stem Cells

It has been widely accepted that not all cells within tumors are equally able to produce tumor instead only a small minority of cells within the tumor are capable of forming tumor. These cells are commonly named as cancer stem cells (CSCs) or cancer stem-like cells (CSLCs). The CSCs are the tumor cells with unlimited self-renewal and multi-lineage differentiation potential capable of forming the tumors in immunodeficient mice that recapitulate the heterogeneity of tumors from which they were originally derived [20]. The concept that tumors arises from stem cells was first put forward in 1875 by Cohnheim who proposed the hypothesis that stem cells misplaced during the embryonic development are the source of the tumors that formed later in life. The CSCs share two main features with normal stem cells: self-renewal capacity (generate more CSCs) and multi-lineage differentiation capacity. Both CSCs and normal stem cells coexist in the tissue microenvironment called the "niche." From this perspective, the main difference between the normal and the CSC niche lies in the finely tuned homeostatic equilibrium. In the normal stem cell niche, the coordination exists in rates of self-renewal, symmetric and asymmetric cell division, cell proliferation, migration, differentiation, and apoptosis through strict regulation by multiple signaling pathways. However, in the CSC niche subtle defects in any of the above cell functions disrupt the homeostatic equilibrium, resulting in unlimited cell growth, and thereby lead to the formation of the tumor which further fuels the process of accumulating gene mutations and/or activating signaling pathways that regulate normal stem cell niche. For example, the self-renewal of normal stem cell is commonly regulated by the Oct-4 [20–23], Wnt/*β-catenin* [24, 25], Notch [26, 27], TGF beta [28, 29] and Hedgehog pathways [30]. Mounting evidence showed that self-renewal and proliferation of CSCs are likely due to dysregulation of pathways that are involved in maintaining the normal stem cell self-renewal [27, 31–37]. Identification and isolation of CSCs are becoming complicated because malignancies have been known to be highly heterogeneous in nature [38]. The CSCs in different tissues exhibit exclusive differences in morphology, marker expression, self-renewal and proliferative potential, and therapy response. Currently, the identification and isolation of CSCs from various tumor tissues or tumor cell lines has been mainly based on cell-surface marker expression. However, cellular origin of cancer is still debatable although lineage tracing studies published recently clearly suggest the origin and existence of CSCs [39–42].

The Cellular Origin of Cancer

Although compelling evidence has indicated that cancers initiate from rare population of cells named cancer stem cells (CSCs) or cancer stem-like cells (CSLCs) that are capable of self-renewal and predetermined differentiating capacity, the cellular origin of cancer is still elusive. The cellular origin of cancer could arise

from long-lived normal stem cells that transformed by mutations and alternations in multiple cell signaling pathways. In support of this concept, Bonnet et al. have demonstrated that the cells capable of initiating human acute myeloid leukemia (AML) in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) exhibited self-renewal potential, and their differentiation and proliferative capacities, suggesting the true nature of leukemic stem cells. These cells from all subtypes of AML were exclusively $CD34^{++}CD38^{-}$, which is similar to the cell-surface phenotype of normal SCID-repopulating cells, suggesting that normal primitive cells, rather than committed progenitor cells, are the cellular origin of human acute myeloid leukemia [43]. Barker et al. also found that deletion of adenomatous polyposis coli (APC) in the long-lived intestinal stem cells resulted in micro-adenomas that showed unimpeded growth and developed into macroscopic adenomas within 3–5 weeks. However, deletion of APC in short-lived transit-amplifying cells led to very rare large adenomas in these mice [44]. These results suggest that intestinal cancer could indeed be initiated from long-lived intestinal stem cells mediated by Wnt-pathway-activating mutations through deletion of (APC) but not short-lived transit-amplifying progenitor cells. The most primitive cells, stem cells, have been the favorite candidate for transformation targets because these cells possess their inherent capacity for self-renewal and their longevity, which could make them subjected to the accumulation of genetic or epigenetic mutations that are required for oncogenesis. However, transformation of distinct breast epithelial cells resulted in tumors with different phenotype [45]. These results suggest that any cell in the cell hierarchy of various tissues with proliferative capacity could serve as a cell of origin for cancer development if it acquires mutations with propensity for self-renewal capacity and limiting differentiation to a post-mitotic state through dedifferentiation or trans-differentiation process. Moreover, the targeting cell of malignant transformation is an important determinant of tumor phenotype because their molecular characterization would lead to the identification of therapeutic targets.

The Cellular Origin of Cancer in Hematological Malignancies

Cancer stem cells (CSCs) were first identified from human acute myeloid leukemia (AML). Bonnet et al. found that the cells capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) exhibited $CD34^{++}CD38^{-}$ phenotype, which is similar to normal stem cells as discussed above. Thus, they proposed that long-lived normal primitive cells, but not short-lived progenitor cells, are the cellular origin of human acute myeloid leukemia [43]. However, Cozzio A et al. showed that isolated stem cells and myeloid progenitor populations with granulocyte/macrophage differentiation potential could be transduced with a leukemogenic MLL fusion gene, which resulted in the rapid onset of acute myeloid leukemia. These results suggest that acute myeloid leukemia not only arises from normal hematological stem cells (HSCs) but also is committed to

progenitors that lack unlimited self-renewal potential [46]. These studies identified and proved the existence of CSCs, which could initiate the human acute myeloid leukemia based on the limited dilution transplantation analysis. Somervaille and Cleary demonstrated that colony-forming cells (CFCs) are also leukemia stem cells (LSCs). These cells are characterized by their self-renewal capacity and also show the expression of mature myeloid lineage-specific antigens with ability to generate a phenotypic, morphologic, and functional leukemia cell hierarchy. Moreover, these cells are found frequently, accounting for 25–30 % of myeloid lineage cells at late-stages of the disease [47].

The Cellular Origin of Cancer in Solid Tumor

Following the identification of CSCs from hematological malignancies, CSCs were also identified and isolated from various solid tumors such as breast [48], brain [49–51], prostate [52], pancreatic [53], and colon cancer [54, 55] as well as menaloma [56, 57]. In the following sections, we will catalogue the evidence in support of the role of CSCs isolated and characterized from different solid tumors.

The Cellular Origin of Cancer in Breast Cancer

Breast cancer is the most common malignancy in women of the USA. Identification of breast cancer cells, which could initiate and maintain tumor growth, is a key step to eliminate breast cancer through therapeutic targeting of these cells. Al-Hajj M et al. demonstrated that as few as 200 cells identified and isolated tumorigenic cells as $ESA^{+}CD44^{+}CD24^{-/low}$ Lineage-cells were able to form tumors in NOD-SCID mice, whereas 2,000 cells with $ESA^{-}CD44^{+}CD24^{-/low}$ Lineage-cells or 20,000 $CD44^{+}CD24^{+}$ Lineage-cells failed to form tumors [48]. Aldehyde dehydrogenase (ALDH) has been found to be expressed in both normal stem cells and CSCs [16, 58–62]. Ginestier and colleagues found that human normal and cancer mammary epithelial cells with stem/progenitor properties showed increased aldehyde dehydrogenase activity (ALDH). The cancer cells with high ALDH activity displayed increased self-renewal capacity and were able to generate tumors that could recapitulate the heterogeneity of the parental tumor. Moreover, the expression of ALDH1 was correlated with poor prognosis [15]. This finding is consistent with the results showing that ALDH-positive cells are responsible for mediating metastasis [63]. Therefore, these studies have clearly documented the potential stem cell markers of breast cancer.

The Cellular Origin of Cancer in Brain Tumors

In 1997 CSCs from human leukemia were the first to be identified; in the following years, little evidence has been obtained to support the existence of CSCs in solid tumors, except for breast cancer [48]. Singh and colleagues, however, identified

cancer stem cells from human brain tumors of different phenotypes by taking advantage of the expression of cell surface marker CD133 that was used to isolate normal neural stem cells from fresh human fetal brain tissue [64]. These CSCs from the brain tumor expressed the neural stem cell surface marker CD133 and lacked the expression of neural differentiation markers. These cells possessed a marked capacity for proliferation, self-renewal, and differentiation in culture and gave rise to tumor cells that phenotypically resembled the original tumor from the patient. Moreover, the increased self-renewal capacity of the brain tumor stem cell (BTSC) was associated with aggressiveness of clinical behavior of the tumors in patient [49]. Although $CD133^+$ cells from brain tumor displayed stem cell signatures in vitro, the true measures of CSCs were further supported by their capacity for self-renewal, which exactly recapitulated the original tumor in vivo. Using a xenograft model, Singh and colleagues showed that the cells developed brain tumors when injected into NOD-SCID (non-obese diabetic, severe combined immunodeficient) mice. Moreover, they found that as few as 100 $CD133^+$ cells could form a tumor and these tumors recapitulated the heterogeneity of the patient's original tumor. However, injection of 10^5 $CD133^-$ cells into in NOD-SCID did not yield any tumors [50]. These findings strongly support the existence of stem cells within brain tumors, which could initiate brain tumor, and associated with tumor aggressiveness. Furthermore, the identification of brain tumor initiating cells provides specific cellular target for more effective cancer therapies [49–51].

The Cellular Origin of Prostate Cancer

Prostate Cancer (PCa) is the second most frequent cause of cancer-related death in men in the USA. In recent years, there have been significant improvements in the surgical treatment options for patients diagnosed with localized PCa and adjuvant therapy such as androgen deprivation therapy (ADT) to reduce local and distant disease for patients [65]. However, more than 50 % of patients do recur and metastasize several years after adjuvant therapy. Therapy resistance after an initial seemingly successful treatment commonly occurs, which is usually explained by the presence of a resistant subpopulation of cells. This drug-resistance is believed to be mediated by an acquired mutation/genetic alterations of cancer cells occurs de novo (intrinsic) or due to acquired resistance (extrinsic) after therapy. However, emerging evidence has suggested that CSCs are responsible for drug-resistance [66, 67]. Thus, identification and isolation of CSCs from the patient-derived tumor or cell lines has great importance for specific targeting the CSCs through advanced molecular understanding.

Collins and colleagues, for the first time, reported the identification and characterization of a putative basal cancer stem cell population from human prostate tumors. These cells with a $CD44^+$/integrin$\alpha2\beta1^{hi}$/$CD133^+$ phenotype possessed a significant capacity for self-renewal and were able to regenerate the phenotypically mixed populations of non-clonogenic cells expressing androgen receptor (AR) and prostatic acid phosphatase. The CSCs accounted for approximately 0.1 % of cells based on the above mentioned phenotype. However, no correlation was found

between the number of $CD44^{+}$/integrin$\alpha2\beta1^{hi}$/$CD133^{+}$ cells and tumor grade [52]. The markers of $CD44^{+}$/integrin$\alpha2\beta1^{hi}$/$CD133^{+}$ were used for the isolation of stem cells from normal prostate epithelium based on high surface expression of integrin α2β1 and CD133 on human prostate epithelia [68, 69]. These results suggested that CSC and their normal counterparts share many phenotypic markers. Lawson and colleagues have prospectively identified populations of murine basal/stem cells with $Lin^{-}Sca\text{-}1^{+}CD49f^{hi}$ phenotype and luminal cells with $Lin^{-}Sca\text{-}1^{-}CD49f^{lo}$ phenotype, and then introduced a series of genetic alterations into these cells. They found that basal/stem cells, but not luminal cells, displayed efficient capacity for cancer initiation and could produce luminal-like disease characteristics of human prostate cancer in multiple models. This finding provides strong evidence supporting the fact that basal epithelial stem cells are the cells of origin for prostate cancer [70]. However, these basal epithelial stem cells were isolated from murine prostate, and thus further studies are required in order to prove that the basal epithelial stem cell from human prostate is the cells of origin for the development of prostate cancer.

Goldstein and colleagues have identified populations of basal stem cells with $CD49f^{hi}Trop2^{hi}$ phenotype expressing high levels of Keratin-5 and the basal transcription factor p63. They also identified luminal cells $CD49f^{lo}Trop2^{hi}$ phenotype expressing high levels of the luminal keratins including keratin-8 and keratin-18, low or negative levels of basal keratins such as keratin-5 and keratin-14, and high expression of AR and PSA, Nkx3-1, and TMPRSS2. They have demonstrated that basal cells with $CD49f^{hi}Trop2^{hi}$ phenotype from primary benign human prostate tissue could initiate prostate cancer in immunodeficient mice mediated through the introduction of AKT, ERG, and AR into basal cells. The resulting prostate cancers recapitulated histological and molecular features of human prostate cancer, with loss of basal cells and expansion of luminal cells expressing prostate-specific antigen and alpha-methylacyl-CoA racemase. However, the combination of AKT, ERG, and AR did not result in the development of adenocarcinoma from luminal cells [71]. These results confirmed that basal epithelial stem cells from human prostate represent the cell of origin for the development of prostate cancers.

Findings from Wang and colleagues, however, strongly support prevailing theories suggesting that luminal cells are the origin of prostate cancer because these cells are histologically defined by the loss of basal cell and expansion of malignant luminal cells. Wang and colleagues found that castration-resistant cells from mice expressing Nkx3-1, a known regulator of prostate epithelial differentiation, exhibited stem cell signatures. These castration-resistant Nkx3-1-expressing cells (CARNs) with luminal markers cytokeratin-18 and AR expression, and negative basal cell marker p63 expression, gave rise to both basal and luminal cells during regeneration and displayed self-renewal capacity in vivo. Moreover, they found that CARNs could reconstitute prostate ducts in renal grafts using single-cell transplantation assays. Furthermore, deletion of the Pten tumor suppressor gene in these cells resulted in rapid formation of high-grade prostatic intraepithelial neoplasia (PIN) and carcinoma characterized by increased proliferation and loss of basal cells after androgen-mediated regeneration [72]. This observation clearly supports the idea

that a luminal stem cell population isolated from mouse prostate could be an efficient target for oncogenic transformation toward the development of prostate cancer, supporting the notion that luminal stem cells are the cell of origin for the development of prostate cancer. The findings from Germann and colleagues also support the idea that prostate cancer originates from luminal stem cells. They used the human prostate cancer xenograft BM18 model, and found that castration-resistant BM18-cancer cells showed significantly increased mRNA expression of SOX2, NANOG, BMI1, LGR5, CD44, and ALDH1A1 as well as expression of luminal markers such as NKX3-1, PSA (lower levels) and AR (lower levels) but not basal or NE markers. Moreover, they confirmed that these castration–resistant cells are the preexisting CSLCs, which were selected by castration and survived as a quiescent cell population. However, these castration-resistant luminal CSLCs could reinitiate BM18 tumor growth after androgen replacement [73]. These findings confirmed that luminal stem cells are the cell of origin for the development of human prostate cancer and may have important clinical implications for castration-resistant prostate cancer therapy.

The Cellular Origin of Colon Cancer

Colon cancer is one of the best-understood neoplasms from a genetic perspective. It remains the second most common cause of cancer-related death in the USA in both males and females combined. Identification of colon cancer initiating cells may provide specific therapeutic target for eliminating colon cancer. O'Brien et al. used renal capsule transplantation in immunodeficient NOD/SCID mice to identify a human colon cancer-initiating cell (CC-IC) that was $CD133^+$. They found that as few as 262 $CD133^+$ cells produced tumor with heterogeneity upon transplantation. However, the $CD133^-$ cells that comprised the majority of the tumor were unable to initiate tumor. These results provided strong support showing that targeting the colon CSCs could become effective therapeutic strategies for colon cancer patients [54]. Similar results were reported by Ricci-Vitiani and colleagues [55]. Mounting evidence revealed that CSCs are the cells of direct progeny of mutated normal stem cells [44]. Zhu et al. demonstrated that normal stem cells in the intestine are susceptible to cancer-causing mutations. They used an inducible Cre, nuclear LacZ reporter allele knocked into the Prom1 locus [Prom1(C-L)] and conducted lineage-tracing studies of adult Prom1(+/C-L) mice. They found that Prom1(+) cells are located at the base of crypts in the small intestine and could generate the entire intestinal epithelium. The activation of endogenous Wnt signaling in Prom1(+/C-L) mice by mutation of beta-catenin resulted in focal high-grade intraepithelial neoplasia and crypt adenoma formation. These data indicates that Prom1 represent stem cells in the adult small intestine which are susceptible to transformation [74]. Further studies have revealed that colon cancer can originate from long-lived normal stem cells following activation of Wnt signaling; this was not true for short-lived transit-amplifying progenitor cells [44].

The Cellular Origin of Pancreatic Cancer

Pancreatic cancer has the worst prognosis of any major malignancy and is the fourth most common cause of cancer death in the USA. Delayed diagnosis, relatively high resistance to chemotherapy and radiation, and an intrinsic biological aggressiveness, contributes to the high death rate of pancreatic cancer. Emerging evidence have suggested that resistance to chemotherapy and radiation, and an intrinsic biological aggressiveness are all attributed to the existence of stem cells, which are associated with tumor initiation and progression. Following the identification of CSCs from human blood [43], breast cancers [48], brain [49–51] and prostate cancer [52], Li and colleagues have identified pancreatic CSCs using cell surface markers CD44, CD24, and epithelial-specific antigen (ESA) in a xenograft model, in which primary human pancreatic adenocarcinomas were grown in immunocompromised mice. They found that as few as 100 pancreatic cancer cells with the $CD44^+CD24^+ESA^+$ phenotype could form tumors that were histologically indistinguishable from the original human tumors. The highly tumorigenic $CD44^+CD24^+ESA^+$ cells not only produced additional $CD44^+CD24^+ESA^+$cells but also produced phenotypically diverse non-tumor inducing cancer cells, suggesting that the pancreatic CSCs display self-renewal capacity, and also have the ability to produce differentiated progeny, which is consistent with the stem cell properties. Moreover, increased expression of the developmental signaling molecule sonic hedgehog could also be involved in maintaining the CSCs signatures of $CD44^+CD24^+ESA^+$cells [53]. Identification of pancreatic CSCs and further elucidation of the signaling pathways that regulate stem cell signatures may provide novel targets for designing effective treatment modalities for pancreatic cancer.

The Cellular Origin of Lung Cancer

Lung cancer is the leading cause of cancer deaths worldwide [75]. However, the cellular origin of lung cancer is largely unknown. Identification of tumor initiating cells or CSCs and the signaling pathways that could regulate self-renewal and proliferation of stem cells in lung cancers could provide therapeutic targets for the treatment of lung cancer. Kim and colleagues have identified bronchioalveolar stem cells (BASCs) from the bronchioalveolar duct junction. BASCs exhibited self-renewal capacity, and were multi-potent in vitro. Moreover, the activation of the oncogenic protein K-ras caused BASCs expansion in culture, and resulted in the development of precursor lesions of lung tumors in vivo. These results suggest that BASCs as the putative cells of origin for the development of adenocarcinoma [76], which provides the basis for possible therapeutic interventions to combat lung cancer [77].

EMT Phenotypic Cells as a Source for Cancer Stem Cells

EMT was first recognized as a feature of embryogenesis during embryonic development [78], which has been shown to be responsible for the plasticity of epithelial cells and that the EMT phenotypic cells are also reminiscent of CSCs [79].

It is also involved in adult tissue remodeling and wound healing in response to injury, and has also been implicated in the conversion of early stage tumors into advanced invasive malignancies [80, 81]. During tumor progression, cancer cells acquire EMT phenotype characterized by the loss of epithelial marker expression and up-regulation of mesenchymal molecular markers, leading to increased cell motility and invasion, which allows cancer cells to metastasize in distant sites [82–85]. These processes are consistent with the acquisition of "cancer stem-like cell (CSLC)" or cancer stem cell (CSC) characteristics [83]. Increasing evidence has suggested that the cells with EMT phenotype could also serve as a source for CSCs [86–89].

Mani and colleagues found that the induction of EMT phenotype from non-tumorigenic, immortalized human mammary epithelial cells by the overexpression of either twist or snail resulted in the loss of epithelial phenotype and the acquisition of mesenchymal phenotype. These EMT phenotypic cells acquired $CD44^{high}/CD24^{low}$ expression pattern, which was consistent with increased self-renewal capacity as characterized by enhanced mammosphere-forming ability in vitro and tumor initiating capacity in vivo. Whereas, the stem-like cells with $CD44^{high}/CD24^{low}$ phenotype isolated from normal and neoplastic human mammary cells displayed a mesenchymal morphology consistent with increased expression of mesenchymal markers such as vimentin and fibronectin [87]. These findings strongly support the hypothesis that induction of EMT by expressing EMT-related transcription factors in human mammary epithelial cells could lead to the generation of stem-like cells. Morel and colleagues found that the induction of EMT by activation of signaling pathways that regulate EMT could also produce stem-like cells [90]. They have demonstrated that the activation of Ras/MAPK signaling pathway in $CD44^{low}CD24^{+}$ cells, non-tumorigenic mammary epithelial cells produced the cells with EMT phenotype as characterized by the loss of E-cadherin expression and gain of vimentin expression. During this process, stem-like cells with $CD44^{+}CD24^{-/low}$ phenotype were also generated from $CD44^{low}CD24^{+}$ cells. They hypothesized that the induction of EMT could be responsible for switching $CD44^{low}CD24^{+}$ cells to $CD44^{+}CD24^{-/low}$ stem-like cells. To confirm this hypothesis, they treated $CD24^{+}$ cells with TGF-β, a potential inducer of EMT, which resulted in the generation of cells with $CD24^{-}$ phenotype 8 days after treatment and it was concomitant with enrichment of EMT phenotypic cells as characterized by the loss of E-cadherin and the gain of vimentin expression [90]. Cancer stem cells or stem-like cells exhibit unlimited self-renewal and multi-lineage differentiation potential, and also possess the capacity of forming tumors in immunodeficient mice, and these tumors could recapitulate the heterogeneity of primary tumors. Santisteban et al. observed that the induction of EMT by an immune response against an epithelial breast cancer leads to the outgrowth of tumor in vivo [89]. Interestingly, as few as 100 cells of the resulting mesenchymal tumor cells with $CD44^{+}CD24^{-/low}$ phenotype could form the tumor, whereas $>10^{6}$ cells with epithelial phenotype could form tumors. Moreover, mesenchymal tumor cells with $CD44^{+}CD24^{-/low}$ phenotype generated the epithelial cells with $CD44^{+}CD24^{hi}$ phenotype and showed increased drug resistance, which is consistent with breast CSCs [89]. These results are consistent with the findings by Gupta et al. showing that increased population of

$CD44^{high}CD24^{low}$ cells were generated through the induction of EMT using transformed HMLER breast cancer cells by shRNA-mediated knock-down of E-cadherin expression, and these cells displayed an increased drug resistance associated with CSCs signatures [91]. These studies strongly suggest that the induction of EMT by deregulation of various factors and signaling pathways that regulate EMT could generate stem-like cells in breast epithelial cells. Scheel and colleagues found that signaling pathways, involving transforming growth factor (TGF)-β and canonical and noncanonical Wnt signaling, that collaborate not only to induce the activation of the EMT program but also maintain the EMT phenotype, resulting in mesenchymal state and stem cell traits through autocrine regulation of multiple signaling pathways [32].

Generation of stem-like cells through induction of EMT was not only observed in breast epithelial cells but in other epithelial cells, especially in prostate epithelial cells. Klarmann and colleagues found invasive prostate cancer cells through Matrigel assay underwent EMT phenotypic changes and displayed CSC-like signatures as characterized by increased expression of CD44. Moreover, invasive cells from DU145 and primary prostate cancer cells are more tumorigenic in NOD/SCID mice compared with noninvasive cells [86]. In our studies, we found that platelet-derived growth factor-D (PDGF-D), a newly recognized growth factor, induced EMT in PC3 PCa cells, which were consistent with the up-regulation of ZEB1, ZEB2 and slug with corresponding down-regulation of epithelial markers such as E-cadherin, stratifin, EpCAM, F11R, and connexin 26, and increased expression of mesenchymal marker such as vimentin [92–94]. Interestingly, we found that these cells with EMT-phenotype displayed stem-like cell signatures as characterized by increased clonogenicity, self-renewal capacity and increased tumorigenicity in SCID mice, and these results were consistent with increased expression of stem cell markers such as Notch-1, Sox2, Nanog, Oct4, and Lin28B [95]. $ARCaP_M$ cell with EMT phenotype is the subclone of the ARCaP cells that were originated from the ascites fluid of a patient with prostate cancer bony metastasis [96, 97]. We also found that $ARCaP_M$ cells with EMT phenotype shared stem-like cell signatures showing enhanced clonogenic and self-renewal ability, which was consistent with increased expression of Notch-1 compared with control cells ($ARCaP_E$ cells) with epithelial phenotype [95].

Androgen deprivation is currently used as a standard treatment for advanced prostate cancer. Sun Y et al. demonstrated that androgen deprivation could induce EMT phenotype in normal prostate and prostate cancer cells associated with CSCs signatures, whereas the mouse prostate stem cells with $Lin^-CD44^+CD133^+Sca\text{-}1^+CD117^+$ phenotype expressed multiple mesenchymal-related markers including vimentin, ZEB1, ZEB2, Twist1, Snail1, and slug [98]. Albino A et al. showed that the loss of ESE3/EHF that regulates prostate epithelial cell differentiation and have stem-like potential, could induce EMT which is consistent with stem-like features due to deregulation of Twist1, ZEB2, Bmi1, and Oct4 expression. The loss of ESE3/EHF led to increased tumorigenic potential of prostate cancer cells, and was associated with increased biochemical recurrence in prostate cancer patients with reduced overall survival after prostatectomy [99]. Armstrong AJ and colleagues revealed

that circulating tumor cells (CTC) from patients with progressive metastatic prostate cancer and breast cancer co-expressed epithelial, mesenchymal, and stem cell markers [100]. These reports strongly support that EMT phenotypic cells are a source for CSCs. Moreover, committed differentiated cells can also undergo trans-differentiation leading to the acquisition of stem-like cell characteristics induced through the processes of EMT.

The Molecular Connection and Distinction Between Normal Stem Cells and Cancer Stem Cells

Cancer stem cells could be initiated from normal stem cells through mutation or reactivation of signaling pathways that control the normal stem cell function. The function of normal stem cells and cancer stem cell or tumorigenic cancer cells is conceptually similar in that both cell types possess self-renewal capacity, and are capable of producing differentiated progeny. The pathways that regulate self-renewal of normal stem cells also frequently mediate the regulation of self-renewal in cancer stem cells. Although cancer stem cells and normal stem cells are similar in some ways, they are also fundamentally different in other ways. Understanding both shared and differences in the molecular mechanisms that regulate normal stem cells and cancer stem cells is an important challenge in cancer biology and treatment toward eradicating the tumor initiating cells without destroying normal stem cells. Krivtsov and colleagues demonstrated that isolated leukemia stem cells (LSC) from the leukemia, initiated in committed granulocyte macrophage progenitors through the introduction of the MLL-AF9 fusion protein, which could maintain the global identity of the progenitor from which they arose. However, a subset of genes highly expressed in normal hematopoietic stem cells was reactivated in LSC including Hox genes and Mef2c, which are associated with self-renewal signature in leukemia stem cells, and are important for LSC development. These activated genes are associated with leukemia self-renewal signature [101]. Therapeutic strategy for specific targeting of the unique capacity of self-renewal of stem cells could have significant impact in designing novel strategies for the treatment of human malignancies with much improved therapeutic outcome.

Recent finding has provided clear evidence showing that selective targeting of CSCs by targeting the pathways that are dysregulated without damaging the normal stem cells, is feasible [102, 103]. Yilmaz and colleagues have demonstrated that the deletion of the Pten in adult hematopoietic cells led to myeloproliferative disease within days and transplantable leukemias within weeks, suggesting that inactivation of Pten is necessary for function of the leukemia-initiating cells. However, Pten deletion in hematopoietic stem cell (HSC) resulted in short-term expansion, but long-term decline of HSC via a cell-autonomous mechanism [102]. These results are consistent with findings by Zhang and colleagues [103]. Moreover, Yilmaz and colleagues found that rapamycin, an mTOR inhibitor, not only depleted leukemia-initiating cells but also restored normal HSC function. Therefore, targeting

mechanistic differences between normal stem cells and cancer stem cells could have therapeutic benefit toward the elimination of CSCs without damaging normal stem cells.

Cancer Stem Cells and Drug Resistance

Cancer stem cells play important roles in drug resistance of cancer cells due to deregulation of multiple signaling pathways [4]. Mounting evidence shows that cancers including hematological malignancies and solid tumors are initiated from a rare population of cells capable of unlimited self-renewal capacity that is necessary for cancer maintenance and progression. Therefore, eradication of these CSCs by novel approaches is critical for successful treatment of human malignancies. However, conventional cancer therapies are often effective in killing the bulk of the differentiated cancer cells, resulting in the reduction of tumor burden, but not effective in the killing (eradicating) rare stem cell population with quiescent behavior. These drug-resistant cells are responsible for tumor relapse after treatment as *summarized in* Fig. 16.1. Chen and colleagues have identified a subset of relatively quiescent endogenous tumor cells with properties of CSCs that were responsible for tumor regrowth after temozolomide (TMZ) administration using a genetically engineered mouse model of glioma [104]. Creighton and coworkers have demonstrated that tumor cells surviving after conventional treatments displayed increased expression of mesenchymal markers such as vimentin, consistent with CSCs features with the $CD44^{+}/CD24^{-}$ phenotype [105]. In colon cancer Xenograft mice model, CSCs are enriched in the tumors after chemotherapy [106]. A similar scenario occurs for prostate cancer therapy. The enriched castration-resistant Nkx3-1-expressing cells from mice prostate tissues displayed self-renewal capacity in vivo and could reconstitute prostate ducts in renal grafts using single-cell transplantation assays which resulted in rapid formation of high-grade prostatic intraepithelial neoplasia (PIN) and carcinoma through deletion of the Pten tumor suppressor gene in these cells [72]. The similar findings from Germann and colleagues showed that castration-resistant BM18-cancer cells isolated from human prostate cancer xenograft significantly increased mRNA expression of stem cell related markers including Sox2, Nanog, Bmi1, LGR5, CD44, and ALDH1A1, and these authors have confirmed that the castration-resistant cells were preexisting cancer stem-like cells and selected by castration, survive and maintain quiescent state [73].

Drug resistant CSCs should be targeted for their elimination in order to achieve complete cures for patients after therapy. However, targeting cancer stem cell microenvironments or niches are not yet available for patients although it is quite clear that tumor microenvironment or niches are important in supporting and maintaining cancer stem cell self-renewal [32], and thus novel therapeutic strategies much be developed for targeting the tumor microenvironment and the CSCs. Vermeulen and colleagues showed that differentiated colorectal cancer cells could reacquire a CSC phenotype, including the capacity to induce new tumors, upon exposure to factors secreted by myofibroblasts. Myofibroblasts are prominent cells in the stroma of

colorectal cancers, and these authors have identified hepatocyte growth factor (HGF) as the mediating factor [31]. Calabrese and colleagues demonstrated that increasing the number of endothelial cells or blood vessels in orthotopic brain tumor xenografts escalated the fraction of self-renewing cells and promoted the initiation and growth of tumors, while blocking vascular endothelial growth factor (VEGF) signaling decreased the CSC fraction by disrupting the vascular endothelial CSC niche in glioblastoma [107]. Therefore, agents with multiple targets may become novel therapeutic approach in the pursuit of overcoming drug resistance, and such strategy will improve the treatment outcome of patients in the future.

Conclusion

Adult normal stem cells consist of two kinds of stem cells: one is the quiescent long-lived stem cells that are responsible for regenerative response upon tissue injury; another one is involved in amplifying progenitor cells that are responsible for daily turnover to maintain the tissue homeostasis. The reprogramming of these normal stem cells is likely the cause of tumor development and the maintenance of tumors due to the presence of cancer stem cells (CSCs). Overall, it is believed that cancers arise from a rare population of CSCs with limitless self-renewal capacity, and these cells are capable of multi-lineage differentiation. The cellular origin of cancer could originate from long-lived stem cells or transit amplifying progenitor cells through oncogene-mediated transformation (Fig. 16.1). The cellular origin of cancer could also arise from committed progenitor cells through dedifferentiation or from terminally differentiated cells through trans-differentiation that is consistent with a process commonly known as EMT phenotype (Fig. 16.1), which are characterized by the loss of epithelial markers and gain of mesenchymal phenotype. Normal stem cells and CSCs exist within the microenvironment, which allows for maintaining the “stemness” niche regulated by multiple genes and pathways. Therefore, it is tantalizing to speculate that novel agents must be developed for targeting this niche, which will lead to eliminate CSCs for achieving the dream of tumor eradication and cure. In conclusion, mounting evidence has provided a ray of hope for eradicating tumors and thus achieving a cure in patients diagnosed with malignancies; the future looks much brighter than ever before.

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Chapter 17
Mechanisms of Metastasis

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Abstract Metastatic disease is the culmination of cancer and its most common life-threatening manifestation. The highly complex process by which cancer cells disseminate to and successfully colonize organs distant from the primary tumor has been divided into stages, collectively termed the metastatic cascade. Decades of research into metastasis biology has yielded several proposed models, each of which address experimental and clinical observations and contribute mechanistic insight to the metastatic cascade. Despite major advances in dissecting and identifying associated molecular pathways, many details remain to be clarified about the mechanisms that enable tumor cells to form these life-threatening lesions. The lack of a comprehensive understanding of the mechanisms of metastasis has thus delayed advancement of therapeutic strategies for late stage cancer. Here, we review the leading models describing tumor progression and provide an overview of the current state of the scientific community's understanding of metastasis.

Introduction

Despite recent advances in cancer biology and therapeutics, disseminated metastatic disease persists as an insurmountable challenge in the oncology clinic. It is estimated that 577,000 Americans will die of cancer in 2012 accounting for 25 % of all deaths in the USA [1], the vast majority of which will be the result of metastatic disease.

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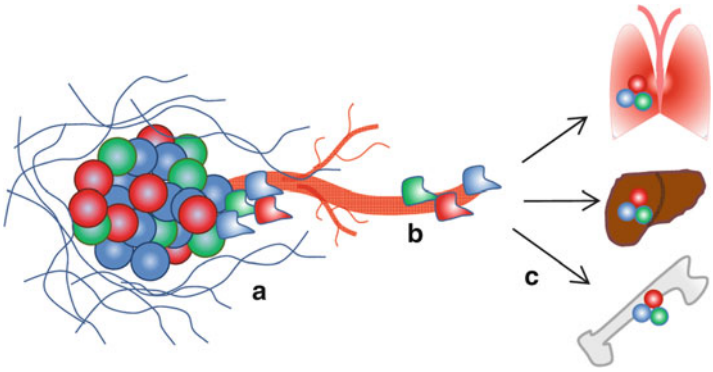

Fig. 17.1 Simplified steps of the invasion–metastasis cascade. (*a*) Invasion of cells into the surrounding tissue. (*b*) Entrance into the bloodstream. (*c*) Extravasation, entrance into distant organ parenchyma, and successful establishment of metastasis

The process by which a tumor that has initiated at a primary site, such as the breast, colon, or prostate gland, spreads to secondary organs is termed the invasion–metastasis cascade. The steps of the invasion–metastasis cascade are poorly understood but evidence suggests that it involves the complex interplay between tumor-intrinsic and host-derived factors. In order for a tumor cell of epithelial origin to form a clinically relevant metastatic lesion, cells from the primary tumor are thought to invade beyond the epithelial basement membrane, gain access to vasculature, survive in the lymphatic or blood stream, and arrest at the target organ, all the while evading innate and adaptive host immune surveillance mechanisms [2, 3].

Upon arrival at this secondary site, the tumor cell must adapt to survive and proliferate in an environment distinct from its tissue of origin, a step commonly termed metastatic colonization (Fig. 17.1). Further adding to the complexity of metastasis are observations that tumors originating in distinct organs have differential organ tropism and that metastases of the same primary tumor employ distinct cellular mechanisms and gene expression programs to colonize different secondary sites [4, 5]. Evidence suggesting that fewer than 0.01 % of cells that reach the bloodstream form macrometastases highlights the extreme inefficiency of the metastasis cascade [6]. Yet despite this inefficiency, metastasis continues to be the primary cause of cancer-related death.

While this admittedly simplistic model of the metastatic process has gained wide acceptance and many of the cellular and molecular determinants of metastasis are continually being identified, the persisting dearth of therapies for metastatic disease underlines the remaining gaps in our understanding of metastasis. Several models elaborating on the invasion–metastasis cascade have been proposed, each of which addresses particular clinical and experimental observations and provides detailed mechanistic insight. Here, we review the previously and recently proposed models of metastatic progression and provide an overview of the current state of the community's understanding of metastasis.

Clonal Selection Model

The most widely accepted model of metastasis is the clonal selection model. Successful dissemination to a secondary site involves many steps and it is thought that tumor cells acquire traits that allow them to initiate invasion, survive within the blood stream, extravasate, and grow in a foreign tissue environment. The clonal selection model postulates that genomic instability in the primary tumor leads to stochastic mutations that result in clonal selection of highly metastatic tumor cells [7–9]. This provides an explanation for why only a subset of cells succeeds in forming lesions at the secondary site. Since genomic instability within the primary tumor results in heterogeneity of neoplastic cells, not all tumor cells will acquire the necessary advantageous mutations and therefore only a small subset of them obtains full metastatic potential (Fig. 17.2a). This model thus attempts to explain the heterogeneity observed in primary tumors as well as the inefficiency of the metastatic process.

Based on the concept of tumor heterogeneity, Fidler and Kripke were the first to suggest this model by showing in animal models that a minute proportion of tumor cells were capable of successfully disseminating to the distant organ [10]. They demonstrated that clones from a parental murine tumor varied in their metastatic potential indicating the existence of metastatic heterogeneity within the primary tumor. In a separate study, using irradiation to induce unique chromosome rearrangements in cells, it was shown that each metastatic lesion was clonal [11]. Thus, these studies concluded that the ability to survive this process was not due to random selection. Since then, many other studies have validated these findings, further supporting that clonal selection occurs, which allows only specific cells to gain full metastatic potential. A recent paper provided support for the clonal selection model in medullablastoma [12]. By using the sleeping beauty transposon method, the authors recapitulated tumorigenesis and subsequent metastasis in mice. Analysis of the primary tumor and the matched metastatic tumor revealed only a small overlap indicating that they are genetically different. However, since common insertion sites existed in the primary tumor and the metastasis, they concluded that the secondary tumor arose from a common progenitor cell which had undergone divergence. Furthermore, the investigators showed analogous findings in human medullablastoma samples. These and other studies indicate that the clonal selection model provides a general mechanism for metastasis [13–15].

Additional evidence for the clonal selection model came with the birth of genomics: microarray analyses of primary tumor samples showed that genetically heterogeneous cellular populations exist within any given tumor [9, 16, 17]. These analyses demonstrated that particular genetic signatures underlie organ-specific metastasis and that not only do tumors exhibit heterogeneity but also that certain genetic mutations account for specific tissue tropism of tumor cells [5]. Further, recent advances in sequencing technology have enabled the analysis of whole genome sequencing of tumors [18]. Using second generation sequencing Ding et al. examined a single patient's peripheral blood, primary basal-like breast tumor, and matched brain metastasis [19]. They found

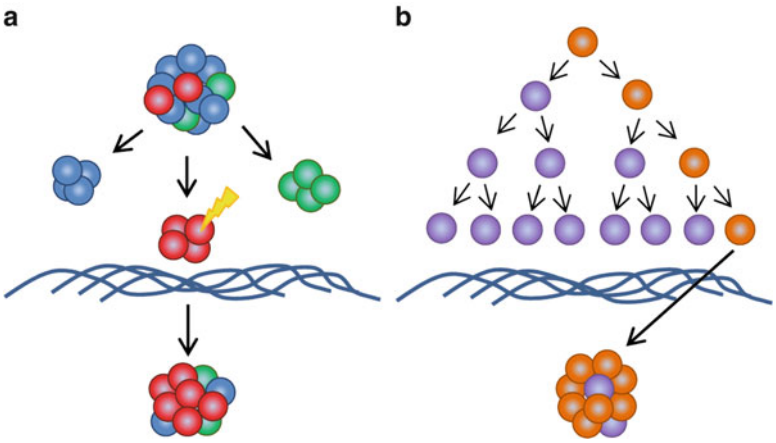

Fig. 17.2 Clonal selection model and cancer stem cell model. (**a**) The clonal selection model proposes that genomic instability within the primary tumor results in tumor cell heterogeneity such that only a subset of tumor cells acquire the mutations (*lightning bolt*) that endow them with metastatic capability. (**b**) The cancer stem cell model suggests that only cancer stem cells (*orange*) have the capability to form metastatic lesions

a wide range of mutations in the primary tumor supporting genetic heterogeneity within the sample. Analysis of the metastasis showed an enrichment of a subset of mutations, suggesting a subset of cells within the primary tumor had metastasized to the brain. The xenograft derived from the patient's primary tumor was shown to contain a mutational profile that overlapped with the metastasis, further supporting the notion that a minority population of cells arose within the primary tumor with an enhanced metastatic capability.

Similarly, in a study by Navin et al. the investigators inferred tumor evolution using single-cell sequencing [20]. Employing flow-assisted cell sorting with subsequent genome amplification and sequencing of 100 single cells from a heterogenic breast cancer sample, the authors revealed three distinct subpopulations that shared genomic alterations. When analyzing single cells from a homogeneous breast tumor and matching liver metastasis samples, the authors found a single subpopulation of aneuploid cells, indicating that the metastasis formed from a single subpopulation with little further evolution.

These data provide compelling evidence that clonal selection occurs in metastatic progression. While this model gives important insight to the possible mechanisms for metastasis, some observations cannot be explained by clonal selection theory. If all metastases occurred in the manner described by this model, the metastasis should be entirely composed of only a subset of cells observed in the primary tumor; however it has been shown that metastatic tumors phenotypically resemble their cells of origin [21, 22]. Another inconsistency arises from reports showing that metastatic gene expression signatures can be derived from expression profiles of the primary tumors [16, 23]. If a minority of genetically divergent tumor cells gain metastatic ability, it is unlikely that their gene expression profile would be detectable by

expression profiling of the bulk tumor. Nonetheless, the clonal selection model was the first to show a potential mechanism through which metastasis occurs. These insights by Fidler and subsequent studies have filled major gaps in metastasis biology.

Metastatic Cancer Stem Cell Model

In addition to the clonal selection model, another theory has been proposed to explain the observation of cell heterogeneity in primary tumors. Based on the concept of stem cells, this theory has postulated the existence of a niche of cancer stem cells. Stem cells are specialized cells that self-renew by asymmetric division to produce two daughter cells. While one cell remains a stem cell and retains self-renewal capacity, the other becomes a progenitor cell that differentiates [24, 25]. With these characteristics, stem cells provide life-long cell growth for tissue homeostasis and provide regenerative capacity for tissue repair.

Cancer stem cells are thought to have similar properties that allow them to sustain constant tumor growth. Due to their properties of self-renewal and differentiation, cancer stem cells are thought to provide the heterogeneity observed in the primary tumor [21, 24, 26]. Further, it has been hypothesized that cancer stem cells are tumor-initiating cells capable of forming new tumors at distant sites [21, 27]. Similar to the clonal selection model, the cancer stem cells hypothesis states that only cancer stem cells can colonize distant organs: only a distinct subset of cells is thought to have the ability to successfully metastasize—the cancer stem cell population [28, 29]. In contrast to the clonal selection model, cancer stem cells are the cells intrinsically programmed to have this advantage rather than metastatic cells being stochastically selected in the context of genomic instability (Fig. 17.2b).

The first evidence for the cancer stem cell hypothesis was demonstrated in acute myeloid leukemia where it was observed that a small percentage of leukemia cells were capable of proliferating extensively [30]. Dick took this finding and isolated a small subpopulation of acute myeloid leukemia cells that resembled normal hematopoietic stem cells and introduced them into immunodeficient mice. Upon transplantation, the cells from that subpopulation were able to induce leukemia in the mice whereas other cells found in the acute myeloid leukemia cell population were not, suggesting that the cancer stem cell hypothesis could indeed be true, at least in the case of hematologic malignancies. Since this study, cancer stem cells have been reported in solid tumors including breast, pancreas, colon and prostate, although these findings are less clear and continue to be controversial [31–34].

Breast cancer was the first solid tumor to show the existence of cancer stem cells. Al-Hajj et al. identified and isolated a subgroup of breast cancer cells using specific cell surface markers and showed that a few of these cells were needed to initiate new tumor formation while thousands of cells of other subtypes did not [31]. These tumor-initiating cells were identified to be $CD44^{+}CD24^{-/low}$ lineage and resembled stem cells. It was demonstrated that subpopulations within the tumorigenic cells

upon serial transplantation into nude mice continually gave rise to the same subpopulations of cells in new tumors. These data led Al-Hajj et al. to conclude that the isolated subset of cells had stem cell capacity and were alone responsible for the initiation of these tumors.

The identification of cancer stem cells in solid tumors has given rise to the possibility of these cells being involved in the metastatic process. Different theories regarding metastasis and cancer stem cells have been proposed. One hypothesis proposes that tumor cells undergoing epithelial-to-mesenchymal transition (EMT) gain stem cell-like properties. EMT and its counterpart, MET, are processes dictated by distinct signaling pathways during embryonic development that allow cells to migrate to appropriate regions of the body and develop into various tissue types [35]. These processes are defined by the loss of epithelial markers such as E-cadherin, and the gain of mesenchymal features such as vimentin and myosin, which lead to reduced attachment to the extracellular matrix and increased in cellular motility [36]. Such temporary phenotypic shifts in progenitor cells play an important role in embryonic development.

EMT and MET have also been observed in tumor cells and some evidence exists that they may play a role in invasion and metastasis [26, 37, 38]. During EMT, cells undergo a transition in gene expression programs that alter cell morphology and behavior that endows tumor cells with invasive properties that enable metastatic progression to commence. Cells lose their adhesion to the basement membrane by downregulation of E-cadherin, and upregulation of vimentin allowing for reduced adhesion and increase in motility [39]. Further, upregulation of membrane-degrading genes, such as matrix metalloproteinases enables cancer cells to escape the primary tumor and disseminate [40]. Since cancer stem cells appear to be the tumor-initiating cells at the distant site, it is hypothesized that the EMT process could bestow cancer cells with stem cell-like properties in order for them to successfully metastasize. This was demonstrated in a study by Mani et al. in which the authors showed EMT-induced cells acquired stem cell-like phenotypes [37]. Data from this study indicates a potential link between cancer stem cells and EMT, a process that may initiate dissemination.

Another theory hypothesizes that metastatic cancer stem cells directly derive from cancer stem cells. Data from a study led by Hermann et al. demonstrated the existence of metastatic cancer stem cells in pancreatic tumors [32]. First, the investigators analyzed pancreatic cancer samples and found a distinct population of cancer stem cells. Upon further analysis they discovered a second population of stem cell-like cells at the invasive front of the tumors. These tumors were found to be $CXCR4^{+}$, which is a specific receptor for SDF-1, a mediator of cell migration. Inhibition of CXCR4 with a receptor-specific inhibitor reduced the metastatic capability of these cells, indicating that this distinct population of cancer stem cells is important in cell dissemination. These data therefore demonstrate that metastatic stem cells may derive from cancer stem cells.

The identification of cancer stem cells in primary tumors sets forth the possibility that they are the drivers of tumorigenesis and metastasis. Evidence for cancer stem cells in solid tumors has provided a large body of knowledge for potential

mechanism of tumor initiation, progression, and metastasis. Whether cancer stem cells can explain all aspects of the invasion–metastasis cascade requires further elucidation but sufficient data suggest that they may play a role in metastasis in at least some solid tumors.

Transient Compartment Model

The transient compartment model is an extension of the dynamic heterogeneity model and was first proposed by Weiss [41]. This theory attempts to explain the observation that secondary tumors, although having successfully metastasized, do not necessarily have an increase in metastatic capacity over primary tumor cells. Having observed this in a number of experimental systems (reviewed in ref. 41), Weiss proposed that all cells within the primary tumor have the ability to metastasize. However, due to spatial or epigenetic factors only a few cells will successfully disseminate. Therefore, as depicted in Fig. 17.3, only cells that have a positional advantage (i.e., have adequate blood supply) will gain the required capability to metastasize. Similarly, throughout the steps to successful metastasis, cells may undergo transient epigenetic changes that provide them further advantage to disseminate. Furthermore, it is thought that when cells from the secondary tumor are injected into the primary site, they revert to their original phenotype, indicating that the disseminated cells do not permanently acquire an enhanced metastatic ability. Unlike the clonal selection or cancer stem cell model, in which only a certain cell population gains advantage through somatic mutations or stem cell characteristic, respectively, the changes in the transient compartment model is temporary and may affect any cell in the primary tumor.

The hypothesis that epigenetics plays a role in the transient compartment model is supported by studies demonstrating that methylation inhibitors modulate the metastatic capacity of cell lines [42–46]. Data by Kerbel et al., for example, demonstrated that when non-metastatic cell lines were treated with a demethylating reagent, they obtained small, unstable tumor clones with enhanced metastatic capability [46]. However, while global demethylation may mimic some of the proposed epigenetic events, these agents also cause chromosomal aberrations [47], opening the possibility that the modulation of metastatic capacity was due to mutational rather than epigenetic events.

While the transient compartment model accounts for the observation that not all metastatic cells are consistently more metastatic, this phenomenon is also explained by other models. The inability of cells isolated from metastases to be consistently more metastatic than the primary tumor could be explained by the tumor microenvironment which has been shown to play a significant role in metastasis (reviewed below). Furthermore, the transient compartment model does not explain the clonal nature of metastases [47–49]. Studies have shown that primary tumors are heterogeneous [50, 51] and, therefore, if metastatic capability was only modulated by transient epigenetic events, then it is less likely that significant proportions of secondary tumors would appear to be of clonal origin [12, 17].

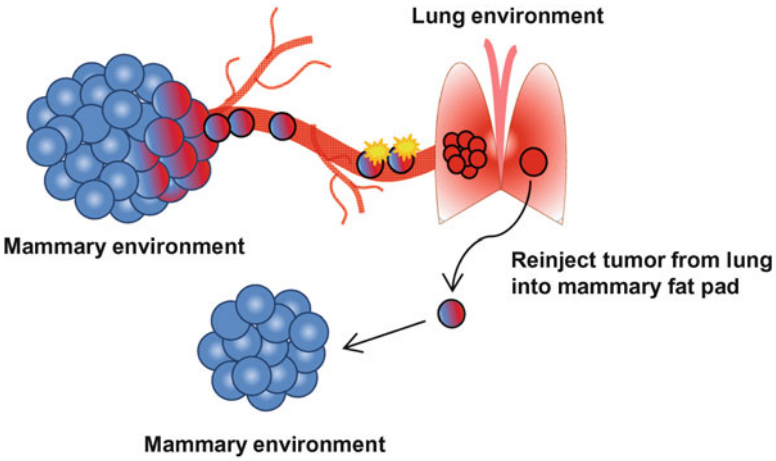

Fig. 17.3 Transient compartment model. Transient compartment model suggests that epigenetic or microenvironmental factors allow cells to become metastatic. All viable cells (*blue*) in the tumor acquire metastatic capacity, but due to positional (*red*/*blue*) and/or random epigenetic (*yellow star*) events only a small fraction is capable of completing the process at a given moment (as depicted by the change from *blue* to *red*)

Fusion Model

Many models have been proposed to explain the mechanism of metastasis, most of which attempt to explain discrepancies between experimental observations and shed light on specific aspects of metastasis. For metastasis to successfully occur, cells must enter and survive in the circulation, then invade and form tumors at a secondary site. The clonal expansion model suggests that somatic mutations contribute to the heterogeneity in the primary tumor while the cancer stem cell model requires dedifferentiation of cells into a more embryonic phenotype, and both genomic instability and anaplasticity are attributes of highly invasive cells [7, 37]. While these phenomena are not characteristic of normal epithelial cells, they are to cells that originate from lymphoid tissue. Cells from myeloid origin are capable of dedifferentiation, migration throughout the body, and survival in many tissue environments [52]. Using features of epithelial and myeloid cells, the fusion model has been proposed to explain the dedifferentiated phenotype observed in primary tumor cells [53, 54]. The fusion model hypothesizes that epithelial cells in the primary tumor fuse with myeloid cells, resulting in the fusion of both cells' nuclei (Fig. 17.4). Lymphoid cells are known to migrate throughout the body and therefore such fusion might allow tumor cells to obtain the necessary characteristics to successfully metastasize.

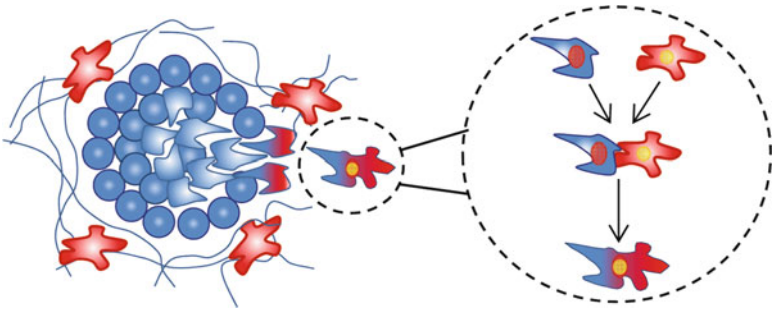

Fig. 17.4 Fusion model. Metastatic potential is achieved by fusion of a primary tumor cell with a lymphoid cell. Nuclear fusion of these two cell types endows tumor cells with gene expression programs of lymphoid cells, which enhances their metastatic potential

The idea that fusion of cells results in genomic instability is not a new one as demonstrated by early studies of fertilization by Boveri and Aichel. It was observed in the early 1900s that eggs experimentally fertilized with multiple spermatozoa underwent abnormal mitosis, which suggested that chromosomal imbalance might result in oncogenesis (reviewed in ref. [52]). Upon this observation, Aichel and Boveri proposed that the mechanism of metastasis could stem from the fusion and hybridization of cells. They hypothesized that this imbalance led to "qualitative differences" in chromosomes and resulted in metastasis.

Many in vivo experiments have supported the notion of cancer cell fusion: animal studies demonstrated that cancer cells have the ability to fuse with epithelial cells, stromal cells and endothelial cells. While the first fusion studies only observed enhanced tumorigenicity, Goldenberg et al. made the first connection between cell fusion and metastasis in 1974 [55]. These investigators injected human astrocytic tumor cells into the cheeks of hamsters and observed the formation of lethal metastases. Upon dissection and analysis of these cells, they found them to be hybrids containing human and hamster cells. Similarly, data from a study by Larizza et al. showed that the fusion of low-metastatic T-cell lymphoma cells with host macrophages resulted in hybrids that were more metastatic in nature than the tumor cells alone [56]. They observed that the hybrid cells expressed the macrophage-specific antigen Mac-1, which was not found in the T-cell lymphoma line or any other tumor cell line except for a macrophage tumor cell line. The investigators concluded therefore that the fusion of the tumor cells with the host macrophages could be a mechanism for genetic alterations leading to metastasis. Furthermore, recent evidence by Carloni et al. suggests that cellular fusion also plays a role in chemoresistance in colon cancer [57]. The authors showed a mechanism by which the expression of ADAM10 on colon cancer cells drives cellular fusion and this, in turn, leads to the development of chemoresistance to 5-fluoro-uracil and oxaliplatin. It is known that metastatic cells are highly resistant to chemotherapy and, therefore, these data could have important implications in understanding therapeutic resistance in metastatic colon cancer.

It is known that metastatic tumor cells target specific tissues, and the fusion model has attempted to explain this phenomenon of organotropism. In a study dating back to 1984, De Baetselier et al. demonstrated that fusion of tumor cells with a particular lymphocyte resulted in differential organ metastasis: the fusion of myeloma cells with B cell lymphocytes led to metastases to the spleen and liver whereas the fusion of plasmacytoma with a macrophage gave rise to lung metastases. While this study did not implicate cell fusion to enhancing metastatic capability, it linked cell fusion to metastatic organotropism [58].

Most of the data for the fusion model so far has occurred in vitro. However evidence for spontaneous cellular fusion in solid tumors, although rare, has been shown in humans. The occurrence of renal cell carcinoma in bone marrow transplant recipients has been described in which the tumor cells contained markers from both donor and recipient [59, 60].

Evidence exists to support the mechanism of cellular fusion in tumorigenesis. Data have shown that hybrid cells exist with features of both tumor cells and macrophage-specific phenotypes. However, there is no clear pathway or evidence suggesting cell fusion to be mechanistically linked to metastatic progression. The role of the tumor microenvironment and bone marrow-derived cells in metastasis has been studied (reviewed below) and shown to be significant in the dissemination of primary tumor cells. Whether this occurs via the fusion of these two cell types requires elucidation in vivo models or from patient samples.

Gene Transfer Model

Similar to the fusion model, a related hypothesis regarding metastatic capacity has been proposed. The gene transfer model is based on a theory observed among nineteenth century physicians who debated whether primary tumors could release unknown substances that then influence normal cells at secondary sites. Years later, in 1965, Bendich et al. revisited these original observations and demonstrated that DNA, indeed, can be found in the circulation of tumor mice [61]. Similarly, a study by Leon et al. demonstrated levels of free DNA in patients with and without tumors. While the levels of DNA did not correlate with the size of a primary tumor in cancer patients, the authors did see a significant correlation in those with metastatic disease as compared to those with no metastases [62]. These studies gave rise to the model of genometastasis which hypothesizes that secreted DNA from primary tumors could be horizontally transferred to susceptible cells in a distant site and therefore give rise to a secondary tumor (Fig. 17.5). To demonstrate this theory, experimental data showed that plasma from tumor-injected animals can transfect cells with DNA in culture [61]. Furthermore, it has been proposed that the circulating DNA can be taken up by stem cells at secondary sites [63]. Thus metastatic lesions may not derive directly from primary tumor cells but rather circulating DNA from primary tumor cells may mediate horizontal gene transfer that may induce tumorigenesis at the distant site.

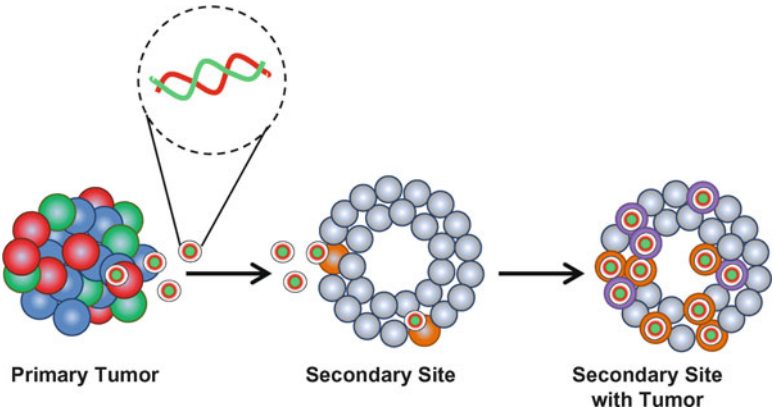

Fig. 17.5 Gene transfer model. The primary tumor secretes DNA (*small green/red circles*) into the bloodstream and is taken up by stem cells (*orange cell*) in the distant organ. This horizontal gene transfer enables stem cells to develop into tumor cells at the secondary site (*orange* and *purple cells*)

Even though experimental data exists to support the validity of this hypothesis, a few caveats must be taken into account. It has been well observed and studied that cancers exhibit specific organ preference for dissemination and colonization [4, 5]. If primary tumors do release oncogenic DNA into circulation that then has access to all tissues in the body, how does it transform only specific cells at specific distant sites? If the genometastasis theory held true, then the DNA would have to contain markers which only certain tissue sites could recognize. While this may be theoretically possible, no in vivo data exist to support this phenomenon. Furthermore, if DNA in plasma is the basis for metastasis, sufficient uptake of the genetic information of the primary tumor by the cells at the distant sites has to occur for reprogramming of the cells to resemble the primary tumor cells. Enough evidence exists for the presence of tumor DNA in blood plasma, however thus far it has only been suggested to be used as a biomarker for disease [64, 65] and has not yet been shown to be mechanistically involved in metastasis promotion.

MicroRNAs and Metastasis

MicroRNAs (miRNAs) are short nucleotide sequences (17–20 nt) of noncoding RNAs each of which are capable of sequence specific binding of numerous mRNA targets. miRNAs regulate mRNA transcript abundance or expression by targeting mRNA for degradation or interfering with its translation, respectively. As such, miRNAs are capable of regulating cellular functions such as development, proliferation, apoptosis, and cell cycle progression, functions critical to cellular homeostasis, tumorigenesis, and metastasis [66–68].

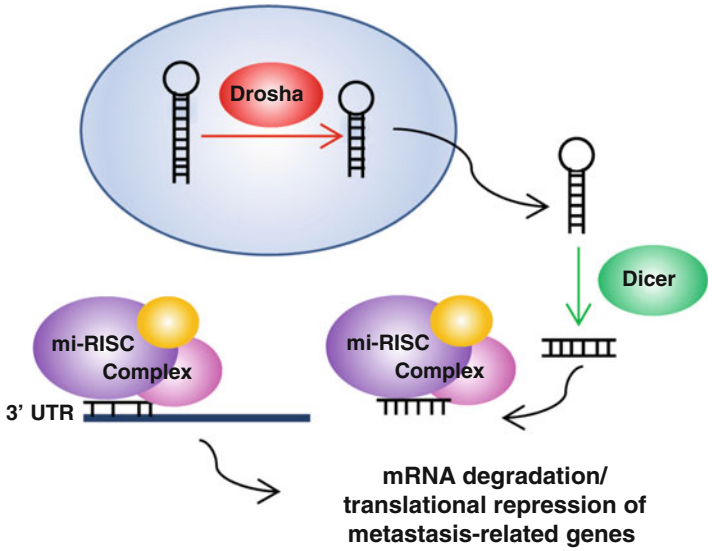

Fig. 17.6 miRNA maturation and function in metastasis

miRNAs are initially transcribed in the nucleus and processed by Drosha into precursor-miRNA structures which are then shuttled into the cytoplasm where they become further processed by Dicer. Upon maturation the miRNA, along with the multiprotein RNA-induced silencing complex (mi-RISC), bind to sequences on the 3′ untranslated region of target genes. Depending on the degree of complementarity of the seed sequence and the target mRNA, miRNA binding leads to the degradation or translational repression of its target transcripts (Fig. 17.6). The seed sequences vary from 2 to 8 nucleotides and, because complete complementarity binding to these sequences is not required for gene regulation, miRNAs can bind to more than one target. Due to this lack of perfect complementarity, it is thought that a single miRNA can influence the expression of hundreds of genes [66, 67]. Their function in gene regulation plays a significant role in cellular physiology and homeostasis but, when aberrantly expressed, they can also be involved in disease progression. Therefore, since their discovery, miRNAs have been a major focus in tumorigenesis and metastasis.

Evidence from miRNA studies has demonstrated that these small RNAs are involved in the suppression or progression of cancer pathways leading to metastasis [69, 70]. From regulation of cellular proliferation to epithelial-to-mesenchymal transition (EMT), one of the initial steps in the distant dissemination process, miRNAs have shown to play crucial roles as oncogenes and tumor suppressors. As an activator of metastasis, miR-21 has been well studied. First discovered in glioblastoma [71], this miRNA has since been shown to regulate gene function in a variety of solid tumors such as breast, colon, lung and prostate cancer [72–75]. In glioblastoma, miR-21 was demonstrated to function as an anti-apoptotic factor by

downregulating genes important in apoptosis. Since then, miR-21 has been shown to play many more roles in tumorigenesis and metastasis. In breast cancer, this miRNA was shown to target tumor suppressor tropomyosin 1 (TPM1), an actin-binding protein that suppresses anchorage-independent cell growth. Upon overexpression of miR-21, TPM1 levels are knocked down, leading to aberrant tumor growth. In metastasis-specific studies miR-21 was demonstrated to increase metastatic capacity by regulating the expression of genes important for cell invasion, such as matrix metalloproteinases (MMPs) [76].

Other metastasis-activating miRNA including miR-10b, a miRNA discovered by the group of Weinberg, can have their expression regulated by a gene involved in EMT, such as TWIST [77]. Overexpression of TWIST induces EMT and subsequent cellular invasion of tumor cells, upstream steps of the invasion–metastasis cascade. TWIST-mediated activation of miR-10b leads to the downregulation of HOXD10, initiating the transcription of various pro-invasion genes including the derepression of RhoC which enhances cellular motility. Identifying miR-10b as a target of TWIST regulation, in addition to other studies, demonstrated that miRNAs are significant contributors to the initiation of metastasis.

Since the discovery of miR-21 and miR-10b, several other miRNAs have been shown to be involved in tumorigenesis and metastasis [72, 74, 78]. While these small RNAs have added another layer to understanding molecular mechanisms of tumor progression, they have also opened a door to many new and interesting questions. Since each miRNA has the potential to target dozens if not hundreds of targets, it is currently difficult to discern the significance of any one particular target in tumor progression. A recent study shows that a miR-126-mediated regulon—a set of transcripts regulated by a single miRNA—non-tumor cell autonomously regulates endothelial cell recruitment to metastatic breast cancer cells [79]. With the recent advent of novel miRNA-based therapeutics, a more complete understanding of the roles of miRNA in tumor progression and metastasis may provide another avenue to clinically target metastasis.

The Tumor Microenvironment and Metastasis

While historically most work on tumor progression has focused on the role of tumor-cell-autonomous mechanisms, it is now widely accepted that the tumor non-autonomous microenvironment also plays a significant role in progression and metastasis. The tumor microenvironment consists of the untransformed cell types in the immediate surroundings of tumor cells including myoepithelial cells, endothelial cells, lymphocytes, myeloid cells, and fibroblasts. It also includes noncellular components such as the extracellular matrix, as tumor-stroma interactions have been shown to alter the composition of extracellular matrix deposition [80, 81]. It has been known that epithelial cells communicate with the surrounding stroma to maintain tissue homeostasis [81]. Conversely, the stromal cellular environment secretes factors that modulate epithelial behavior such as proliferation. In addition

to the cells' own regulation, factors from cells in the microenvironment can send stimuli to further regulate homeostasis [82]. This concept can also be applied to the tumor environment, such as the stromal cells and primary cancer cells. During tumorigenesis, therefore, aberrant signaling from these cells can stimulate tumor cells to disseminate as well as prepare the secondary site for successful colonization by the disseminated tumor cells.

An important factor in the metastatic process is the extracellular matrix (ECM), which can regulate cell behavior. This matrix can act as a physical barrier which, when degraded, allows cells to leave their surroundings. It is also a repository for growth factors and cytokines that stimulate growth and modify cellular behavior. During tumorigenesis, cancer cells can degrade the proteins that normally allow them to stay in place so that they can leave their primary organ and enter the blood stream, which are the initial steps of dissemination.

Evidence for the interplay between tumor cells and the microenvironment initially came from studies showing that teratoma cells injected into blastocysts of a different cohort of mice gave rise to genetically normal mice [83]. The data from this study suggested that a non-tumorigenic cell microenvironment could suppress and reverse the cancerous phenotype of the injected cells. Since then, many other investigators have applied this concept to their research. Olumi and others have demonstrated that cancerous cells can reverse their aggressive tumor phenotype when cocultured with the ECM of normal cells. When added to a tumorous microenvironment, however, cancer cells can become more aggressive, leading to an increase in migration and invasion [84, 85]. Studies like these lead to the hypothesis that the interaction of tumor cells with their surrounding environment can activate or repress metastasis.

Recently, a set of observations have led to the concept of the premetastatic niche, which proposes that primary tumors produce factors that remodel the microenvironment of the secondary site to make it more amenable for colonization prior to the arrival of metastatic cells. The first study inspecting the premetastatic niche suggested that bone marrow-derived hematopoietic progenitor cells positive for vascular endothelial growth factor 1 ($VEGFR1^+$) arrive at the secondary site and alter the tissue microenvironment by upregulating integrins and cytokines. Interestingly, subsequent to the implantation of tumor but prior to its colonization by ($VEGFR1^+$) hematopoietic progenitor cells, an upregulation of fibronectin was observed at the distant site, suggesting that the primary tumor somehow communicated with the distant site to alter its gene expression to allow for the arrival of metastasis-promoting ($VEGFR1^+$) hematopoietic progenitor cells. Importantly, the functional role of ($VEGFR1^+$) hematopoietic progenitor cells in promoting metastasis was directly queried by demonstrating that the metastatic potential of tumor cells was abrogated by treating of ($VEGFR1^+$) hematopoietic progenitor cells with anti-VEFGR1 prior to implantation into irradiated mice [86].

To further add to the role of the microenvironment, one of the most recent findings is the theory of priming bone marrow-derived cells (BMDCs) towards a metastatic phenotype via exosomes. Exosomes are vesicles that are secreted from a variety of cells. These structures mainly carry cellular cargo such as proteins, mRNAs, and miRNA which can be transported from one cell to another [87, 88]. Such transfer of information may be a type of intercellular communication.

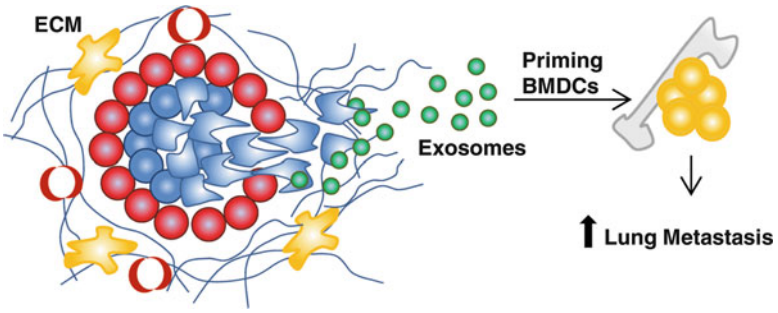

Fig. 17.7 The role of exosomes in metastasis. Exosomes released by metastatic cells prime bone marrow-derived cells (BMDCs) to potentiate lung metastasis

As stated above, BMDCs have been shown to play a significant role in adjusting the microenvironment to be more suitable for successful dissemination and it is possible that tumor cells communicate with distant sites to form an amenable premetastatic niche by secreting such exosomes.

Integrating the above findings, data by Peinado et al. showed that melanoma-derived exosomes have the ability to prime BMDCs to develop a pro-metastatic environment (Fig. 17.7). The authors of this study showed the interplay of tumor cells and their microenvironment at a molecular level [89]. First, the investigators determined the significance of exosomes by measuring their levels in various clinically staged melanoma patients and found that a positive correlation between tumor stage and exosome protein levels. They then demonstrated that introducing exosomes from highly metastatic melanoma cells into naïve mice resulted in exosomes localizing to sites at which metastasis is commonly observed. These initial experiments indicated that exosomes could play a role in metastasis. Furthermore, the authors showed that these exosomes carry proteins important in the formation of a pre-metastatic environment. Such proteins included the Met oncoprotein, heat shock protein 90 (HSP90) and tyrosinase-related protein 2 (TYRP2). This study therefore demonstrated the importance of primary tumor communication with cells needed for metastasis. By secreting exosomes that contained pro-metastatic proteins, tumor cells could prime the environment of metastatic sites before dissemination occurred.

Among many others, the aforementioned studies showed that an intertwined network of communications mechanisms exists between tumor cells, the primary tumor microenvironment, and the microenvironment at the distant secondary site. In the context of physiological function one can imagine that distant and disparate organs concertedly regulate homeostasis. In the context of cancer these same mechanisms can be exploited by the tumor to promote its own dissemination and virulence. While the exact mechanisms of bone marrow cell-derived education by tumors remain to be worked out, studies like these highlight the importance of understanding tumor biology on a scope beyond tumor cell intrinsic mechanisms. And though these studies cast an unanticipated layer of complexity to tumor progression, they also suggest an entirely novel set of molecular and cellular targets for the development of therapeutics.

Genetic Susceptibility

While the previous models propose that somatic mutations drive metastasis, our laboratory focuses on the genetic susceptibility to metastasis encoded within the germ line. Germ line polymorphisms contribute to defining each person as an individual. Differences such as eye color, height, or responses to drugs can be explained by polymorphisms within the germ line. Similarly, this concept also appears to hold true to the susceptibility of an individual to develop metastasis [90]. Studies in our laboratory, among others, have demonstrated that germ line polymorphisms modify cellular properties leading to tumorigenesis, invasion, and metastasis [91–93].

This concept was first developed with the observation that inbred mice of distinct genetic backgrounds showed differential susceptibilities to lung metastasis (Fig. 17.8). The initial experiment was conducted by crossing female mice of various inbred strains (such as FVB, NZB, C58BL/6, AKR, DBA, etc.) to male FVB mice transgenic for the mouse mammary tumor virus promoter driving mammary tissue-specific expression of the polyoma middle T antigen (MMTV-PyMT) oncogene. All female transgene positive F1 progeny acquired mammary tumors; however progeny of different maternal genetic backgrounds showed distinct pulmonary metastatic burdens. Since the oncogenic driver and paternal genotype were constant in all mice, this study clearly demonstrated that, in mice, polymorphisms in the maternal germ line contribute to metastatic susceptibility [94]. Extending this observation to humans led to the hypothesis that the genetic make-up of an individual can predispose him or her to be more vulnerable to metastatic progression upon tumor initiation and has opened the door to epidemiological studies to support it. In mice, the idea that metastatic susceptibility is a quantitative (polygenic) heritable trait has given way to quantitative trait loci (QTL) mapping, crossing mice with significantly different metastatic susceptibilities and tracking genotype and phenotype, to identify regions of the genome associated with—and therefore likely containing elements regulating—metastasis.

Data from our laboratory first identified a candidate polymorphic gene whose differential expression resulted in modulation of metastatic capability of murine tumor cells and that could be used to successfully stratify patients into poor and good survival groups. Since metastasis is the primary determinant of survival, it can be inferred that this polymorphism modified metastatic potential in patients [95]. By mapping the loci potentially responsible for differences in metastasis, we found *Sipa1* on the *Mtes1* locus. Ectopic expression of *Sipa1* was shown to enhance metastasis while knockdown of *Sipa1* reduced the metastatic capacity of tumor cells in a mouse model of metastasis. Furthermore, the same polymorphism was identified in a cohort of human breast cancer samples and, as predicted, it was a marker of poor outcome in estrogen receptor-positive (ER^+) breast cancer. These data were particularly exciting as this was one of the first studies to show that genetic background can influence susceptibility to metastasis in humans.

With recent advances in global transcript analysis, further investigations of gene networks and their role in metastatic progression became possible [96]. A study from

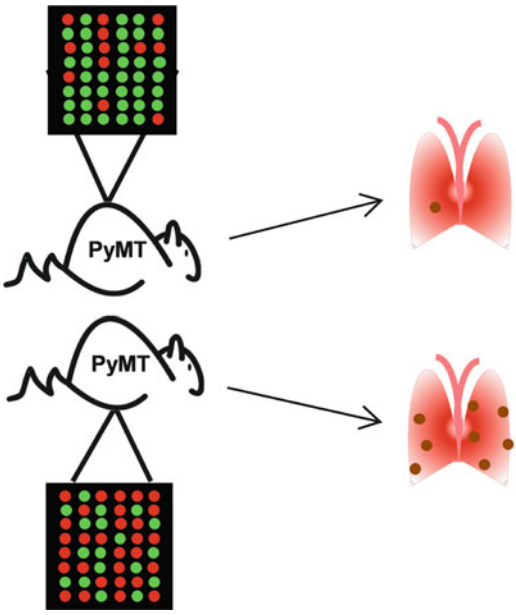

Fig. 17.8 Genetic susceptibility to metastasis. Variability in the germ line genome and in gene expression networks contribute to determining an individual's metastatic susceptibility

our laboratory demonstrated that the global transcript network analysis of human as well as mouse samples identified co-expressed gene networks capable of predicting metastasis-free survival in independent human breast cancer cohorts. Interestingly, these networks also suggested that the differences in breast cancer subtypes were either due to tumor-cell-autonomous behavior or the microenvironment. Estrogen receptor-positive (ER^+) breast cancers, for example, were shown to be tumor-driven while estrogen receptor-negative (ER^-) breast cancers appeared to be influenced by the host-derived stroma [97].

As an example of this concept, a recent study from our laboratory identified *Cadm1*, a gene whose over- and under-expression influenced metastatic outcome of breast cancer cells. This gene was identified after analyzing the quantitative trait loci (QTLs)—regions of the genome that segregate with the phenotype of interest subsequent to introduction of genetic and phenotypic diversity by breeding genetically and phenotypically distinct mice—of NZB and FVB, mice with significantly different susceptibilities to pulmonary metastasis, and then searching for polymorphic genes that were also differentially expressed in tumor tissue. Although *Cadm1* was differentially expressed in both tumor and untransformed tissue between NZB and FVB, it showed no coding-level polymorphisms between the two strains. Similar to the network analyses above which queried samples based on differential expression, it appeared in this case that differential expression of *Cadm1* was the significant factor. This was confirmed by showing that overexpression of this candidate metastasis modifier gene resulted in the suppression of lung metastases while knocking down *Cadm1* increased the ability of breast cancer cells to colonize the lungs. Furthermore, this study showed that this difference in metastatic

susceptibility, though resulting from tumor-cell autonomous differential expression of *Cadm1*, had a tumor nonautonomous component as the metastasis suppressive effects of high *Cadm1* expression was lost in mice lacking functional T-cell-mediated immunity. This study therefore showed that polymorphisms in germ lines can not only predict the susceptibility to metastasis but that such genes can also play a role in tumor-nonautonomous factors [98]. Importantly, because T-cell-mediated immunity was essential to *Cadm1*-mediated effects on metastasis, the use of metastatic human tumor cell lines in athymic mice would have been incapable of detecting *Cadm1* as a metastasis suppressor. In this regard, the study by Faraji et al. underlines the essential role mouse mammary tumor cell lines in immune-competent mice play in modeling metastasis biology.

To emphasize the differences in gene network and breast cancer outcome based on subtype, another of our studies recently showed that tumor-autonomous genes influence metastasis. QTL analysis of PyMT crossed with the AKXD panel of recombinant inbred mice identified another metastasis susceptibility gene that influenced the dissemination of breast cancer cells to the lungs. We showed that polymorphisms in *Arib4b* on AKR/J and DBA/2J alleles had different metastatic phenotypes. Furthermore, while analyzing gene networks, we discovered that this gene regulated many genes of the *Tpx* network, which previously showed to be useful in predicting metastasis-free survival in ER^+ breast cancers. As expected, the levels of ARID4b predicted of ER^+ breast cancers. This data provided further evidence that germ line polymorphisms in tumor-autonomous genes play a role in predicting metastasis progression in specific subsets of breast cancer [99].

Taken together, these studies demonstrate that a germ line component exists that influences a tumor's ability to successfully form metastatic lesions. Additionally, polymorphisms in genes that regulate or are within the global gene networks can predict metastasis-free survival in subsets of breast cancer. While these data are promising and have been supported by epidemiological studies, additional studies could further confirm that this concept directly applies mechanisms of metastasis in humans. In contrast to the clonal selection model which bases the ability of metastasis on somatic mutations, genetic susceptibility to distant dissemination of cancer cells is inherited. This not only provides a novel approach to dissect molecular pathways involved in metastasis to find novel therapeutic targets, it also provides insights into predicting patient outcomes using gene expression signatures based on metastasis susceptibility-specific markers.

Conclusion

The invasion–metastasis cascade continues to be poorly understood, particularly with regard to therapeutically targeting metastatic lesions. Over the past century, clinical and anatomical insights into metastasis coupled to technical advances in cellular and molecular biology and animal modeling have shed light onto the mechanisms of metastasis. Yet cellular determinants and gene expression programs

mediating tumor cell dissemination continue to be incompletely understood, as evidenced by the largely refractory nature of metastatic lesions to classical and targeted chemotherapeutics. In this review, we have summarized the leading models and recent conceptual advances that provide a framework for our understanding of the invasion–metastasis cascade. The fact that several disparate models, each illuminating one layer of biology involved, have been proposed to describe key aspects of metastasis is a testament to the complexity of the metastatic process. The emerging challenge is now to link the relevant aspect of each model to identify rate-limiting steps of the invasion–metastasis cascade, which may reside at conceptual interfaces between models, for therapeutic targeting. The development of successful therapeutics against metastatic disease necessitates elucidating clear links between intracellular protein and RNA signaling pathways in tumor cells and in non-neoplastic components of the microenvironment while considering the vast genetic heterogeneity within the tumor. Such knowledge will pave a path for the development and strategies for implementation of a arsenal of novel therapeutics against the largely untreatable and final stage of cancer.

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Part V
Reaching Cancer Cells

Chapter 18
Cancer-Specific Ligand–Receptor Interactions

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Abstract The concept of cancer targeting, which exploits the abundance of specific molecular epitopes on cancer cells, has been proposed as a strategy to enhance the efficacy and specificity of cancer therapy and diagnostics. Although many promising results have been obtained with this approach, the research experience of the last decades demonstrates clearly the challenges that the clinical application of cancer-targeted approaches faces. This can be attributed to both the complexity of targeted probe–cell interactions as well as the multitude of additional factors, which influence the efficacy of the targeting process. The aim of this chapter is to address the key steps involved in the cellular pathway of ligand-functionalized probes for cancer targeting. Special attention is given to nanoparticulate delivery systems as the most commonly exploited formulations for cancer targeting. Their interaction with target cells is initiated by ligand binding to the cell surface receptor, which is frequently followed by endocytosis of ligand–receptor complex and, in the final phase, by lysosomal degradation. All the aforementioned processes are presented in view of the pathophysiological and molecular features of the biological system as well as the physicochemical and biological properties of targeted probes. Importantly, we discuss the implications of these intracellular events for the therapeutic activity and diagnostic capabilities of targeted agents.

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Introduction

This chapter describes the state-of-the-art in the exploitation of tumor-specific molecular and cellular targets for advancing diagnostic imaging and chemotherapeutic interventions in the field of oncology. Following an account of the rationale for tumor-directed targeting, the concepts underlying ligand–receptor interactions that form the prime basis of the sought-after tumor specificity are extensively described. The emphasis is on the use of cell-surface receptors, as these are readily accessible from the extracellular compartment. Next, the molecular details of ligand–receptor interactions, the options for the design of specific ligands as well as the biological consequences of the formation of the ligand–receptor complex are reviewed. Since many targeting strategies in oncology involve the use of nanoparticles, considerable attention is paid to the interaction between ligand-conjugated nanostructures that can be equipped with a high payload of imaging and/or therapeutic agents. The role of multivalent interactions between the nanoparticles and the target receptors, and the opportunities they provide for optimizing the efficacy and specificity of the tumor targeting are described at length. The initial cell surface interaction is often followed by internalization of the ligand–receptor complex. Therefore, we next highlight the intracellular fate of the internalized materials as well as the consequences of the processes involved for the efficacy of diagnostic imaging and antitumor therapy. The chapter ends with a brief outline of the future perspectives of cancer-targeted diagnostic and therapeutic agents.

Rationale for Targeted Strategies in Oncology

Over the past 20 years, we have witnessed intensive developments in the field of targeted therapy and diagnostics of cancer. This can be attributed to the arsenal of molecules that has been identified for selective interaction with tumor cells and remarkable advances in engineering of targeting ligands and delivery platforms. In this section, we aim to give an outlook on the versatility of established molecular markers of cancer as well as on the development and application of cancer-specific ligands. Furthermore, we outline main trends in the design of nanocarrier-based delivery platforms.

The tumor microenvironment comprises two interdependent compartments: the parenchyma composed of neoplastic cells and the stroma formed by host cells [1]. Neoplastic cells are the primary source of malignancy. However, the nonmalignant supporting elements, including connective tissue, blood vessels and in many cases also inflammatory cells, are equally crucial for cancer cell survival and tumor progression. For that reason, both types of tumor tissue components are important therapeutic and imaging targets.

Cancer cells present a number of specific molecular epitopes, which are actively involved in the development and spread of malignant lesions. The human epidermal

growth factor receptor (HER) family of receptor tyrosine kinases control critical pathways involved in epithelial cell differentiation, growth, division and motility [2]. Two members of the HER family: the epidermal growth factor receptor (EGFR) and HER-2 are currently the most exploited molecular cancer cell targets, both for therapeutic and imaging purposes. EGFR is overexpressed in approximately 80 % of all epithelial carcinomas [3], whereas the 10- to 100-fold upregulation of HER-2 is found mainly in breast cancer [4, 5]. Overexpression of the folate receptor (FR), reaching even 200-fold higher levels compared to normal tissue, is also a characteristic feature of many malignancies [6]. FR binds folate and folic acid derivatives, which participate in the biosynthesis of nucleic and amino acids, thus playing an essential role in cell survival. The transferrin receptor [7] and hyaluronan receptor (CD44) [8] are also important molecular markers present in the plasma membrane of malignant cells. Steroid hormone receptors, which belong to the group of cytoplasmic receptors, have been also implicated in malignant growth, e.g., the androgen receptor in prostate cancer [9], the progesterone receptor in ovarian and breast cancer [10, 11], and the estrogen receptor in breast cancer [12]. This category of receptors can be reached, however, only after ligand penetration into the cytoplasmic compartment.

Tumor angiogenesis, the process of tumor vessel formation from pre-existing host vessels, has become an important target for many therapeutic and diagnostic strategies in oncology [13, 14]. The activated endothelium is particularly suitable for the application of molecular recognition strategies since it overexpresses a variety of specific molecules that are virtually absent in the normal vasculature and it is of vital importance in tumor progression [15–17]. Additionally, the endothelium is in contact with blood, making it directly accessible for an intravenously injected agent. The vascular endothelial growth factor (VEGF) family and VEGF receptors (VEGFR) play an essential role in angiogenesis and lymphangiogenesis. VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF [18]. VEGFR-1 seems to be not directly implicated in mitogenesis and angiogenesis. However, growing evidence supports the idea that VEGFR1 has important roles in hematopoiesis [19, 20]; in the recruitment of monocytes and other bone marrow-derived cells that might also be incorporated in the tumor vasculature [21, 22]; in the induction of matrix metalloproteinases [23]; and in the paracrine release of growth factors from endothelial cells [24]. Cell–extracellular matrix interactions, which are mediated by transmembrane cell adhesion receptors, are particularly important during vessel sprouting. Among the adhesion molecules, $\alpha_v\beta_3$ integrin was found to be particularly active in the process of endothelial cell migration and apoptosis [25]. Arg-Gly-Asp (RGD) is a well-documented peptidic sequence [26, 27] with a high affinity for this angiogenic marker, and its different stereochemical variants have been exploited as targeting ligands [28–30]. Furthermore, galectin-1 has been recently identified as an important molecule in the signaling of endothelial cell proliferation, migration, and apoptosis [31].

As mentioned above, VEGFR signaling is also actively involved in the process of lymphangiogenesis, i.e., the formation of lymphatic vasculature [32], which has been suggested as a key promoter of tumor metastases in the lymph nodes [33–35].

Two VEGF factors i.e., VEGF-C and VEGF-D, are the key lymphangiogenesis propagators. They act via VEGFR-3, the activation of which triggers lymphatic endothelial cell proliferation, migration and survival. Therefore, VEGFR-3 has been proposed as a molecular target for anti-lymphangiogenic therapy [36]. LYVE-1, the receptor for the extracellular matrix mucopolysaccharide hyaluronan, is a second important marker of lymphatic endothelium [37]. Furthermore, podoplanin, a transmembrane glycoprotein found in the cell membrane of lymphatic endothelial cells, plays a regulatory role in lymphatic vasculature formation [38, 39]. It should be stressed, however, that expression of the latter two markers is not exclusive to newly formed lymphatics.

In addition to the endothelial layer, tumor-associated fibroblasts and myofibroblasts play an important role in the maintenance of the functional vasculature [40]. These cells migrate to areas of angiogenic activation to support the endothelium of the newly formed tumor vessels. The production of growth factors, chemokines and extracellular matrix by the fibroblasts facilitates the angiogenic recruitment of endothelial cells and pericytes. The cell-surface serine protease known as fibroblast activation protein (FAP) emerges as a promising candidate for specifically targeting tumor-associated fibroblasts [41]. FAP is not expressed by mature somatic tissues except for activated fibroblasts during wound healing and within tumor stroma [42].

Inflammation is a common feature of the tumor microenvironment [43, 44]. Tumor-associated macrophages (TAMs), classified as immunosuppressive macrophages (M2), are the largest population of immune cells present in malignant tissue. They actively contribute to tumor development, by releasing various inflammatory mediators that promote tumor angiogenesis. Both the macrophage scavenging receptor 1 (CD204) and hemoglobin-scavenger receptor (CD163) are considered as specific M2 macrophage markers [45, 46]. Strongly pro-angiogenic TAM's that reside in hypoxic tumor areas have been shown to highly express macrophage mannose receptor (MMR, CD206) [47]. Furthermore, the targeting of p32/gC1qR receptor by using LyP-1 peptide was shown to produce a selective recognition of TAM's [48].

Compositional changes in the extracellular matrix (ECM) are one of the hallmarks of tumor angiogenic activation. These include the degradation of macromolecules, such as collagen, decorin, thrombosponin 1 and 2, and hyaluronan, to yield low-molecular weight fragments that exert pro- or anti-angiogenic activity [15]. The expression of ECM enzymes, e.g., hyaluronidase, metalloproteinase-2 and -9, and heparinize, can therefore serve as an indication of the angiogenic status. Furthermore, the level of enzymatic activity in the ECM has been correlated to tumor aggressiveness and metastatic potential [49, 50].

The growing number of identified cancer molecular markers goes hand in hand with developments in the bioengineering of targeting agents. In general, targeting ligands can be classified as proteins, i.e., antibodies and their fragments, nucleic acids (aptamers), and other receptor ligands, such as peptides, vitamins, and carbohydrates. The key feature of a targeting ligand is the presence of a unique sequence of amino acids or other molecules that can bind with high affinity and specificity to the target receptor. In antibodies and antibody fragments, the antigen-binding site is

called the Fab region. Both natural and engineered agents can be used for selective targeting of a given antigen. Targeting of cancer with monoclonal antibodies (mAb) for therapeutic purposes was initiated by Milstein in 1981 [51] and followed by clinical approval of several different mAbs. Rituximab (Rituxan) was the first mAb approved for treatment of patients with cancer, namely with non-Hodgkin's lymphoma [52]. In 1998, trastuzumab (Herceptin), an anti-HER2 mAb that binds to ErbB2 receptors, was approved for the treatment of breast cancer [53]. The first angiogenesis inhibitor, bevacizumab (Avastin), which is an anti-VEGFR mAb, was approved for treatment of colorectal cancer in 2004 [18]. Antibodies may be used in their native state or as fragments. The use of whole mAbs is advantageous because of the presence of two binding sites, which results in higher binding avidity and stability. However, the Fc domain of an intact mAb is responsible for increased immunogenicity and enhanced liver and spleen uptake. Recent developments in the field of antibody engineering have resulted in the production of chimeric mAbs, humanized mAbs, and antibody fragments, which are characterized by lower immunogenicity compared to mAbs. The group of antibody fragments consists of antigen-binding fragments (Fab), dimers of antigen-binding fragments (F(ab′)2), single-chain fragment variables (scFv) and other engineered fragments. For efficient screening of targeting antibodies and their fragments, phage display libraries have been developed [54, 55]. This method generates a multitude of potentially useful antibodies that bind to the same cells but to different molecular epitopes. For example, through this high throughput approach, scFv antibody fragments have been identified with superior binding to and internalization by prostate cancer cells [56]. Moreover, inspired by the high efficacy of multivalent ligand–receptor interactions, homo- and hetero-multivalent ligands have been engineered [57]. Antibody fragments have been covalently linked to form dia-, tria- and tetrabodies, which display increased functional affinity compared to the monomers [58]. Furthermore, peptides have been found to be very useful as targeting ligands. They possess many advantages as compared with antibodies, such as small size, relatively easy synthesis and modification, and good biocompatibility. The selection of proper peptide ligands can be achieved by screening peptide libraries produced by phage display [59]. In addition to bioengineered or synthetic ligands, natural compounds can be employed as targeting moieties, e.g., components of the extracellular matrix, such as heparin sulfate or hyaluronan, which have high affinity to ECM receptors overexpressed on cancer cells [60, 61].

The ligands described above can mediate target-specific therapy and/or diagnostics only when coupled to a relevant entity. This can be achieved either by direct conjugation of a ligand to a drug or an imaging agent, as in the case of targeted radiotherapeutics and radiotracers, or indirectly by conjugating a ligand to the surface of a carrier/delivery system, which contains the therapeutic or diagnostic cargo. The latter method is the most widely applied since it does not require chemical modifications in biologically active molecules, which may affect their therapeutic properties. Moreover, it enables the delivery of a high payload of a drug or an imaging agent to the target site using relatively low quantities of costly ligands. In recent years, we have witnessed a true explosion in the versatility of delivery platforms.

Generally, these can be defined as nano-size materials (typically 1–200 nm in diameter) that can carry drugs and/or imaging agents. Their surface should be suitable for functionalization with ligands to enable their interaction with the site of interest. Currently, both natural and synthetic polymers and lipids are used as drug delivery vectors. The nanocarrier family is very heterogeneous as it includes polymer conjugates, polymeric nanoparticles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles, including nanoshells and nanocages. The choice of the nanocarrier material and shape depends on the physicochemical properties of a drug or imaging agent. A high stability of the construct is essential to assure site-specific delivery to the tumor tissue. However, in the therapeutic setting, the stability requirement should not hinder the release of the drug once the target tissue is reached. The balance between these two features is the key to obtaining a successful formulation.

Polymers are widely explored materials for constructing nanocarriers. Polymeric nanoparticles can be made from synthetic polymers, including poly(lactic acid) (PLA) and poly(lactic co-glycolic acid) (PLGA), or from natural polymers such as chitosan and collagen [62]. The drug encapsulation does not require chemical modification and the drug release can be fine-tuned. Several multifunctional polymeric nanoparticles are currently in various stages of preclinical and clinical development [52, 63]. Unfortunately, due to the inherent structural heterogeneity of polymers, polymeric nanocarriers display a high polydispersity, which leads to significant pharmacokinetic and pharmacodynamic variability within a single dose of particles.

Lipid-based nanocarriers, such as liposomes, micelles, and nanoemulsions, are very attractive delivery platforms. This is due to their general biocompatibility, biodegradability, protection of drugs from the surrounding environment, and the ability to entrap both hydrophilic and hydrophobic agents. Furthermore, the physicochemical properties of lipid-based carriers, such as their size, charge, and surface functionality, can easily be modified. Among different lipid-based formulations, liposomes, i.e., vesicular structures formed by one or several lipid bilayers with inner aqueous phase, were found to be particularly useful delivery platforms for water-soluble agents. Today, liposomes are clinically approved to carry a range of chemotherapeutics [64]. In contrast, polymeric micelles, which are spherical particles with a hydrophobic core and hydrophilic shell, have been successfully applied as pharmaceutical carriers for water-insoluble drugs [65].

Organic nanoparticles include dendrimers, viral capsids, and nanostructures made from biological building blocks such as proteins. The latter type is clinically represented by the albumin-bound paclitaxel nanoparticle formulation Abraxane, which is approved as a second-line treatment for metastatic breast cancer [66]. Dendrimers are synthetic, branched oligomers that form a tree-like structure. Particularly, polyamidoamine dendrimers have shown promise for biomedical applications. The favorable properties of these carriers include the availability of functional groups for conjugation with targeting molecules, imaging agents, and drugs; high water solubility and well-defined chemical structures; biocompatiblity; and rapid blood clearance via the kidneys.

Inorganic nanoparticles are primarily metal-based and they may also be functionalized to introduce targeting molecules and drugs. Among them, iron-oxide and semiconductor particles have been extensively studied for magnetic resonance imaging and high-resolution superconducting quantum interference devices, respectively [67]. The recently developed inorganic nanoparticles include nanoshells, which are composed of a silica core and a metallic outer layer, and gold nanoparticles [68, 69].

Considering the very broad arsenal of potential molecular targets, targeting ligands and delivery platforms, which enable practically endless combinations of materials, we can achieve a multitude of targeted delivery systems. This trend can be clearly observed in the current research, where numerous types of molecular-recognition platforms based on antibodies, their fragments, peptides and other targeting devices are evaluated in preclinical and clinical trials [52]. In view of all the available options, it is crucial to approach the development of a delivery system in a systematic manner. To this aim, high-throughput technologies, such as combinatorial libraries of biomaterials and formulations varying with respect to the physicochemical parameters, have been explored [70–74]. These strategies are useful screening and optimization tools, which can help to define the most optimal characteristics of a delivery system.

Ligand–Receptor Complex Formation

Ligand Binding to the Receptor

Many intracellular biological processes are regulated by means of ligand–receptor interactions. Cell receptors can be either embedded in the cell membrane or present in the cytoplasm or the nucleus of the cell. In line with the main focus of this book, the cell surface receptors will be of our particular interests. As described in the previous section, ligands can be classified as proteins, i.e., antibodies and their fragments, nucleic acids, and other biomolecules, such as peptides, vitamins, and carbohydrates. The key property of a receptor ligand is the presence of a unique sequence of amino acids or other entities that can bind to the active or allosteric site of a given receptor. The interaction between the binding domain of a ligand and the acceptor site of a receptor leads to the establishment of a receptor–ligand complex (Fig. 18.1). The binding occurs via intermolecular forces, such as ionic bonds through electrostatic interactions, hydrogen bonds and van der Waals forces, and is usually reversible. The stability of the formed receptor–ligand complex, i.e., the binding affinity, is determined by the thermodynamic strength of the binding interaction between a single antigen and a single binding region of a ligand. Alternatively, the complex's stability can be dependent on the avidity or, so-called, functional affinity, which is the accumulated strength of multiple affinities summed up from multiple binding interactions. The equilibrium between receptor-bound and free ligand is expressed as the association constant (K_A). Moreover, the ligand binding is

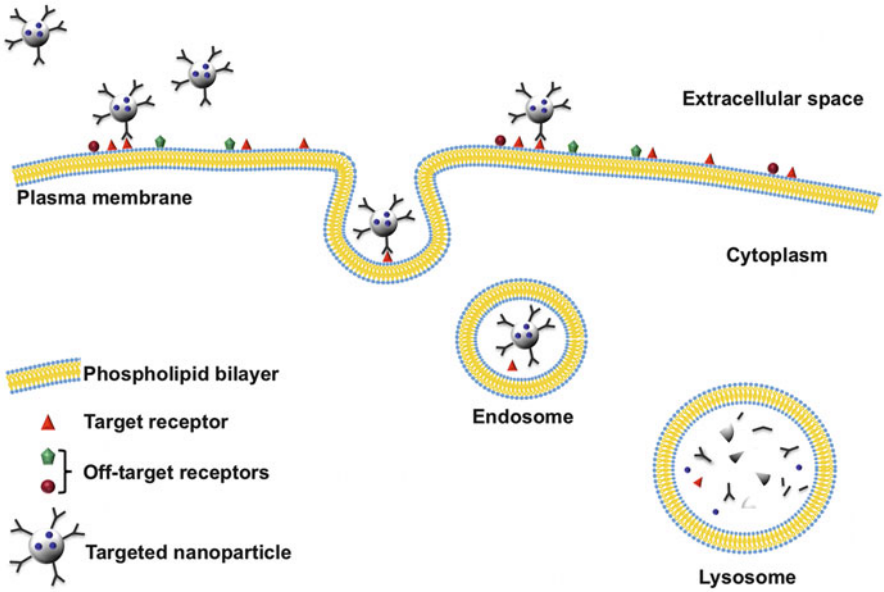

Fig. 18.1 Schematic representation of the receptor-mediated intracellular delivery of nanocarriers. In the initial phase of this multistep process, the interaction between the target receptor and receptor-specific ligand present on the nanocarrier leads to the establishment of receptor–ligand complex. This is followed by conformational changes in the plasma membrane, which trigger its invagination. The completion of this process results in the formation of cytoplasmic vesicles, e.g., endosomes, containing the endocytosed material. In endosomes, the ligand-functionalized nanoparticle can dissociate from the receptor–ligand complex to be further metabolized separately from the receptor. Eventually, the internalized nanomaterial undergoes enzymatic degradation in lysosomes. This process yields break down products, which can be either reused by the cell or excreted from the body. The co-internalized receptor enters either the recycling or degenerative endocytic pathway

often characterized in terms of the concentration of ligand at which half of the receptor binding sites is occupied, known as the dissociation constant (K_D). High affinity ligands are therefore associated with low K_D values. Optimally, the ligand should bind to only one receptor type, which is referred to as the binding specificity. Frequently, however, it is very difficult to achieve 100 % specificity, as ligands tend to interact, to different extents, with off-target receptors as well. In this case, we rather refer to ligand binding selectivity. In summary, the ligand affinity and specificity play a critical role in the ligand–receptor interactions.

In many cases, ligand–receptor interaction triggers internalization of the complex. The relationship between the ligand affinity and receptor internalization efficacy has been an important subject of the basic research on receptor–ligand interactions, also in relation to cancer targeting. To this aim, Adams et al. [75] tested three anti-HER2 IgGs, e.g., G98A, C6.5, and H3B1, having different HER2 receptor affinities. After 24 h incubation of radioiodonated IgGs with SKOV-3 human

ovarian adenocarcinoma cells, quantitative analysis of iodine in cell supernatants revealed that the lowest affinity mAb, G98A, had the highest dissociation and least catabolism of 80 and 6.5 %, respectively. In contrast, the highest-affinity mAb, H3B1, had the least dissociation and most catabolism of 14.4 and 50.1 %, respectively. These data demonstrated a clear trend of increased internalization with higher intrinsic affinity.

However, we can also find conflicting reports, showing that a high binding affinity does not always result in a high cellular uptake. For example, Cressman et al. [76] compared the binding affinity of cyclic RGD peptide with that of the $\alpha\nu\beta3$ integrin-specific antibody LM609X. The dissociation constant of the peptide was found to be in the micromolar range, which is much higher than the nanomolar range of LM609X, indicating lower binding affinity of the RGD peptide. Nevertheless, the RGD ligand showed greater cellular uptake following incubation at the temperature that permits endocytosis. A 7.4-fold increase in the cellular uptake of the RGD peptide was observed following 1 h incubation with HUVEC at 37 °C (an endocytosis permissive temperature), as compared to that at 4 °C (an endocytosis prohibitive temperature). In contrast, only a 1.9-fold increase in cell-associated fluorescence was observed for LM609X in the same conditions. According to the authors, the enhanced endocytosis of the RGD ligand, despite its less favorable binding characteristics, may be related to the different conformational and functional consequences of RGD binding as compared to antibody binding to the integrin receptor.

A number of studies proposed a specific role of ligand affinity in the efficacy of tumor penetration, which was extensively reviewed by Rudnick and Adams [77]. It has been traditionally considered that the tumor penetration by a targeted agent is determined by tumor-specific features, such as the antigen expression, structure and function of the vasculature, interstitial pressure and tumor viability, as well as pharmacokinetic properties of the targeted probe. However, it has recently been postulated that the binding affinity of a ligand can, in part, influence the intratumoral localization. This hypothesis was based on the commonly observed heterogeneous, often perivascular, distribution of mAbs within a tumor [78]. Fujimori at al. [79], by using a model algorithm for studying both full mAb and mAb fragments, showed that binding of the mAb to its antigen can limit the tissue penetration. The model predicted an inverse relationship between the affinity and penetration, which has been termed the binding-site barrier. The binding affinity, antigen density, mAb internalization and metabolism were predicted as the critical factors that affect the extent of the binding-site barrier. Empirical support for the binding-site barrier principle has been provided by several in vivo studies, which compared the distribution of tumor cell-specific and nonspecific radiolabeled mAbs after intravenous administration [80, 81]. In these studies, the nonspecific mAb was found to be diffusively distributed throughout the tumors, thereby demonstrating that it can penetrate essentially freely in the tumor meeting no biological barriers. In contrast, high concentrations of the specific mAb were retained on the periphery of antigen-rich regions when given at a low dose. At very high dose, the distribution of specific mAb was much more homogeneous and very similar to the pattern of antigen

expression, showing that dosing can be used to overcome the binding-site barrier. Similarly, studies on scFv, the binding affinity of which can be fine-tuned, further supported the hypothesis of binding-site barrier, by showing that slow dissociation rates (high affinity) of scFv limit the concentration of free scFv and, thus, reduce the tumor penetration [75, 82]. The described inverse relation between the ligand affinity and its ability for tumor penetration may have major consequences for the targeted therapy and intratumoral delivery of therapeutics. Due to the poor penetration, only small areas of the tumor may be exposed to elevated concentrations of a drug, while other regions may receive very little drug or no drug at all. This heterogeneous distribution might thus affect the overall treatment efficacy and increase the risk of drug resistance. Therefore, the penetration profile of ligands with intermediate affinity might be more favorable for drug delivery purposes.

Ligand Affinity: Impact on Targeted Delivery Strategies

The aforementioned concept of binding-site barrier is not particularly recognized in the field of targeted nanomedicine. At the same time, the inverse relation between the avidity of a delivery system and tumor penetration has been frequently reported in the literature. Mulder et al. [83] designed MRI and fluorescence-detectable liposomes functionalized with $\alpha_v\beta_3$ integrin-specific cyclic RGD peptide (Fig. 18.2a) to achieve specific in vivo recognition of tumor angiogenesis for diagnostic purposes. Liposomes equipped with RAD peptide, which has virtually no affinity to the target integrin, were used as a negative control. The differences between the target-specific and nonspecific liposomes were manifested mainly by the distinct spatial distribution of MRI signal enhancement throughout the tumor rather than by its magnitude. After injection of RGD-conjugated liposomes, the contrast-enhanced pixels were mainly located in the rim of the tumor (Fig. 18.2b, upper panel), whereas the enhancement induced by nonspecific RAD-conjugated liposomes was more evenly distributed through the tumor area (Fig. 18.2b, lower panel). Ex vivo fluorescence microscopy revealed a different mechanism of accumulation in the tumor; predominant association with the endothelium in the case of RGD-conjugated liposomes (Fig. 18.2c, upper image) and considerable extravasation in the case of RAD-conjugated liposomes (Fig. 18.2c, lower image). The preferential accumulation of RGD-liposomes at the tumor rim, which is characterized by the most pronounced angiogenic activity, has been proposed as a hallmark of their targeting specificity. The same trend of intratumoral distribution was observed by other authors working with angiogenesis-targeted delivery platforms [28, 84, 85].

Interestingly, a similar intratumoral distribution pattern was observed by Yang et al. [86] for magnetic iron oxide nanoparticles functionalized with the amino-terminal fragment of urokinase type plasminogen activator (uPA). In this case, however, mammary carcinoma cells were the cellular targets for the nanoparticles. Transversal relaxation time (T2) maps of mammary tumor-bearing mice as obtained by MRI 48 h after intravenous administration showed that uPA-targeted

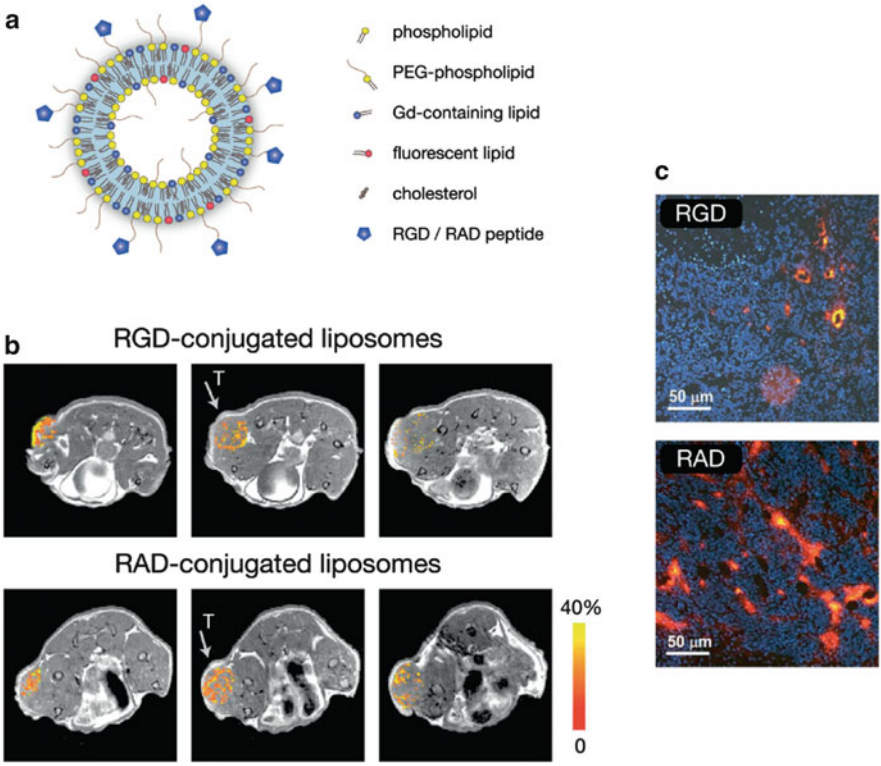

Fig. 18.2 Influence of active tumor-targeting on the intratumoral distribution of nanoparticles. (**a**) Magnetic resonance (MR) and fluorescence-detectable liposome for bimodal imaging of activated tumor endothelium. The building blocks of the liposome are shown on the right. (**b**) T1-weighted MR images of three slices through a subcutaneous xenograft human LS174T colon carcinoma tumor in an athymic mouse after intravenous injection of either RGD-conjugated liposomes (*upper panel*) or RAD-conjugated liposomes (*lower panel*). The tumor is indicated with the letter *T*. After administration of RGD-conjugated liposomes, contrast enhancement, indicated by the pseudo color scale on the right, was mainly found at the tumor rim (*upper panel*). In contrast, injection with nonspecific RAD-conjugated liposomes resulted in contrast enhancement more evenly distributed throughout the tumor (*lower panel*). (**c**) Ex vivo fluorescence microscopy of tumor sections obtained 1.5 h after liposome administration. Tumor-accumulated liposomes and cell nuclei are shown in *yellow* and *blue*, respectively. RGD-conjugated liposomes were predominantly co-localized with the tumor endothelium (*upper image*). In contrast, massive extravasation was observed for RAD-conjugated liposomes (*lower image*). Reproduced with permission [83]

nanoparticles were not uniformly distributed inside the tumor mass, as concluded from the heterogeneous T2 decrease in the tumor. Areas with the greatest T2 decline were found primarily in the periphery regions of the tumor mass. This preferential accumulation of uPA-targeted nanoparticles was linked to the previously reported high level of uPA receptor expression at the invasive edge of the tumor mass [87]. Furthermore, the authors considered the impact of dense vasculature at the tumor rim, which makes this area particularly accessible for nanoparticles.

There are several factors that may contribute to the restricted tumor penetration by the targeted delivery systems. Tumor-specific features appear to be of great relevance. Among these, the heterogeneous expression of target receptor within the tumor is the most frequently implicated factor. The tumor periphery, being a hot-spot of tumor viability and proliferation, is a site of intensive biological activity, involving not only angiogenesis-associated molecules but also other cancer-specific epitopes involved in the process of tumor invasion. This spatial imbalance in tumor molecular machinery may be therefore an underlying reason for the preferential peripheral accumulation of targeted agents. At the same time, the published work provides only scarce information on the receptor expression in tumor areas that displayed no or low nanoparticle uptake, which would help to assess the true relevance of the aforementioned factors. In addition to a high concentration of molecular targets, the tumor periphery is usually well vascularized, which facilitates its accessibility to the systemically administered agent. Next to that, a high interstitial pressure in the tumor forms a barrier to transcapillary transport [88]. This parameter would, however, be expected to affect the intratumoral accumulation of both targeted and non-targeted formulations in the same manner, which is frequently not the case. Also, it is important to stress the impact of probe-specific factors, such as the pharmacokinetic properties. It has been previously reported that modifications of the particle surface can lead to enhanced blood clearance compared to non-modified control particles [89]. This can limit the tumor exposure time to the circulating agent, leading to restricted tumor penetration. Finally, in line with the principle of binding-site barrier, a rapid binding to and intracellular turnover by the first-line target cells may limit the retention of targeted particles to the periphery of antigen-rich regions.

Multivalent Receptor Recognition

Multivalent Ligand–Receptor Interactions

Ligand-induced receptor oligomerization plays an important role in transmembrane signaling by a large number of receptors for hormones, cytokines, and growth factors. Heterodimerization of the extracellular domains of two members of the same receptor family, or interaction with an accessory molecule, can increase the diversity of ligands recognized by individual receptors.

Heterodimerization of cytoplasmic domains increases the repertoire of signaling pathways that can be activated by a given receptor. Homo- or hetero-ligomerization of cell-surface receptors can occur via simultaneous binding of a multivalent ligand-presenting structure to several receptor molecules. For example, platelet-derived growth factor (PDGF) and colony-stimulating factor-1 are disulfide-linked dimers, which protomers are thought to bind to a single receptor molecule, leading to the effective cross-linking and subsequent activation of the receptor. Intact antibodies

are also naturally occurring multivalent molecules. It has been suggested that bivalent binding of antibodies to cells is essentially irreversible, in contrast to the situation of monovalent interactions [90]. The functional affinity of multimeric ligands can reach dramatically high values compared to that of monomers, e.g., the optimal pentamer of galactose displayed a 10^5-fold increase in functional affinity over that of monomeric galactose [91]. These findings have stimulated the development of multivalent recombinant antibody fragments, e.g., diabodies, triabodies, and tetrabodies, other synthetic multimeric ligands and a broad range of multivalent delivery platforms [57, 58]. By facilitating multivalent ligand–receptor interactions, these novel constructs have been shown to dramatically improve the molecular recognition [92].

Multivalency of Targeted Delivery Systems

Targeted delivery systems, being equipped with multiple targeting ligands, interact with cell surface receptors in a multivalent manner. The cumulative binding to several receptors results in a very high avidity and low receptor off-rate per particle [77]. In this respect, the ligand density is an important factor to be considered. It has been generally assumed that an increase in the density of the targeting moiety results in higher accumulation at the target site, in line with the principle of the cooperative effects of multivalency. A positive correlation between the ligand density and cellular uptake has been reported in vitro by Kok et al. [93] for RGD-targeted paramagnetic/fluorescence liposomes. Similarly, in the recent study by Moradi et al. [94], increasing ligand density on the nanoparticle surface resulted in increased internalization of folate-modified nanoparticles by the cells, up to the saturation level. The authors demonstrated also that surface clustering of the folate enhances cellular internalization of nanoparticles, relative to a more homogeneously dispersed surface distribution of the ligand. On the other hand, Bandyopadhyay and coauthors [95], whose work was focused on vaccine delivery systems, demonstrated that internalization of nanoparticle functionalized with C-type lectin receptor-specific DEC-205 mAb (for schematic representation see Fig. 18.3a) was independent on the antibody surface density (Fig. 18.3b). Despite the same cellular uptake levels, high-density formulations increased the expression of anti-inflammatory cytokines, e.g., IL-10, by dendritic cells (DCs) and splenocytes, compared to that induced by low-ligand density particles (Fig. 18.3c, d). The authors proposed that the correlation between DC production of IL-10, which is the desired effect, and the density of anti-DEC-205 is due to the cross-linking of the DEC-205 receptor.

In another study exploring the importance of the ligand density for targeted delivery systems, Elias et al. [96] showed that an intermediate ligand density provides statistically significant improvements in cell binding in comparison with higher and lower ligand densities. Superparamagnetic iron oxide (SPIO) nanoparticles labeled with HER2/neu targeting affibodies at differing ligand densities were used as a model platform. The advantages of intermediate ligand density with

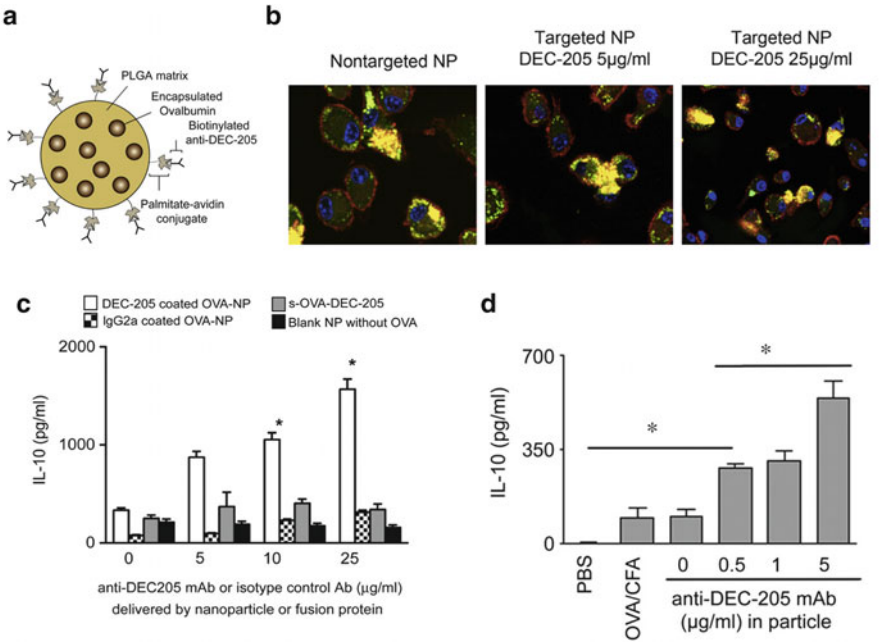

Fig. 18.3 Influence of ligand density on the cellular uptake and therapeutic effects of targeted drug delivery system. (**a**) Schematic representation of poly(lactic-co-glycolic) (PLGA)-based nanoparticles (NP), which encapsulated the immunotherapeutic ovalbumin (OVA) and were functionalized with different concentrations of anti-DEC-205 monoclonal antibody (anti-DEC-205 mAb). (**b**) Fluorescence microscopy images of macrophages incubated with either non-targeted NP (*left image*) or targeted NP functionalized with 5 μg/ml (*middle image*) or 25 μg/ml of anti-DEC-205 mAb (*right image*). Incubation with both targeted NP formulations resulted in a similar level of cellular uptake as that observed for the non-targeted NP. (**c**) Comparison of the interleukin-10 (IL-10) expression level in dendritic cells induced by different drug delivery strategies, i.e., DEC-205-conjugated and OVA-encapsulating NP (*white bars*), isotype control Ab (IgG2a)-conjugated and OVA-encapsulating NP (*black* and *white bars*), DEC-205-conjugated OVA (*grey bars*) and control NP without encapsulated OVA (*black bars*) that were functionalized with different concentrations of either anti DEC-205 mAb or IgG2a. *Significantly increased IL-10 level compared to the control groups, which was observed at higher anti DEC-205 mAb concentrations exclusively for the NP system ($P < 0.05$). (**d**) Cytokine IL-10 response of splenocytes after secondary immunization with either emulsified OVA (OVA/CFA), the non-targeted OVA-NP (0 μg/ml anti-DEC-205 mAb) or targeted OVA-NP modified with different concentrations of anti-DEC-205 mAb (0.5–5 μg/ml anti-DEC-205 mAb). PBS was used as a control. Higher IL-10 production correlated with the greatest density of anti-DEC-205 mAb on the particle surface. Reproduced with permission [95]

respect to the cellular uptake were demonstrated using flow cytometry and MRI analysis. The intermediate, optimal ligand density was found across NPs with differing hydrodynamic diameters, different HER2/neu targeting ligands and also when studying cells with lower receptor densities. Additionally, an intermediate optimal ligand density was also evident when NPs were labeled with folic acid. The authors pointed out several factors that might contribute to the observed ligand

density effect. Steric interference between ligands and competition for a single receptor molecule can potentially limit the binding of high-ligand density formulations. Furthermore, targeting "receptor clusters", which is a common form of HER2 residence in the cell membrane, with very low ligand densities may not provide a high enough NP avidity to result in stable cell binding. In contrast, NPs with a high ligand density may collect too many receptors in each cluster and thus hinder other NPs from binding to the same cluster. Similar findings on the intermediate optimal ligand density have been reported for several other targeted delivery platforms, including folate receptor-targeted liposomes [97] and micellar NPs [98], and has been simulated for general spherical particles [99].

Fonge et al. [100] studied the influence of ligand density on the in vivo performance of targeted nanoparticles. ^{111}In-labeled block copolymer micelles were conjugated with either 1 or 5 mol% of human epidermal growth factor (hEGF). In mice bearing MDA-MB-468 human breast cancer xenografts, the high hEGF density micelles displayed faster clearance kinetics compared to that of low-density counterparts. Furthermore, the tissue biodistribution of particles depended on the surface density of hEGF. The tumor accumulation of 1 % hEGF-modified 15 nm micelles was higher compared to that of 5 % hEGF-modified counterparts. The latter formulation displayed a similar tumor accumulation level as non-targeted particles. The 5 % hEGF micelles of 60 nm in diameter accumulated in the tumor 5.7-fold less effectively than non-targeted micelles of the same size. The relatively poor in vivo performance of the high-density hEGF formulation has been attributed to the accelerated removal of the nanoparticles from the circulation.

The studies cited above underline the critical role of ligand density in the design of targeted delivery systems. This parameter should be carefully optimized to most effectively benefit from the presence of targeting ligand on the vehicle's surface. Furthermore, the translation from in vitro to in vivo conditions appears to be a critical step in determining the optimal ligand density. To some extent, this can be predicted based on the physicochemical and biological properties of a ligand and delivery platform. Nevertheless, due to multiple influential pathophysiological factors, the empirical optimization of targeted delivery systems remains the most fruitful pathway.

Heteromultivalency of Targeted Delivery Systems

Heteromultivalent ligand–receptor interactions are frequently observed in biological systems [101]. They involve simultaneous binding of a heteromultivalent ligand to two or more types of molecular epitopes on the same target cell. Inspired by the efficacy of this type of interactions, several research groups explored this concept for the delivery of therapeutics and imaging agents. Their common goal was to improve the recognition of the cellular target, and, ultimately, to enhance the efficacy and specificity of a given therapeutic or diagnostic approach.

To potentiate the cytotoxic effects of liposomal doxorubicin in cancer cells, Laginha et al. proposed functionalization of doxorubicin-carrying liposomes with

two antibodies, αCD19 and αCD20 [102]. The receptor binding, cellular uptake and cytotoxicity were tested in B-cell lymphoma cells. At similar antibody densities, the binding and uptake of the dual-targeted liposomes were greater than that of either individually targeted liposomes alone, and showed additivity. One to one mixtures of individually targeted liposomes gave, however, similar results to dual-targeted liposomes, suggesting that co-functionalization of liposomes is not crucial to achieve an additive targeting effect. Moreover, the authors reported that a dual-targeted formulation with a higher density of ligands on the liposomes was taken up in a subadditive manner, which was attributed to steric hindrance between antibodies on the particle surface.

Simultaneous targeting of two cancer cell-specific molecular epitopes has been also demonstrated to improve the selectivity of chemotherapy. Saul et al. [103] designed liposomal nanocarriers loaded with doxorubicin and bearing both folic acid and a monoclonal antibody against the epidermal growth factor receptor (EGFR), which were tested in the FR- and EGFR-positive human KB cell line. To assess the targeting selectivity, either one or two target receptors were blocked in KB cells with excess of free ligand. Dual-ligand liposomes reduced viability exclusively in target cells (KB) expressing both targeted receptors. The viability of cells bearing one or none of the targeted receptors remained unaffected. These data demonstrate that the multi-targeting strategy offers an improved therapeutic selectivity towards the cells that express the target set of receptors.

Instead of using two types of targeting ligands, Meng et al. [104] developed a dual-targeted, single peptide containing an α_v integrin-specific and a neuropilin-1-specific motif. The hybrid peptide exhibited two- to threefold greater cellular uptake than separate α_v integrin- and neuropilin-1-specific peptides in vitro. The liposomal formulation of paclitaxel targeted with the dual peptide resulted in significantly enhanced cellular uptake and cytotoxic effects in HUVEC and A549 lung carcinoma cells compared to the single-targeted paclitaxel liposomes. Moreover, the treatment of lung carcinoma xenografts in vivo with the dual-targeted formulation inhibited the tumor growth most effectively.

Multi-targeting strategies have been also exploited for molecular imaging purposes. Despite the use of powerful multifunctional contrast agents that are equipped with target-specific ligands, in vivo molecular imaging remains very challenging. This is due to a number of reasons, such as a low amount of target-associated contrast material, difficulties with the quantitative assessment of the targeting efficacy and the presence of unbound contrast agent in the tumor, producing an unspecific background signal. The simultaneous targeting of multiple molecular epitopes has been proposed as a promising strategy to enhance the efficacy and specificity of an imaging agent to the target cell. For activated endothelium, which overexpresses a diverse set of molecular markers, this appears to be a particularly attractive approach [16, 17]. In the study by Willmann et al. [105], two angiogenesis-specific antibodies, e.g., anti-VEGFR2 and anti-$\alpha_v\beta_3$ integrin, were coupled to microbubbles (MB) for ultrasound imaging of tumor angiogenesis. The attachment of dual-conjugated contrast agent to tumor cells overexpressing target receptors

was found to be significantly higher than association of either anti-VEGFR2- or anti-$\alpha_v\beta_3$ integrin-targeted MB. The mean number of dual-targeted MB per cell was 0.74, whereas separate targeting of VEGFR2 and $\alpha_v\beta_3$ integrin resulted in 0.58 and 0.42 MB/cell, respectively. The same trend was observed in the in vivo experiments in tumor-bearing mice. The average difference in video intensity in the tumor induced by administration of MB was increased from 11.3 for VEGFR2- and 7.8 for $\alpha_v\beta_3$ integrin-conjugated particles to 16.7 when both receptors were targeted simultaneously with dual-conjugated agent.

In addition to the aforementioned positive, yet subadditive improvement, dual-receptor targeting has been shown to produce synergistic targeting effects [106]. In the latter in vitro study, the authors proposed simultaneous targeting of two receptor populations, i.e., $\alpha_v\beta_3$ integrin and galectin-1, to improve the recognition of activated endothelial cells by a liposomal MRI contrast agent. To evaluate this approach, paramagnetic and fluorescent liposomes were functionalized with two ligands, anginex (Anx) and RGD peptides, binding with high affinity to galectin-1 and $\alpha_v\beta_3$ integrin, respectively. The cellular uptake of Anx and RGD dual-conjugated liposomes (Anx/RGD-L) was compared to that of single-targeted counterparts, i.e., Anx- (Anx-L) and RGD-functionalized liposomes (RGD-L), using MRI and optical methods. The schematic representation of the studied single- and dual-targeted systems is displayed in Fig. 18.4a. Fluorescence microscopy images obtained after 3 h incubation of the human umbilical vein endothelial cells (HUVEC) with the investigated liposomal formulations show the obviously enhanced cellular uptake of Anx/RGD-L compared to both Anx-L and RGD-L (Fig. 18.4b). Quantitative measurements of liposome association with HUVEC using MRI and flow cytometry revealed that the dual-targeting approach produces synergistic targeting effects with dramatically elevated cellular uptake of nanoparticles as compared to targeting with single ligands (Fig. 18.4c, d). The observed superadditive uptake efficacy was found exclusively for high-ligand density dual-targeted formulation, indicating an important role of heteromultivalency and no signs of steric hindrance for these relatively small peptidic ligands. At the same ligands concentration, a mixture of single-targeted liposomes resulted in lower cellular uptake than dual-targeted counterparts, showing only an additive and no synergistic effect (Fig. 18.4c, d).

Interestingly, under the in vivo conditions, the same strategy improved significantly the specificity of contrast agent association with the tumor endothelium, while its targeting efficacy was lower compared to RGD-L [107] (Fig. 18.5). By comparing pre- and 24 h post-administration T1 maps, Anx/RGD-L and Anx-L were found to be localized predominantly at the tumor rim, whereas RGD-L were distributed throughout the entire tumor area (Fig. 18.5a, two left panels). Fluorescence microscopy of tumor sections revealed that all the investigated formulations displayed specific association with tumor endothelium as well as undesired liposome extravasation (Fig. 18.5a, two right panels). Importantly, however, semiquantitative assessment of the colocalization between the fluorescence signal of liposomes and that of endothelial cells revealed a significantly higher specificity of Anx/RGD-L association with the tumor endothelium compared to both single-targeted formulations (Fig. 18.5b).

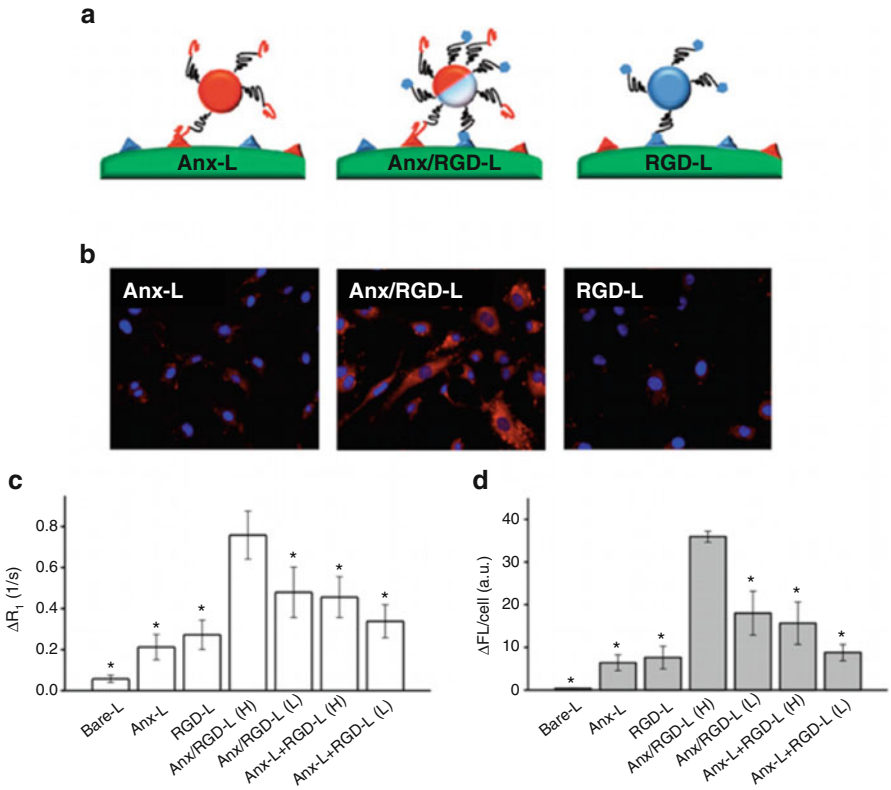

Fig. 18.4 Improved cell recognition by heteromultivalent nanoparticles. (**a**) Schematic representation of the investigated targeting strategies, i.e., single-targeted liposomes functionalized with either anginex (Anx-L) (*left panel*) or RGD peptide (RGD-L) (*right panel*), and dual-targeted liposomes conjugated with both anginex and RGD peptide (Anx/RGD-L) (*middle panel*). (**b**) Fluorescence microscopy images of human umbilical vein endothelial cells (HUVEC) acquired after 3 h of incubation with Anx-L (*left image*), Anx/RGD-L (*middle image*) and RGD-L (*right image*). HUVEC (cell nuclei are shown in *blue*) that were incubated with Anx/RGD-L showed the highest uptake of liposomes (*red fluorescence*). (**c**) and (**d**) display the quantitative comparison of the cellular uptake level between the non-targeted liposomes (Bare-L), single-targeted Anx-L and RGD-L, dual-targeted Anx/RGD-L conjugated with either high (H) or low (L) concentration of peptides, and a mixture of single-targeted formulations (Anx-L + RGD-L) containing either high (H) or low (L) concentration of peptides. With respect to both parameters i.e., increase of the longitudinal relaxation rate compared to control cells (ΔR_1) (**c**) and increase of mean fluorescence intensity/cell compared to control cells (ΔFL/cell) (**d**), dual-ligand functionalized Anx/RGD-L showed significantly increased cell association compared to single-ligand functionalized counterparts and their mixtures. *Significantly lower values compared to Anx/RGD-L (H). Reproduced with permission [106]

At the same time, a more efficient endothelial targeting, as deduced from the fraction of the total endothelial area that colocalized with liposomes, was observed for RGD-L (Fig. 18.5c). The apparent discrepancies between the in vitro and in vivo findings were attributed to the faster blood clearance of Anx/RGD-L compared to that of RGD-L (Fig. 18.5d), which strongly reduced the interaction time between

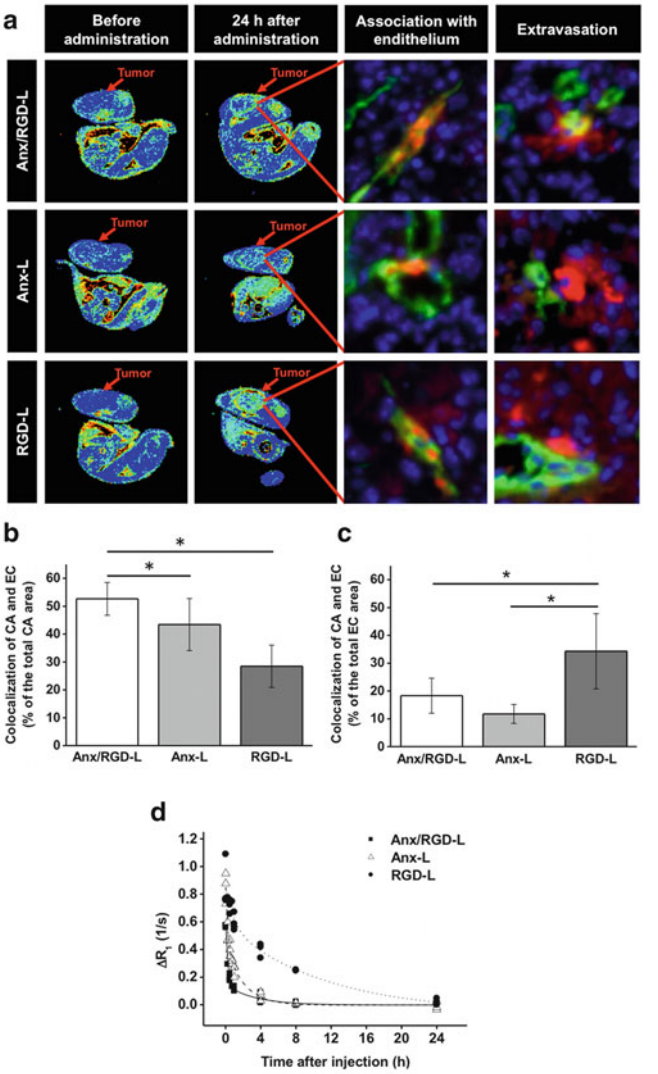

Fig. 18.5 In vivo targeting performance of dual-ligand functionalized nanoparticles. (**a**) Representative T1 maps of tumor-bearing mice (*two left panels*) acquired before and 24 h after administration of Anx/RGD-L (*upper panel*), Anx-L (*middle panel*) and RGD-L (*lower panel*), where *red arrows* indicate the tumor location. For both Anx/RGD-L and Anx-L, decreased T1 pixel values (changes from *blue* to *green* color) were found primarily at the tumor periphery. In contrast, RGD-L-induced contrast was found throughout the entire tumor area. Fluorescence microscopy images of tumor sections obtained 24 h after liposome administration (*two right columns*) revealed, in addition to the specific liposome association (*red*) with endothelial cells (EC) (*green*), the undesired localization of liposomes in the extravascular space. The targeting specificity and efficacy of the formulations to the tumor endothelium was determined ex vivo by quantifying the fraction of the total liposomal contrast agent area (CA) that colocalized with endothelial cells (EC) (**b**) and the fraction of the total EC area that colocalized with CA area (**c**), respectively. The highest colocalization between the CA and EC was found for Anx/RGD-L, showing thus the best targeting specificity (**b**). RGD-L were associated with the largest EC area, which indicated the most efficient endothelial targeting (**c**). *Significant difference between the experimental groups at $P < 0.05$. (**d**) Measurements of changes in the blood longitudinal relaxation rate ($\Delta R1$) after administration of the liposomes over time revealed very different blood clearance kinetics of the investigated formulations, i.e., a rapid clearance of Anx-containing liposomes and long blood circulation of RGD-L. Reproduced with permission [107]

the Anx/RGD-nanoparticles and tumor endothelium. These very different pharmacokinetic properties of Anx/RGD-L have been correlated to the presence of Anx on the surface, since Anx-L showed similarly rapid blood clearance (Fig. 18.5d). Nevertheless, the influence of a high ligand density per Anx/RGD-particle on the clearance kinetics cannot be excluded.

An interesting application of the dual-targeted approach has been proposed by Zhou et al. [108]. The authors prepared a dual-ligand nanoparticle array (DLNA), based on gold nanoparticles (GNPs), which served the identification of cells that have different surface receptor profiles surrounding a common primary receptor. The concept was tested in different cancer cell lines that overexpress folate receptor (FR). At the same time, apart from FR, the cell lines had different receptor profiles due to their different origin. Using 30 members of DLNA, which differed with respect to the type and molecular density of the secondary ligand next to the primary ligand (folic acid), the cellular uptake of dual-targeted GNPs was studied. Diverse secondary ligands on dual-ligand GNPs generated different cell recognition patterns, suggesting that the microenvironmental receptor profiles surrounding FRs in these cells are indeed different and that the DLNA approach is highly effective in identifying selective ligands when the cell receptor profile is unknown.

All the aforementioned studies show consistently that the multi-targeting approach can significantly improve the efficacy and specificity of intracellular delivery of nanocarriers and thus could be useful for cancer therapy and molecular imaging. The reported levels of improvement compared to the conventional single-receptor targeting varied between the studies, which can be attributed to considerable differences in experimental setups, including targeting ligands, delivery platforms, cell lines and evaluation methods. Despite that, the cellular uptake exceeding even tenfold the level of single-targeting approach [108] and improved therapeutic outcome [104], show a great promise for the future of multi-targeting in cancer nanomedicine.

Biological Effects

Receptor-Mediated Signaling and Its Modulation

In this section, we focus on the biological signaling induced by the binding of a ligand to the target cell surface receptor. In general, ligands can act either as receptor agonists (activators) or antagonists (inhibitors). Growth factors, such as EGF and VEGF, belong to the group of receptor agonists, which trigger the receptor-mediated biological response. Signal transduction through the EGFR or other receptor tyrosine kinases involves a series of dynamic and reversible processes [109]. Upon ligand binding, tyrosine kinases form homo- or heterodimers with other receptors, activating their intrinsic kinase activity. Multiple tyrosine autophosphorylation sites in their cytoplasmic domains are modified with phosphates, which can be added and

removed at various rates. These phosphotyrosine residues can then engage and activate specific cytosolic signaling proteins, i.e., phospholipase C (PLC), guanosine triphosphatase (GTPase) belonging to the Ras superfamily and phosphoinositide 3′-kinase (PI3K). Activated forms of the aforementioned proteins modify other relevant cytoplasmic proteins, initiating the cell proliferation, migration and survival cascades.

On the other hand, ligands that have affinity to the receptor, yet exert no biological action, are denoted as receptor antagonists. By blocking the active or allosteric site of receptors, they inhibit the agonist-induced responses. The vast majority of cancer-targeted therapeutics belongs to the group of receptor antagonists. Among them, anti-VEGF (bevacizumab), anti-EGFR (cetuximab), anti-HER2 (trastuzumab) monoclonal antibodies and low-molecular weight tyrosine kinase inhibitors (gefitinib, erlotinib, sunitinib) have been shown to produce additive therapeutic effects in combination with conventional chemoradiotherapy in different cancer types [110]. Receptor agonists represent a minority among cancer therapeutics. Cytokines, e.g., interleukin 12 (IL-12), which belong to the latter group, have been proposed as anticancer vaccines, which role is to stimulate the immune response against cancer [111]. In line with the growing number of phase II and III clinical trials [112, 113], the clinical application of targeted cancer therapeutics becomes more important. Several of them, e.g., cetuximab and bevacizumab, have been recommended as a first line treatment in metastatic disease [114–116], which readily stimulates further development in the field of targeted medicine.

Receptor-Mediated Biological Effects of Targeted Delivery Systems

The therapeutic role of the nanocarrier-based drug delivery systems can take several forms. It can be mediated by biologically active material, such as chemotherapeutics, anti-inflammatory agents or genetic material, which is preferentially delivered and released at the tumor site. Secondly, the imaging-detectable nanoparticles can guide tumor resection surgery by highlighting the tumor location and borders, or be used as a surrogate marker of local drug delivery for optimization purposes. Finally, the therapeutic role may be in disrupting a cellular or metabolic pathway. This approach utilizes a ligand coupled to a nanocarrier to disturb the tumor molecular regulation. In this section, we focus on the latter therapeutic strategy as it exploits ligand–cell surface receptor interactions, which is the leading topic of this chapter. In the case of molecular imaging agents, there is a general rule that the concentration of a ligand should be much below its therapeutic dose. There is, however, another group of agents that combine both treatment and diagnostic purposes. These, so called, theranostics are designed to enable monitoring of the drug delivery and/or release at the target site [117]. In this setup, the application of the same agent as both a molecular

recognition moiety and a therapeutic would enable the assessment of the expression level of molecules, which are directly involved in the mechanism of therapeutic action. The provided molecular information could be very valuable in therapy prediction and monitoring.

Kluza et al. [106] proposed a combined imaging and therapeutic strategy, where the same molecule acted as both the targeting moiety and the therapeutic agent. In this study, two types of peptidic angiogenesis inhibitors—RGD [25] and Anx [118, 119]—were conjugated to multifunctional paramagnetic and fluorescent liposomes [120, 121]. The targeting efficacy and the anti-angiogenic activity were studied in vitro using activated endothelial cells. It was demonstrated that, by combining two different ligands on the same nanoparticle, a synergistic targeting effect could be achieved that led to an enhanced imaging readout, which is more extensively discussed in the section "Heteromultivalency of Targeted Delivery Systems". Moreover, cell-cycle analysis revealed significant inhibition of endothelial cell proliferation induced by these peptide-functionalized nanoparticles. Interestingly, the inhibitory effects were stronger for liposome-conjugated than for identical doses of free peptides. Considerably enhanced anti-proliferative activity, similar as observed under serum deprivation, was found for Anx and RGD dual-targeted liposomes. The study shows therefore that the conjugation of two populations of angiogenesis inhibitors to the same particle resulted in potent multifunctional nanoparticles for combined imaging and therapy.

Furthermore, the therapeutic effects of an $\alpha_v\beta_3$-integrin-specific peptidomimetic, which was employed as a targeting ligand for endothelial delivery of another anti-angiogenic drug, fumagillin, have been reported by Winter et al. [122]. In this study, nanoemulsion particles, consisting of a perfluorocarbon core and a PEGylated lipid corona, were equipped with a peptidomimetic $\alpha_v\beta_3$-integrin antagonist. Fumagillin, a hydrophobic mycotoxin with anti-angiogenic properties, was incorporated in the lipid corona. For MRI readout of angiogenic activity, a paramagnetic Gd-containing $\alpha_v\beta_3$-targeted nanoemulsion was used. The animals were treated with $\alpha_v\beta_3$-targeted fumagillin-containing nanoparticles 16 days prior to imaging. Almost no signal enhancement was observed in the tumor periphery, demonstrating suppressed angiogenic activity. In contrast, in animals treated with $\alpha_v\beta_3$-targeted nanoparticles without fumagillin or non-targeted fumagillin-containing nanoparticles, a significant signal enhancement was visible in the tumor periphery. Importantly, however, the enhancement was significantly lower compared to that observed in saline-administered animals, which indicated some degree of angiogenic inhibition. In view of these findings, the largest suppression of angiogenesis by $\alpha_v\beta_3$-targeted fumagillin-containing nanoparticles may have partly originated from the additive effect of ligand-induced $\alpha_v\beta_3$-integrin suppression.

According to the presented studies, targeting ligands conjugated to nanovehicles can exert some or even a high level of biological activity. Although the surface area of spherical nanoparticles is very favorable for efficient ligand conjugation, their capacity usually is not as high as that of the aqueous particle interior, which is conventionally used for drug incorporation. Therefore, the targeting agent

concentration might be the limiting factor in reaching the optimal therapeutic effect. Furthermore, increased particle multivalency can lead to the steric hindrance between neighboring ligands and unfavorable pharmacokinetic properties. These issues have been extensively reviewed in section "Multivalency of Targeted Delivery Systems". Considering the aforementioned findings, it is relevant to investigate the expression level of the target receptor and receptor-specific response to assess the potential role of the specific targeting ligand in the therapeutic outcome of targeted drug delivery.

Cellular Internalization

Receptor-Mediated Endocytosis

The establishment of the ligand–receptor complex in the cellular membrane is frequently followed by the process of endocytosis, i.e., the internalization of extracellular material into intracellular vesicles (Fig. 18.1). This cellular pathway enables plasma membrane receptors to transfer the extracellular stimuli to the cell interior and to initiate signaling. Initially, the activated receptors undergo post-translational modifications, e.g., phosphorylation, on their cytoplasmic side. The modified receptor–ligand complexes cluster in the pitted membrane regions lined by either clathrin or caveolin-1, which promotes their internalization. The transfer to early endosomes occurs within a few min. Due to the low pH of circa 5.5–6.0 in the endosomal interior, the ligand can be released from the receptor and transported further in distinct endosomes. Early endosomes are also responsible for so-called receptor sorting, which determines the fate of the receptor. If the cell needs to be desensitized, i.e., the stimulus needs to be eliminated, the receptors are sorted to late endosomes and subsequently to lysosomes for degradation. If the cell needs to be resensitized, i.e., the response to the stimulus should be sustained, the receptors are rerouted back to the plasma membrane directly or via recycling endosomes. The receptor sorting pathway is determined by the receptor type and its level of activation.

There are two main endocytic pathways: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) [123, 124]. In the former case, clathrin oligomers and associated proteins facilitate the formation of the clathrin-coated vesicles. Cell adhesion molecule (CAM)-mediated endocytosis, found in endothelial and neuronal cells, is considered as a subtype of CME. The CIE mechanism of plasma membrane invagination includes the caveolin-mediated and, still incompletely understood, lipid raft-mediated endocytosis. The internalization mechanism is determined by the type of receptor–ligand complex, e.g., the folate–folic acid complex is internalized via caveolae, $\alpha_v\beta_3$ integrin-RGD via CAM-mediated endocytosis, whereas EGFR-EGF is taken up through CME [124]. Interestingly, in the latter case both CME and CIE pathways are possible [123]. In the presence of low

EGF concentrations, EGFR is internalized via CME, while its exposure to high EGF concentrations triggers the CIE mechanism. CME results in EGFR recycling and signal propagation, whereas CIE leads to receptor degradation in lysosomes and signal attenuation.

Without the attachment of a receptor-specific ligand, macromolecules can only enter the cell via fluid phase endocytosis (FPE), which is significantly slower than receptor-mediated internalization. Nevertheless, FPE leads eventually to the same vesicular propagation as receptor-mediated internalization. Molecules that undergo FPE have frequently positive charge and hydrophobic properties, which promote the interaction with the cell membrane. FPE accompanies the receptor-mediated internalization, predominantly CME, during which the concentrated receptor–ligand complexes are entrapped in the endosome together with the extracellular fluid.

Endocytosis of the Targeted Delivery Vehicles

The above described process of endocytosis has important implications for the targeted delivery of therapeutics and diagnostics. During the design of a delivery system, several relevant aspects should be considered. The choice of the molecular target will determine whether internalization occurs and which internalization pathway is exploited. Moreover, the concentration of targeting ligand can modulate the efficacy of endocytosis and receptor-mediated signaling. The physicochemical properties of the carrier should be balanced in order to limit the nonspecific cellular uptake. Crucially, the influence of endosomal entrapment on the therapeutic effects and/or imaging signal needs to be taken into account.

In the majority of drug delivery systems, targeting is applied to increase the intracellular cargo of a therapeutic, which is incorporated in the carrier. In this case, the targeting of non-internalizing receptors misses the main goal of the delivery system. Therefore, a more logical choice is to target a cell surface receptor that undergoes post-activation endocytosis. For example, Sapra and Allen [125] showed that targeting doxorubicin-containing liposomes to the internalizing CD19 receptor results in significantly greater survival times of B-lymphoma-bearing mice than targeting to the non-internalizing CD20. Irrespective of the internalization mechanism of the given receptor, similar vesicular propagation takes place. However, one can expect differences in the internalization kinetics and the intracellular fate of the receptor. CME has been recognized as a highly efficient internalization mechanism, in contrast to CIE [124]. However, exceptions to these general principles have been reported, e.g., the kinetics of CIE mediated by the interleukin-2 receptor is similar to those of CME. These generally distinct kinetics have been related to the involvement of compositionally different membrane microdomains in the internalization process. Furthermore, CME has been associated predominantly with lysosomal degradation, whereas CIE with endosomal accumulation and sorting to the non-degradative path. Nevertheless, the research of recent years has shown that there is no straightforward correlation between the internalization mechanism and intracellular destination.

Physicochemical properties of the delivery systems can also influence the endocytic pathway. The majority of currently developed vehicles has no net charge and is equipped with hydrophilic poly(ethylene glycol) (PEG) coating, which limits the direct contact between the carrier shell and cell membrane. The cellular uptake of this type of formulations is very low [126]. Nevertheless, under in vivo conditions, many non-targeted nanoparticle formulations, including FDA-approved liposomal formulations of chemotherapeutics, e.g., Doxil, have been shown to be as effective as their free drug counterparts, while having, importantly, a more favorable toxicity profile [127]. The observed therapeutic efficacy has been attributed to the accumulation of nanoparticles in the tumor extracellular space and subsequent release of the encapsulated drug, which can penetrate the cancer cells and exert its biological activity [128]. Cationic delivery systems have been extensively exploited for gene delivery [129]. By facilitating the cell transfection, the gene silencing was achieved. However, the transfection of off-target cells, which is a consequence of enhanced unspecific interaction with negatively charged cell membranes, remains a major concern [130, 131]. To reduce the unwanted effects, shielded cationic delivery systems have been introduced, which limited the unspecific cellular uptake [132]. Negatively charged particles, containing phosphatidylserine (PS) or phosphatidylglycerol (PG), have been shown to be preferentially recognized by macrophages, which is due to the high affinity interactions of negatively charged phospholipids with scavenger receptors on the macrophage surface [133].

The particle size can also be a limiting factor during the transmembrane transport. Gao et al. [134] and Zhang et al. [135] reported that the optimal nanoparticle radius for endocytosis is approximately 25–30 nm for ligand-functionalized formulations, which was based on two independent mathematical models. They estimated that the shortest internalization time of one particle is 20 min. Chitharini et al. [136, 137] investigated experimentally the influence of the nanoparticle size and shape on the endocytosis rate in mammalian cells. The authors found the same optimal particle size as in the aforementioned reports, which was independent on the cell line. Small particles of 7 nm could only undergo endocytosis after clustering of few of them together. Zhang et al. [135] suggested that the existence of a lower threshold of nanoparticle radius of ~20 nm can be explained by too low adhesion energy, which cannot sufficiently compensate the energy of bending that is necessary to initiate the membrane invagination. At the same time, the authors report a higher radius threshold of 60 nm, above which the endocytosis activity drops to zero. It needs to be pointed out, however, that the model assumes a relatively large cell-surface concentration of NPs, for which tension-mediated competition among NPs is the governing mechanism. In the case of low cell-surface NP concentration, the same thermodynamic model predicts that endocytosis can occur for very large NPs with diameters up to a few micrometers, thus, also including the most commonly exploited diameter range of 150–250 nm. Under the in vivo conditions, however, large particles (>500 nm) are prone to be rapidly cleared by the reticuloendothelial system (RES) of the liver and spleen, so they might not have a chance to reach the target site [138]. With respect to the shape, spherical particles were found to be internalized more efficiently than rod-shaped particles [136].

Endocytosis: Implications for Targeted Drug Delivery

The majority of drug delivery systems exploit the targeting ligand as a mediator of the cellular uptake of the vehicle-encapsulated drug. The benefits of targeted drug delivery have been demonstrated in numerous studies on different therapeutic strategies [139–141]. Interestingly, some studies report either no or minor improvement with respect to the therapeutic outcome, despite that the cellular uptake of targeted drug delivery vehicle was frequently increased as compared to non-targeted controls. In the work by Fens et al. [142], the antitumor effects of RGD-targeted liposomes containing the vascular disrupting agent ZD6126 were compared to those induced by a non-targeted formulation of the same drug. Similar accumulation pattern and comparable degree of necrosis in B16F10 murine melanoma were observed after treatment with either targeted or non-targeted liposomes. Furthermore, no statistically significant difference was noted with respect to the tumor growth inhibition and mice survival. In conclusion, the so-called active targeting to activated endothelial cells did not provide therapeutic advantages over the non-targeted drug delivery approach. This is simply because the presence of targeting ligand does not necessarily increase the chance of reaching the target. It is also possible that the receptor-mediated endocytosis limited the accessibility of ZD6126 to its target, cytosol-located tubulin, whereas extracellularly accumulated PEG-liposomes may have served as a depot of free ZD6126, which can transverse cellular membranes and reach its intracellular target.

In gene delivery, in particular, the entrapment in endosomes, followed by the lysosomal degradation, has been recognized as a limiting step in achieving the desired therapeutic amplification. This has been attributed to the separation of the biologically active material from the site of its activity, which is usually either the cytoplasm or nucleus. Slow diffusion of a drug through the endosomal and lysosomal membrane is possible in certain cases, however, it may not be sufficient to assure the optimal therapeutic efficacy. To overcome this issue, several strategies for achieving the endosomal escape have been proposed: (1) pore formation in the endosomal membrane [143], (2) pH-buffering effect [144], (3) fusion into the endosomal lipid bilayer [145], and (4) photochemical disruption of the endosomal membrane [146]. The aim of all the aforementioned strategies is the destabilization of the endosomal membrane and subsequent release of the endocytosed material in the cytoplasm.

Mastrobattista et al. [147] showed that the co-encapsulation of the influenza virus-derived synthetic fusogenic peptide diINF-7 enhances the cytosolic delivery of liposome-entrapped proteins. The diINF-7 peptide was co-encapsulated in liposomes with the catalytic A-domain of diphtheria toxin (DTA), which inhibits protein synthesis when delivered in the cytoplasm, resulting in cell death. CLSM images collected after 1 h of incubation with EGFR-targeted DTA and diINF-containing liposomes showed their efficient internalization by ovarian carcinoma cells. The same formulation showed high cytotoxicity, whereas targeted liposomes containing either DTA or diINF-7 alone did not have any cytotoxic effect. In another study,

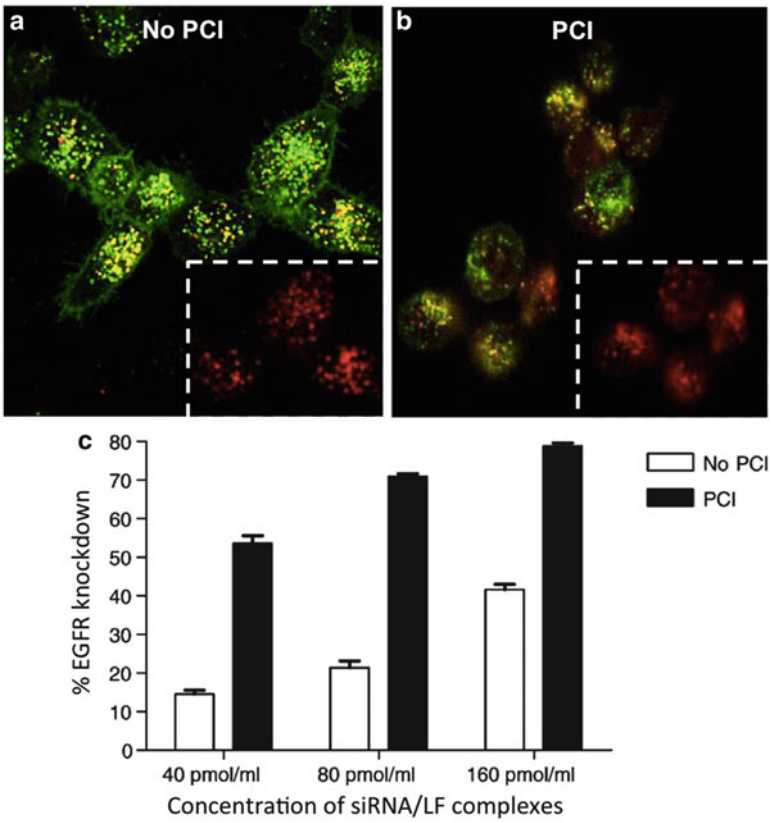

Fig. 18.6 Photochemical internalization (PCI) improves the cytoplasmic delivery and transfection efficacy of gene delivery system. (**a**, **b**) Confocal laser scanning microscopy images show human epidermoid carcinoma cells incubated for 4 h with siRNA-Alexa633/lipofectamine (LF) complexes (*red*) and EGF-Alexa488 (*green*) (no PCI) (**a**), and cells that were pre-incubated with photosensitizer and illuminated with light after the 4 h incubation with siRNA-Alexa633/LF and EGF-Alexa488 (PCI) (**b**). The particle incubation alone resulted in the punctuated fluorescence pattern, indicating endosomal localization of both agents (**a**). In PCI-treated cells, a more diffuse fluorescence pattern throughout the cell cytoplasm was observed, suggesting the release of both siRNA–lipid complex and EGF to the cytoplasm (**b**). (**c**) PCI greatly improved the transfection efficacy (% of EGFR knockdown) of different concentrations of anti-EGFR siRNA/LF complexes. Reproduced with permission [148]

Oliveira et al. [148] applied photochemical internalization (PCI) to enhance the cytoplasmic delivery and transfection efficacy of EGFR-silencing siRNA (Fig. 18.6). The incubation of A431 human epidermoid carcinoma cells with cationic liposome-siRNA complex resulted in the punctuated intracellular pattern of fluorescently labeled lipid-siRNA complex (red), suggesting the endosomal localization of the complex (Fig. 18.6a). In contrast, after 75 s of illumination of the incubated cells, which were pretreated with a photosensitizer (meso-tetraphenylporphine with two

sulfonate groups on adjacent phenyl rings), a more diffuse distribution of the transfection complex, throughout the cytoplasm, was observed, indicating the PCI-induced endosomal escape of siRNA (Fig. 18.6b). Importantly, a tenfold increased efficiency in knockdown of the EGFR protein was obtained when anti-EGFR siRNA treatment was combined with PCI as compared to siRNA treatment alone (Fig. 18.6c). The aforementioned studies illustrate perfectly that tuning the intracellular delivery by endosomal escape strategies can tremendously improve the therapeutic efficacy of the drug delivery systems. Alternatively, the dose of a therapeutic agent can be reduced, which consequently may limit the unwanted side effects that often occur.

Endocytosis: Implications for Molecular Imaging of Cancer

In vivo molecular imaging requires both the spatial localization and quantification of the cell-associated contrast agent. This is necessary to provide the relevant information on the distribution of the target molecule throughout the tumor as well as its expression level. The research of recent years has shown that the cell internalization of the molecular imaging agents may have important impact on their properties, particularity in the case of MRI-detectable probes. This is due to the fact that the relaxivity of Gd-based MR contrast agents (Gd-CA), which is a measure of the MR contrast agent potency, depends on the accessibility of bulk water protons to the Gd ion. In the cellular environment, the water exchange is limited by the presence of natural barriers i.e., the semi-permeable phospholipid bilayers, including the cell membrane and endosomal membranes. The effects of CA entrapment in the cell cytoplasm and endosomes on the relaxation properties have been investigated by Terreno et al. [149]. In that study, hepatocarcinoma cells were incubated with Gd-HPDO3A, a low-molecular weight CA, which resulted in the intravesicular CA location in cells (Fig. 18.7a). As a parallel experiment, the authors applied electroporation to assure the cytoplasmic distribution of the CA. The CA entrapment in endosomes resulted in less effective MRI contrast changes compared to that induced by cytosol-located CA (Fig. 18.7b). At the same Gd concentrations [Gd^{3+}], the longitudinal relaxation rates (R_1) of the endosomal CA were markedly lower than those observed for the cytosolic CA (Fig. 18.7c). Furthermore, the saturation of the R_1 increase was observed at the higher concentrations of the endosomal CA, which was not the case for the electroporated cells (Fig. 18.7c). The reported dramatic drop in the relaxation properties of the endosome-entrapped CA compared to the cytosol-distributed probe has been attributed to the limited water exchange across the vesicular membrane. The number of vesicles per cell and the probe concentration in the vesicles are important variables in this compartmentation or "quenching" effect.

In another study on this topic, Kok et al. [93] investigated the effects of cell internalization on the relaxation properties of RGD-conjugated, integrin-targeted and non-targeted (NT) paramagnetic liposomes. In this study, both formulations were entrapped in endosomal structures of activated endothelial cells by incubating the cells with liposome-containing medium. The cellular uptake of RGD-liposomes was dramatically higher compared to that of NT-liposomes. Furthermore, $\alpha_v\beta_3$

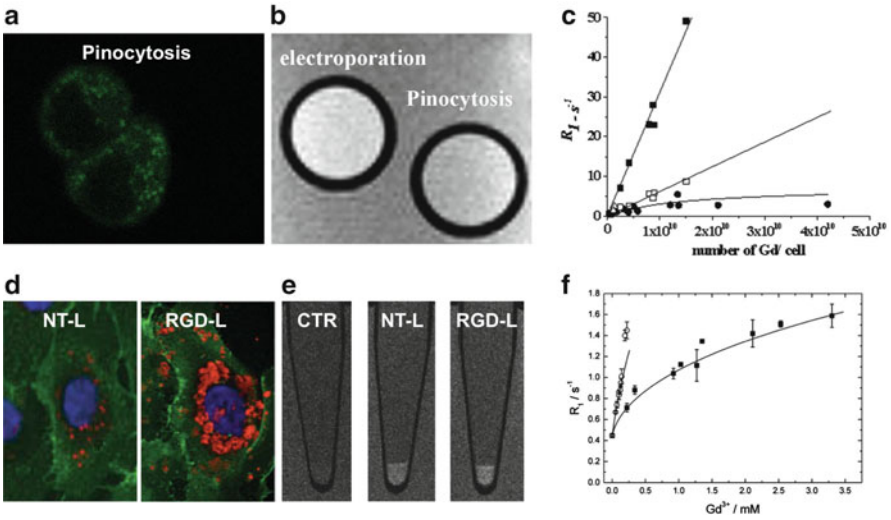

Fig. 18.7 Effects of endocytosis on the relaxation properties of MR contrast agents. (**a**) Confocal laser scanning microscopy (CLSM) image of rat hepatocarcinoma (HTC) cells showing that the fluorescent Eu-HPDO3A taken up via pinocytosis accumulates in the subcellular vesicles around the nucleus. (**b**) T1-weighted images of an agar phantom containing HTC cells labeled with GdHPDO3A internalized either by electroporation or pinocytosis. The electroporation-mediated cell uptake resulted in a higher MRI contrast compared to that achieved by pinocytosis. (**c**) The longitudinal relaxation rate (R_1) of the cells labeled by pinocytosis showed a saturation effect upon increasing of the amount of internalized probe (circles). R_1 values for electroporated cells are markedly higher than the corresponding values for the pellets labeled by pinocytosis and they are linearly dependent on the amount of the internalized complex (*open* and *filled squares*). (**d**) CLSM images of the human umbilical vein endothelial cells (HUVECs) incubated 8 h with NT-liposomes (NT-L) (*left image*) or RGD-liposomes (RGD-L) (*right image*). After incubation with RGD-L, HUVECs contained large accumulations of rhodamine-labeled liposomes (*red*) in the perinuclear region in spherical 1–5 μm diameter vesicles. Incubation with NT-liposomes, on the other hand, resulted in much lower liposome uptake in sharply defined 0.4–1.0 μm diameter spherical vesicles located in the perinuclear cell region. CD31-stained endothelial cells and DAPI-stained cell nuclei are shown in *green* and *blue*, respectively. (**e**) Typical example of T1 weighted images of cell pellets of HUVECs after control incubation (CTR) (*left image*), 2 h incubation with NT-L (*middle image*) or 2 h incubation with RGD-L (*right image*). The images show no difference between NT-L and RGD-L with respect to the produced MR contrast. (**f**) The relationship between R_1 and gadolinium concentration [Gd^{3+}] in HUVECs incubated with either of the investigated formulations, which reflects the effective longitudinal relaxivity (r_1) of the internalized contrast agent, was found to be linear for NT-liposomes (*open circles*). In contrast, cells incubated with RGD-L displayed a nonlinear relationship between R_1 and [Gd^{3+}] throughout the range of [Gd^{3+}], which indicated a reduced effective r_1 of internalized RGD-L (*solid squares*). Reproduced with permission [93, 149]

integrin-mediated endocytosis resulted in the labeling of many large vesicles of 1–5 μm in diameter (Fig. 18.7d, right panel), whereas the non-specific uptake of NT-nanoparticles resulted in labeling of considerably smaller endosomes ($\phi = 0.4$–1.0 μm) (Fig. 18.7d, left panel). Despite the differences in the achieved intracellular Gd concentration, the produced MRI contrast was similar for both formulations

(Fig. 18.7e). The effective longitudinal relaxivity (r_1) of the internalized NT-liposomes, $r_1 = 4.7\ mM^{-1}\ s^{-1}$, expressed as a slope of the R_1 versus [Gd^{3+}], was found to be considerably higher than that of RGD-particles (Fig. 18.7f). For the latter formulation, a nonlinear relationship was found between the R_1 and [Gd^{3+}]. Accordingly, at the low [Gd^{3+}] range, r_1 was 1.3 $mM^{-1}\ s^{-1}$, whereas high cellular [Gd^{3+}] was associated with a remarkably lower r_1 of 0.1 $mM^{-1}\ s^{-1}$. The authors identified two key factors that influence the relaxation properties of internalized agent i.e., the CA concentration in the intracellular vesicles and the size of vesicles. The lowered efficacy of the T1 relaxation of water protons at high endosomal CA concentrations has been attributed to the low volume fraction of the endosomal water and limited water exchange across the endosomal membrane. Moreover, an increase in the vesicle size reduces the surface-to-volume ratio of the vesicle and, thus, lowers the water exchange flux across the membrane. These observations are in agreement with findings by Stijkers et al. [150], who provided convincing evidence, using mathematical modeling, that the intracellular r_1 decrease is due to the confinement of paramagnetic material in a low-volume fraction intracellular compartment, from which water proton exchange with the bulk is too slow to achieve the intrinsic r_1. Similarly to Gd-CA, superparamagnetic iron-oxide nanoparticles (SPIO-NPs) were shown to form clusters after being internalized by cells [151]. However, in contrast to Gd-CA, this intravesicular clustering of SPIO-NPs was found to positively influence their relaxation properties, as deduced from the increased effective transversal relaxivity r_2.

The aforementioned findings have serious implications for the future of molecular imaging based on MRI approaches. The revealed mismatch between the detected MRI contrast and true CA concentration does not allow for the reliable quantification of the cell internalized CA. Nevertheless, the excellent spatial resolution and multiparametric readout possibilities of MRI are important factors that continuously stimulate the development of novel probe designs. Kok et al. [152] introduced an emulsion-based probe that contained two MRI-detectable components, i.e., Gd-DTPA-lipid, which was incorporated in the lipid membrane, and perfluoro-15-crown-5-ether (PFCE), which was encapsulated in the emulsion interior (Fig. 18.8a). The combination of Gd- and fluor (F)-containing agents provides multispectral detection possibilities by using ^{1}H MRI as well as ^{19}F MRI and ^{19}F MRS, respectively. While ^{1}H MRI can provide a high-resolution detection of CA accumulation at the site of interest, ^{9}F MRI or ^{19}F MRS can facilitate an absolute quantification of the accumulated probe. In the proof-of-concept study, the uptake of RGD-targeted emulsion by activated endothelial cells was compared to that of the NT-emulsion. In agreement with the previous report [93], the cell internalization of the targeted emulsion resulted in twofold lower r_1 compared to that produced by the incubation with NT-emulsion and that measured in the extracellular environment (Fig. 18.8b). This confirmed the challenges related to the accurate assessment of the Gd-CA concentration. In contrast, ^{19}F MRI and ^{19}F MRS signals for both targeted and control nanoparticles were linear and quantifiable as function of nanoparticle concentration (Fig. 18.8c).

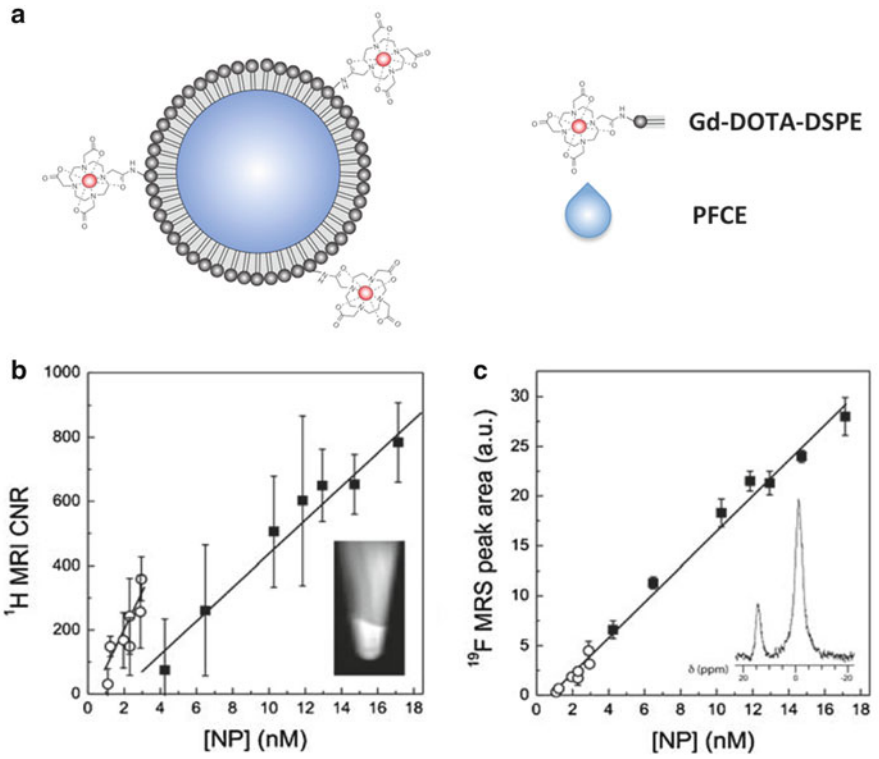

Fig. 18.8 Nanoparticulate delivery system for multimodal detection. (**a**) Schematic drawing of perfluoro-15-crown-5-ether (PFCE)-based nanoemulsion coated with a gadolinium chelate-containing lipid (Gd-DOTA-DSPE). Quantitative proton (**b**) and fluorine MRS (**c**) readouts as function of the nanoparticle concentration ([NP]) in cell pellets obtained after incubation of HUVECs with RGD-emulsion (*solid squares*) or NT-emulsion (*open circles*). (**b**) ^{1}H MRI contrast-to-noise ratio (CNR) measurements showed that the association of emulsion with the cells resulted in different proton longitudinal relaxivity values, represented as different slopes of linear fits (*solid lines*) to the data, for RGD-emulsion and NT-emulsion. (**c**) 19F MRS peak area, normalized to the pellet volume, showed the same linear relation with the [NP] for both RGD- and NT-emulsion. Data are means ± SD ($n = 3$). Reproduced with permission [152]

De Vries et al. [153] recently proposed another probe quantification concept, which was based on multimodal imaging. Namely, MRI- and SPECT-detectable agents were incorporated into the membrane of liposomes to enable imaging with both MRI and SPECT. The authors showed that MRI signal is of limited value for the assessment of intracellular Gd concentration and can rather serve the spatial localization and understanding the cellular location of the contrast agent. On the other

hand, the radiotracer is perfectly suited for the quantification of the cell-associated Gd. Similarly, we observe intensive development of multimodal PET-MRI [154, 155] and SPECT-CT [156] probes, combining the strengths of two imaging modalities.

The aforementioned hybrid strategies fit perfectly within the current trend of multifunctional and multimodal imaging, which is observed in both preclinical and clinical research. Hybrid or multimodality imaging takes advantage of the unique and complementary strengths of individual imaging modalities, providing critical information on anatomy, physiology as well as molecular biology. The recent advances in image fusion techniques and hybrid imaging systems enables efficient multimodal imaging. However, to access the full potential of these hybrid techniques, new multimodal contrast agents are required.

Intracellular Degradation

Lysosomal Degradation

Lysosomes are cellular organelles responsible for intracellular degradation of complex exogenous and endogenous structures into their building blocks. These are able to leave the lysosome to be utilized for the resynthesis of complex molecules or to undergo further degradation. Lysosomes have been first recognized by Christian de Duve in the 1950s as vacuolar structures that contain various hydrolytic enzymes, which function optimally at an acidic pH [157, 158]. The enzymes are isolated from the cytoplasm by a phospholipid membrane, which protects the cellular content from the degradation machinery. Both exogenous and endogenous molecules as well as cellular organelles undergo lysosomal digestion. Exogenous molecules are transported to lysosomes through receptor-mediated endocytosis, phagocytosis or pinocytosis, whereas endogenous components are delivered by micro- and macro-autophagy. The digestive process involves numerous stages of lysosomal maturation i.e., primary lysosomes that have not yet been engaged in any proteolytic process; early autophagic vacuoles that might contain intracellular organelles; intermediate/late endosomes and phagocytic vacuoles that contain extracellular content; and multivesicular bodies which are the transition structures between endosomes/phagocytic vacuoles and the digestive lysosomes. Moreover, the digestive pathway also includes structures that are free of hydrolases, for example, early endosomes, which contain endocytosed receptor–ligand complexes and pinocytosed/phagocytosed extracellular contents, and the residual bodies, which are the end products of the completed digestive processes.

Lysosomes contain about 50 types of hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases, which break down different molecules [159]. Proteolytic enzymes are responsible for degradation of proteins. These are usually tagged for selective destruction in proteolytic complexes called proteasomes, by covalent attachment of a small

protein, i.e., ubiquitin. However, some proteins may be degraded by proteasomes without ubiquitination. Phospholipids originating from lipoproteins or cellular membranes are degraded by phospholipases. Water-soluble glycosidases sequentially cleave off the terminal carbohydrate residues of glycoproteins, glycosaminoglycans, and glycosphingolipids. All these enzymes have their optimal activity around pH 5.0, which is generated by an H^+ATPase located in the lysosomal membrane.

Lysosomal Degradation of Targeted Delivery Systems

Lysosomal degradation is a consecutive step following the endocytosis of targeted delivery systems. Under the influence of lysosomal enzymes, complex nanoparticulate structures are broken-down to simple building blocks (Fig. 18.1), which can be reused by or excreted from the cell. As in the case of endosomes, the entrapment in lysosomes and concomitant degradation have been recognized as limiting steps in achieving the optimal therapeutic activity by targeted drug delivery systems. Consequently, the research efforts are focused on delaying the process of lysosomal degradation. In principle, the same escape strategies that have been proposed for the endosomal pathway, and which are described in the section "Endocytosis: Implications for Targeted Drug Delivery", can be applied to the lysosomes as well. This is possible due to the compositional similarities between the vesicular membranes. We might therefore consider the application of membrane-disturbing agents and processes as combined endo-lysosomal escape strategies. Moreover, the composition of the delivery system itself can facilitate endo-lysosomal escape. Panyam et al. [160] reported a rapid (<10 min) endo-lysosomal escape of nanoparticles formulated from the copolymers of poly(DL-lactide-co-glycolide) (PLGA). To study the intracellular compartmentalization of these particles, the authors performed confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM) experiments on human arterial smooth muscle cells exposed to the nanoparticle suspension. After 2 min incubation, the internalized fluorescently labeled particles were found to colocalize only partly with transferrin-positive early endosomes, while the majority of them were localized in the LysoTracker Red-positive late endosomes and lysosomes. This, however, changed dramatically as early as 10 min after start of the incubation. At this time point, the nanoparticles were evenly distributed throughout the cytoplasmic compartment. This localization became dominant over time. The aforementioned results were supported by TEM experiments. Additionally, TEM images revealed the adherence of NPs to the inner wall of late endosomal/lysosomal vesicles, but were not seen in the early endosomes. This suggested some interaction between NPs and the membrane of the endocytic vesicles prior to the relocation to the cytoplasm. The escape mechanism was attributed to the selective reversal of the surface charge of nanoparticles, from anionic to cationic, under the acidic conditions of the endo-lysosomal compartment. In earlier studies, cationization of poly(lactide) or PLGA microparticles under conditions of low pH was attributed to the transfer of excess protons from the bulk liquid to the

NP surface or hydrogen bonding between carboxyl groups of poly(lactide) or PLGA and hydronium molecules [161, 162]. A positive charge on the particle surface facilitates the interaction with the vesicle membrane and escape into the cytosol. Furthermore, PLGA nanoparticles were also shown to provide sustained therapeutic effects of the encapsulated DNA and dexamethasone.

The prevention from lysosomal degradation can be also achieved by using pharmacological agents that interfere with lysosome activity. Muro and colleagues [163] demonstrated that the endothelial cell trafficking of nanoparticles targeted to the intercellular adhesion molecule-1 (ICAM-1) can be prolonged by suppressing the lysosomal enzyme activity and disturbing the microtubule function with chloroquine and nocodazole, respectively. Before incubation with the anti-ICAM nanoparticles, the human umbilical vein endothelial cells (HUVEC) were pretreated with either chloroquine or nocodazole. Both agents inhibited anti-ICAM nanoparticle degradation, which was demonstrated by means of fluorescence microscopy and densitometric analysis. In the control condition (no treatment), specific fluorescence- and radio-labeling of anti-ICAM revealed rapid depletion of the intracellular anti-ICAM pool over time. In contrast, in the pretreated cells, the degradation of the internalized anti-ICAM particles was markedly delayed. Furthermore, chloroquine and nocodazole prolonged the duration of anti-oxidant protection by catalase-encapsulated anti-ICAM nanoparticles. Considering these results, the combination therapy with the inhibitors of lysosome activity appears to be an attractive strategy of delaying the degradation of targeted nanoparticles, which can have a positive impact on the therapeutic outcome.

In contrast to the aforementioned lysosome-avoiding strategies, some drug delivery systems take advantage of the native intracellular trafficking routes. In line with this trend, Morachis et al. [164] developed nanoparticles composed of a dual pH responsive, random copolymer (poly-β-aminoester ketal-2), which can undergo a two-step response to low pH. The first step is a hydrophobic–hydrophilic switch, which is followed immediately by rapid degradation. These structural perturbations were proposed to increase the cytoplasmic delivery of a nanoparticle-encapsulated drug. To study the nanoparticle-mediated transfection efficacy, the particles were encapsulated with Cy5-labeled pDNA encoding enhanced green fluorescent protein (EGFP). The performance of this gene delivery system was tested in vitro on the HCT116 colon carcinoma cell line. Flow cytometry revealed an increasing number of Cy5-DNA positive cells over time. Furthermore, the pH responsive formulation produced superior (threefold higher) EGFP expression compared to PLGA nanoparticles. Inhibition of V-ATPases by bafilomycin A1 resulted in the decreased expression of EGFP, which demonstrated that the system's performance is dependent on low endo/lysosomal pH. The proposed mechanism of rapid nanoparticle disintegration, even though occurring in the endo/lysosome compartment, has been therefore shown to facilitate the cytoplasmic exposure of the encapsulated agent.

Furthermore, in view of the excessive proteolytic activity in some cancer cell types, the lysosomal compartment can also serve as an attractive therapeutic target [165]. Among the lysosomal proteases, cysteine protease cathepsin B has been implicated in the process of malignant progression [166]. Obermajer et al. [167]

introduced a drug delivery system composed of poly(D,L lactide-coglycolide) nanoparticles, a specific anti-cytokeratin monoclonal IgG and cystatin, a potent protease inhibitor. By using fluorescence microscopy and flow cytometry, this drug delivery system was shown to efficiently recognize breast tumor cells, by binding to cytokeratins on their membranes. The capability of these nanoparticles to inhibit intracellular proteolytic activity in living cells was tested by using specific cathepsin B fluorogenic substrate. Under the control conditions, a strong fluorescence of the degraded substrate appeared in the endocytic vesicles, indicating a high concentration of the lysosomal enzyme. In contrast, pre-incubation of cells with cystatin-loaded and cytokeratin-targeted nanoparticles almost completely abolished the substrate fluorescence, showing that cathepsin B activity was strongly inhibited. Furthermore, the receptor-mediated internalization of cystatin nanoparticles resulted in significantly stronger inhibition of the in vitro cell invasiveness compared to that induced by free cystatin.

Next to the therapeutic implications, an important issue related to the lysosomal degradation is the biocompatibility of the internalized material. Optimally, the degradation of a delivery vehicle should lead to the formation of simple breakdown products that can be reused by the cell and/or easily removed from the cell and the body. Alternatively, the material should be exocytosed in its unchanged form from the cell. However, the efficacy of the latter process is known to be rather poor. Some materials exert excellent biocompatibility, e.g., the degradation products of PLGA nanoparticles are lactic and glycolic acids, which are easily metabolized in the body via the Krebs cycle and subsequently eliminated [168]. However, for other delivery systems, such as mesoporous silica nanoparticles, the intracellular degradation path remains unclear. Recently, Zhai et al. [169] have studied the intracellular degradation of silica nanoparticles (SNPs) using a range of methods, i.e., transmission electron microscopy (TEM), fluorescence microscopy, enzymatic proteolysis and inductively coupled plasma atomic emission spectroscopy. SNPs were found to undergo degradation both in the cytoplasmic and lysosomal compartment of human umbilical vein endothelial cells. TEM images acquired 48 and 96 h after start of the incubation, which are displayed in Fig. 18.9a, revealed the on-going degradation of SNPs. This was concluded from the changes in the particle size, i.e., the majority of residual particles appeared smaller than the original particle diameter of 200 nm (Fig. 18.9a). For fluorescence microscopy purposes, the particles were labeled with FITC. After 7 days of culture, besides fluorescent-labeled small SNPs, low-density fluorescence was observed throughout the cytoplasm, which indicated the release of FITC molecules from the particles. The Si content in the culture medium increased over time, as shown in Fig. 18.9b, suggesting that the degradation product was excreted from the cells. The degradation rate was fast in the first 2 days and slow over the following days (Fig. 18.9b). The study proved therefore the intracellular breakdown of SNPs. At the same time, the duration of the entire process, further metabolism and excretion remain to be investigated.

In addition to the type of building material, other physicochemical factors of delivery vehicles may influence the degradation pathway. Akagi et al. [170] investigated the impact of particle size on the intracellular fate of nanoparticles. Forty and two-hundred

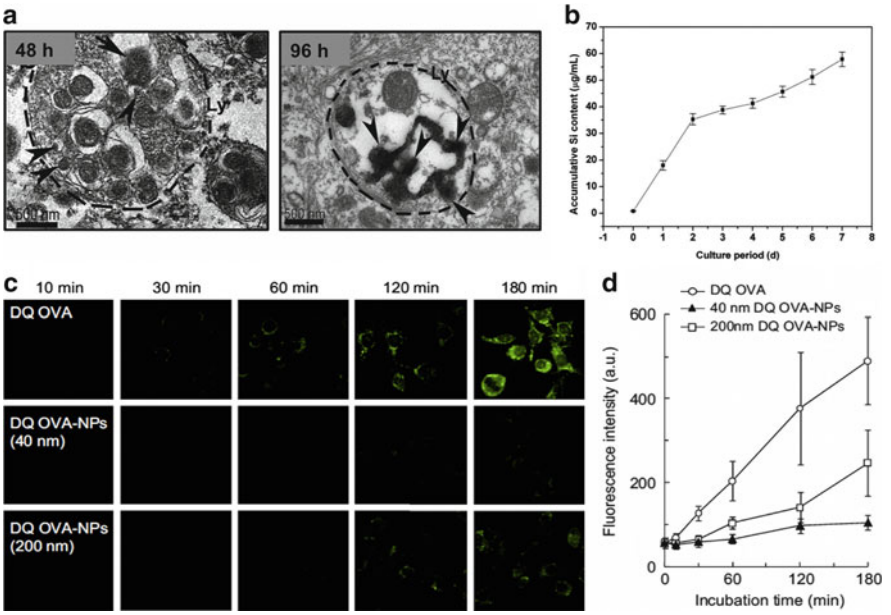

Fig. 18.9 Lysosomal degradation of nanoparticles. (**a**) Transmission electron microscopy images showing degradation of silica particles in HUVECs at 48 and 96 h of incubation. *Black arrow* points to the non-degraded particle of the original size, whereas *black arrow heads* indicate partly degraded particles. (**b**) The release of silica (Si) into the culture medium, quantitatively assessed by atomic emission spectrometry, was particularly rapid during the first 2 days after incubation and slower yet persistent over the following period of 5 days. (**c**) Fluorescence microscopy images of macrophages (RAW264 cells) incubated with self-quenched ovalbumin conjugate (DQ OVA) (*upper panel*), which generates bright-green fluorescence upon proteolytic degradation, 40 nm-sized DQ OVA-poly(amino acid) nanoparticles (NPs) (*middle panel*) or 200 nm-sized DQ OVA-NPs (*lower panel*). The uptake of DQ OVA alone by the cells resulted in the early degradation of OVA, increasing over the incubation time (*upper panel*). The degradation of NP-encapsulated DQ OVA, although attenuated for both NP formulations as compared to the free DQ OVA, occurred faster in the case of 200 nm-sized NPs (*lower panel*) compared to 40 nm-sized counterparts. (**d**) Similarly, the fluorescence intensity measurements of the degraded DQ OVA showed a rapid degradation of free DQ OVA (*circles*). Low fluorescence intensity values reported for both NP formulations indicated the delayed degradation of the NP-encapsulated DQ OVA. At the same time, higher fluorescence intensity over time, thus faster degradation, was observed for the cells incubated with 200 nm-sized NPs (*squares*) compared to those treated with the 40 nm-sized NPs (*triangles*). Reproduced with permission [169, 170]

nanometer sized ovalbumin-encapsulated polymer nanoparticles (OVA-NPs) were compared with respect to the cellular uptake efficacy, intracellular distribution and degradation kinetics in macrophages (RAW264 cells). The cellular uptake of FITC-labeled OVA-NPs increased with increasing particle size. At the same time, the authors found no obvious differences in the intracellular distribution of the nanoparticles. Both formulations were predominantly located in the endo/lysosomal compartment. The intracellular degradation of OVA-NPs and the effect of size on this process were investigated using a pH-insensitive self-quenched OVA conjugate (DQ OVA) that exhibits

bright-green fluorescence upon proteolytic degradation. Fluorescence microscopy images obtained at different time points during incubation of RAW264 cells with either free DQ OVA or DQ OVA-NPs of 40 or 200 nm are displayed in the Fig. 18.9c. As the incubation time increased, the fluorescence of soluble DQ OVA became more intense inside the cells (Fig. 18.9c, upper panel). As expected, the degradation of DQ OVA encapsulated into the nanoparticles was attenuated as compared to the free DQ OVA. Interestingly, the size of the nanoparticles affected the intracellular degradation of the encapsulated DQ OVA, i.e., the degradation of small nanoparticles was slower (Fig. 18.9c, middle panel) than for the larger ones (Fig. 18.9c, lower panel). The quantitative assessment of the fluorescence intensity of the degraded DQ OVA per cell showed the same trend (Fig. 18.9d). Concerning the mechanism responsible for this size effect, it has been hypothesized that the polymer density of the nanoparticles varies for different particle sizes, thereby influencing the kinetics of proteolytic degradation.

Finally, we should consider the potential cellular toxicity induced by internalized nanoparticles and their breakdown products. Many reports have shown that the cell-internalized nanocarriers can persist in the endo/lysosomal compartment for extended periods of time i.e., days, weeks or even months [171, 172]. This long residence may disturb the intracellular balance, eventually leading to cell death. As an example, we can consider iron oxide-based contrast media, which are recognized as highly biocompatible. Nevertheless, once internalized, they have been shown to induce cellular toxicity. Lunov et al. [172] studied extensively the effects of internalized carboxydextran-coated superparamagnetic iron oxide nanoparticles (SPIO) on murine hepatic Kupffer cells and human macrophages. In mice, intravenous injection of SPIO led to a rapid accumulation of the particles in phagocytes and to long-lasting increased iron deposition in liver and kidneys for at least 3 weeks. The uptake of nanoparticles by Kupffer cells triggered apoptosis, as deduced from active caspase 3 staining, and the subsequent depletion of Kupffer cells. In vitro cultured human macrophages incorporated SPIOs in vesicles containing α-glucosidase, which colocalized with lysosomes. Three days after internalization, the carboxydextran shell of the particles was degraded, as concluded from the decreased intracellular fluorescence signal originating from the fluorescent shell-label. At the same time, no signs of intracellular iron depletion were observed. The exposure to TNF-α, which regulates iron metabolism, increased the apoptosis rate, the reactive oxygen species production and the activation of c-Jun N-terminal kinase. The study shows therefore that internalized SPIOs are retained intracellularly for extended time periods, where they induce delayed cell death.

Furthermore, the exposure to lysosomal conditions, i.e., low pH and high enzymatic activity, may lead to the formation of toxic breakdown products. This is a major concern for MRI-detectable nanocarriers containing Gd-chelates, which are either incorporated in the particle shell of encapsulated in its interior. It has been previously demonstrated that low pH, corresponding to that of the lysosomal compartment, promotes Gd^{3+} release from the chelate [173, 174]. This is of high clinical relevance since the systemic exposure to free Gd^{3+} ions has been implicated in the etiology of nephrogenic systemic fibrosis (NSF), a severe disorder that is predominantly diagnosed in patients with end-stage renal disease [175, 176]. The study on protein-conjugated

Gd-DTPA showed that the macromolecular backbone of the probe undergoes efficient metabolism in the lysosomal environment, leading to the formation of single amino acid-Gd-DTPA conjugate [174]. This metabolite was released from the cell and recovered intact in the kidney and colon. At the same time, Gd^{3+} readily dissociated from the protein/amino acid-DTPA under the acidic conditions. The accumulation of free Gd^{3+} ions was particularity prominent in the liver and skeleton, which was also reported in other biodistribution studies [177–179]. In contrast to the aforementioned Gd-DTPA, which belongs to the group of linear chelates, macrocyclic complexes, such as Gd–DOTA, have much higher thermodynamic and kinetic stability [176, 180]. Therefore, there is a clear trend towards the latter chelates in both extracellular and cell-targeted imaging applications. Furthermore, none of the chemical groups that are involved in the formation of coordination bonds with Gd^{3+} ion should be used for the conjugation with macromolecules. This is believed to largely improve the extra- and intracellular stability of Gd-containing constructs.

The lysosomal compartmentalization and degradation are the final and important steps of the intracellular pathway of targeted delivery systems. They are responsible for the metabolic turnover of internalized material, leading to either its recycling or elimination. At the same time, lysosomal processing can limit the potency of a therapeutic or imaging cargo and/or propagate the cytotoxic effects. Therefore, during the in vitro and in vivo evaluation of novel intracellular delivery systems, both positive and negative implications of endo/lysosomal compartmentalization and degradation should be always considered.

Future Perspectives for Targeted Delivery Systems

This chapter aimed to illustrate the remarkable variety of tumor targeting strategies that are explored to improve the specificity and the efficacy of cancer-specific imaging read-outs as well as cytotoxic and/or cytostatic therapies. The emphasis was on ligand-conjugated nanoparticles directed towards cell-surface receptors as these provide both exciting opportunities for the delivery of large amounts of imaging contrast agents and antitumor drugs. There are many examples of the successful use of fabricated nanostructures in preclinical imaging and therapy studies on small-animal cancer models.

Several nanoparticle-based formulations of antitumor agents, such as liposome-encapsulated doxorubicin and daunorubicin or albumin-bound paclitaxel nanoparticles, have been approved for therapeutic use in the clinic. Their utility is often based on the exploitation of the EPR effect [181], causing relatively high concentrations in the tumor compartment while keeping the systemic levels of free drug relatively low. The EPR effect has been frequently observed in mouse models, however, has not been proven in human, yet. If it exists in human, it is expected to reduce the manifestation of unwanted side effects. The clinical utility of ligand-conjugated nanoparticles for cancer imaging and therapy has to be awaited. The translation of newly developed diagnostic and therapeutic agents is a lengthy and

costly process, with an uncertain outcome. This is partly due to the fact that there are no consensus guidelines for optimizing ligand-conjugated nanostructures to achieve most effective cancer imaging and therapy. Recently, Hrkach et al. [70] have presented a framework for optimizing the utility of ligand-conjugated docetaxel-loaded PLA- and PLGA-based polymeric nanoparticles that may be generally applicable. Docetaxel is a potent chemotherapeutic. Hrkach's particles were equipped with a ligand that targets prostate-specific membrane antigen, which is expressed on the surface of prostate cancer cells and the neovasculature of many non-prostate solid tumors. The authors used combinatorial and high-throughput technologies to explore the vast multifactorial parameter space, with respect to size, surface hydrophilicity, ligand density, and drug loading as well as drug release characteristics. Basic nanoparticle makeup was restricted to a clinically validated set of biomaterials to enhance the opportunities for clinical approval. A library of more than 100 distinct nanoparticle compositions was explored, which was followed by kilogram scale GMP production of the most promising material. The in vivo performance of the targeted, drug-loaded nanoparticles was tested in multiple animal species and several mouse xenograft tumor models, while also a clinical study was initiated to evaluate the tolerability and pharmacokinetics of the agent in patients with advanced and metastatic solid tumors. The initial results of the phase 1 clinical trial were promising. Shortly more extensive clinical tests are foreseen. The study by Hrkach et al. was performed with non-imageable nanoparticles, necessitating the use of invasive one-off methods to assess the efficacy of docetaxel delivery in the mouse tumors, thus excluding longitudinal follow-up of the relation between local drug delivery and tumor growth inhibition. The addition of imaging label obviously poses additional challenges in terms of clinical translation, both with respect to cost and safety profiles. For these reasons, the use of imageable, drug-loaded tumor-targeted nanoparticles will probably remain largely restricted to preclinical research, in which these tools provide powerful tools for steering the optimization of tumor imaging and therapy.

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Chapter 19
Targeting Drugs to Cancer: A Tough Journey to the Tumor Cell

Shiran Ferber, Galia Tiram, and Ronit Satchi-Fainaro

Abstract Chemotherapeutic agents continue to represent the preferred therapeutic option for most malignancies. Despite major therapeutic potential, their use is limited due to severe side-effects and inefficient delivery to the tumor site. In the last four decades, researchers investigated the use of nano-sized drug delivery systems (i.e., nanomedicines) for targeting of anticancer agents. Using a nano-sized macromolecule as scaffold for drug delivery to tumors is an efficient approach to improve the delivery of drugs by ameliorating biodistribution, reducing toxicity, preventing degradation, and enhancing cellular uptake. Nevertheless, in some cases, nonselective targeting is insufficient and the incorporation of a ligand moiety is required for improved accumulation of the drug in the tumor cell. This chapter discusses the different targeting strategies used for delivery of nanomedicines to cancer cells.

Introduction

Cancer remains one of the major causes of death worldwide and the incidence of most cancers increases every year (http://www.cancerresearchuk.org). An estimated 12.7 million new cancer cases were diagnosed in 2008 and 7.6 million deaths were reported. Even with remarkable advances in medical sciences over the last two decades, the overall survival of cancer patients with advanced and/or metastatic cancer remains extremely poor. Among these are lung, female breast, colorectal, stomach, pancreatic, and prostate cancer. When surgical removal is impossible, chemotherapy is widely used in attempt to control cancer progression. However, conventional chemotherapy utilizes low-molecular-weight molecules which lack

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selective toxicity to cancerous tissues and thus results in severe off-target effects in normal tissues. This limits the use of these drugs and may even lead to treatment termination. Administration of drugs using a highly selective delivery system can solve most of the above mentioned limitations.

The rationale for using nano-sized molecules as drug carriers relies on the specific pathophysiological characteristics of both the tumor cell population and the components that form the tumor microenvironment. Addition of antibodies or other ligands, that bind to antigens or receptors that are usually abundant or uniquely expressed on the surface of tumor cells and/or components of the tumor microenvironment (endothelial cells, fibroblasts, immune cells, etc.), to a nano-sized delivery system, along with the distinctive tumor vasculature, allows the superior extravasation to the tumor interstitium and therefore represents a promising approach for cancer therapy.

The purpose of using nanomedicines is to improve the therapeutic index of the drug by increasing the half-life of low-molecular-weight or easily degraded compounds, enhancing their solubility and facilitating controlled release at the target site, while reducing their toxicity. In these delivery systems, the therapeutic agent is incorporated, adsorbed, complexed, or chemically conjugated to the carrier. Nanomedicines are usually composed of three basic elements: (1) a macromolecular backbone (i.e., carrier), (2) therapeutic agent, and (3) a spacer for non-encapsulating systems. The selected carrier should ideally be water-soluble in order to increase drug solubility, biocompatible, non-immunogenic and finally be degraded or eliminated from the organism [1, 2]. Drugs can be conjugated to the carrier either directly or via a bio-degradable linker which allows control of the rate and site of release [3].

The delivery system can be designed for nonselective and/or ligand-based targeting. The nonselective targeting approach relies on the pathophysiological characteristics of the tumor vasculature. The receptor-specific or ligand-based approach relies on a receptor or antigen specifically expressed by either the tumor-cell population, tumor microenvironment or tumor tissue. Segal et al. [4] and Miller et al. [5, 6] were able to target calcified tissue of bone primary neoplasms and bone metastasis by conjugation of the amino-bisphosphonate alendronate (ALN) to *N*-(2-hydroxypropyl)methacrylamide (HPMA copolymer) or poly(ethyleneglycol)-polyglutamic acids (PEG-PGA) as a targeting moiety to the bone mineral hydroxyapatite. The concept of such drug delivery systems based on nano-sized polymeric macromolecules was first proposed by Ringsdorf in 1975 [7].

DaunoXome—a self-assembling liposomal daunorubicin [8], OncoTCS—a liposomal vincristine [9], and Doxil—a PEGylated (polyethylene glycol-coated) liposomal doxorubicin [10, 11] were the first nanomedicines approved by the US Food and Drug Administration (FDA) for the treatment of cancer. The development of Doxil and PEG incorporation to nano-sized molecules in general was a milestone in drug delivery systems for cancer and allowed them to circulate for remarkable longer periods of time in the systemic circulation [12, 13]. Currently, many other nanoparticles, liposomes, micelles and other vesicles are in clinical trials (www.clinicaltrials.gov). However, most of these nanomedicines, while exhibiting great therapeutic potential in animal models, eventually result in poor outcome in

the clinic. In a recently published manuscript, the authors found profound differences in EGFR inhibitor-treatment efficacy according to genetic background, sex and diet [14]. Hence, the model system may dramatically impact preclinical results as it disregards patients' heterogeneity. Better designed preclinical studies would lead to more reflective predictions of therapeutic response in the clinic.

In this chapter, we will briefly discuss several aspects of tumor targeting. We will describe the barriers and limitations in targeting drugs to cancer.

Nonselective Targeting to the Tumor Site

Nonselective targeting is achieved by exploiting the enhanced permeability and retention (EPR) effect phenomenon for macromolecules typical for tumor vasculature. In this phenomenon, that was first described in 1986 by Matsumura and Maeda [15], tumor blood vessels are typically characterized by enlarged vessels with wider lumen, to which Dvorak refered to as "mother vessels" [16], leakage, highly tangled, sluggish, noncontinuous blood flow and blunt ends [17–19]. This allows nano-sized drug carriers to easily extravasate into the interstitial matrix at the tumor site, while potentially reducing drug release in normal tissues resulting in toxicity. These nanocarriers cannot penetrate the impermeable vasculature that characterizes normal and healthy tissues. Only molecules up to 2–4 nm can penetrate the normal vasculature [20, 21] (Fig.19.1). Once the nanomedicine enters the interstitium, it is retained by diminished intratumoral lymphatic drainage and accumulates at high concentrations [19]. This results in decreased clearance of the nanomedicines and sustained drug release in the tumor site. As opposed to nanomedicines, low-molecular-weight drugs diffuse rapidly into both tumor and normal tissues through the endothelium of blood capillaries, therefore causing undesirable systemic side-effects followed by rapid renal clearance. Consequently, as suggested by Maeda and coworkers [21], exploiting the EPR effect by nanomedicines has become the "gold standard" for targeted cancer therapy. This includes low-molecular-weight cytotoxic agents, gene delivery, antibody therapy, proteins, and theranostic agents [22]. It is noted, however, that the quantity of the nanomedicine delivered to a target tumor site is still very low, accounting for only ≤ 5 % of the total administered dose. Clearly, the EPR effect alone cannot achieve successful cancer therapy.

The use of nanomedicines enables overcoming drug resistance mediated by membrane transporters, like P-glycoprotein (Pgp/MDR1). Active internalization of ligand-based nanomedicines to cells restrains the rapid segregation that occurs in passively diffusing low-molecular-weight drugs.

Considerations for Rational Design

Since nanomedicines accumulate at the tumor site according to their nanometric size, when designing a delivery system, the carrier size and hydrodynamic volume

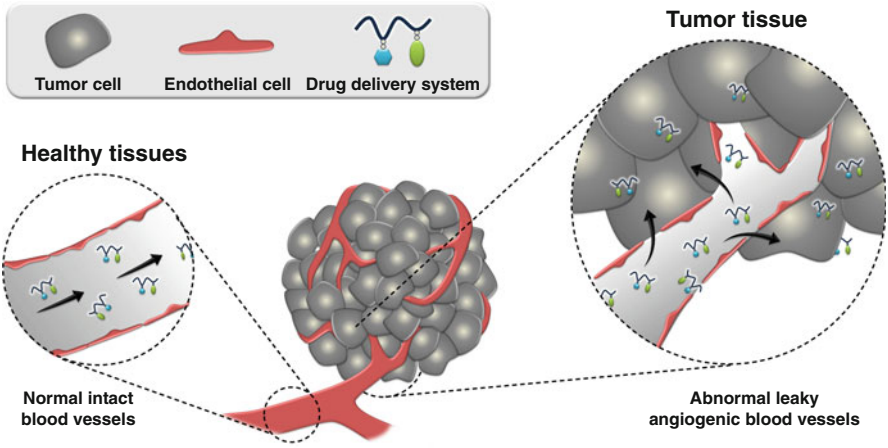

Fig. 19.1 Targeting tumor cells using nanocarriers of anti-cancer agents. Schematic illustration of the EPR effect allowing extravasation of nanomedicines through the hyperpermeable tumor blood vessels and their accumulation at the tumor site. Nanomedicines cannot penetrate normal intact blood vessels as found in healthy tissues

are important parameters that will determine its pharmacokinetics. In order to achieve an optimal accumulation of the drug at the tumor site, two main size-related factors should be considered. First, the size of the nanomedicine should be lower than the normal renal threshold, which its upper limit is in the range of 30–50 kDa [23]. Second, the size cut-off threshold between endothelial cells varies among tumor types, though permeability and extravasation of nano-sized molecules up to 400 nm through endothelial gaps has been observed in mouse xenograft models [23, 24]. Additional factors dictating the biodistribution of the nanomedicines are charge, conformation, hydrophobicity, and immunogenicity [23].

Excessive production of EPR-enhancing factors (i.e., vascular permeability factors) by the tumor cell population may occur, in a signal transduction process triggered by hypoxia and orchestrated by hypoxia-inducible factors (HIFs), as a result of rapid growth and thus the need for nutrients and oxygen. Enhanced angiogenesis may result in interstitial hypertension, hypoxia, and acidosis, and all promote tumor progression and interfere with the delivery of low-molecular-weight drugs to tumors [25]. These vascular permeability factors include bradykinin, nitric oxide (NO), prostaglandins, angiotensin-converting enzyme (ACE) inhibitors, vascular endothelial growth factor (VEGF) and other cytokines [26–30]. In general, tumor angiogenesis is affected by factors regulating the permeability (e.g., VEGF, NO, prostaglandins, MMPs, TNFα, and IL-2) and the anatomy of the newly formed vessels (e.g., extensive angiogenesis and high vascular density, lack of vessel stabilizers like smooth muscle and pericytes coating, irregular blood flow, poor lymphatic drainage and venous return). Inflammatory processes are also mediated by these

factors and it is therefore not surprising that the EPR effect is manifested in other angiogenesis-dependent diseases like psoriasis, macular degeneration, diabetes, and arthritis [21]. Different therapeutic agents can also promote the generation of these factors such as doxorubicin that upregulates VEGF and pro-inflammatory anticancer agents that promote the activation of matrix metalloproteinases (MMPs) like mitomycin C and nitrosourea [26, 27, 31, 32]. In this case, delivery may be improved by combining anticancer with anti-angiogenic therapeutics in the delivery system. Vessel normalizing anti-angiogenic agents (e.g., Bevacizumab, TNP-470, and caplostatin) reversibly reduce the EPR effect and improve penetration of cytotoxic chemotherapy drugs and oxygen to the tumor site, thus resulting in enhanced efficiency [21, 33–37]. Incorporation of an anti-angiogenic moiety will not be beneficial for nanomedicines lacking sustained release mechanism. Certain nano-sized drug delivery systems demonstrate rapid burst release post administration leading to premature drug release and consequently lacking effective accumulation.

The opposite is correct when dealing with large tumors which contain necrotic tissue at the core. Blood vessels-deprived areas lead to reduced accumulation and decreased therapeutic effect of nano-sized molecules. In this case, several studies have shown that co-administration of nanomedicines with NO [21, 38], bradykinin, VEGF [39], and TGF-β [40] enhance their extravasation. Nagamitsu and colleagues showed that by using angiotensin II as a blood pressure inducer, it is possible to improve SMANCS (conjugate of neocarzinostatin and poly(styrene-comaleic acid anhydride) delivery to poorly vascularized tumors [38].

An additional factor that should be considered is the relatively rapid opsonization which results in decreased half-life that nanomedicines face in the systemic blood circulation. Plasma proteins in the bloodstream recognize nanomedicines as foreign objects and incorporate opsonin, which is recognized by the mononuclear phagocytic system (MPS) located in the liver and spleen [41]. Consequently, nanomedicines undergo rapid clearance from the systemic circulation, therefore resulting in reduced therapeutic effect. One option to bypass the fast recognition by the innate immune system is by coating nanomedicines with hydrophilic polymers, such as poly(ethylene glycol) (PEG) or PEG-copolymer (poloxamers, poloxamine, and polysorbate). For example, Doxil holds an advantage over DaunoXome, since the surface incorporation results in MPS escape, sustained drug accumulation at the tumor site, resulting in increased antitumor activity and reduced toxicity.

Although PEGylation is a useful method for achieving biocompatibility and longer circulation time for delivery systems, it strongly inhibits cellular uptake and endosomal escape. The aqueous phase formed by PEG interferes with the nano-sized molecule-membrane interaction required for endocytosis into the cancer cell and thus the drug is released in the tumor interstitium [42]. For successful design of an anticancer nanomedicine, this crucial issue referred to as “PEG dilemma” must be addressed [43]. Furthermore, administration of PEGylated nanomedicines has led to the generation of PEG-specific antibodies causing a rapid clearance which further diminish the treatment efficiency [44, 45]. However, it can be improved by careful tuning of the drug regimen. Recent study on the accelerated blood clearance of the PEG-conjugates showed that the PEG chain length and the

surface area of the carriers are important factors for the rapid clearance by the PEG-specific antibodies [46].

To summarize, some of the limiting factors for the therapeutic success of nanomedicines are: tumor size, degree of vascular permeability and negative pressure gradient within tumor interstitium (high at tumor core and lower towards the periphery). These factors restrict their effective penetration and equal distribution in the tumor. Furthermore, nonselective targeting does not promote internalization of drugs into cancer cells. The conventional approach to circumvent problems discussed above and to improve cellular uptake is by ligand-based targeting of molecules to the surface of cancer cells.

Ligand-Based Targeting to Cancer Cells

As mentioned above, passive accumulation at the tumor site is probably insufficient for most drugs. In such cases, ligand-based targeting should be considered as an alternative strategy. Ligand-based targeting is the incorporation of a ligand with specific affinity to the target site. A targeting moiety can be designed to target a tumor cell population, components of the tumor microenvironment (endothelial and non-endothelial cells or enzymes like MMPs) and/or the tissue from which the tumor originates or where it is located. Targeting the tumor environment is broadly described in the "General Tumor Physiology and Microenvironment Issues" part in this book. Ligand-based targeting can be achieved either by conjugation of a targeting moiety to the surface of the nanomedicine or by direct conjugation to the drugs. Small ligands, antibodies and their derivatives, nucleic acids and aptamers, peptides and proteins can be used as targeting elements. The targeting moiety may possess therapeutic potential by itself, either by scavenging key molecules in cancer progression or by serving as an agonist or antagonist, activating or blocking a signal transduction pathway. Eldar-Boock et al. used the recognition peptide E-[c-(RGDfK)$_2$] (Arginine–Glycine–Aspartic acid peptidomimetic-PM) for the integrin $\alpha_v\beta_3$ that is overexpressed in both breast cancer cells and tumor-endothelial cells selectively during angiogenesis, to deliver poly-glutamic acid (PGA) conjugated to paclitaxel [47]. Integrin $\alpha_v\beta_3$ is a key element in cellular adhesion, migration and invasion; hence, by using RGD-PM as a targeting moiety, Eldar-Boock and colleagues were able to target migrating endothelial cells during the angiogenic process and to prevent $\alpha_v\beta_3$ interaction with other cells at the microenvironment.

There are two main drawbacks associated with the ligand-based targeting of drugs to cancer cells. First, most targeting elements are directed to molecules overexpressed by the cancer cells, rather than molecules uniquely expressed by cancer cells, but these are mostly unknown or absent. Targeting molecules that are uniquely expressed by cancer cells will greatly increase the "signal to noise ratio" of the nanomedicines biodistribution (examples to be discussed later). The second issue relates to the intrinsic genetic diversity of tumors. It is well established that a single tumor contains multiple cell subpopulations; however, tumor heterogeneity also

relates to diversity between one organ and another and even between patients bearing tumors from the same origin [48, 49]. This variability is present at multiple levels of cell functions and results in variability in prognosis. Moreover, genes that control the growth, metabolism, migration, and invasion of tumor cells tend to undergo extensive mutations. Analysis of 22 breast and colorectal cancers revealed that a single tumor exhibit an average of 90 mutated genes out of 13,023 genes tested and 189 genes that are mutated at significant frequency [48]. In a recent study, whole-genome deep sequencing of tumors showed that a single tumor may contain up to 50,000 different mutations affecting hundreds of genes [50]. Therefore, a targeted nanomedicine can efficiently accumulate at the target site only in the appropriate model system. Furthermore, in most cases, targeting elements are directed to general cancer-related molecules. For example, only in the last 3 years, research groups developed 34 folic acid (search "folic acid based-cancer therapy"), 12 anti-EGFR (aptamer or antibody), and 12 RGD-based nanomedicines. Hence, it cannot be expected that the same targeting moiety will be equally efficient to breast tumor in one patient and lung tumor in another, or even to primary and metastatic lesions in the same patient. This has led to an era in which a particular type of tumor would be treated with a targeted drug delivery system tailor-made according to its distinct characteristics which derive from its origin tissue.

In the following, we will discuss targeting strategies for specific types of cancerous tissues including breast, lung, brain and bone cancers (Fig. 19.2).

Brain Targeted Therapy

Out of all nano-sized drug delivery systems that are approved for clinical use, none are targeted to the brain [51–53]. Currently, there is only one clinical trial involving a nanomedicine for glioblastoma treatment and three for head and neck cancers (www.clinicaltrials.org). This is probably due to the fact that in a drug journey to the brain tumor-cell population, the delivery system must cross two biological barriers: first, it needs to cross the blood–brain barrier (BBB) in order to extravasate from the systemic blood circulation to the brain tissue. This process is followed by diffusion in the brain extracellular space, where the nanomedicine is required to cross the cancer cells membrane for internalization. In a normal brain, a nanomedicine will cross a tight junction between cerebral endothelial cells that build the BBB only when it carries a targeting moiety for these cells, which facilitates endocytosis and transcytosis [54]. Conversely, in a glioblastoma-bearing brain or secondary brain metastases from peripheral tumors (i.e., lung, melanoma, breast, gastrointestinal, and renal), the BBB is disrupted. This enables the extravasation of larger molecules, up to 12 nm as measured in mice models [54, 55]. Yet, it is only to a limited extent, since in micrometastasis or infiltrating glioblastoma cells inside the brain, the BBB might remain intact [56–58]. Delivery across the BBB is broadly discussed in the "Central Nervous System Cancers" part in the book, so we will not elaborate on it further.

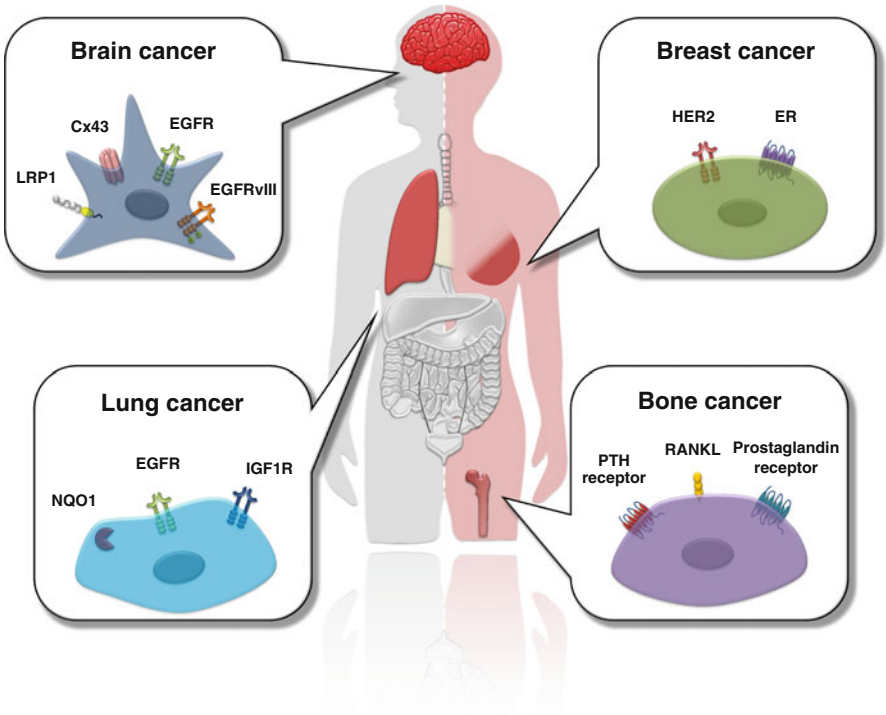

Fig. 19.2 Schematic presentation of tumor tissue-specific molecular targets. *LRP1* indicates low density lipoprotein receptor-related protein 1, *Cx43* indicates connexin 43, *EGFR*/*EGFRvIII* indicates epidermal growth factor receptor/variant III, *HER2* indicates human epidermal growth factor receptor 2, *ER* indicates estrogen receptor, *NQO1* indicate NAD(P)H dehydrogenase, *IGF1R* indicates insulin-like growth factor 1 receptor, *PTH* indicates parathyroid hormone, *RANKL* indicates receptor activator of nuclear factor kappa-B ligand

Diffusion across the extracellular space in the brain is also limited by additional hurdles. On its way to the tumor site, a nanomedicine might extravasate towards cells different than the tumor-cell population, and it is subjected to high cerebrospinal fluid turnover rate and efflux system which results in its elimination from the brain [59, 60]. Furthermore, glioblastoma is different from other cancers due to its diffused invasive nature to the surrounding normal brain tissue, which makes complete removal of glioblastoma impossible by conventional surgery and hence leads to very high recurrence from residual tumor cells [61]. Glioblastoma chemotherapy treatment is also limited owing to poor BBB penetration and lack of efficient targeting to glioblastoma cells [62].

Targeted nanomedicines should typically include a targeting moiety with high affinity to a ligand expressed only or mainly in the brain-tumor cells. Most targeted nanomedicines that are currently in preclinical or phase I trials are, in fact, designed to target BBB-related moieties (e.g., systems conjugated to ligands or antibodies to transferrin receptor, insulin, low-density lipoprotein (LDL) and LDL

receptor-related protein-1 and -2), rather than brain-tumor related moieties [55, 63]. Nevertheless, in recent years there is a tremendous effort in discovering brain specific biomarkers. In 2008 The Cancer Genome Atlas (TCGA) research network performed a large-scale multidimensional analysis on molecular characteristics of 206 human glioblastoma tumors. The most frequent gene amplifications in which the resulting protein is located at the cell membrane, were found in two growth factor receptors with tyrosine kinase activity—epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor α (PDGFR-α), and to a smaller extent MET (or HGFR) [64]. In the same study, EGFR was also found among the most frequently mutated genes (41 out of 91 tumors tested for nucleotide sequence aberration). Human Epidermal Growth Factor Receptor 2 (HER2, Neu, ErbB-2, CD340) was also found to be mutated in 7 out of 91 samples analyzed. The activation of these receptors initiates signaling pathways involving Phosphatidylinositol 3-kinases (PI3K), p53 and retinoblastoma (RB1) that are known to be substantially altered in glioblastoma. A different analysis used a beads-based method for screening the phosphorylation status of 62 out of 90 tyrosine kinases in the human genome in 31 primary glioblastoma human samples [65]. This analysis showed data similar to the analysis performed by TCGA, with extensive phosphorylation of EGFR and MET, but also revealed new key players like proto-oncogene tyrosine-protein kinase (SRC), fibroblast growth factor receptor 3 (FGFR3), protein tyrosine kinase 2 (PTK2, also known as focal adhesion kinase—FAK), LCK and LYN (two SRC-family kinases). Additional studies showed the extended involvement of EGFR in glioblastoma, with overexpression in approximately 50 % of tumors and constitutive expression of the active form of EGFR in 25 % of primary glioblastomas [66, 67]. The EGFR variant III (EGFRvIII) is a glioblastoma-tumor specific mutation that is not expressed by the normal brain and encodes a constitutively active form of EGFR that enhances tumorigenicity and accounts for radiation and chemotherapy resistance in glioblastomas [68, 69]. Furthermore, transgenic mice expressing *v-src* kinase were found to develop brain and spinal cord tumors with morphological and molecular characteristics of human glioblastoma [70]. SRC and SFK are regulators of several signaling pathways regulating proliferation, adhesion, migration and invasion, all key elements in tumor progression and metastasis. Overexpression of PDGFR-α was shown in all grades of astrocytoma [71].

Since most of the membrane-expressed proteins discussed above are also key factors for tumor progression and survival, it is natural to target them. Tyrosine kinase and growth factor receptors-targeted therapeutics are already in the clinic for the treatment of different types of cancer. Currently, two major types of systemic anti-EGFR therapeutics have entered clinical trials and were FDA-approved: anti-EGFR antibodies (Cetuximab) and small molecules EGFR tyrosine kinase inhibitors (gefitinib and erlotinib) [72–74]. Unfortunately, these agents only exhibited modest efficacy in patients with glioblastoma for several reasons, including poor BBB penetration, challenging systemic delivery, and insufficient potency. Besides anti-EGFR therapeutics, there is a variety of approved drugs with available preclinical data in glioblastoma: Dasatinib is a SRC and SFK inhibitor, but also inhibits c-Kit

and PDGFR [75]; Imatinib and Sorafenib are targeted to PDGFR, although Sorafenib possess affinity to VEGF-R and MAPK as well.

Three papers were published in the past year using Angiopep-2 (ANG) as a targeting moiety to glioblastoma [76–78]. Angiopep-2 serves as an attractive dual targeting moiety since it is the ligand of low-density lipoprotein receptor-related protein 1 (LRP1) that, compared with the normal brain, is overexpressed on malignant glial cells and brain endothelial cells [79]. In previous studies, nanoparticles modified with Angiopep-2 efficiently crossed the BBB by transcytosis and accumulated in the cancer cells following receptor-mediated endocytosis [80, 81]. Xin et al. [76] utilized Angiopep-2 to deliver paclitaxel (PTX)-loaded poly(ethylene glycol)-co-poly(ε-caprolactone) (PEG-PCL) copolymer (ANG-PTX-PEG-PCL). PTX is a microtubule stabilizing agent with potent antitumor activity that has not been used for the treatment of glioblastoma due to poor BBB penetration [82, 83]. In this study, intravenous (i.v.) administration of non-targeted PEG-PCL nanoparticle (NP) showed only a slight accumulation around orthotopic glioblastoma tumor region, which was attributed to the EPR effect, while a significant higher tumor accumulation was observed following administration of ANG-PEG-PCL, with undetectable accumulation in the normal brain. This was accompanied by inhibition of tumor growth by 65.6 % compared to saline treated mice and 29.5 % compared to the non-targeted NP. Although the median survival of tumor-bearing mice was only 7 days longer for those treated with the targeted NP, compared to those treated with the non-targeted, it was statistically significant. Toxicity following i.v. administration of ANG–PEG–PCL (without PTX) was not evident and the NP was able to escape the MPS system and thus did not induce any inflammatory reaction in healthy tissues. Ren et al. [78] constructed an Angiopep-2-dependent dual drug delivery system of oxidized multi-walled carbon nanotubes (O-MWNT) PEGylated with 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (DSPE)-PEG in order to deliver doxorubicin (DOX) to glioblastoma. Carbon nanotubes hold unique physical and chemical properties that enable high loading of multiple molecules alongside the nanotube wall on account of ultrahigh surface area. DOX-O-MWNT-PEG-ANG designed in this study had DOX-loading coefficient of 80 % which permitted low carrier dose. DOX release was pH-sensitive with higher release rate in acidic environment, typical for the tumor site. Biodistribution analysis revealed that O-MWNT-PEG-ANG greatly increased DOX accumulation in the brain and glioblastoma site compared to DOX alone and, more important, compared to the non-targeted carrier, with high intensity of DOX at 2 h post i.v. injection that was retained for 24 h. Also, cardiac toxicity, which is a well-known disadvantage of DOX treatment, was reduced as seen by improved myocardial fiber rupture. Overall, DOX-O-MWNT-PEG-ANG showed improved antitumor activity and median survival time, negligible toxicity and high biocompatibility following systemic administration, compared with free DOX and non-targeted carrier. Finally, dual targeting to intracranial glioblastoma was achieved by Huang et al. who designed a 110 nm bifunctional PEGylated high-branching polyamidoamine (PAMAM) dendrimer complexed with plasmid DNA expressing TRAIL. TRAIL, tumor necrosis factor (TNF) related apoptosis-inducing ligand, is a signaling molecule that induces apoptosis upon binding to an agonist TRAIL

receptor found on glial cells [84, 85]. Normal cells express the antagonist TRAIL receptor and thereby avoid apoptosis when introduced with TRAIL-expressing plasmid DNA. Other studies have shown TRAIL-induced apoptosis only in tumorigenic or transformed cells but not in normal cells [86, 87]. Indeed, there was a significant high level of apoptosis detected in intracranial glioblastoma cells in mice treated with PAMAM-PEG-Angiopep bearing TRAIL plasmid DNA, which was spread throughout the tumor mass. In contrast, low levels of apoptosis that was detected mostly at the tumor edge were observed in mice treated with free plasmid, non-targeted dendrimer with or without PEGylation, and temozolomide. This is also due to the fact that the non-targeted NP hardly accumulated in the brain and tumor site. An increased tumor accumulation was observed for the PEGylated non-targeted NP, but still considerably lower compared to the targeted one. Interestingly, all compounds, except the PEGylated non-targeted NP, accumulated at the liver, but not in other organs. The authors did not refer to this observed phenomenon, but an explanation might be due to prevention of PEG shield from MPS recognition by peptide incorporation. Therefore, while PAMAM-DNA and PAMAM-DNA-PEG-ANG are recognized by the MPS, PAMAM-PEG escapes from the MPS and does not accumulate in the liver. The targeted NP exhibited higher median survival time compared to the non-targeted NP and temozolomide (61 days compared to 49 days).

Chekhonin et al. [56] designed a PEGylated immunoliposome targeting connexin 43 (Cx43) or glial fibrillar acidic protein (GFAP). Cx43 is an integral membrane protein forming hexamers (connexons), which in turn form gap junctions allowing both cell adhesion and exchange of various intracellular messengers [88]. It is expressed by rapidly migrating glioblastoma cells, by reactive astrocytes surrounding the peritumoral zone and by vascular endothelium in the central nervous system [89]. GFAP, is the main astroglial marker also expressed by peritumoral reactive astrocytes [90]. GFAP positive reactive astrocyte forms heterologous gap junctions with glioblastoma cells which induce the migration of peritumoral Cx43 positive glioblastoma cells [89, 91]. Therefore, Cx43 and GFAP are attractive targets for glioblastoma tumors. The PEGylated immunoliposomes harbored either monoclonal antibody targeting the E2 extracellular loop of Cx43 (MAbE2Cx43), or against GFAP (MabGFAP). Both immunoliposomes accumulated in peritumoral astroglial cells of C6 intracranial tumors 48 h following intravenous injection, but differed in the accumulation site. MabGFAP liposomes were observed as small intracellular inclusions, whereas MAbE2Cx43 liposomes were observed as heterogeneous staining of the cytoplasm. Control liposomes, without a targeting moiety or harboring a nonselective mouse immunoglobulin, did not show any accumulation at the peritumoral zone of the invasion of high-grade glioblastomas. However, it is unknown how the MabGFAP liposomes were able to effectively accumulate at the peritumoral site since GFAP is an intracellular protein. The authors suggest the presence of undefined mechanisms of internalization of the water-soluble form of GFAP.

Another aspect of ligand-based tumor targeting is site-specific drug release, i.e., where the drugs are only released from the nano-scaled delivery system by enzymes overexpressed in the cancer cells themselves or their microenvironment [92].

Gu et al. [93] recently designed a PEG-PCL based delivery system conjugated to PTX via a pH-sensitive linker and to a cationic cell penetrating peptide (CPP), activatable low molecular weight protamine (ALMWP). ALMWP contains a sequence of polycationic CPP, a MMP-sensitive peptide linker and a polyanionic inhibitory domain. Therefore, at the MMP rich tumor environment, the linker is cleaved, the polyanionic inhibitory peptide dissociates and the polycationic CPP delivers its "cargo" into the tumor cells. In this work, the authors used a MMP-2/9-cleavable linker (PLGLAC) since these two have been previously reported to be dramatically upregulated in glioblastoma cells and blood vessels, governing glioblastoma angiogenesis and invasion when the degree of malignancy increased [94, 95]. Indeed, the 120 nm ALMWP-PEG-PTX exhibits high cellular uptake by glioblastoma cells, its activity is suppressed by MMP inhibitors, and it has no effect on the non-activatable LMWP. Biodistribution and pharmacokinetic studies showed higher accumulation and higher PTX concentration of ALMWP-PEG-PTX in intracranial glioblastoma tumor site with markedly delayed blood clearance, compared to LMWP-PEG-PTX following i.v. administration. Both LMWP-PEG and unmodified PEG showed similar low accumulation in the central tumor site and nonselective distribution in the normal brain, which is attributed to the EPR effect. These results indicate that the non-activatable CPP can facilitate delivery of NP to both normal brain and glioblastoma tissues without selectivity, whereas ALMWP provides selective glioblastoma targeting. This observation is strengthened by improved antitumor activity of ALMWP-PEG-PTX with prolonged median survival time of approximately 24 days compared to saline and PEG-PTX, and 13 days compared to LMWP-PEG-PTX.

Since the 5-years survival rate for glioblastoma is still a dismal 4 % for the past few decades, significant breakthrough in treatment of glioblastoma derived from rational targeting strategies, as presented here, is urgently needed.

Bone Targeted Therapy

Excluding primary bone tumors, bone metastases are highly common in patients with lung, thyroid, kidney, but mostly breast and prostate cancer. Cancer of the bone, primary or secondary, causes significant pain, morbidity and severe decrease in patients' quality of life. The mechanisms involved in predisposition to bone metastasis of many solid tumors include several parallel pathways. Understanding the mechanisms that predispose tumor metastases to the bone will improve therapeutic options and will unravel novel attractive targeting moieties to these tumors.

Osteosarcoma, the most common type of primary bone cancer, typically develops in the femur, tibia and humerus. It is most often diagnosed in children and young adults, while bone metastases are typically common in adults. Approximately 20 % of patients with osteosarcoma will have metastatic disease at diagnosis. In a study of patients with newly diagnosed high-grade osteosarcoma, 202 patients had more than five metastatic lesions that were associated with a 5 years overall survival rate of 19 % [96]. Other common types of primary bone cancer are chondrosarcoma and

Ewing's sarcoma. Cancer of the bone possesses a range of surface-specific targeting opportunities. Most of them are directed to the bone tissue itself using tetracyclines, acidic oligopeptides, chelating compounds, salivary proteins, and most commonly—bisphosphonates [97]. Bisphosphonates bind strongly to hydroxyapatite, the main mineral component of the bone, and serve as an attractive targeting moiety since hydroxyapatite is exposed to the blood in local inflammation. The use of bisphosphonates as targeting ligands holds several more advantages: (1) relatively easy to conjugate due to the presence of a primary amine group in the case of aminobisphosphonates, (2) possess an anti-angiogenic activity, (3) may produce a synergistic effect in combination with other drugs when conjugated via a degradable linker, (4) long half-life in the bone that results in long-term osteoclasts inhibition and osteoblasts formation, and (5) thermodynamic absorption to hydroxyapatite is favorable over kinetic absorption [98–102].

Miller et al. [5, 6, 103] designed a bone-targeted HPMA copolymer with synergistic antitumor and anti-angiogenic activity by combining ALN and paclitaxel (PTX) via a cathepsin B-degradable linker. HPMA copolymer-PTX–ALN exhibited a notable anti-angiogenic effect by decreasing microvessel density within mammary adenocarcinoma tumors inoculated into mice tibia, mimicking breast cancer metastasis to the bone. Treatments with the targeted copolymer demonstrated improved efficacy with up to 60 % inhibition in tumor growth, compared to 37 % inhibition when treated with the free drugs at equivalent dose. Also, the targeted conjugate showed better tolerance and water-solubility compared with the clinically used PTX. In a parallel study, Segal et al. [4, 104] designed an HPMA copolymer conjugated to ALN and TNP-470 to treat primary bone tumors inoculated into mice tibia. TNP-470 is a highly efficient anti-angiogenic agent that failed clinical trials due to its many side-effects [105]. This HPMA copolymer-ALN-TNP-470 conjugate consisted of drugs conjugated via a linker cleaved by cathepsin K, overexpressed in bone resorption sites. To conclude, bone neoplasm targeting was achieved using three levels of targeting: (1) nonselective targeting by using a nano-scaled polymer exploiting the EPR effect, (2) ligand-based bone targeting by using ALN, and (3) site-specific drug release by using cathepsin K-degradable linkers. HPMA copolymer-ALN-TNP-470 conjugate exhibited synergistic anti-angiogenic and anti-tumorigenic activity of ALN and TNP-470 by remarkably decreasing osteosarcoma tumor growth by 96 %, compared to 45 % with the free drugs. Moreover, the conjugate was able to diminish TNP-470 side effects and exhibit reduced toxicity.

Apart from bone tissue specific ligands, osteoblasts express on their surface several molecules that are important for bone regulation, including parathyroid hormone (PTH) receptor, prostaglandin receptors and receptor activator of nuclear factor κB ligand (RANKL) that controls osteoclast differentiation. RANKL binding to its receptor, found on the surface of monocytes, promotes cellular fusion of several monocytes to form a multinucleated osteoclast [106, 107]. Many of the drugs that are used for the treatment of osteoporosis may be used as target moieties when targeting nanomedicines to the bone. These drugs are often designed to target a specific receptor on the surface of osteoblasts in order to activate bone-building signaling pathways. Prostaglandin E (PGE) regulates bone turnover by binding

mainly to two receptor subtypes expressed on the surface of osteoblasts, EP2 and EP4 [108–110], that activate p38 and ERK signaling pathways [111]. Several studies used modified PGE2 specific to EP2 or EP4 in order to promote bone formation [112–115]. Fragment 1–34 of PTH is also used for the treatment of osteoporosis and can be adapted to targeting osteoblasts. It should be taken under consideration that while periodic administration of fragment 1–34 of PTH promotes anabolism and reduces apoptosis, chronic elevated levels promote catabolism of the bone [116, 117]. A more advanced research is performed on RANKL as a target for the treatment for osteoporosis with the first RANKL inhibitor to receive FDA approval—Denosumab [118, 119]. Denosumab is a fully human monoclonal antibody that binds and neutralizes human RANKL. Therefore, incorporation of Denosumab to a nano-scaled delivery system will be beneficial since it will improve the antibody's delivery in the bloodstream, which is known to be challenging.

Still, there is a need for osteosarcoma-associated/specific markers that hinders development of targeted nanomedicines. Perhaps, this is the reason why currently, to best of the authors' knowledge, there are no targeted nanomedicines specific to osteosarcoma tumor-cell population under investigation. All published bone-targeted nanomedicines include general cancer markers, like IGFR, or bisphosphonates.

Lung Targeted Therapy

Lung cancer has been the most common cancer in the world for several decades with approximately 1.61 million cases in 2008, accounting for 13 % of all cancers (www.iarc.fr). Incidence of lung cancer is more than double in men than in women. It is also the most lethal cancer (18 %) with 1.36 deaths in 2008 worldwide. Non-small cell lung cancer (NSCLC) comprises approximately 80 % of all lung cancers and a 5-years survival rates of 5–14 % [120].

Commonly overexpressed or mutated tyrosine-kinase receptors on lung cancer cells surface, similar to those observed in glioblastomas, include EGFR, IGFR, HER2, cMET, and Reactive Oxygen Species 1 (ROS1) [121]. Clearly, signaling pathways controlled by these receptors are also commonly dysregulated in lung cancer and in turn trigger multiple signaling pathways including RAS/RAF/MEK, PI3K/AKT, and STAT (Signal transducer and activator of transcription 3). Based on this knowledge, two tyrosine-kinase inhibitors have been approved by the FDA for the treatment of NSCLC—Crizotinib (ROS1 inhibitor) and Erlotinib (EGFR inhibitor).

EGFR is overexpressed or exhibits aberrant activation in 50–90 % of NSCLC [122–124]. It is usually associated with a more aggressive phenotype of disease not only in NSCLC, but also in bladder, breast and hand and neck cancers [124, 125]. In a screening for EGFR mutations in 2,105 patients bearing lung cancer tumors, EGFR was found to be mutated in 16.6 % of tumors [126]. Mutations were more frequent in women (~70 %), in nonsmoker patients (~67 %) and in adenocarcinomas (~81 %). NSCLC classic mutations in EGFR, like exon 19 deletion or exon 21

L858R mutation, account for approximately 45 % of aberrant EGFR each and exhibit hyperactive EGFR [127]. These results are strengthened by another study where exon 19 deletion and exon 21 L858R mutation are observed in 33 and 48 % of tumors, respectively, but only 3 % of tumors express both mutations [128]. Interestingly, these mutations have a preferential activation of PI3K/AKT/mTOR pathway and STAT3/5 pathway, over RAS/RAF/MEK [129]. The most common mutations in exon 19 of EGFR are clustered around the catalytic intracellular domain and therefore cannot be specifically targeted. Mutations in exon 19 and 21 also influence the sensitivity to tyrosine-kinase inhibitors, unlike mutations in exon 18 and 20, that represent 10 % of mutations in EGFR [126, 130]. In an analysis testing differential expression of biomarkers in primary and metastatic NSCLC, 33 % of tumors exhibited discordance in EGFR status, with a significant trend towards down-regulation in metastatic sites [131].

Overexpression of IGF1R, which leads to dysregulation of its signaling pathway, was found in up to 70 % of all NSCLC cases [132, 133]. Increased IGF1R signaling results in tumor growth and drug resistance, and was found to be associated with increased risk of lung cancer [134–136]. IGF1R can form homodimers or heterodimers with insulin receptor (IR) or HER2, also overexpressed in NSCLC. Interestingly, both IGF1R and HER2 have been found to be overexpressed rather than mutated in cancer. Unlike EGFR, tyrosine-kinase inhibitors have only limited success against IGF1R due to a high degree of homology of the TK domain with the insulin receptor (IR), and thus will not serve as an attractive moiety to target IGF1R [137].

In a bright-field in situ hybridization (BISH) of MET and c-MET/phospho-MET expression levels in 906 NSCLC tumor, positive cells were observed in 10.9, 5.6, and 22.2 % of NSCLC, respectively [138]. However, another study utilizing a whole-genome amplification method, found c-MET sequence variation only in 1 % of samples [128]. In this study, although c-MET mutations did not occur frequently, they strongly correlated with decreased survival. In contrast, EGFR, the most frequently mutated protein in lung cancer, had no impact on survival. Mutations in HER2 are found in 2 % of NCLCs and were found to be most frequent in nonsmokers, women, Asians and in adenocarcinomas [139, 140]. Mutations in HER2 are inframe insertions leading to constitutive activation of the receptor and are mutually exclusive to EGFR or KRAS mutations harboring tumors. Overexpression of HER2 is found in 25 % of NSCLCs. Younger age, non-smokers or light smokers and adenocarcinoma patients correlate with ROS1 translocation that represent 2 % of NSCLCs [141]. ROS1 translocation results, like HER2 mutations, in constitutive kinase activity, but also sensitivity to tyrosine kinase inhibitors. Therefore, it can be concluded that targeting c-MET, HER2 or ROS1, although broadly discussed in NSCLC context, will not be beneficial for a targeted nanomedicine.

Improved understanding and characterization of NSCLC has led to the development of drug targeting cell-specific markers as discussed above. EGFR-targeted inhibitors have been tested in the clinic for the treatment of NSCLC [142–146]. Kim et al. [147] designed a 145 nm PEGylated pH-sensitive liposome (DOPE and CHEMS 6:4 molar ratio) conjugated to EGFR antibody for the delivery of gemcitabine to NSCLC. Gemcitabine is a highly potent and effective chemotherapy agent for the treatment of

NSCLC, but its use is limited due to severe hematological toxicity and other side effects [148, 149]. The targeted gemcitabine-encapsulating liposomes exhibited high antitumor effect with approximately 80 % growth inhibition, compared to 40 % inhibition with the non-targeted liposome. Tumor volume in mice treated with the targeted liposome remained constant 1 week following the last drug i.v. injection, while it continuously increased with the non-targeted liposome. Tumor growth inhibition was attributed to improved delivery to the cancer cells. Overall, the delivery of gemcitabine for the treatment of NSCLC was greatly improved with the conjugation of anti-EGFR antibody to liposomes, as shown by increased circulation time of the delivery system and increased apoptosis of tumor cells compared to the non-targeted liposome.

Liu et al. [150] developed a PAMAM dendrimer-based delivery system specifically targeting lung cancer by conjugation of a NSCLC-targeting peptide (LCTP; RCPLSHSLICY) chosen based on a phage display library. Phage display peptide library enables the screening and identification of ligands binding to a target protein through biopanning of a library, which contains more than a billion peptides. This can be done on whole cell, tissue samples, live animals, and human bodies regardless of whether the receptor is known [151–153]. These peptides possess high affinity and specificity to target sites, accompanied by better tissue penetration due to their small molecular weight, low immunogenicity, acceptable stability and integrity in vivo [154, 155]. In vitro results with LCPT-PAMAM dendrimer showed that it could be easily taken by human NSCLC cells. In vivo tissue biodistribution revealed that LCTP can also effectively facilitate the targeting of the NP to NSCLC tumors.

Blanco et al. [156] used a different approach to achieve specificity in NSCLC by using a prodrug that is bioactivated upon cleavage by an enzyme overexpressed exclusively in NSCLC [157]. The anticancer prodrug used was β-Lapachone (β-lap) that is bioactivated by NAD(P)H:quinone oxidoreductase1 (NQO1). In cells overexpressing NQO1 enzyme, β-lap undergoes cyclization, resulting in the generation of reactive oxygen species (ROS) that causes DNA single-strand breaks, hyper-activation of poly(ADP-ribose) polymerase-1 (PARP) [158], loss of NAD+ and ATP pool and a unique form of cell death called "necroptosis" [159]. Necroptosis, programmed necrosis, is a caspase-independent regulated cell death that has the morphological features of necrosis and is activated by death receptors signaling. Although a promising cytotoxic effect on tumor tissue compared to normal tissue was observed, poor solubility and non-specific drug distribution limited the clinical usage of β-lap. Blanco et al. increased the solubility by entrapping β-lap in 30 nm micelles composed of PEG-co-poly(D,L-lactic acid) creating a core–shell architecture. The resulting β-lap-PEG-PLA exhibited favorable pharmacokinetics with prolonged blood circulation (half-life of 28 h) and increased accumulation in subcutaneous NSCLC tumors attributed to the EPR effect. Furthermore, a significant decrease in tumor growth and increased survival were achieved in orthotopic tumors with the β-lap-PEG-PLA nanoconjugate.

Breast Targeted Therapy

Breast tissue is unique in a way that it remodels and develops postnatally with the cyclical influence of hormones from the reproductive system and reaches full epithelial-cell differentiation only with completion of full-term pregnancy, lactation and involution cycles [160]. This continuous renewal of the cells and the maintenance of progenitors cells, contributes to the heterogeneity of the breast cancerous tissue [161]. Breast cancer heterogeneity reflects intratumorally in the presence of independent tumor clones, in marked differences between primary and metastatic tumors and in molecular subtypes [162–165]. The most common classification to molecular subtypes is based on upregulation of the key growth factor receptors—estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), and classifies breast cancer into six subtypes that differ from one another in incidence, survival and most important—response to therapy. Other key cell-surface receptors are EGFR, androgen receptor, FGF-R2, insulin receptor, IGFR and Glucose transporter 1 (GLUT1) [166–168]. Breast cancer unique heterogeneity is probably the reason that even with new and sophisticated targeted nanomedicines (like the PEGylated liposomal doxorubicin, Doxil), breast cancer mortality has still increased in the last decade [10, 169–171]. Breast cancer holds the highest incidence rate of approximately 23 % of all cancer types in women and represents the second cause of death in females with cancer (http://www.cancerresearchuk.org).

HER2 is overexpressed in about 20–25 % of breast cancer cases and is indubitable one of the major targets for the design of breast cancer treatment. In the last 2 years, more than 30 HER2-targeting nanoparticles bearing various drugs have been developed for breast cancer therapy. The most common targeting moiety is trastuzumab, a monoclonal antibody against HER2 that is approved by the FDA for the treatment of HER2-positive breast cancer [172]. However, 66–80 % of HER2-overexpressing breast cancers develop resistance to trastuzumab [173]. Colombo et al. [174] recently reviewed various types of nanoparticles conjugated with trastuzumab for the treatment of breast cancer showing promising preclinical results. One outstanding example was recently published by Inoue et al. [175] with a remarkable over 90 % inhibition in tumor growth of orthotopic mammary HER2-positive tumor, following systemic administration of a multifunctional nanoparticle. In this study, a poly(β-L-malic-acid) (PMLA) nanoparticle was designed to target HER2 and block receptor activity simultaneously by using trastuzumab, to target tumor vasculature using transferrin receptor antibody and to block new HER2 synthesis using antisense oligonucleotides. In vivo imaging demonstrated selective accumulation of the dual targeted NP in tumor cells, with enhanced apoptosis of tumor cells, inhibition of HER2 expression and Akt phosphorylation, compared with the controls.

Kumar et al. [176] designed a dextran-coated superparamagnetic iron oxide NP targeting underglycosylated mucin-1 (uMUC-1)-expressing cells in order to deliver siRNA that targets the tumor specific anti-apoptotic gene BIRC5, encoding survivin. uMUC-1 is a tumor-specific antigen found in more than 90 % of breast

adenocarcinomas and was targeted in this study using a uMUC-1-targeting peptide (EPPT) [177]. MN-EPPT-siRIBC5 NP exhibited preferential accumulation at the tumor site, but not in adjacent muscle tissue, following systemic administration and was retained there. Histological staining of tumor sections revealed fivefold increased levels of apoptosis and twofold increased antitumor effect, compared to control NP bearing scrambled siRNA.

Estrogen receptor has an amplified expression over 85 % in breast cancer [178, 179] and the selectivity of estrogen-anchored liposomes for breast cancer targeting was previously described by Paliwal et al. [180]. Paliwal et al. [181] used pH-sensitive PEGylated liposomes (PEG-DSPE) for the targeted delivery of doxorubicin to estrogen receptor-expressing breast cancer cells by surface-decoration with estrogen derivative (ES). The pH-sensitive liposomes (150 nm) showed irreversible change in vesicle size with decreased pH in vitro and exhibited longer systemic circulation time in vivo, relative to free DOX and non-pH-sensitive liposomes. When administered systemically, pH-sensitive liposomes exhibited reduced cardiac toxicity and high antitumor activity with more than 80 % inhibition in tumor growth, compared to 60 % with non-pH-sensitive liposomes and 20 % with free DOX. No data were shown regarding antitumor activity of non-targeted pH-sensitive liposomes.

*Theranostics (*Thera*py and Diag*nostics*)*

Apart from targeted therapy, early detection is another major challenge in cancer treatment. A relatively new emerging approach is the promising field of theranostics that, as its name implies, integrates therapeutics with diagnostics to address these challenges. The combination of novel imaging contrast agents and targeting approaches on the same nano-sized delivery system is a potential multifunctional clinical tool that can provide a high diagnostic capability and versatility by different imaging systems (i.e., MRI, PET and CT) and therapeutic intervention systems. Most theranostic NPs can enable monitoring by either incorporation of different imaging moieties (e.g., fluorophores for fluorescent imaging or radio-labeling for PT) or exploiting of the intrinsic properties of the carrier (e.g., superparamagnetic NP for MRI).

Theranostics is essential for addressing challenges commonly encountered by physicians in real-time. It will allow the adjustment of drugs type and dosing for individual patients and will lead to reduced off-target effects by prevention of over-treatment and optimization of cancer remission by prevention of under-treatment. Moreover, it will allow monitoring adaptive resistance, will enable clinicians to detect tumor margins during surgery and enable guided therapy by verifying cancer biomarkers in the tumor tissue.

Hadjipanayis et al. [182] targeted the EGFR using an antibody selectively binding to the aforementioned EGFR deletion mutant, EGFRvIII, typical for glioblastoma, in order to deliver 10 nm iron oxide nanoparticle (EGFRvIIIAb-IONP).

Their work provides a proof of concept of therapeutic targeting and MRI contrast enhancement after convection-enhanced delivery (CED) for glioblastoma cells and infiltrating cells in the brain. By a minimally invasive surgical procedure, CED provides fluid convection in the brain by a pressure gradient that bypass the BBB and thereby a mean to deliver therapeutic agents while avoiding toxicity to normal tissues [183, 184]. The use of IONP holds several advantages: (1) in a size range of 10–25 nm, IONPs have unique magnetic properties that generate significant transverse T2 relaxation time shortening resulting in strong T2-weight contrast on MRI, (2) IONPs are biodegradable, have low toxicity and are able to evade the immune system and target cancer cells for destruction, and (3) the IONP-surface coating provides a stable hydrophobic inner layer around a single crystal of IONP with carboxylate group in the outer layer allowing the conjugation of different moieties [185–187]. In an intracranial human xenograft model of highly tumorigenic glioblastoma tumor (U-87ΔEGFRvIII), CED of EGFRvIIIAb-IONP resulted in an increased animal survival and antitumor activity in vivo following intravenous administration, compared to the control treated with free EGFRvIIIAb and to human glioblastoma cells that do not express EGFR. However, free IONPs also exhibited pronounced antitumor activity. The authors attribute it to nonspecific uptake by the glioblastoma tumor cells that have been shown both in vitro and in vivo previously [188, 189]. The influence of surface functionalization, as presented in this IONP by amphiphilic surface coating, has recently been shown to enhance the internalization of magnetic nanoparticles into cancer cells [190]. In vitro, EGFRvIIIAb-IONP exhibited significant decreased cell survival with lower EGFR phosphorylation and without any toxicity in human astrocyte. Also, EGFRvIIIAb-IONPs initial distribution was observed within or adjacent to intracranial tumors and continued dispersion days later. Consequently, EGFRvIIIAb-IONPs provide both selective MRI contrast enhancement and targeted therapy of glioblastoma tumor cells after CED.

Guthi et al. [191] designed an MRI-visible polymeric micelle (PEG-PLA) modified with lung cancer-targeting peptide (LCP) and loaded with superparamagnetic iron oxide (SPIO) as a contrast agent and doxorubicin for MR imaging and therapeutic delivery to lung cancer. The LCP, isolated from a phage-displayed peptide library, binds to the restrictively expressed integrin $\alpha_v\beta_6$ cell and possesses a broad specificity for NSCLC that was found to bind to 18 out of 39 human NSCLC cell lines tested [192]. In a microarray performed on tumors from 311 lung cancer patients, it was found that $\alpha_v\beta_6$ integrin is upregulated in NSCLC compared to normal lung tissue and correlates with poor survival [193]. $\alpha_v\beta_6$ integrin expression is also observed in other cancers, like ovarian, breast, colon, gastric, cervix, and oral squamous cell cancer [91, 194–198]. Unlike traditional T1-based contrast agents (e.g., Gd-DTPA) that have only mM detection levels, SPIO-clustered polymeric micelles used in this study have a decreased MR detection limit of less than nM, which is more suitable for imaging tumor markers at lower concentrations, due to increased T2 relaxivity and higher SPIO loading [199]. In vivo studies revealed significantly decreased cardiac toxicity and increased survival following i.v. administration of the targeted micelle compared to DOX at equivalent dose.

Fu et al. [200] established a novel approach of fluorescent magnetic nanoparticles (FMNP) for cancer imaging and enhanced dual targeting. Magnetic NPs, containing 8 nm superparamagnetic iron oxide core, were targeted to tumor site by incorporation of RGD targeting $\alpha_v\beta_3$ integrin, overexpressed by many cancer cells and tumor vasculature. The NPs were given in combination with an external permanent magnet to produce a moderate magnetic field and embedded micromesh, to induce a very strong magnetic field gradient that attracts the MNPs. The fluorescence of the MNPs allows intravital noninvasive imaging, while the magnetic properties are suitable for targeting. The MNPs are assembled from a SPIO core coated with a biocompatible siliceous shell covalently linked to Cy5.5 resulting in ~97 nm NPs. The novelty in such system lies in the combination of two magnetic entities. Indeed, FMNP-RGD exhibit high accumulation at the tumor site when utilizing the two magnetic entities and demonstrated binding specificity to glioblastoma cells. Systemic administration of FMN-RGD results in fast, stable and high antitumor activity when combined with an external magnetic targeting (~90 % inhibition within 5 days) and at a slower rate without magnetic targeting (~75 % inhibition within 15 days). These results are in accordance with a previous observation of apoptosis of tumor blood vessels and tumor regression by RGD binding to $\alpha_v\beta_3$ integrin [201]. Furthermore, antitumor activity enhanced with doubling FMNP-RGD dosage, suggests that retention at the tumor site is due to the magnetic targeting.

Conclusions

The nano-sized-based drug delivery system goals, whether relies on nonselective or ligand-based targeting, are primarily reaching the target tissue by simple blood circulation and extravasation and then reaching the target site in the cancer cell (i.e., surface membrane, cytoplasm or nucleus). Although the Polymer Therapeutics research field has 40 years of clinical experience, it is aiming for better validation of preclinical models and a better quality of design. That is to say, there is a need in a profounder pharmacokinetic profiling that will demonstrate the link between the biodistribution, cell targeting and activity. Toxicity and low efficiency of a delivery system might be due to the fact that the drug does not reach its target site. Besides, most biodistribution experiments include the use of a fluorescent probe conjugated to either a therapeutic agent or a polymer, while assuming that the integrity of the delivery system is retained. The fact that the delivery system reaches its target site by simple blood circulation and extravasation does not necessarily mean that the drug will be released from the scaffold at the right place and time and will internalize to the cancer cell. When designing a polymer or a nano-sized particle system, one should bear in mind that the model system needs to be adjusted according to the target. Not all tumor types show the same rate of enhanced permeability and retention effect. Consequently, a nanoparticle-based drug delivery system might not

always be the treatment of choice. Targeted nanomedicines will only yield substantial improvement in the therapeutic response of a specific group of patients with a particular type and even a specific subtype of cancer. Choosing patients with the appropriate tumor tissue, suitable sub-tumor type and appropriate enzyme-expressing cells is crucial for treatment success.

Most of the clinically approved nanomedicines and research currently focuses on EPR effect dependent delivery to solid tumors. Although this is an excellent strategy, in order to accomplish complete tumor remission, reaching the entire tumor cell population is needed. However, finding a protein expressed in the entire tumor cell population and uniquely expressed by the cancerous tissue is very challenging in account of tumor heterogeneity. Alternatively, incorporation of a moiety that recruits the immune system to the tumor site and induces antitumor activity is also beneficial, if possible. This may be achieved by multifunctional targeted nanomedicines.

High affinity of the targeting moiety may also be a drawback for a targeted nanomedicine. Incorporation of a targeting element with a very high affinity and a very low dissociation constant may result in kinetic binding of the targeted nanomedicine to the first target it encounters, causing off-target effect. Limiting the adsorption to the tumor site may be achieved by (1) modification of the targeting moiety loading, (2) choosing a ligand favoring thermodynamic adsorption over kinetic adsorption, and (3) using a ligand with a reduced dissociation constant. Another strategy is indirect targeting of tumor cells by targeting the tumor microenvironment cells that in turn will influence the tumor cell population.

This realization has led to the understanding and recognition of the importance of combining personalized medicine with targeted nanomedicine—personalized targeted therapeutic delivery systems to treat cancer. In this "tailored made" therapy approach, individual differences will be recognized and the appropriate treatment will be matched, providing better efficacy with less undesired side effects. Today's main challenge is developing specific cancer therapeutic delivery systems to treat the individual patient, or subgroup of patients, with treatment selection being driven by a detailed understanding of the genetics and biology of the patients and their cancer. Integration of genome-wide and proteomic tumor analysis with clinical data of response to therapy with different drugs will provide new possibilities for improved targeted therapeutics.

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Chapter 20
Long Circulation and Tumor Accumulation

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Abstract Nanoparticles have been employed in cancer management as vectors to deliver chemotherapeutic and/or imaging agents to tumors. Enhanced tumor accumulation occurs by virtue of the long circulation properties of the nanocarrier and the enhanced permeability and retention effect that is characteristic of solid tumors. The versatility of the nanoparticle platform has enabled the design and development of various nanocarriers differing in physicochemical properties such as surface composition, size, charge, and shape. While such properties can influence the pharmacokinetics and biodistribution of a formulation, total tumor deposition can be further impacted by inherent pathophysiology of the tissue. This chapter presents the nature and impact of nanoparticle design on tumor accumulation, particularly in the context of the tumor microenvironment. In vivo barriers, such as opsonization, impaired tumor blood flow, heterogeneous vascular and interstitial permeability

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impede the effective delivery of nanocarriers and their cargo and are discussed herein, while strategies to overcome them and enhance the effective delivery of nanoparticles are presented.

Introduction

Nanoparticles in Cancer Diagnosis and Treatment

The emergence of nanoparticles as viable alternatives to small molecule agents for cancer therapy can be largely attributed to three fundamental principles. First, the size and surface properties of nanoparticles can be tailored to achieve favorable pharmacokinetics that enables their use in applications requiring longitudinal imaging or site-specific drug delivery. Second, their extended circulation lifetime permits exploitation of the enhanced permeability and retention (EPR) effect resulting in a greater target-to-background signal ratio at extended time points for imaging applications and an enhanced therapeutic index when used as a treatment vector. Third, the high payload of imaging and/or therapeutic agents encapsulated by nanoparticles can be exploited for amplification of imaging signal or therapeutic effect particularly when used with a lower sensitivity imaging agent or a less cytotoxic drug, respectively. Despite these promising characteristics, to date, only five chemotherapeutic nanoformulations have been approved for clinical use.

Nanoparticle-based chemotherapeutic formulations generally achieve an improved toxicity profile relative to conventional small molecule therapeutic agents. Yet, realizing substantial gains in therapeutic efficacy has proven more elusive. For example,

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Doxil® is well known to reduce cardiotoxicity and other side effects commonly associated with doxorubicin, but achieves significant improvements in response rates and survival times only in certain patients and for specific indications [1]. Indeed, considerable heterogeneity in liposome uptake has been observed among patients with the same and different tumor types [2]. In addition, liposomes often fail to completely release their cargo resulting in poor bioavailability and limited distribution of the drug throughout the tumor mass [3]. In general, in order to maximize tumor localization, the drug should remain encapsulated within the carrier while in the systemic circulation and be released after the nanoparticles have sufficiently accumulated in the tumor. However, in reality, drug delivery systems must strike a balance between drug retention in the carrier and bioavailability of the drug at the tumor site. This balance requires careful consideration of drug toxicity and the demands of a specific therapy which may benefit from more rapid or more sustained release of the therapeutic agent. In some cases, achieving significant improvements in drug solubility by replacing toxic solubilizing excipients with inert colloidal carriers may be sufficient to reduce the severity of side effects or allow for the implementation of a more aggressive dosing strategy. However, truly tumor-specific drug delivery systems hold the greatest promise in terms of achieving significant improvements in treatment outcomes.

Realization of revolutionary advancements in therapeutic efficacy will only be achieved by accounting for important pathophysiological barriers which currently limit the efficacy of nanoparticle-based therapies. In particular, the primary mechanism of tumor accumulation of nanosystems through the EPR effect may not be as ubiquitous as once thought. In fact, heterogeneous vascular permeability and transient vascular perfusion may significantly hinder the effective accumulation and intratumoral distribution of nanosystems by this mechanism [4]. Precise investigation of these aberrant processes within the context of the heterogeneous and complex tumor microenvironment (TME) will enable the design of advanced nanosystems capable of exploiting or circumventing these barriers.

Tumor Microenvironment: Friend or Foe?

Effective delivery of a chemotherapeutic agent from the site of administration to its cellular target is a multistep process requiring accumulation at the tumor, distribution via the tumor vascular network, extravasation and permeation of the interstitial space. Each of these steps is associated with a number of complex physiological barriers which may limit the efficacy of therapy. In recent years, the TME has been recognized for its vital role in promoting both tumor growth and resistance to anticancer therapies [5]. In solid tumors, the irregular arrangement of the vascular network and its sporadic blood flow give rise to large regions with a limited supply of oxygen, nutrients, and systemically administered therapies [6, 7]. Indeed, the toxicity of many chemotherapeutic agents is diminished in the hypoxic and acidic microenvironments of poorly perfused tissues which may contain large populations of quiescent cells [6, 8]. Elevated hydrostatic fluid pressure and the dense, fibrotic

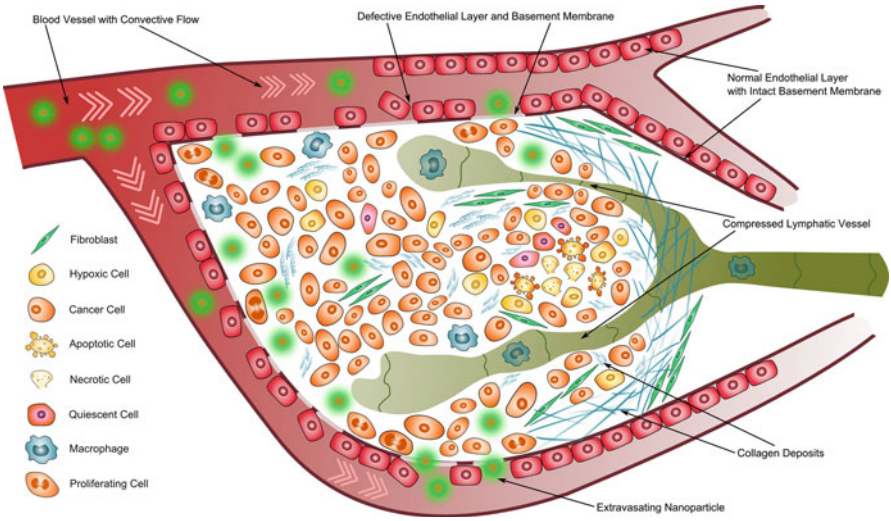

Fig. 20.1 Accumulation and extravasation of long circulating nanoparticles in solid tumors by the EPR effect. The heterogeneity of the tumor microenvironment presents several barriers to the effective accumulation and penetration of nanocarriers

nature of the interstitial compartment further inhibit the efficacy of chemotherapy by limiting the penetration of drugs through the intervascular space [9] (Fig. 20.1).

As our understanding of the implications of the TME on therapeutic efficacy continues to grow, so do the opportunities to design novel treatments capable of altering its properties in a manner favorable to the efficient delivery of nanocarriers. As such, so-called "promoter" compounds have recently been explored which complement anticancer agents by altering the TME to enhance the efficacy of the encapsulated drug [10]. Importantly, tumor physiology is heterogeneous on an intra- and inter-tumor basis (i.e., within a single tumor and throughout a patient population) and this variability can have a profound effect on the efficacy of EPR-mediated therapies and other forms of tumor-targeted therapies [2, 4, 11, 12]. Thus, nanoparticles need not be tailored specifically to a rigid representation of a particular cancer, rather one that may potentially be altered, or *modulated*, to maximize drug delivery to these tumors.

Tumor Accumulation of Nanoparticles

The Need for Long Circulation

Tumor targeting via the EPR effect, first described by Matsumura and Maeda in 1986 [13], is increased if the particles possess prolonged vascular residency time (i.e., maintain high plasma levels for >6 h in preclinical models [13–15]). Indeed, the enhanced circulation time of drugs conferred by poly(ethylene glycol)-coated

(PEGylated) nanoparticles exploits the inherent pathophysiology of tumor vasculature. In particular, the characteristically tortuous, leaky and immature blood vessels found in solid tumors facilitate the extravasation of nano-sized entities into the tumor interstitium. Conversely, low-molecular-weight agents are distributed systemically following administration and are rapidly cleared from the circulating blood via renal clearance [16], while their tumor accumulation is only transient (on the order of minutes) [17]. If the concentration gradient is not maintained, their small size may allow them to return to the circulating blood system following extravasation [15, 16]. It has been established that the degree of macromolecule accumulation in tumors is directly proportional to the blood AUC (or exposure) and inversely proportional to the rate of urinary clearance [18–20]. Once the prerequisite of high exposure has been achieved, homogeneous penetration of nanoparticles is greatly impeded throughout tumor tissue, resulting in its resistance to the delivered chemotherapy. Importantly, poor drug efficacy has been linked to poor drug penetration from tumor capillaries [6, 17]; a phenomenon which has been linked to high interstitial fluid pressure (IFP) in preclinical [21, 22] and clinical settings [23]. Other factors impeding drug penetration include dense extracellular matrix (ECM) [24], high tumor cell packing density [25, 26], and extracellular and intracellular consumption and sequestration of drug [6, 27, 28]. Furthermore, the subsequent intratumoral retention of nanoparticles is a function of their speed of venous return (usually slower in tumors than in normal tissue) and lymphatic clearance [29]. The impairment of the tumor's lymphatics effectively results in the retention—and over time, the accumulation—of the extravasated macromolecules. Taken together, these tumor-specific phenomena contribute to the EPR effect [13, 20], which has been thoroughly exploited since its discovery in the late 1980s as the prime strategy for delivering small-molecule chemotherapeutic drugs via nanoparticulate carriers. Through the ability to significantly increase the accumulation of nanoparticles in tumor versus healthy tissue, enhanced therapeutic ratio can be achieved during treatment while amplified target-to-background signal ratio is equally realizable during imaging applications. Consequently, EPR has become the hallmark of nanoparticle-based delivery of diagnostic and therapeutic agents to tumors [20].

Most nanomedicine formulations, such as liposomes and micelles, have been designed to deliver cargo to tumors by relying on the EPR effect. In fact, owing to their physicochemical characteristics and the pathophysiological properties of the tissue, nanoparticle-based drugs are able to accumulate at the tumor more efficiently than the control formulation. Furthermore, functionalizing the nanoparticle surface with ligands has emerged as a design strategy to increase treatment efficacy by promoting the delivery of chemotherapeutic agents into target tumor cells [30]. However, challenges have plagued the translation of nanomedicines into the clinic, largely due to greatly attenuated results in humans in comparison to those obtained in preclinical models. At present, certain nanoformulations currently available significantly improve the toxicity profile of chemotherapeutics, but fail to demonstrate clinical efficacy that is superior to the free drug alone. Advances in nanotechnology have involved the addition of stimuli-sensitive materials or cancer-specific recognition sequences. While such sophistication undoubtedly constitutes a major step

towards confident control of cancer, the seemingly precipitated integration of new features into nanoformulations may be perceived as a few steps backwards. Notwithstanding, long circulation remains a critical aspect to be upheld in the design of nanoparticle-based formulations. The resulting accumulation of nanocarriers enables the delivery of a higher payload of drugs to the tumor site with reduced off-target side effects in comparison to the control free drug. However, the in vivo fate of a nanoformulation is dependent upon a number of factors which impact its structural and/or functional integrity, circulation time and ultimately, deposition at the target site. In particular, the pharmacokinetic profile and biodistribution of nanosystems will rely on properties of the carrier including its surface chemistry, surface charge, stability, size and morphology among others [31–33].

Optimization of Physicochemical Properties

Perhaps the most important feature of long-circulating nanoparticles is their ability to avoid adsorption of plasma proteins, or opsonins, and subsequent clearance by the mononuclear phagocyte system (MPS). Immediately following intravenous (i.v.) administration, plasma proteins bind to nanoparticles leading to potential nanoparticle destabilization, extraction of the encapsulated drug and/or uptake by phagocytic cells [33, 34]. In particular, surface adsorption of plasma proteins facilitates the identification of nanoparticles by circulating phagocytes, macrophages of the spleen and hepatic Kupffer cells [35–37]. In addition, protein binding can trigger secondary aggregation of nanoparticles which may increase their hydrodynamic size resulting in entrapment in the capillary beds of the lungs [38].

Safe and effective delivery to tumors requires that nanoparticles be able to circulate stably for prolonged periods of time. Opsonization is one of numerous in vivo barriers which test the nanoparticles' physicochemical properties upon systemic administration. As such, incorporation of "steric-stabilizing" hydrophilic polymers, such as PEG on the surface of nanoparticles is a well-established technique for enhancing their circulation lifetime [39–41]. For instance, circulation half-lives range from 5 h [42] to over 20 h [43] for PEGylated, or "stealth," liposomes while the $t_{1/2}$ of their non-PEGylated counterparts can be less than 30 min. The ability of PEG to limit opsonization depends on several factors including the chain length, surface density, and conformation of the polymer [44–47]. In general, studies have reported the greatest improvement in circulation longevity for nanoparticles containing longer PEG chains up to ~20 kDa likely due to greater chain flexibility and better surface coverage of the nanoparticle [48–50]. Significantly, Gref et al. have shown that 20 kDa PEG-coated nanospheres reached 30 % injected dose (ID) in the liver after 5 h while 66 % ID of non-coated particles were found in the liver only 5 min post-injection [48]. Surface PEG chain density and conformation are also important factors in achieving prolonged nanoparticle circulation. In general, greater surface density of PEG imparts greater stealth characteristics to the nanoparticle, although corresponding

changes in chain conformation (e.g., "mushroom" vs. "brush") can also enhance or diminish surface coverage and steric repulsion of proteins at very low or very high PEG densities [51]. The long circulation conferred by PEGylation, however, is threatened by the administration of multiple doses of stealth nanoparticles. Specifically, accelerated blood clearance (ABC) may result following the production of anti-PEG IgM in response to subsequent doses [52]. An evaluation of the differential induction and effectuation of the ABC phenomenon using polymeric micelles was recently published [53].

Nevertheless, the function of the PEG layer is contingent upon a number of factors that call into question the rational design of nanoparticles, notably nanoparticle composition, size, and charge.

Nanoparticle composition may influence its rate of clearance from the circulation as a function of particle material (e.g., lipid-based vs. metal-based), with more biocompatible components requiring a less "stringent […] PEGylation protocol." [54]. Additionally, considerations into the length of the PEG chain are made based on the size of the uncoated particle, with the trend being inversely proportional. As such, nanoparticles in the range of 50–100 nm often bear PEG grafts of 3,400–10,000 Da [55]. Effectively, increasing particle size is positively correlated with serum protein absorption, which in turn leads to greater hepatic uptake and reduced circulation half-life [56].

In general, nanoparticles of intermediate size (10–100 nm) can achieve extended residence time in the blood by minimizing renal filtration and accumulation in the liver and spleen. However, colloidal carriers commonly demonstrate elevated accumulation in the liver due to passage through sinusoidal endothelial fenestrations (~100 nm in diameter) and uptake by Kupffer cells [57]. Larger nanoparticles may be removed from circulation by the spleen or become entrapped in the small capillary beds encountered shortly after venous administration (e.g., in the lungs) [58]. For nanoparticles to be eliminated from circulation by glomerular filtration in the kidneys, they must generally have a molecular weight of less than 50,000 Da [19, 59]. Choi et al. defined the renal filtration threshold in rodents using quantum dots by demonstrating that a hydrodynamic diameter of $\leq$5.5 nm was required for rapid urinary excretion [60].

It is important to note that proper selection of nanoparticle size for drug delivery requires careful consideration of the balance between residence time in the blood, vascular permeability, and interstitial penetration. In general, larger nanoparticles within the range of ~10–100 nm with similar surface properties demonstrate superior circulation lifetimes and provide more time for extravasation within the tumor [61]. However, larger nanoparticles may have limited vascular permeability and/or demonstrate poor penetration into the tumor interstitium [17, 62]. Therefore, the size of the carrier should be adjusted so as to strike an appropriate balance between circulation longevity, deposition at the tumor via the EPR effect, uptake in the liver and spleen and tumor penetration. This is particularly important when acknowledging the high degree of intertumoral variability. Thus, selection of an optimal size will depend both on the physicochemical properties of the nanoparticle and unique physiological properties of the tumor.

Surface charge is another property which can also influence biodistribution and the rate of systemic clearance. In general, the circulation lifetime is highest for neutral nanoparticles while a strong positive or negative surface charge results in rapid systemic clearance. In a recent study, Arvizo et al. found that neutral and zwitterionic gold nanoparticles demonstrated extended circulation profiles ($AUC_{0-}\infty = 2.65 \times 10^4$ and 2.98×10^4 mg/ml min, respectively) following i.v. administration relative to nanoparticles with highly positive or highly negative surface charges ($AUC_{0-}\infty = 1.02 \times 10^3$ and 3.03×10^3 mg/ml min, respectively) resulting in greater tumor accumulation [63]. Gessner et al. found an increase in plasma protein adsorption with increasing negative surface charge density on polymeric nanoparticles [64]. It is likely that charge-dependent differences in circulation longevity are a result of interactions of nanoparticles with plasma proteins and differences in the rate of cellular uptake by phagocytic cells of the MPS [65, 66]. In certain cases, surface charge may also influence tissue biodistribution. For example, Yamamoto et al. demonstrated that the biodistribution of PEG-*b*-poly(D,L-lactide) (PEG-b-PDLLA) copolymer micelles was altered by introducing a slight anionic charge (−10.6 mV) on the micelle surface through the conjugation of charged peptides [67]. Specifically, micelles containing an anionic charge (Tyr-Glu) displayed lower uptake into the liver and spleen.

Recently, the shape of nanoparticles has been shown to play an important role in determining their drug loading capacity, release rate, and in vivo disposition [68–71]. In certain cases, changing the morphology of a nanoparticle can significantly alter its circulation in the blood due to changes in flow characteristics and clearance. For example, a study employing fluorescence imaging has shown that elongated, rod-like micelles, or filomicelles, circulate for up to 1 week (i.e., $t_{1/2} \approx 5$ days) following i.v. administration—10 times longer than their spherical equivalents [69]. This is thought to be a result of enhanced fluid flow in blood and the ability of the rod-like structures to evade filtration and uptake by phagocytic cells. Encapsulation of chemotherapeutic agents in rod-like micelles has also been shown to influence the toxicity of the drug in vivo. Discher's group has demonstrated that encapsulation of paclitaxel in rod-like filomicelles increased the maximum tolerated dose of the drug in mice by nearly twofold compared to paclitaxel-loaded spherical micelles, resulting in a sustained reduction in tumor size of 87 % and a growth phase more than sixfold slower, lasting up to 1 year [72]. In addition, nanoparticle morphology may influence their biodistribution and accumulation in MPS organs such as the liver and spleen likely due in part to their orientation-dependent size [73]. Due to the challenging nature of synthesizing nanoparticles with distinct morphologies in a controlled manner, there remains an incomplete understanding of the role of nanoparticle shape on their performance as drug delivery vehicles [74].

Effective exploitation of the EPR effect necessitates that the nanoparticle-drug system reach the malignant site in its original state. Specifically, it is indispensable that the drug formulation remain stable in vivo, a prerequisite of advanced drug delivery systems that is often overlooked, assumed or underestimated. Nevertheless, importance is equally attributed to the ability of the nanocarrier to release the drug at the tumor site. Thus, the development and optimization of nanoparticle-based

anticancer drugs requires that the formulation be sufficiently stable in vivo to result in enhanced accumulation at the tumor site (for instance, maximum accumulation of liposomes occurs at 24–48 h post-administration), followed by the effective release of the active therapeutic. Accordingly, Drummond et al. have examined the pharmacokinetics and antitumor efficacy of multiple liposome formulations of vinorelbine. The authors tracked the retention of vinorelbine within the liposomes in vivo by monitoring the drug-to-lipid ratio over time and demonstrated significant tumor growth inhibition using a highly stable and active liposome formulation of vinorelbine [75]. Alternatively, drugs which dissociate rapidly from the nanoparticle will display a pharmacokinetic profile more closely resembling that of the free drug. In the latter case, the nanoparticle acts primarily as a solubilizer rather than a true tumor-specific delivery system. For drugs that are well retained in stable nanoparticles, their fate is primarily determined by that of the delivery vehicle. The ideal drug carrier should achieve a balance between drug retention during circulation and the efficient release and bioavailability of drug within the tumor. Similarly, prolonged in vivo stability is critical for nanoparticle-based contrast agents. In particular, retention of imaging agents within nanoparticles is acutely important when tracking their in vivo fate, be it at the whole-body, tissue, or cellular level.

Influence of Surface Ligands on Long Circulation

Molecularly targeted surface ligands have been incorporated onto the outer layer of nanoparticles in an effort to promote intracellular localization and full bioavailability of the delivered therapeutic. Specific ligand-receptor interactions are expected to reduce nonspecific tissue uptake and its resulting toxicity, decreased drug resistance via Pgp efflux pumps, and potentially greater tumor accumulation [12]. Ideally, targeting ligands are designed to have specificity for cell receptors that are present at negligible levels or are entirely absent in normal tissues, as is the case in certain physiological processes such as angiogenesis [76]. While target receptors may exhibit significantly greater expression levels in cancerous relative to healthy tissue [77] (e.g., 40- to 100-fold greater expression of HER-2 in breast cancers [78], or on the order of 10^6 receptors/cell in a HER-2 overexpressing preclinical tumor model [79]), others such as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [80, 81], as well as aminopeptidase N (aka APN/CD13) [76], are found to be selectively expressed in angiogenic tumor vasculature. In addition, the ligands should present high affinity for their cognate receptors and induce receptor-mediated endocytosis [30]. As such, peptide sequences along with antibodies, small molecules, and aptamers have been used for nanoparticle targeting.

Rational design of receptor targeted nanoparticles requires the selection of a ligand which possesses low immunogenicity. In particular, it is critical that the addition of the surface ligand result in no significant reduction in the circulation lifetime of the vehicle [82], as this may lead to decreased accumulation at the tumor site [83]. Still, the influence of targeting moieties on the pharmacokinetics of

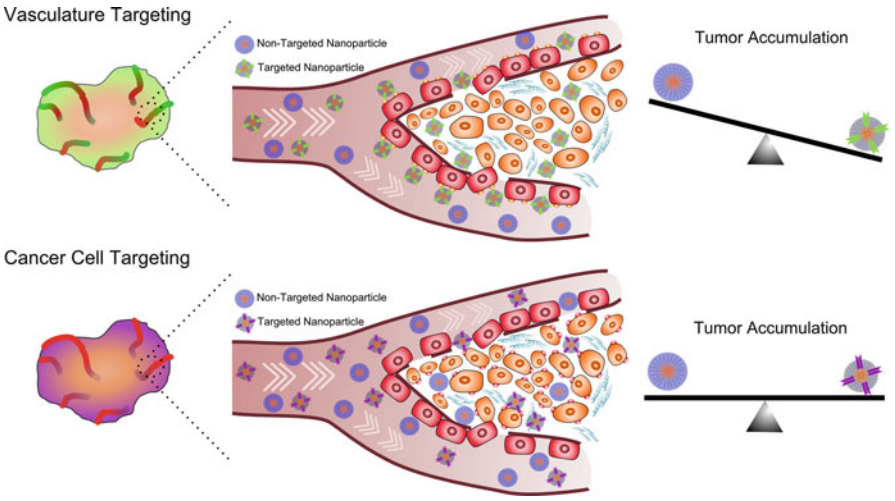

Fig. 20.2 Targeting strategies of nanoparticles to solid tumors. Targeting by the EPR effect and receptor targeting may function as independent processes via homing to the tumor vasculature [82], resulting in an increase in tumor accumulation due to the latter process (*top panel*). Targeting of tumor cells, or other extravascular components, relies on blood circulation and extravasation which, in turn, is reliant upon the degree of vascularization and permeability of tumor vessels [82]. As a result, nanoparticle formulations differing only in targeting capability may exhibit similar levels of accumulation (*bottom panel*)

nanoparticle-based formulations remains unclear. There have been reports of targeting ligands having a negative impact on circulation lifetime (i.e., reduced half-life) of formulations relative to their non-targeted counterparts [84, 85], while others have shown no difference [82, 86, 87].

Studies have shown that tumor cell-targeted nanocarriers do not result in an increase in tumor accumulation [86, 88]. Alternatively, targeting to the relatively unhindered endothelial cells of the tumor vasculature may lead to greater tumor accumulation, as well as therapeutic targeting of both tumor and endothelial cell populations [89]. Figure 20.2 illustrates the potential impact (i.e., increase) on bulk tumor accumulation of targeting the tumor vasculature versus tumor cells. Recently reported data demonstrating an increase in total tumor accumulation using NGR-liposomes targeted to the APN/CD13 receptor expressed on tumor neovasculature may provide evidence that tumor vasculature-targeted nanoparticles can accumulate independently of EPR-mediated targeting [82]. Contrary to targeted nanomedicines directed towards the extravascular compartment, tumor vasculature-targeted nanoparticles are, in part, exempt from the biological and physical barriers that face tumor cell-targeted nanocarriers. However, uptake by activated tumor-associated macrophages may account for a significant degree of tumor accumulation of receptor targeted nanoparticles [90] following deposition at the tumor site via the EPR effect. Therefore, further investigation is warranted into the intratumoral fate of receptor targeted nanoparticles, while their successful development requires

concurrent advances in TME characterization methods (i.e., microvessel density, vessel permeability, perfusion, pericyte coverage, etc.) capable of profiling the spatial and temporal expression of the cell target, as well as the underlying tumor vascular network.

The incorporation of targeting moieties in nanosystems has thus far led to seemingly disappointing results, reflected in the absence of clinically approved targeted nanoparticles and likely a result of the underestimation of the physiological barriers existing between the decorated nanoparticle and its target receptor. These barriers are biological (i.e., cell layers, basement membrane, endosomal sequestration) and physical (i.e., IFP, binding site barrier) [7], impeding both interstitial and cellular delivery of potent therapeutics following their nanoparticle-mediated deposition at the tumor.

Heterogeneity in Tumor Accumulation

Hailed as a universal trait of solid tumors, EPR has become known as somewhat of a "moving target." Effectively, reports have surfaced revealing stark differences in the delivery of nanoparticles to tumors. A pivotal study conducted by Harrington and colleagues critically evaluates the effectiveness of targeting solid tumors using ^{111}In-labeled PEGylated liposomes [2]. Pharmacokinetics and biodistribution were evaluated in 17 patients with locally advanced cancers. In particular, region of interest analysis of gamma camera images of patients revealed levels of tumor uptake ranging from 2.7–53 % ID/kg tumor while, importantly, the pharmacokinetics of the formulations also varied among patients. These data revealed significant variability in liposome uptake among different tumor types and, remarkably, among patients with the same tumor type. Specifically, liposome uptake was highest in head and neck tumors (33.0 ± 15.8 % ID/kg), while intermediate and low levels were found in lung (18.3 ± 5.7 % ID/kg) and breast (5.3 ± 2.6 % ID/kg) tumors, respectively. The authors have hypothesized that these differences could be due in part to variability in the density, structural and functional integrity of the tumor vasculature among different tumor types. Accordingly, Koukourakis et al. have reported observations that vascularization is often higher in head and neck carcinomas, relative to non-small-cell lung cancer (NSCLC). Further, the authors evaluated microvessel density (MVD) in patients with NSCLC who were administered Caelyx™ (PEGylated doxorubicin hydrochloride, aka Doxil®) and found a direct correlation between MVD and liposome accumulation at the tumor [91], suggesting that "highly angiogenic tumors better accumulate liposomal drugs."

Heterogeneity in EPR was also recently discussed by Lammers and colleagues. In their review, they argue that the EPR effect is more pronounced in animal models in comparison to humans, and can be variable both spatially and temporally in the same tumor [11]. Clinically, the influence of the degree of the EPR effect is evidenced by the notable response which patients with Kaposi's sarcoma have to Doxil®. Kaposi's sarcoma, is known to present with extensively

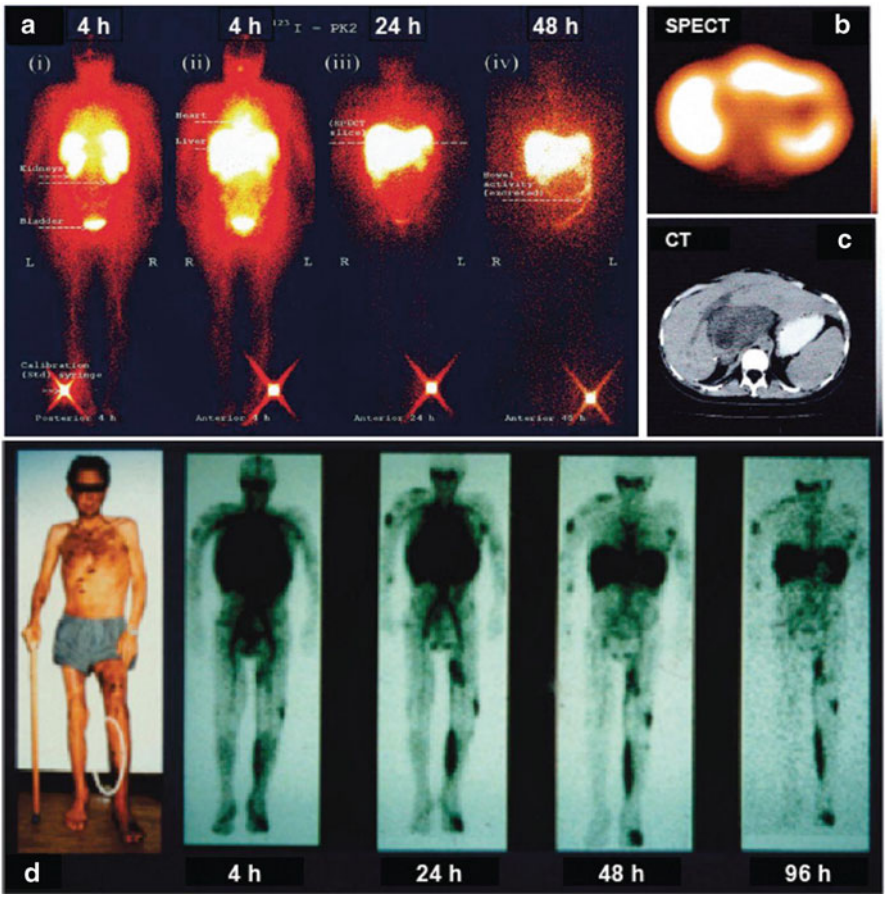

Fig. 20.3 Clinical evidence of the significant heterogeneity in targeting nanocarriers to tumors. An ^{123}I-labeled poly(hydroxypropyl methacrylate) (pHPMA) polymer–drug conjugate, Gal-pHPMA-GFLG-doxorubicin (PK2), was tracked at the whole-body (**a**) and tissue (**b**) levels using gamma camera and SPECT imaging, respectively. Accumulation of PK2 is evident at the liver (**a**, **b**) although inefficient localization within the cancerous lesion (**c**, dark mass) and consequently, anti-tumor activity, were prominent when coupled with anatomic CT imaging. Conversely, efficient localization of ^{111}In-labeled PEGylated liposomes in lesions of Kaposi's sarcoma was associated with effective treatment (**d**). Reprinted by permission from the American Association for Cancer Research from [96]

vascularized and permeable tumors [92] (Fig. 20.3d). Conversely, targeting to hepatocellular carcinoma using polymer–drug conjugate PK2 resulted in inefficient tumor localization and poor efficacy, despite considerable tissue uptake [93] (Fig. 20.3a–c).

The variability in EPR, such as MVD, vessel permeability, perfusion and IFP also leads to significant differences in the intratumoral distribution of nanoparticles. While several microenvironmental factors at the tumor site may influence the bulk

pattern of accumulation of nanoparticles, their ensuing extravasation is particularly impacted by tumor vessel properties, such as integrity of the basement membrane and blood flow [94]. Currently, there is interest in addressing the variability in EPR by means of image-based assessment, whereby contrast agent-labeled nanoparticles reveal potential high/low-responders to nanomedicines based on their high/low degree of EPR [95], however this approach has not been evaluated extensively and further investigation is needed [96]. Alternatively, EPR status may be strategically altered, and nanoparticle extravasation enhanced, with the use of permeabilizing agents. Such agents, discussed below, are pharmacological (e.g., TNF-α, TGF-β inhibitor) or physical (e.g., ionizing radiation, hyperthermia) in nature and have shown promising results in terms of nanoparticle extravasation in various tumor models [62, 94].

Enhancing the Delivery and Distribution of Nanoparticles at the Tumor Site

As the heterogeneity, and ensuing limitations, of the EPR effect come to light, concessions must be made in nanoparticle design, while fundamental features should be upheld and validated. Specifically, the hallmarks of stable long circulation and delivery of high drug payload attributed to nanoparticles should not be sacrificed upon arrival at an uncompromising environment. Rather, in vivo barriers can be overcome via modulation of the TME.

In the following sections, strategies to enhance the EPR effect are discussed, while their methods of evaluation are further presented. Finally, we discuss the clinical applicability of these strategies and the emerging integration of prescreening applications into nanoparticle-based treatment.

Modulating Tumor Vascular and Interstitial Properties

The EPR effect has been the most commonly used rationale to motivate the development of nano-sized drug carriers, despite the significant intra- and intertumoral variations in nanoparticle accumulation which have been observed in almost all preclinical and clinical tumors. It is clear that these variations exist in all tumors and cannot be neglected. They are the result of a hostile microenvironment (e.g., low partial oxygen pressure, low pH and necrosis) [7], and are of critical importance when designing a nanoparticle-based drug delivery system. Given the heterogeneous nature of the EPR effect, it is a difficult task to design such a system that is effective in all tumors. Hence, it is beneficial to pursue strategies which render the TME more conducive to effective nanoparticle accumulation.

Heterogeneous delivery of nanoparticles results from the reduction in transvascular fluid flow and convection-driven extravasation in the central tumor volume.

This is further compounded by the intermittent and spatially heterogeneous blood flow due to increased IFP [97]. While it remains an important component of EPR-mediated nanoparticle delivery, the full effects of IFP on tumor targeting are extensively discussed in another chapter in this book.

Several strategies have been formulated in order to realize the promise of nanoparticle-based drug delivery and achieve greater target tissue uptake, increased extravasation of nanoparticles and improved intratumoral distribution. These include manipulation of the endothelium via vasoconstriction/dilation, enhancement of vascular permeability and normalization of tumor interstitial and vascular compartments. It is important to note that many of the mechanisms of action of agents which are directed towards the aforementioned targets are often implicated in the complex cross-talk that governs the TME. The following sections describe the strategies and agents which have shown considerable clinical promise.

Tumor Blood Flow and Intravascular Pressure

Pharmacologic modifiers of tumor blood flow act directly by vessel constriction or dilation, modifying blood viscosity, microvascular pressure and/or IFP [98]. Effectively, tumor blood flow exhibits unique irregularities in consistency and direction [99]. Such impaired vascular function was shown to be restored under hypertensive conditions with the use of angiotensin-II (AT-II). Both AT-II and nitroglycerin (NG) represent vasoactive agents that have been shown to improve delivery of macromolecules in solid tumors [99–103]. While the mechanism of action of AT-II is complex, it is believed that the agent causes significant vasoconstriction at the host arterial level, leading to increased mean arterial blood pressure (MABP) and tumor blood flow [104], thus restoring the transvascular pressure gradient [105, 106]. Normal tissue is known to remain unaffected by virtue of homeostasis [107]. Increasing blood flow in tumors is deemed an effective means of enhancing extravasation of macromolecules [108] and has accordingly shown a 1.2- to 1.8-fold increase in tumor accumulation in a system employing albumin-bound SMANCS [103]. Indeed, AT-II has been successfully used in the clinic to improve the accumulation and therapeutic index of SMANCS administered with lipiodol in several primary and metastatic human cancers [109]. Additionally, an infusion of AT-II for 25 min was found to significantly improve the accumulation, intratumoral distribution, and antitumor activity of liposomal doxorubicin in a poorly vascularized lung carcinoma xenograft model, as well as a well vascularized colon carcinoma xenograft model [100]. Conversely, NG, a nitric oxide (NO)-releasing agent, has been shown to induce vascular permeability. Specifically, NG acts through vasodilation which leads to decreased MABP and IFP [106]. Effectively, pretreatment with NG led to a two- to threefold increase in accumulation of two macromolecular agents, Evans blue/albumin complex and PEG-conjugated zinc protoporphyrin (PZP), in a panel of preclinical tumor models [110]. The same study showed that the enhanced permeability of tumor blood vessels was sustained for more than 24 h following a

single application of NG, translating into the enhanced therapeutic effect of PZP. Therefore, augmentation of the EPR effect via vascular mediators such as AT-II and NO holds promise yet clinical validation is needed [102].

Tumor Vascular Permeability

The permeability of the vasculature is a key determinant of extravasation of nanoparticles into the tumor interstitium and therefore presents an attractive vascular property for modulation. In one study, Corti et al. showed an increase in chemotherapeutic response through combination therapy of various drugs with NGR-TNF [111, 112], hypothesizing that "endothelial permeabilizing factors" may improve tumor perfusion and drug penetration in tumor tissues [113]. Tumor necrosis factor (TNF)-α, is a major inflammatory cytokine which causes hemorrhagic tumor necrosis at high doses, but has been shown to promote vascular permeability and improved drug delivery at low doses [114]. Several studies have demonstrated that TNF-α treatment can increase the accumulation of Doxil® by threefold in preclinical tumor models [115, 116]. Specifically, TNF-α was shown to increase both tumor penetration and response to Doxil® as a result of an increase in the number of permeable vessels and consequently, a more homogeneous intratumoral distribution [94]. Conversely, one study found that TNF-α did not improve the bulk tumor accumulation of Doxil® in a rat osteosarcoma model, but did in fact augment its antitumor activity [117]. While the systemically administered cytokine may result in severe toxicity [114], its administration at low doses has produced encouraging results clinically with superior antitumor activity achieved in patients presenting with carcinomas confined to the extremities which has led to the licensing of TNF-α (tasonermin) in Europe [113].

Cabral and colleagues recently exposed the size restriction associated with the use of clinically approved Doxil® through evaluation of the accumulation and antitumor activity of the formulation in poorly permeable tumors. Previously, the same group had shown that a low-dose transforming growth factor (TGF)-β inhibitor (TGF-β-I) was able to enhance vascular permeability via a reduction in pericyte coverage and thus enhance the accumulation of Doxil® in tumors [118]. This important finding was further confirmed by demonstrating equivalent levels of accumulation of both 70 and 30 nm micelles in the hypopermeable BxPC3 tumor model, after TGF-β-I-mediated permeabilization [62].

Tumor vascular permeabilization has also been achieved with co-administration of iRGD, which Sugahara et al. have shown to be selective towards a panel of tumors [119]. Such a strategy also alleviates design constraints on nanocarriers such as alterations in pharmacokinetics, activity and penetration that may accompany the inclusion of a targeting moiety. Nevertheless, the promise of greater intratumoral drug accumulation achieved in preclinical models remains to be confirmed in human patients.

Vascular permeability can also be modulated via physical means such as hyperthermia (HT) and radiotherapy (RT). HT is a mechanical heating technique that has

been shown to increase vascular permeability and blood flow [120]. Notably, HT has been found to significantly increase the accumulation of liposomes between 100 and 400 nm in diameter in preclinical tumors [121]. Interestingly, in one study, Lammers et al. reported that vasoactive effects due to HT only led to an increase in pHPMA copolymer concentration in one out of the three tumor models evaluated [122]. Indeed, the impact of HT can vary due to its dependence on several factors including tumor model, degree of host tissue support, as well as temperature and duration of heating [123–125].

Additionally, RT has been shown to result in enhanced vascular permeability and overall tumor accumulation. For example, the tumor accumulation of pHPMA copolymer-based drug delivery systems was evaluated by Lammers et al. as a function of RT, delivered as a single dose of 20 Gy administered 24 h prior to injection of copolymers varying in molecular weight [122]. The resulting increase in tumor uptake of the copolymers was hypothesized to have been enabled by the production of permeability-enhancing factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are known to be upregulated following RT [126–128]. Further, Giustini et al. have shown a twofold increase in tumor accumulation of iron oxide nanoparticles following a single 15-Gy dose in an experimental breast cancer model [129]. Effectively, the authors demonstrate that the increase in accumulation correlated with a decrease in tumor IFP and an increase in vascular permeability. Evidence supports the clinical use of RT to potentiate the efficacy of nanoparticles. Koukourakis et al. have found that co-administration of radiolabeled Caelyx™ (25 mg/m^2 every 2 weeks) with 70 Gy RT (2 Gy/fraction, 5 fractions/week) resulted in a 1.8- to 4.7-fold increase in accumulation of nanoparticles in 7 sarcoma patients [130]. This result was mirrored preclinically in human osteosarcoma xenografts where the distribution and accumulation of Caelyx™ was improved two- to fourfold after either 8 Gy was delivered as a single fraction or 3.6-Gy fractions administered over 3 days [131]. Given the prevalence of RT in many healthcare institutions, this strategy offers a practical avenue for the improvement of nanoparticle-based treatment.

Vascular Normalization

Vascular normalization has been conceived as a means to restore tumor blood vessels to their original functional state and thus improve the delivery of anticancer therapeutics. Anti-angiogenic effects imparted directly or indirectly by small-molecule inhibitors have been shown to improve both functional and structural integrity of tumor vasculature (e.g., less tortuosity, leakiness and greater pericyte stabilization), further leading to reduced IFP [97] and restoration of a transvascular pressure gradient [132, 133]. However, the benefit to nanomedicines remained unclear until a recent report by Chauhan et al. The authors conducted this investigation using clinically approved nanoformulations, Doxil® and Abraxane®. Despite achieving similar efficacy in mice bearing orthotopic E0771 mammary tumors as monotherapies given their distinct sizes (~100 and ~10 nm, respectively), the

combination of vascular normalization-inducing anti-VEGFR2 antibody DC101 led to enhanced penetration of the 10-nm nanomedicine, relative to its larger counterpart. Therefore, this study supported the hypothesis that vascular normalization reduces vessel pore sizes [134], thus providing significant evidence to influence the design of nanocarriers when administered in conjunction with vascular normalizing therapies. Additionally, the failure of other studies to detect and exploit the vascular normalization window has hampered the adoption of this strategy. Tailor et al. found that the administration of the anti-angiogenic pazopanib did not improve the accumulation of Doxil®, and rather, significantly decreased the penetration distance of liposomes relative to the untreated control group [135]. Others have found that, treacherously, normalization of the vascular bed in gliomas was able to restore the once-defective blood-brain barrier, therefore obstructing the delivery of nanoparticles [136]. Given the transient nature of the normalization window, its apparent size restriction on nanoparticles and the spatial and temporal interplay between various factors of the TME, it is clear that greater optimization is needed to achieve successful therapeutic gains using this technique.

Tumor Matrix Normalization

Enhanced permeability of the interstitial matrix may favor greater accumulation and distribution of nanoparticles. In fact, tumors have an increased presence of stromal cells and ECM proteins including collagens, glycosaminoglycans (GAG), proteoglycans, fibronectin, and glycoproteins [137] relative to normal tissue. The dense mosaic of interstitial structures results in an interstitial space that is not completely connected throughout the tumor volume and one that is filled with the viscous hydrogel-like GAG hyaluronan. The dense and irregular space serves as a tortuous, viscous, and steric barrier that hinders diffusive nanoparticle transport. Elevated IFP abrogates convection-driven flow and diffusive transport of nanoparticles through the interstitial space is sterically hindered by the level, organization, and orientation of fibrillar collagen. Finally, nanoparticle interaction with matrix and cellular components also provide substantial barriers to their delivery. While tumor matrix normalization strategies have shown much promise in the preclinical setting, there have been few translatable approaches.

For example, hyaluronidase, a modulator of the ECM, led to a fourfold increase in tumor uptake and intratumoral distribution of liposomal doxorubicin in a human osteosarcoma xenograft [138]. However, the clinical translation of ECM degradation has been slow due to findings linking ECM degradation to cancer progression, invasion and metastasis [139, 140].

Conversely, a promising approach lies in the elimination of tumor cells using conventional chemotherapeutic agents, termed “tumor priming” [141]. Reduction of the cellular volume increases the interstitial space, improves transvascular and interstitial transport, reduces IFP, and improves perfusion, likely by reducing solid stress-induced vessel compression [9]. In one study, tumor priming with paclitaxel was shown to significantly enhance the accumulation and penetration of Doxil®,

resulting in improved tumor regression and prolonged survival in mice bearing human pharynx FaDu tumor xenografts [141]. This strategy is attractive due to immediate translatability into patients; however, studies have yet to be performed to determine the optimal timing between tumor priming and the administration of nanoparticles.

Despite these key findings about the governing rules of the TME, an integral understanding of the intratumoral fate of advanced drug delivery systems remains deficient. While drug release strategies become more and more embedded into the design of nanocarriers, familiarization with the TME in conjunction with formulation testing becomes crucial in selecting which neoplastic characteristics to exploit.

Intratumoral Evaluation of Nanoparticle Fate: A Look Within and Beyond

Nanoparticle extravasation within the TME was first evaluated by Yuan and colleagues using intravital fluorescence microscopy. Significantly, they found that liposomes predominantly accumulated in the perivascular region of tumor microvessels. Based on a series of observations, the authors also hypothesized that liposomes were being internalized by cells, and were in fact localized intracellularly for several days [142]. This hypothesis was supported in work performed by Seynhaeve et al. [94]. As discussed earlier (cf. Tumor Vascular Permeability), the authors implemented a strategy of "abnormalization"—increasing the leakiness of tumor vasculature using low-dose TNF-α to increase the tumor accumulation of Doxil® by five- to sixfold. Intravital fluorescence microscopy was similarly employed and not only revealed the abundant extravasation of liposomes into the tumor interstitium but also provided critical insight into the mechanism of action of TNF-α, thus far largely obscure. Indeed, the authors found no change in structural microvascular properties such as vessel density, number and diameter, postulating that the impact of TNF-α on the TME is an increase in the number of permeable vessels. Notably, this imaged-based study was the first to demonstrate uptake of doxorubicin within whole liposomes by tumor cells in vivo, previously thought to be internalized following interstitial release [143–146].

The impact of TGF-β inhibition on the intratumoral distribution and efficacy of Doxil® was also revealed using ex vivo imaging methods. Indeed, the intervention was found to increase the recruitment and incorporation of perivascular cells while normalizing the interstitial matrix by decreasing collagen I content. Furthermore, the blockade was found to increase the proportion of perfused blood vessels, leading to greater intratumoral levels of Doxil®, relative to the non-TGF-β blocked tumors. Functional normalization of the TME, measured as percent of positivity of immunofluorescently stained interstitial components, was found to be the basis for enhanced transvascular transport of both free doxorubicin and Doxil® [147].

Nevertheless, there remains a need for investigation of the intertwined role and regulation of TME parameters as a whole in nanoparticle uptake and distribution. This endeavor necessitates the detection of multiple components and therefore relies on a number of systems and analytical techniques.

A number of models have been tested to measure nanoparticle and drug distribution, both in vitro and in vivo. In vitro models, such as multicellular tumor spheroids and multilayered cell cultures, are advantageous in that they take into account the three-dimensional geometry of tissue penetration; however, they fail to account for all parameters of the TME—notably, tumor vasculature and stromal cells. In vivo models include window chamber models, which allow direct visualization of a fluorescently labeled drug as a function of time [17, 148] but remains limited in terms of multicomponent detection. A more universal method, however, constitutes histologically examining the tumor tissue post-administration of the drug and either directly visualizing the drug fluorescently or indirectly by staining for inhibition of cell proliferation or induction of apoptosis [149]. Effectively, histology enables the identification of various cell populations based on their unique cell-surface antigens. Nevertheless, tissue harvesting suffers from limited spatial and temporal information [150]. Such ex vivo studies enlist the aid of computational methods for objective and systematic analysis [151, 152].

Evaluation of the tumor vasculature for the efficient delivery of nanoparticles is possible, as known factors in the regulation of tumor vessel angiogenesis and permeability may provide useful surrogate markers via histological interrogation. Alternatively, angiogenic gene expression levels can be quantified and correlated with EPR status, as was shown in a study by Karathanasis et al. Using digital mammography to track an iodinated nanoprobe in a rat breast tumor model, the authors found a strong correlation between the tumor signal enhancement conferred by the probe and the gene expression levels of VEGF and VEGFR-2 in individual tumors [153].

Differences arising as a result of inter-patient variability can be reasonably addressed using combined therapeutic and imaging, or "theranostic," methods. The vast heterogeneity in liposome accumulation observed in the study by Harrington et al. revealed that "some breast cancers will fail to be targeted by PEGylated liposomes" [2], which prompted the authors to suggest the clinical inclusion of a pretreatment scan, using ^{111}In-labeled liposomes, as a means to identify patients that are likely to respond to the corresponding liposome-based therapy. Indeed, in recent years there has been considerable interest in the integration of imaging in preclinical and clinical drug development (Fig. 20.4). An additional study by Karathanasis et al. sought to noninvasively determine the EPR status of breast tumors in rats with an iodinated liposomal probe using digital mammography. Relative contrast enhancement (RE) arising from the extravascular accumulation of the probe in tumors served to categorize the tumors into good-prognosis (RE $\geq$50 digital units, DU) or bad-prognosis subgroups (RE $\leq$50 DU) prior to treatment with an equivalent liposomal formulation of chemotherapy. The authors found that tumors possessing "leakier" vasculature (i.e., good-prognosis) were associated with a slower growth rate as a result of greater uptake of liposomal doxorubicin. This study

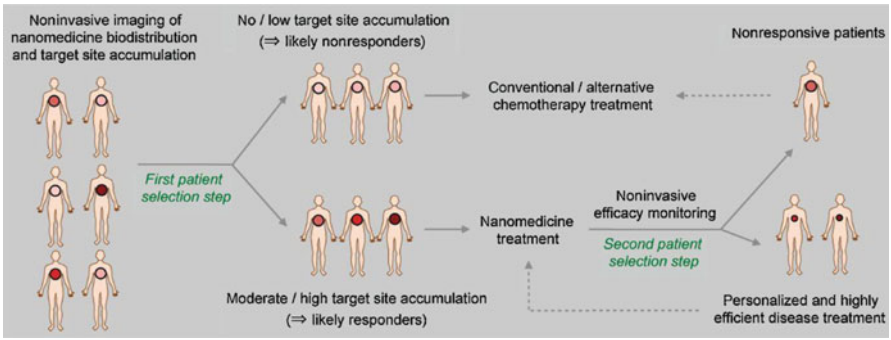

Fig. 20.4 Image-guided treatment planning using nanomedicines. Responders are identified based on their degree of nanoparticle accumulation and stratified into distinct groups. Continuous monitoring enables further adjustment of treatment regimen and/or patient reclassification according to individual response, leading to the implementation of personalized nanomedicine. Reprinted by permission from the American Association for Cancer Research from [96]

highlighted the spatial and temporal variability in tumor vascular permeability among different subjects of the same experimental tumor model. Importantly, the use of a clinically available image-based methodology as an a priori evaluation of the likelihood that a nanoparticle-based therapy will succeed is highly valuable, and merits further investigation in additional tumor models and ultimately in human patients [95].

It is worth noting, however, that, in the design of theranostic tools, great attention must be given to the unavoidable "blows" incurred by the combination of therapeutic and imaging capabilities of nanoparticles. As aptly stated by Cheng et al. "the combination of imaging and therapeutic agents is not a natural fit." While sacrifice is imposed on the loading capacity of nanoformulations, resulting in sub-optimal contrast and/or therapeutic efficacy, the long circulation times necessary for therapeutic formulations are incompatible with the desire for high signal-to-background ratio in diagnostic imaging applications. As such, the accurate detection and characterization of cancerous lesions may only be feasible if separated from therapy [154] and long-circulation is forfeited for the enhancement of signal-to-background ratio [155]. Conversely, longitudinal imaging studies employing nanomaterials which possess or combine both therapeutic and contrast enhancement properties enable noninvasive monitoring of treatment efficacy [154, 156].

Conclusions

In the design of nanoparticle-based therapeutics, effective tumor accumulation and subsequent intratumoral distribution necessitate long circulation of nanocarriers as a primary strategy to enable extravasation and penetration throughout the harsh

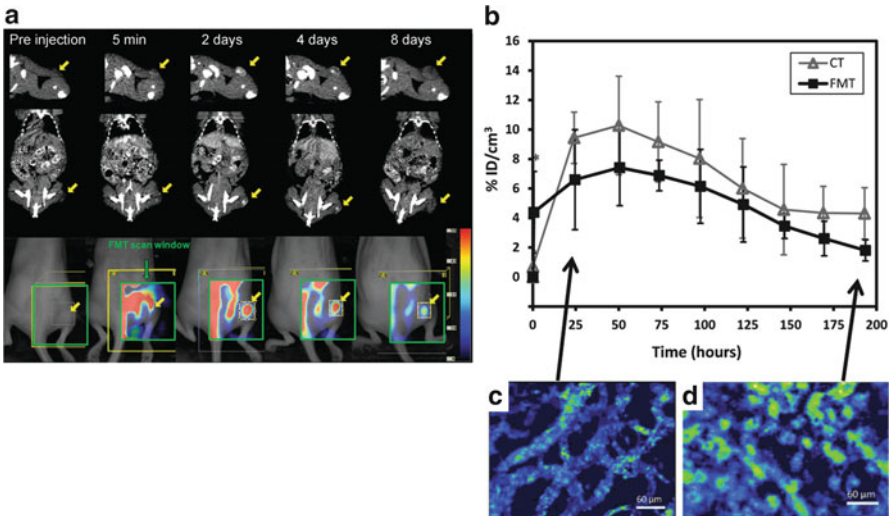

Fig. 20.5 Noninvasive, multimodal imaging of the distribution of CT/optical liposomes in a tumor xenograft model of non-small-cell lung cancer over 8 days (**a**). Micro-CT imaging enabled quantification of tumor accumulation over time (**b**) while in vivo endoscopy revealed the intratumoral microdistribution of liposomes (**c**, **d**). Liposomes are located within the intravascular space at 24 h post-injection (p.i.) (**c**), followed by interstitial and cellular uptake by 8 days p.i. (**d**). Figure adapted with permission from [157] (mi.deckerpublishing.com)

tumor interstitium [7]. The preclinical development of nanomedicines calls for careful consideration into their level of acceptable complexity. Rather, important advances can be made by building on both the success and failure of late-stage/approved formulations through, for example, characterization of the TME via noninvasive imaging techniques and subsequent, careful combination of these promising agents with the appropriate chemical or physical modulators of the TME.

The integration of imaging and drug delivery in nanosystems is likely to grow as valuable insight is gained into the TME and fate of nanoparticles in vivo. Indeed, noninvasive imaging techniques yield invaluable data on the distribution of nanoparticles (Fig. 20.5a, b) while concurrently providing important information regarding the responder status of a patient to a given nanoformulation.

As new nanoformulations continue to be developed, key features such as long circulation and stability will provide a robust platform for clinical applicability, complemented by their in vivo interrogation via multimodal imaging (Figs. 20.3b, c and 20.5a). In spite of the drawbacks tainting its portfolio, nanoparticle-based drug delivery presents great potential for clinical success; time will tell which new avenues outlive our current methods.

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Chapter 21
Convective and Diffusive Transport in Drug Delivery

Abraham R. Tzafriri and Elazer R. Edelman

Abstract Spatiotemporal distribution profiles of drugs in solid tissues are determined by a complex interplay of convective and dispersive forces with mechanisms of drug sequestration, clearance, and metabolism. Understanding how these processes couple with drug delivery modality, kinetics, and dose to determine the fate of delivered drugs en route to and in target tissue has been challenging. Drawing upon examples in local intratumoral and endovascular drug delivery, this chapter reviews how such understanding can be achieved through a combination of reductive experiments, computational modeling, and dimensional analysis.

Introduction

More often than not, proposed pharmacological therapies that show promise *in vitro* do not succeed in animals or humans. It is just as often not clear whether *in vivo* failures reflect a lack of potency in the diseased tissue milieu or whether drug deposition and distribution within tissues is ultimately inadequate. Detailed quantitative pharmacokinetic analyses are required to differentiate these failure modes, and future study designs must include such analyses to ensure that costly animal experiments and clinical trials are not doomed by the technical aspects of limited

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distribution and retention of drug. These issues are especially acute with local modes of drug delivery, which by their nature impart large concentration gradients across tissues and can give rise to regions of toxic-overdosing alongside regions of inadequate dosing and transient efficacy. This need has spurned the development of in vivo, in vitro, and computational models for characterizing and predicting drug distribution patterns in solid tissues.

Studies with a wide range of tissues and drugs have revealed that the spatiotemporal distribution profiles of drugs in solid tissues are determined by a complex interplay of pure transport forces of convection and diffusion and process of sequestration, clearance, and metabolism. Understanding how these processes couple with drug delivery modality, kinetics, and dose to determine the fate of drugs in target tissue has been challenging. This chapter will review how current understanding has been achieved through a combination of reductive experiments, computational modeling, and dimensional analysis. Indeed, though the title highlights convection and diffusion, these processes dominate only in extreme cases, many of which are specifically contrived to measure the underlying parameters.

We will begin by describing the experimental and theoretical underpinnings of these extreme scenarios in sections "Continuum tissue-pharmacokinetics framework" and "Convection and diffusion as sole mediators of drug distribution," and illustrate the roles of drug molecular weight, mode of delivery and location of delivery relative to the source of drug and pressure gradients in the tissue. Then in section "Microvascular clearance limits drug penetration into tissues" we consider the sensitivity of drug distribution to first order drug elimination due to blood clearance or metabolism, and the dependence of the clearance barrier on drug properties. We end with a consideration of the influence of reversible binding interactions with tissue proteins and receptors on drug distribution and retention, for systemic and local delivery (section "Drug binding: a dose dependent barrier to drug distribution"). Coupling of transport and binding is particularly important as it bridges between drug pharmacology and transport and therefore impacts both pharmacokinetics and pharmacodynamics. The dose dependence of drug binding to specific receptors is shown to manifest as pronounced dependence of tissue distribution and receptor binding state on the delivered dose and rate of drug delivery.

Continuum Tissue-Pharmacokinetics Framework

Classical pharmacokinetics provides a powerful framework for drug development, but is limited by the assumption that drug distribution into tissues is a fast process relative to systemic distribution and that persistence of gradients in the tissue is short lived relative to the duration of drug delivery. The focus of the current review is precisely on those scenarios where this assumption breaks down and issues of drug distribution dynamics take center stage. This has been found to be the case not

only for neoplastic tissue targets [1–3] but also in delivery to the brain [4, 5] and cardiovascular tissues [6–18]. We draw on this vast experience to illustrate how coupling of continuum reaction equations to continuum transport equations in the tissue provides a flexible theoretical framework for understanding transport barriers to drug delivery and generating strategies for overcoming or circumventing these barriers. The term continuum stands in contradistinction to a compartmental schema. The presence of a continuum defies defined boundaries and concentration discontinuities and is used to imply a sufficiently high drug concentration that ensures negligible stochastic effects. A continuum perspective justifies the validity of differential and partial differential equations to describe apparent dynamics of drug distribution in solid tissues.

In the continuum framework, the concentration of drug (C) is averaged over a length scale that is large relative to the dimensions of tissue structures like pores and cells that may affect transport but small relative to the tissue diameter. The rate of change in C with time is then related to the local flux of transporting drug (F) through the material balance equation [19, 20]

$$\frac{\partial C}{\partial t} = \mathrm{div}\left(F\right) + S + \sum_{i=1}^{n} R_i. \tag{21.1}$$

Here "div" denotes the divergence operator, S denotes the density of volumetric sources and sinks, e.g., drug extravasating from capillaries or released from microparticles, and R_i denote biochemical rate equations that account for volumetric drug clearance, hydrolytic degradation, binding to tissue proteins and receptors, and receptor mediated drug metabolism. For simplicity, this formulation of the transport problem assumes that only soluble drug is mobile and that metabolite concentrations are negligible—when they must be considered similar local balance equations can be introduced to account for modified forms of the drug. The formulation of each transport problem is completed by the specification of a constitutive relationship between the local flux of drug (F) and the local concentration (C), a statement of the initial concentration distributions of all drug species in the tissue (soluble, solid, bound, or metabolized), and assignment of boundary conditions that account for all surface sources and sinks of drug.

In considering the constitutive relationship between transport flux and local drug concentration it is important to distinguish between convection which is driven by macroscopic gradients in mechanical forces such as hydrostatic pressure and electromagnetic fields [21–23], and those that are driven by thermal fluctuations (Brownian motion) and microscopic dispersion (e.g., hydrodynamic mixing at the pore scale). The local drug flux can then be expressed as a sum of what are for the moment two independent forces, a convective flux (F_v) and a dispersive or diffusive flux (F_D)

$$F = F_v + F_D. \tag{21.2}$$

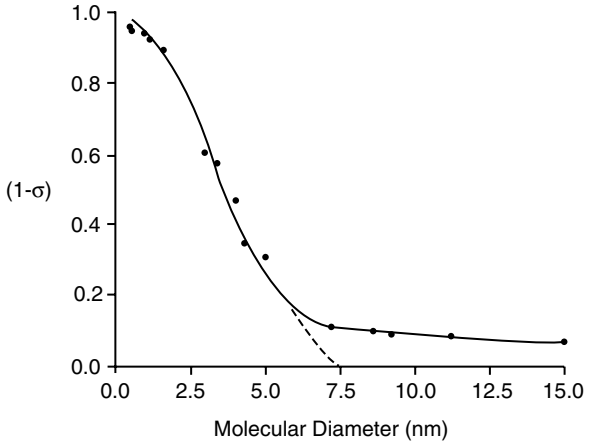

Fig. 21.1 The retardation of drug convection through microvessels relative to water filtration, $R_F = 1 - \sigma$, decreases with increasing molecular diameter. The steeply declining section of the curve (continued as a *broken line*) is consistent with transport through pores of 7.5 nm diameter. The slowly declining section of the curve at molecular diameters ≥7.2 nm suggests a specialized transport pathway for these molecules. Reproduced from Michel 1996 [26] with permission

Convective Flux

For the purposes of this chapter, we shall limit the discussion to applications where drug convection is solely driven by pressure gradients and can be envisioned as the process by which solubilized drug is carried along by an aqueous fluid from regions of high pressure to regions of low pressure. Mathematically, if **v** denotes the velocity of the fluid then the convective flux of the drug through a unit area section is proportional to the product of the fluid velocity and the concentration

$$F_v = R_F \mathbf{v} C \tag{21.3}$$

where R_F is a retardation coefficient that accounts for the hindered convection of solutes in tissue pores relative to that of the small water molecules. Theoretical models [24] and drug transport studies in various bulk tissues [25] and across blood capillary walls [26] (Fig. 21.1) suggest that retardation due to steric and hydrodynamic interactions with pore elements becomes more pronounced as the size of the drug molecule approaches the dimensions of water filled pores in the tissue.

Typically, fluid velocities through porous tissues are sufficiently low to justify the validity of Darcy's law, which in analogy to steady-state (Poiseuille) flows in solid pipes, states that the vector components of fluid velocity are proportional to associated vector components of the hydrostatic pressure gradient [21, 27, 28]

$$\mathbf{v} = -K \cdot \text{grad } P. \tag{21.4}$$

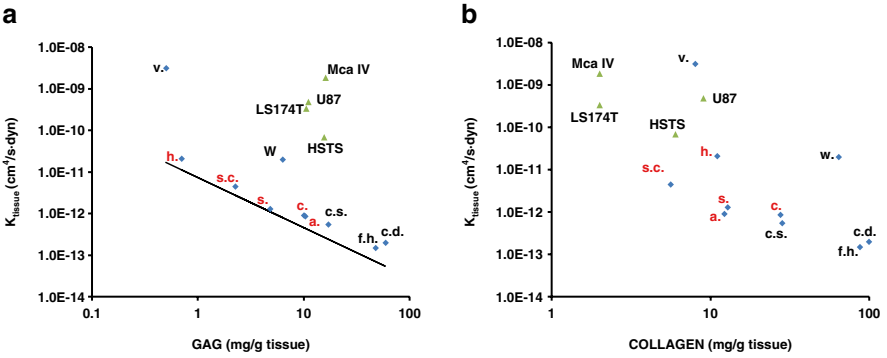

Fig. 21.2 Hydraulic conductivity of various tissues as a function of total contents of GAG (**a**) and collagen (**b**). *Diamonds*: data compiled by Levick [33]. *Triangles*: data from Netti et al. [34]. Continuous line in panel a: theoretical prediction based on GAG content as derived by Swabb et al. [25]. v. = vitreous body; h. = hepatoma; w. = Wharton's jelly; s.c. = subcutaneous tissue slice; s. = sclera; c. = corneal stroma; a. = aorta; c.s. = femoral condylar cartilage, superficial layer; c.d. = femoral condylar cartilage, deep layer; f.h. = femoral head cartilage. *Red letters* are used to denote the subset of tissues that were originally used by Swabb et al. [25] in deriving their correlation of K tissue with GAG content

Here P is the local pressure, K is the hydraulic conductivity of the porous medium and "·" denotes the scalar product. Consistent with Starling's hypothesis, the pressure P is equal to the difference between the hydrostatic pressure and the osmotic pressure [29, 30]. Theoretical considerations suggest a dependence of K on the volume fraction and structure of pore space [24] that can manifest as a dependence on tissue composition. Early experimental studies [25, 31, 32] suggested that tissue dependence of K tracks the relative content of glycosaminoglycans [GAGs] in the tissue (Fig. 21.2a). As the coefficient of drug retardation R_F has also been reported to correlate inversely with GAG content [25], GAG tissue content has been touted as an important determinant of convective drug transport in solid tissues. However, with the assembly of more extensive data sets, it became clear that GAG content does not solely predict hydraulic conductivity as other fibrous proteins such as collagen [33, 34] and elastin [12, 33, 35, 36] also resist fluid flow through the tissue (Fig. 21.2b). Notably, both GAG content and collagen content vary not only between different tissues, but also in the same tissue due to injury and disease [37–39], suggesting that drug convection may well depend on tissue state [12].

As forcefully highlighted by Jain and his collaborators [1, 30, 40], hydrostatic pressure gradients and osmotic pressure gradients will both vary between normal and neoplastic tissues, due to a lack of functional lymphatics in the latter [41]. Consequently, fluid flow is typically low in the bulk of the tissue and increases sharply at the periphery of vascularized tumors (Fig. 21.3). Drugs that are delivered regionally to the tumor will therefore have to overcome this convective outflow barrier en route to the target tissue, whereas drugs that are directly infused into the

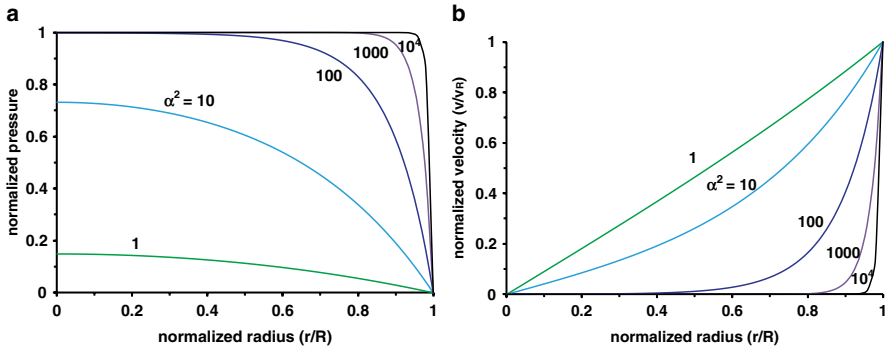

Fig. 21.3 Model predicted normalized interstitial fluid pressure (**a**) and filtration velocity v/v_R (**b**) as a function of the normalized radial distance from the center of the tumor (r/R) and the hydraulic conductivity ratio $\alpha^2 = R \times (L_P/K) \times (S/V)$. Here R is the radius of the tumor, v_R is the filtration velocity at the tumor edge, K and L_P are the interstitial and vascular hydraulic conductivities, and (S/V) is the exchange vessel surface area per unit volume. Results for an isolated tumor are plotted using the analytical solutions derived by Baxter and Jain [28] and are similar to that seen for subcutaneous tumor. Model predictions were subsequently validated by Boucher et al. [40] in subcutaneous mammary adenocarcinoma with $\alpha^2 = 1{,}200$

tumor bulk can harness fluid outflow to homogenize drug distribution. As we shall see below, the impact of such fluid flow patterns on drug delivery will depend on the molecular weight of the drug.

Diffusive Flux

In the absence of bulk flows, drug transport is driven by random Brownian collisions between drug molecules, solvent molecules and tissue proteins. Though Brownian collisions have no preferred directionality, macroscopically they tend to transport drug from regions of high concentrations to regions of low concentrations. In the continuum limit this manifests as Fick's first law which states the rate of transfer of a diffusing substance through a unit area of section is proportional to the concentration gradient measured normal to that section

$$F_D = -D \cdot \mathrm{grad} C \tag{21.5}$$

where D is the diffusion coefficient averaged over a length that is long relative to pore and cell length scales and "·" denotes the scalar product of two vectors. Namely, drug diffusivity in non-isotropic tissues is a vector rather than a scalar, with a magnitude and direction, reflecting the differential steric hindrance and tortuosity associated with various connective tissue layers [4, 7, 19]. Consistent with theoretical descriptions of the tissue as a multiscale porous medium, diffusivity in real and model tissues (Fig. 21.4) decreases with drug molecular weight and depends on

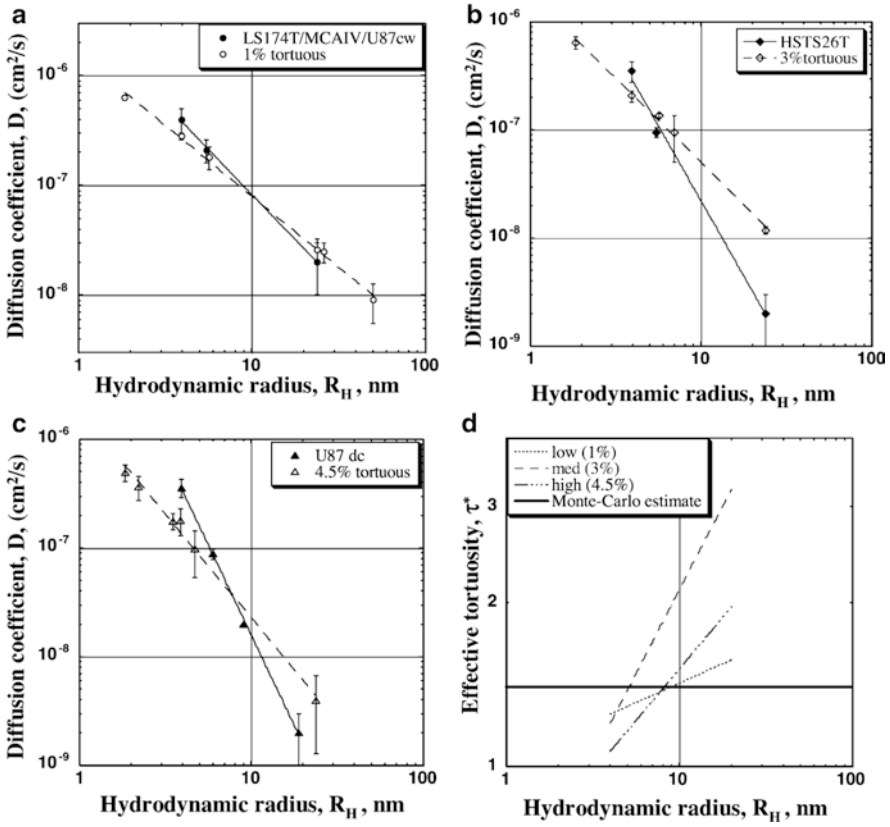

Fig. 21.4 Diffusion coefficients of macromolecules (4.4–2,000 kDa) in collagen gels closely matches previous measurements in tumors. (**a**)–(**c**) Comparison of tortuosity-corrected diffusion data in gels to diffusion data in tumors. Corrected diffusion coefficient is calculated as D/τ^2, using estimate $\tau=2^{1/2}$. Comparisons are show between: 1 % gels (*open circle*) and data for LS174T, MCAIV, and U87cw (*filled circle*) (**a**); 3 % gels (*open diamond*) and HSTS26T (*filled diamond*) (**b**); and 4.5 % gels (*open triangle*) and U87dc (*filled triangle*) (**c**). (**d**) Effective tortuosity necessary to account for discrepancy between uncorrected gel data (D_{gel}) and tumor data (D_{IM}) as a function of tracer molecule hydrodynamic radius. Values were calculated as $\tau=(D_{gel}/D_{IM})^{1/2}$ from linear fits of D_{gel} and D_{IM} data. Reproduced from Ramanujan et al. [42] with permission

microstructural composition of the tissue [5, 7, 21, 25]. In particular diffusive hindrance of macromolecules (Fig. 21.4) has been correlated with the content of fibrous proteins and the tortuosity due to tissue micro geometry [7, 42, 43]. More specifically, diffusive hindrance of macromolecules is largely explained by steric interactions with unassembled collagen [42], whereas diffusion of nanoparticles is also hindered by the macro-porous networks of fibrillar collagen [44] and elastin [7]. Thus, diffusion-limited drug distribution can be improved through optimization of drug physicochemical properties [6, 7] or through the controlled modulation of tissue microstructure [45]. In a striking example of the latter concept, McKee et al. [44]

illustrated that matrix modification with bacterial collagenase co-injection can significantly improve the initial range of viral distribution within the tumor and lead to enhanced therapeutic outcomes. Similarly, drug distribution can also be improved through the mechanical [12, 16] or pharmacologic [46] disruption of resistive cell layers, though such interventions may induce unintended consequences or be short lived as the tissue remodels in response to the induced change.

Convection and Diffusion as Sole Mediators of Drug Distribution

In certain applications drug is delivered to the tissue only through its periphery, and distribution inside the tissue is solely governed by convection and diffusion. In the absence of volumetric sinks and biochemical interactions, (21.1) reduces to a form where its diffusive term is governed solely by the drug concentration and convective element determined by the product of the concentration with the gradient in pressure

$$\frac{\partial C}{\partial t} = -\mathrm{div}\left(D \cdot \mathrm{grad} C + R_F C K \cdot \mathrm{grad} P\right). \tag{21.6}$$

For example, this may be the case when drug is delivered to the region surrounding a vascularized tumor [47, 48], or locally to the blood vessel wall [10, 16], and is also the mode by which systemically delivered drug accesses vascularized tissues by extravasating across capillary walls [30]. Depending on the tissue target and the site of drug application, convection or diffusion might dominate drug transport. This section therefore examines the dynamic signatures of convection and diffusion alone or together, and derives criteria for prioritizing their relative roles. To illustrates these concepts in the simplest possible manner, we shall consider a slab-shaped tissue that extends between $x=0$ and $x=L_{\text{tissue}}$, and derive solutions for the scenario of a constant drug concentration at the inlet ($x=0$) and perfect sink conditions at the outlet ($x=L_{\text{tissue}}$). Such idealized settings are encountered in the study of drug transport across thin tissues [16, 49] and capillaries [29].

Pure Convection

In the absence of other bulk transport forces, the one dimensional convection equation in a homogeneous tissue takes on the simple form

$$\frac{\mathrm{d}C\left(x + R_F \mathbf{v} t\right)}{\mathrm{d}t} = \frac{\partial C}{\partial t} + R_F \mathbf{v} \frac{\partial C}{\partial x} = 0. \tag{21.7}$$

Namely, convection tends to translate drug concentration profiles along the direction of the pressure gradient at a velocity $R_F \times \mathbf{v}$ (see Fig. 21.5a)

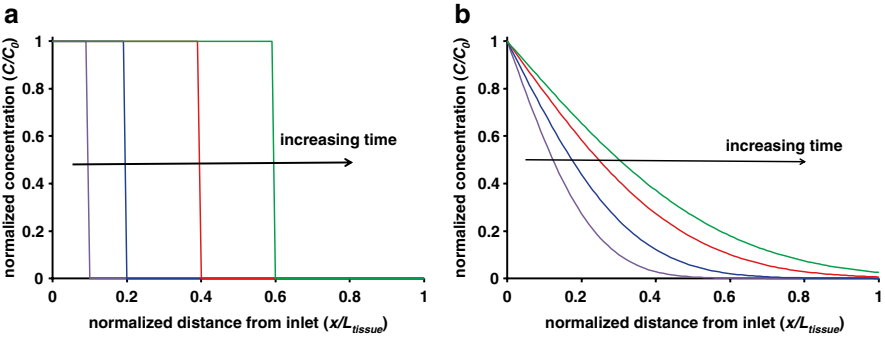

Fig. 21.5 Temporal evolution of drug concentration profiles as determined by pure convection (**a** (21.8)) or diffusion (**b** (21.11)) at dimensionless times $\tau = 0.1$ (*purple*) 0.2 (*blue*), 0.4 (*red*) and 0.6 (*green*). Drug concentration is normalized to the inlet concentration, distance from inlet (x) is normalized to the thickness of the tissue slab (L_{tissue}) and dimensionless time is defined as $\tau = t/t_{\text{v}}$ fro pure convection and $\tau = t/t_{\text{D}}$ for pure diffusion

$$C(\mathbf{r},t) = C(\mathbf{r} + R_F \mathbf{v} t). \tag{21.8}$$

This naturally defines a convective time scale for drug penetration into the tissue, as the ratio of the transport length scale L_{tissue} and the convective velocity

$$t_v = \frac{L_{\text{tissue}}}{R_F \mathbf{v}} = \frac{L_{\text{tissue}}}{R_F(-K \text{ grad } P)} \approx \frac{L_{\text{tissue}}^2}{R_F(-K \ \Delta P)}. \tag{21.9}$$

Importantly, this convective time scale can be traced to the dimensional scaling of the convective term in the continuum balance equation (21.6) which has the units of concentration divided by time (e.g., C/t_{v}) and involves a single spatial derivative so that

$$\text{div}(R_F \mathbf{v} C) \approx \frac{R_F \mathbf{v} C}{L_{\text{tissue}}} \propto C / t_{\mathbf{v}}.$$

Thus, result (21.9) can be used in more general settings to prioritize the role of convection relative to diffusion and binding.

Pure Diffusion

In the absence of other bulk transport forces, the one dimensional diffusion equation in a homogeneous tissue takes on the simple form we commonly refer to as Fick's second law

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}. \tag{21.10}$$

At early times, diffusion fronts are closer to the inlet source ($x=0$) than to the outlet sink ($x=L_{\text{tissue}}$) and it is justified derive an approximate solution under the assumption of a semi-infinite tissue ($L_{\text{tissue}} \to \infty$)

$$C \approx C_0 \text{erfc}\left(\frac{x}{2\sqrt{Dt}}\right). \tag{21.11}$$

Thus, whereas pressure gradients transport drug molecules in well-defined fronts that propagate at a constant velocity (Fig. 21.5a), diffusion smoothens the concentration profile and transports drug at a decelerating rate as local gradients become shallower (Fig. 21.5b). Defining the diffusion time scale t_{D} as the time at which the outlet concentration is just under 10 % of the inlet concentration provides the estimate

$$t_D \equiv \frac{L_{\text{tissue}}^2}{6D}. \tag{21.12}$$

Though derived under simplifying conditions, these results are typical of diffusion controlled drug transport. Indeed, up to the constant of proportionality, the estimate of t_{D} can be traced to the dimensional scaling of the diffusive term which involves two spatial derivatives, so that

$$\frac{C}{t_D} \propto D\frac{C}{L_{\text{tissue}}^2} \Leftrightarrow t_D \propto \frac{L_{\text{tissue}}^2}{D}.$$

At times of order t_{D}, the diffusion front reaches the outlet and shortly afterwards steady state is attained. In stark contrast to convection which establishes a uniform steady-state drug distribution, diffusion tends to maintain a constant steady-state drug concentration gradient. As we shall illustrate in the next section, this distinction becomes important in settings where convection and diffusion coexist as it facilitates the identification of the dominant transport mechanisms through measurement of the drug distribution profiles.

Under constant inlet concentration and zero outlet concentration steady state concentration takes the form

$$C \approx C_0\left(1 - x / L_{\text{tissue}}\right) \tag{21.13}$$

and steady-state diffusive flux is proportional to the concentration difference between inlet and outlet

$$F_D = \left(D / L_{\text{tissue}}\right)C_0. \tag{21.14}$$

The latter result forms the basis for the definition of the steady-state diffusive permeability of a tissue as D/L_{tissue}.

Thus, though convection and diffusion distribute drug differentially within the tissue, these differences are not easily apparent from measurements of the steady-state drug fluxes at the outlet surface. Regardless of the dominant transport mechanisms, steady-state flux is proportional to the concentration difference across the tissue, with the diffusive permeability D/L_{tissue}, and drug velocity $R_F v$ playing similar roles. Nevertheless, convection and diffusion will differentially influence the directionality of steady-state permeability as pure convection perfectly transports drug along the pressure gradient and perfectly reflects it in the reverse direction. On the contrary, diffusive permeability (D/L_{tissue}) only depends on tissue properties, its thickness and influence on diffusivity. Below we generalize this important observation to settings where convection and diffusion are coupled and point out its particular significance for transcapillary drug transport.

Coupled Convection and Diffusion

Though convection and diffusion can be studied independently under in vitro conditions, in vivo they tend to coexist. In coupled settings, convection or diffusion will dominate depending on which moves drug to any given distance within a shorter time span. That is, transport will be dominated by the mechanisms with the shorter characteristic time scale. Defining the Peclet number as the ratio of the typical times scales of diffusion and convection

$$Pe \equiv \frac{t_D}{t_v} \approx \frac{L_{tissue} R_F \mathbf{v}}{D} = \frac{L_{tissue} R_F K \ \mathrm{d}P/\mathrm{d}x}{D} \tag{21.15}$$

suggests that diffusion should dominate when $Pe < 1$ and that convection dominates when $Pe > 1$ [13, 16, 25].

Implications for Drug Distribution

The importance of the Peclet number is succinctly illustrated by its influence on steady state drug distributions patterns under conditions where convection and diffusion are co-aligned (Fig. 21.6a)

$$C_{\uparrow\uparrow} = C_0 \cdot \frac{1 - \mathrm{e}^{Pe \cdot (x/L_{tissue} - 1)}}{1 - \mathrm{e}^{-Pe}} \tag{21.16}$$

or counter aligned (Fig. 21.6b)

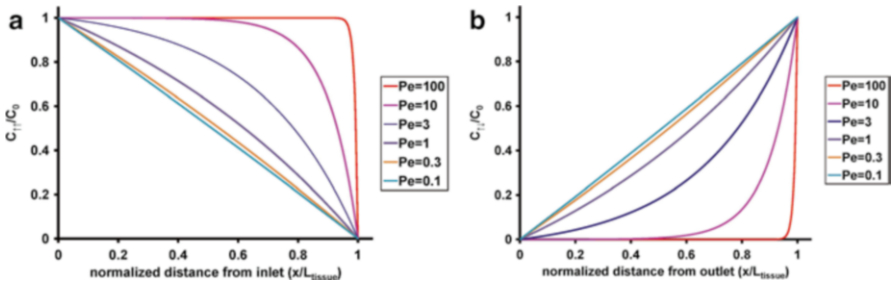

Fig. 21.6 Steady-state drug distribution is determined by the magnitude of the Peclet number and the orientation of convection relative to diffusion. Convection always points from inlet ($x=0$) to outlet ($x=L_{\text{tissue}}$). Diffusion and convection are co-aligned when drug is presented at the inlet (**a** (21.16)) and counter-aligned when drug is presented at the outlet (**b** (21.17))

$$C_{\uparrow\downarrow} = C_0 \cdot \frac{e^{Pe\cdot(x/L_{tissue})} - 1}{e^{Pe} - 1}. \tag{21.17}$$

Note that in deriving (21.17) we have deviated from the convention of assigning $x=0$ as the concentration inlet, to highlight the situation in blood vessels where diffusion and convection are counter-aligned when drug is delivered perivascularly [13, 16].

Evaluation of the steady-state drug distribution profiles affected by convection and diffusion not only confirms the ability of the Peclet number to prioritize the roles of these two processes, but also provides additional insight as to the significance of the Peclet number for drug delivery. When convection and diffusion are co-aligned, as is the case when drug is pressure-infused into a tissue, the fraction of tissue that is sub-optimally dosed at steady-state decreases with increasing Pe, and in the extreme of convection dominated transport scales as $1/Pe$. Namely, at high Pe and long drug exposures, most of the tissue can be optimally dosed by convection and only a thin boundary layer near the outlet is sub-optimally dosed by diffusion. In other circumstances, convection and diffusion can be counter aligned, for example when drug is delivered regionally to a vascularized tumor [47, 48]. In these circumstances where convection is counter-aligned to the concentration gradient, the steady state fraction of tissue that is penetrated by diffusing drug molecules decreases with increasing Pe, and in the extreme of convection domination scales as $1/Pe$. Namely, at large Peclet numbers convection restricts drug distribution to a thin boundary layer around the source.

Appreciation of the significance of the Peclet number as a key determinant of drug distribution naturally raises the question of how and to what extent it can be modulated by varying physicochemical drug properties such as molecular weight. According to (21.15), the drug dependence of the Peclet number is solely due to the factor R_F/D. Though R_F (Fig. 21.1) and D (Fig. 21.4) both decrease with increasing

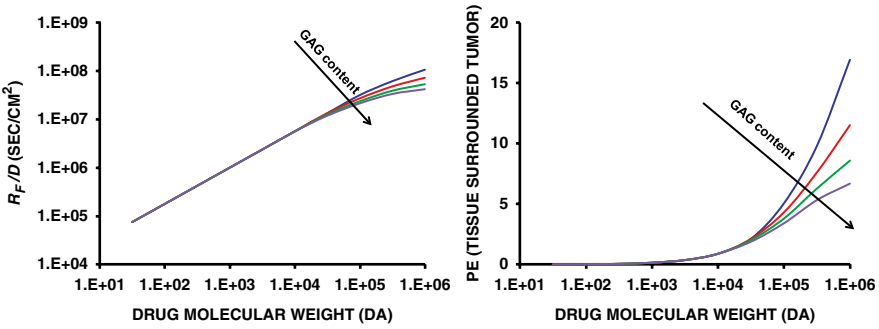

Fig. 21.7 Molecular weight dependence of drug Peclet number in tissues with GAG contents of 2 (*blue*) 4 (*red*) 6 (*green*) or 8 mg g^{-1} (*purple*). (**a**) Relative convective transmittance (R_F/D) increases more than 500-fold as drug MW increases from 32–10^6 Da, irrespective of GAG content. (**b**) Estimated Peclet number at the edge of a 1 cm radius tumor. R_F was set to 1 for MW in the range 32–10^4 Da, and at higher MW was evaluated using the published correlation [25] $R_F = 1.5 \exp(-0.01318 \times MW^{0.366} \times GAG^{0.5})$ ($6.9 \times 10^4 < MW < 2.8 \times 10^9$). Diffusivity was evaluated using the published correlation [25] $D = 1.778 \times 10^{-4}\ MW^{-0.75}$ ($32 < MW < 69{,}000$). Peclet numbers were evaluated using the typical filtration velocity at $v_R = 0.016\ \mu m\ s^{-1}$ for a tumor surrounded by normal tissue [30] and $L_{tissue} = 0.1$ cm, corresponding to 10 % of the tumor radius

molecular weight (MW), evaluation of published correlations [25] for drugs and nanoparticles with MW of 32 Da–3×10^9 Da in tissues with GAG contents in the range 0–8.1 mg per g tissue reveals that R_F/D is virtually independent of GAG content and increases monotonically with drug MW (Fig. 21.7a). The implication is that *Pe* increases with drug MW and consequently that convection plays a greater role in the transport of larger drugs. For example, we can now estimate the relative extent to which outward fluid filtration at the tumor edge limits the tumor uptake of regionally delivered drug (Fig. 21.7b). Estimating the zone of fast fluid filtration in a 1 cm radius tumor as being 1 mm thick (Fig. 21.3b, $\alpha^2 = 1{,}000$) implies that convection will limit the penetration of large molecules and nanoparticles (MW > 69,000 Da) but have little influence on transport of small chemotherapeutics (323–1,000 Da).

Implications for Blood Vessel Permeability

The influence of the Peclet number on steady state distributions patterns of delivered drug has dramatic implications for the degree to which drugs can transport across tissue layers, for example across blood vessel walls. When convection is co-aligned with the concentration gradient the steady state flux of drug across the blood vessel wall is

$$J_{\uparrow\uparrow} = \mathbf{v}C_{\uparrow\uparrow} - D\frac{dC_{\uparrow\uparrow}}{dx} = \frac{\mathbf{v}C_0 e^{Pe}}{e^{Pe} - 1}. \tag{21.18}$$

However, when convection and drug gradients are counter aligned the steady state flux of drug is e^{Pe}-fold lower

$$J_{\uparrow\downarrow} = \mathbf{v}C_{\uparrow\downarrow} - D\frac{dC_{\uparrow\downarrow}}{dx} = -\frac{\mathbf{v}C_0}{e^{Pe}-1} = -e^{-Pe}J_{\uparrow\uparrow}. \tag{21.19}$$

Thus, capillary walls will only allow unidirectional convection dominated ($Pe>3$) extravasation of macromolecules and nanoparticles, while concomitantly allowing bidirectional diffusion dominated ($Pe<0.3$) extravasation of smaller drugs. Consequently, small drugs must rely on binding to overcome clearance forces and ensure their retention in the tissue long after plasma drug levels have dropped, whereas macromolecules and nanoparticles will be retained even in the absence of strong binding interactions. In normal tissues, lymphatic clearance of interstitial drug may compensate for reduced microvascular clearance [50]. However, in tumors the absence of functional lymphatics implies that nanoparticles that extravasate into the interstitium will be retained there, which along with the greater permeability of tumor capillaries provides a mechanistic underpinning for the enhanced permeability and retention (EPR) effect [51, 52]. Due to their large size relative to interstitial pores, macromolecules and nanoparticles will tend to remain localized close to capillaries [53]. Thus, taking advantage of the EPR effect requires a combined delivery approach whereby the extravasated particles slowly release drug within the tissue and allow it sufficient time to distribute more uniformly [54, 55].

Microvascular Clearance Limits Drug Penetration into Tissues

Thus far we have focused on defining the influence of convection and diffusion on drug distribution in the absence of distributed drug sinks and sources within the target tissue. In this section we consider the role of microvessels as distributed conduits that clear interstitial drug and influence drug distribution in well vascularized regions. When the concentration of interstitial drug greatly exceeds the concentration of drug within capillaries, each capillary clears drug at a rate that is proportional to the product of the permeability and the local interstitial concentration (e.g., (21.19)). When intracapillary distances are small compared to the dimensions of the tissue, it is justified to approximate the discrete microvasculature as a continuous distribution of drug sinks that are proportional to the local concentration of interstitial drug [56]

$$S = -kC, \tag{21.20}$$

where the macroscopic rate constant of local drug clearance k is equal to the product of the surface area density (a_{mv}) and permeability of the microvasculature

$$k = P_{mv} \cdot a_{mv}. \tag{21.21}$$

We shall illustrate the fundamental influence of this distributed sink term imn 1D setting with drug source boundary conditions at the inlet ($x=0$) and perfect sink boundary condition at the outlet ($x=L_{\text{tissue}}$)

$$\frac{\partial C}{\partial t} - D\frac{\partial^2 C}{\partial x^2} + R_F \mathbf{v}\frac{\partial C}{\partial x} = -kC. \tag{21.22}$$

Before proceeding with this analysis, we remark that (21.22) has also been used to describe the influence of interstitial hydrolysis or receptor mediated intracellular drug degradation in the limit of receptor excess [56].

Clearance Limited Steady State Drug Distribution

The influence of distributed drug clearance is best illustrated at steady state, for cases where in the absence of clearance drug would distribute throughout the tissue, i.e., under conditions of pure convection, pure diffusion and when the two are co-aligned. Though we are not aware of a practical case where convection is the sole transport mechanism in the tissue, we first analyze this case as it provides the most striking example of the sensitivity of drug distribution to distributed clearance mechanisms. In the extreme of purely convective transport, steady state is defined by

$$R_F \mathbf{v}\frac{\mathrm{d}C}{\mathrm{d}x} = -kC \tag{21.23}$$

and implies that drug that is delivered at the inlet $x=0$ distributes as a declining exponential

$$C = C\left(x=0\right)e^{-x/\ell_v}, \quad \ell_v \equiv R_F \mathbf{v}/k. \tag{21.24}$$

Thus, whereas convection alone would eventually flush the entire tissue thickness with drug (Fig. 21.5a), microvascular clearance would lead to a 90 % decline in drug concentration at a depth of $2.30\,l_v$, where l_v is the distance by which fluid flow moves drug molecule is in time $1/k$. Whether or not this is a significant limitation depends on the magnitude of the length scale l_v relative to the dimensions of the tissue, L_{tissue}.

When diffusion is the dominant transport mechanism, steady state drug distribution is defined by

$$-D\frac{\mathrm{d}^2 C}{\mathrm{d}x^2} = -kC. \tag{21.25}$$

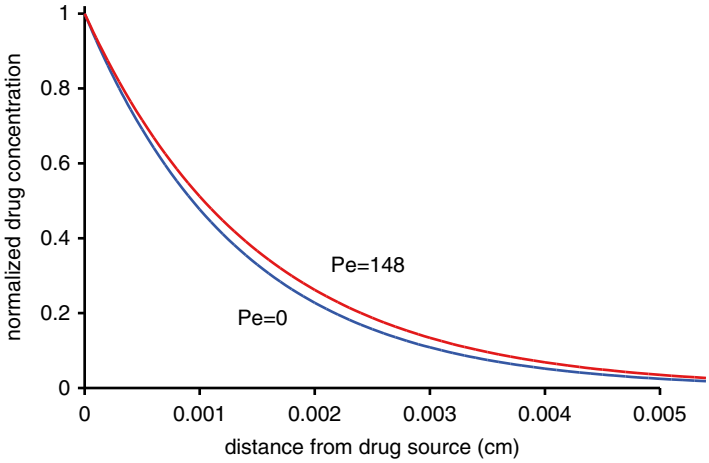

Fig. 21.8 Sensitivity of drug distribution to convection is not predicted by the magnitude of Peclet number. Simulation parameters: $L_{\text{tissue}} = 1$ cm, $D = 0.021$ **μ**m^2 s^{-1}, $k = 1.15 \times 10^{-4}$ s^{-1}, and $v = 0$ ($Pe = 0$) or 0.031 **μ**m s^{-1} ($Pe = 148$)

This equation admits a solution of the form

$$C = c_{-} e^{-x/\ell_D} + c_{+} e^{x/\ell_D}, \quad \ell_D \equiv \sqrt{D/k} \tag{21.26}$$

where l_{D} is the distance by which a drug molecule diffuses is in time $1/k$. Consequently, when tissue dimensions are large relative to l_{D}, the perfect sink condition at the outlet forces the second term to be negligible, resulting in an exponentially declining distribution profile

$$C \approx C(x=0) e^{-x/\ell_D}, \quad \ell_D \equiv \sqrt{D/k} \ll L_{tissue}. \tag{21.27}$$

This result applies equally to situations where drug concentration or drug flux are held constant at the inlet [17] and has been widely applied in the context of regional and local drug delivery [5, 57–59]. Before describing one such example in detail, we note that microvascular clearance can also give rise to an exponentially localized steady-state distribution in the presence of coupled convection and transport and that the magnitude of the Peclet number does not correctly prioritize the importance of convection as the dimensions of the tissue (L_{tissue}) no longer provide an adequate length scale for drug penetration (Fig. 21.8). In the next section we discuss in greater detail how the model of diffusion and capillary clearance can be experimentally validated and used to guide the development of optimized drug delivery strategies.

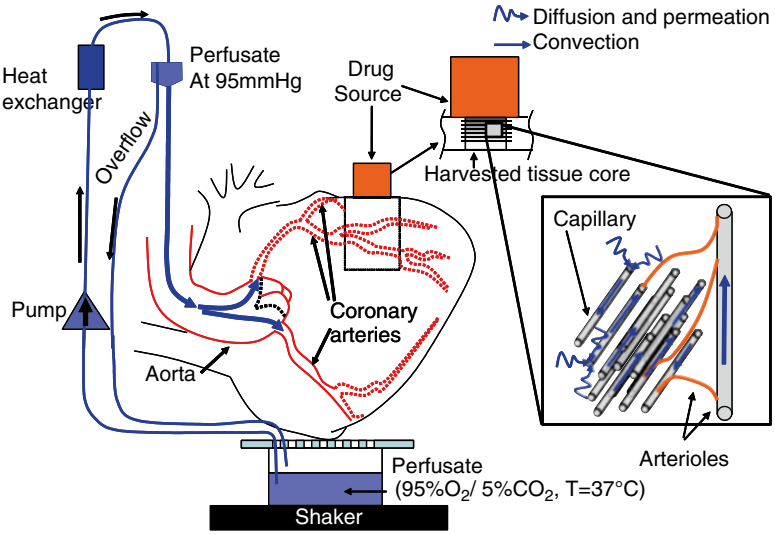

Fig. 21.9 Isolated perfused heart apparatus. Rat coronary arteries were perfused antegrade through an aortic cannula at constant physiologic mean pressure while a constant, well-mixed drug source was applied to the epicardial surface. Drug distribution was quantified in myocardial tissue regions exposed to drug. High magnification schematic illustrates the examined transport forces: drug diffusion within tissue and clearance through convection by intravascular flow after permeation across capillary wall. Reproduced with permission from Le et al. [17]

Growth Factor Delivery to Vascularized Tissues

Angiogenesis plays different roles in normal and neoplastic tissues. In tumors, microvasculature is aberrant and leaky and delivery of anti-angiogenic compounds seeks to either abolish blood supply to the cancerous cells or at least to normalize it. In non-neoplastic tissues, a decline in microvascular density is associated with ischemia and hypoxia. Thus, there has been avid interest in the development of pro-angiogenic pharmacotherapies [60–62]. Yet, as with anticancer therapies, drug pharmacology alone does not guarantee in vivo biological effects. To examine whether limitations of growth factor distribution in the target tissues may explain the lack of efficacy of local delivery modalities, we developed an isolated perfused heart apparatus for studying drug distribution patterns under controlled coronary flow conditions (Fig. 21.9).

Our studies focused on the fibroblast derived angiogenic factors [62] FGF1 and FGF2 labeled with low molecular weight fluorescent or radioactive compounds. We examined the effects of capillary perfusion on myocardial growth factor transport in rat hearts incubated at constant epicardial source concentrations (Fig. 21.9) with and without controlled coronary flow. When delivered to the ex vivo myocardium in the absence of flow, Texas-red FGF2 (TR-FGF2) distributed via diffusion to a penetration depth of 66 μm in 6 h (Fig. 21.10a). Growth factor distribution followed

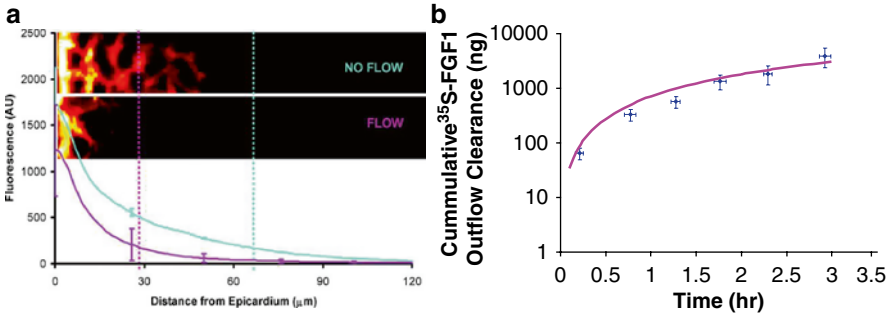

Fig. 21.10 Pericardially delivered growth factor distributes by interstitial diffusion and microvascular clearance. (**a**) Distributions of TR-FGF-2 at 6 h with (*magenta*) and without (*blue*) coronary flow. Data represent mean ± s.e.m ($n = 3$). Vertical dashed lines denote the depth at which concentration has dropped 90 % from its inlet value. (**b**) Ex vivo cumulative outflow of ^{35}S-FGF-1 (*diamonds*) in perfusate is well explained by model predictions (*magenta line*) using TR-FGF-2 parameter estimates. $D = 0.021 \pm 0.001$ **μ**m^2 s^{-1}, $k = 1.15 \pm 0.06 \times 10^{-4}$ s^{-1}. Reproduced with permission from Le et al. [17]

the expected diffusion-controlled pattern at short times (21.11) and provided the estimate $D = 0.021 \pm 0.001$ μm^2 s^{-1}. This estimate is 569-fold lower than Swabb's correlation [25] would imply based on the molecular weight of FGF2 (17 kDa) and also significantly lower than the myocardial diffusivity of Dextran 10 kD (10.24 μm^2 s^{-1}). These findings are consistent with avid binding of FGF2 (but not Dextran) to heparan sulfate proteoglycans (HSPG) in myocardial tissue. Indeed, the experiments of Dowd et al. [49], illustrated that FGF2 binding to HSPG can reduce the diffusivity of this molecule by a factor of 500–2,184.

Restoration of coronary perfusion reduced TR-FGF2 penetration depth more than twofold to 28 μm, localizing growth factor closer to the epicardial drug source (Fig. 21.10a). A fit of the growth factor distribution in the presence of coronary flow to a declining exponent (21.27) provided the estimate $l_D = 13.5$. Combining the estimated diffusivity and penetration length provided the estimate $k = 1.15 \pm 0.06 \times 10^{-4}$ s^{-1}. Since data fits of drug distribution profiles to exponential functions and error functions do not constitute a rigorous test of model validity, we also verified that the model with the estimated diffusivity and clearance rate constant of FGF2 correctly predicts growth factor outflow kinetics (Fig. 21.10b).

Though capillary flow clearly limits drug distribution, even in the absence of capillary clearance myocardial distribution of FGF is limited to the vicinity of the inlet at 6 h. To quantify the influence of capillary clearance in the context of drug diffusion we therefore introduced a time dependent clearance modulus defines as the percent decrease in total uptake due to clearance

$$\%\text{Drug cleared} = 100 \times \left(\frac{M_{k=0}(t) - M_k(t)}{M_{k=0}(t)} \right). \tag{21.28}$$

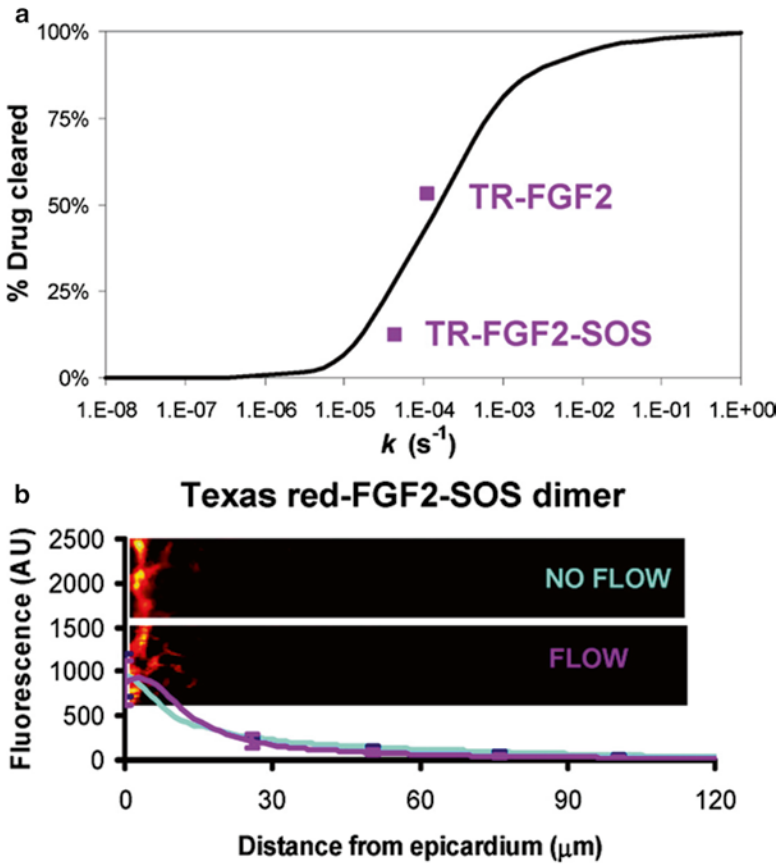

Fig. 21.11 Growth factor distribution is sensitive to the clearance rate constant. (**a**) Percentage of capillary-cleared drug as a function of clearance rate constant, k (*black line* (21.29)). Experimental data points for TR-FGF2 and TR-$(FGF2)_2$-SOS analyzed by (21.11) and (21.27) are superimposed (*magenta squares*) on model predictions providing perspective on the sensitivity of FGF2 to manipulation of its clearance constant. (**b**) Distribution and representative fluorescence microscopy images of TR-$(FGF2)_2$-SOS in rat myocardium with coronary (*magenta*) and without (*blue*) coronary perfusion. Data represent mean ± s.e.m ($n = 3$). Reproduced with permission from Le et al. [17]

Here $M_{k=0}$ and M_k denote, respectively, tissue content without and with coronary flow. Interestingly, the model predicts that the clearance modulus is independent of the magnitude of the diffusivity and increases over time

$$\%\text{Drug cleared} \approx 100\times\left(1-\frac{\lambda_D \operatorname{erf}\sqrt{kt}}{\sqrt{4Dt/\pi}}\right)=100\times\left(1-\frac{\operatorname{erf}\sqrt{kt}}{\sqrt{4kt/\pi}}\right). \quad (21.29)$$

A plot of this predicted dependence (Fig. 21.11a) suggests that percentage clearance of drug with coronary perfusion is most sensitive for clearance rate constants

ranging between 1×10^{-5} and 1×10^{-2}. Notably, the estimated clearance rate constant of TR-FGF2 falls within this range, suggesting that FGF delivery can be optimized by strategies that reduce its clearance rate constant and sustain its delivery to compensate for slow tissue diffusion.

To examine whether the myocardial distribution of FGF might be modulated by altering its molecular weight, we contrasted the distribution of TR-FGF2 alone or in association with sucrose-octasulfate (SOS). SOS was used as it induces FGF dimerization and increases the effective molecular weight of TR-FGF2 [63]. The increase in size was confirmed by size-exclusion chromatography, and should reduce transendothelial permeability, capillary washout, and effective diffusivity. Indeed, in the absence of coronary perfusion, TR-$(FGF2)_2$-SOS penetrated 40 μm into the myocardium, 40 % less than TR-FGF2 under the same conditions and reflecting a 37 % smaller diffusion coefficient ($D = 0.013 \pm 0.001$ μm^2 s^{-1}). Notably, according to Swabb's correlations [25] an increase in molecular weight from 17 to 35 kDa is expected to reduce diffusivity by 42 %, suggesting that dimerization by SOS does not interfere with FGF binding to HSPG. Though the larger compound diffused more slowly it was also less affected by coronary perfusion (Fig. 21.11b), with penetration depth falling only 26 % to 30 μm and total deposition falling by only 12 %. The muted sensitivity of TR-$(FGF2)_2$-SOS to flow (Fig. 21.11a) is consistent with a 62 % reduction in the clearance rate constant ($k = (4.37 \pm 0.33) \times 10^{-5}$ s^{-1}) compared to undimerized FGF and model predictions. These data suggest that the reduction in drug diffusivity with increasing molecular weight may largely negate the benefits of a reduced capillary clearance rate constant. Thus, more specific strategies of decreasing capillary clearance should be explored, such as the manipulation of drug charge. In lieu of such strategies, microvascular clearance can pose a formidable barrier to drug distribution that can only be surmounted by delivering drug carriers uniformly throughout the tissue by injection or some other means.

Drug Binding: A Dose Dependent Barrier to Drug Distribution

Drug transport in tissues is hindered by steric interactions but also by binding interactions with tissue proteins. Such binding interactions may include specific high affinity binding of growth factors or antibodies to cognate receptors on cell surfaces, and nonspecific attraction between hydrophobic groups on drugs and tissue proteins. Both types of binding interactions are increasingly encountered in the context of drug delivery to solid tissues as therapies frequently rely on receptor binding interactions to ensure targeting specificity [64], and interactions with extracellular matrix proteins are relied upon to improve drug retention [65, 66]. Whereas increased antibody affinity correlates with greater and more sustained antigen binding in cell culture, the in vivo benefits of affinity maturation are not clear. Binding does mediate

drug retention [39, 67] but also may act as a barrier to drug distribution [49, 68, 69] since drug that is bound to immobile tissue proteins is not available for convection and diffusion. Consequently, tissue uptake and penetration of the same systemic dose of antibody may scale inversely with antibody affinity [68–71].

However, drug affinity tells only part of the story as drugs with similar affinities can display significantly different retention and transport hindrance. Thus, for example, heparin, paclitaxel, and rapamycin all bind to arterial extracellular matrix with micromolar affinities [18, 72], yet heparin is much more weakly retained in the arterial wall than paclitaxel or rapamycin as the latter compounds bind tissue proteins avidly and heparin does not [67]. Similarly, IgG and the EGFRvIII specific single chain antibody fragment bind to solid tumors with similar binding affinities, yet the transport of the former is only slightly affected by such binding [20], whereas the latter is dominated by it [69, 73]. In estimating the impact of binding on drug transport and retention, molecular affinity must be scaled with the binding capacity. We introduce such scaling in the next section and illustrate how this parameter determines the dose dependence of drug retention and transport hindrance.

Quasi-Equilibrium Transport and Clearance Equations

To illustrate the fundamental concepts we consider the case wherein soluble drug reversibly binds to a single type of immobilized site in the tissue. Denoting the concentration of free and bound drug as C and B we consider transport to be subject to the following equations [18, 56, 74]

$$\frac{\partial C}{\partial t} - D\frac{\partial^2 C}{\partial x^2} + R_F \mathbf{v}\frac{\partial C}{\partial x} = -kC - \frac{\partial B}{\partial t} \tag{21.30}$$

$$\frac{\partial B}{\partial t} = \varepsilon^{-1} k_f \left[C\left(B_{max} - B\right) - K_d B \right]. \tag{21.31}$$

We assume here that binding is bimolecular with a forward rate constant k_f, dissociation constant K_d, and binding site density B_{max}. We further assume that soluble drug is restricted to a volume fraction $\varepsilon \leq 1$ to allow for steric and or hydrophobic restrictions [72, 75]. Finally, where others have also accounted for receptor mediated degradation of antibodies in (21.31) [20, 68, 69], we neglect this term which is unimportant for small chemotherapeutics.

For binding to hinder transport, the time scale of drug–receptor binding

$$t_B \approx \frac{1}{\varepsilon^{-1} k_f B_{max}} \tag{21.32}$$

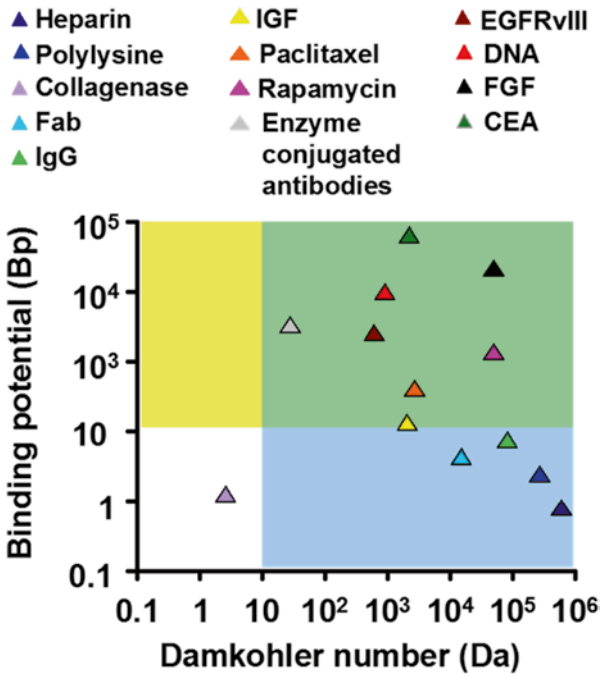

Fig. 21.12 Classification of drug-tissue pairs according to the magnitudes of the diffusion Damköhler number and the binding potential. Damköhler numbers estimate the degree to which transport prolongs binding in the tissue, and tend to be larger (*green* and *blue zones*). The binding potential estimates the tissue's propensity to bind and retain drug and can be low (*white* and *blue*) or high (*yellow* and *green*) independent of the magnitude of the Damköhler number. Binding poses a barrier to drug penetration in the green zone of large binding potentials ($B_p > 10$) and transport limited binding ($Da > 10$). Reproduced with permission from Tzafriri et al. [18]

must be shorter than the time scales of diffusion and convection. The validity of this condition can be assessed through the evaluation of the Damköhler numbers of diffusion

$$\mathrm{Da}_{diff} \equiv \frac{t_D}{t_B} \approx \frac{\varepsilon^{-1} k_f B_{max}}{D / L^2} \tag{21.33}$$

and convection

$$\mathrm{Da}_{conv} \equiv \frac{t_v}{t_B} \approx Pe \cdot \mathrm{Da}_{diff}. \tag{21.34}$$

Small Damköhler numbers may arise in highly porous gels [76] or when the transport path in the tissue is very small. On the contrary, drug binding to most tissues, including arterial tissue and various tumors, is usually characterized by large diffusion Damköhler numbers (Fig. 21.12) implying that binding is diffusion-limited.

When the Damköhler numbers of convection and diffusion are large the concentrations of bound and free drug coexist in a quasi-equilibrium such that

$$B \approx \frac{B_{max} C}{\varepsilon K_d + C}. \tag{21.35}$$

The total local concentration of drug in the tissue, T, is then a function of the local concentration of free drug

$$T(C) \equiv B + C = \frac{B_{max} C}{\varepsilon K_d + C} + C. \tag{21.36}$$

Since the right hand side of (21.36) is an increasing function of free drug, it is invertible as

$$C(T) = \frac{1}{2}\left[-\left(B_{max} + \varepsilon K_d - T\right) + \sqrt{\left(B_{max} + \varepsilon K_d - T\right)^2 + 4\varepsilon K_d T} \right]. \tag{21.37}$$

Thus, the equations of transport (21.30) and binding (21.31) can be combined into a single transport equation written in terms of the total local concentration

$$\frac{\partial T}{\partial t} - \frac{\partial}{\partial x}\left(D_{eff}(T) \frac{\partial T}{\partial x} \right) + v_{eff}(T) \frac{\partial T}{\partial x} = -k_{eff}(T) T. \tag{21.38}$$

This equation is analogous to the transport equation for the soluble drug (21.30) though now drug diffusivity, velocity and clearance rate constant are all nonlinear functions of the total concentration, respectively

$$D_{eff}(T) / D = v_{eff}(T) / v = \left[1 + B_P / \left(1 + C(T) / \left(\varepsilon K_d\right)\right)^2 \right]^{-1}, \tag{21.39}$$

and

$$k_{eff}(T) / k \equiv C(T) / T. \tag{21.40}$$

Here B_p is the binding potential of the drug: tissue pair

$$B_P \equiv k_f \varepsilon^{-1} B_{max} / k_r = B_{max} / \left(\varepsilon K_d\right) \tag{21.41}$$

The significance of the binding potential as a measure of binding strength is evident from its interpretation as the ratio of the time scales of binding dissociation $1/k_r$ and binding association $\varepsilon/(k_f B_{max})$. Thus, a large binding potential implies that drug–receptor binding is fast relative to dissociation of the bound drug, guarantying the stability of the drug–receptor complex. Notably, unlike the diffusion Damköhler number which is large for most drug delivery scenarios (Fig. 21.12), the magnitude of B_p varies significantly with drug and tissue type (Fig. 21.12).

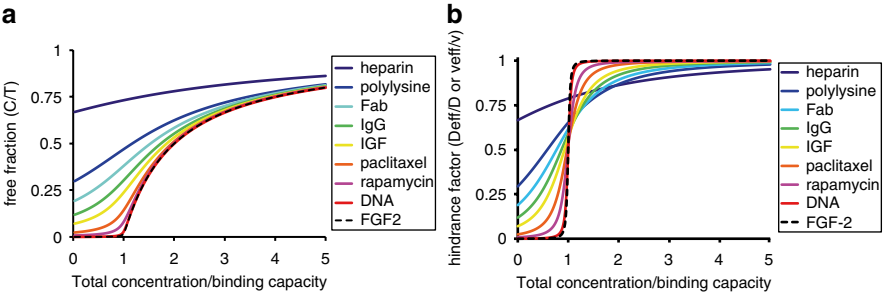

Fig. 21.13 Dose dependence of drug transport and retention is determined by the magnitude of B_p. The fraction of free drug (**a**) and the hindrance factors of diffusion and convection (**b**) are plotted as a function of the total local drug concentration relative to the density of binding sites for a range of drugs and tissues. Drugs are color coded according to their B_p with cold colors (e.g., *blue*) designating small B_p values. Adapted from Tzafriri et al. [18] with permission

Dose Dependence of Drug Transport and Microvascular Clearance

The overarching importance of the binding potential as a determinant of drug retention can be appreciated by plotting the fraction of free drug C/T as provided by (21.37), and scaling the total local concentration of drug (T) to the concentration of binding sites (B_{max}). Such a plot reveals that the fraction of free drug that can be cleared by microvessels is determined by the magnitude of B_p (Fig. 21.13a). Drugs with low B_p such as heparin ($B_p < 1$) are predominantly free regardless of the applied concentration and its duration and therefore relatively sensitive to interstitial clearance mechanisms. Drugs with large B_p such as paclitaxel ($B_p = 40$) and rapamycin ($B_p = 140$) are predominantly bound at states of excess binding sites ($T/B_{max} < 1$) and the fraction of free drug increases appreciably only as the total concentration exceeds the binding capacity ($T/B_{max} > 1$). This analysis correctly captures the differential retention properties of heparin, paclitaxel and rapamycin in arterial tissue, and illustrates that retention is not solely predicted by affinity, as binding capacity and drug dose must also be considered. A practical implication of the sharp dose dependence at large binding potentials, is that drug that is delivered in excess of the concentration of binding sites in the tissue will be cleared more quickly than drug that is bound and must first dissociate. For low binding potential drugs, binding does not confer much of a retention advantage.

As tissue-bound drug is immobilized, the magnitude of B_p also determines the impact of binding on drug transport (Fig. 21.13b). At one extreme are growth factors and antibody fragments with huge binding potentials (>1,000) whose effective diffusivity and convection is at least 3-log orders larger at drug excess ($T > B_{max}$) than at receptor excess ($T \ll B_{max}$). At the other extreme of weakly retained drugs ($B_p < 1$) we find heparin whose effective arterial diffusivity increases by no more

than 30 % with total concentration. Thus, at large B_p, transport and retention both display a pronounced dose dependence that undergoes a qualitative change as total drug concentration achieves parity with the concentration of binding sites. In terms of free drug, total drug concentration is equal to the density of binding sites at the threshold concentration

$$C_{th} \equiv \varepsilon^{-1}\sqrt{B_{max}\varepsilon K_d} = K_d B_p^{1/2}. \tag{21.42}$$

Such a well defined threshold concentration exists only at large B_p (Fig. 21.13a), and is at once much larger than the binding dissociation K_d and much smaller than the binding capacity B_{max}. These dependencies have direct implications for the systemic delivery of drugs with large B_p. At subthreshold plasma concentrations ($C < C_{th}$) transport in the tissue will be strongly hindered, though this may not be evident from the shape of the distribution patterns as the effective transport parameters display a graded concentration-dependence (Fig. 21.13a, b). Indeed, sufficiently far from saturation the influence of binding is limited to a rescaling of the transport parameters by a constant factor

$$D_{eff} / D = v_{eff} / v = k_{eff} / k \approx 1/\left(1 + B_P\right), \quad C \ll C_{th}. \tag{21.43}$$

On the contrary, at supra-threshold plasma concentrations ($C \geq C_{th}$) interstitial transport is weakly hindered near the inlet, but very strongly hindered at greater depths where drug concentrations are no longer saturating. Thus, at saturating plasma concentrations, binding sites within the tissue are saturated up to a well defined front that extends deeper into the tissue with time and increasing surface concentration (Fig. 21.14). These trends set the binding barrier apart from the clearance barrier, as the latter limits steady-state penetration whereas the former can be overcome by sustained delivery of sufficiently high doses.

Rate Dependence of Drug Transport Receptor Saturation

In contrast to systemic drug delivery modalities which control the concentration of luminally delivered drug, local delivery modalities control the rate at which drug is delivered to the tissue. Intuition suggests that the concentration of drug at the delivery site should increase with increasing rates of drug delivery, and therefore that the nonlinear dose dependence of the effective transport parameters will manifest as a nonlinear dependence on the rate of drug delivery to the tissue. Indeed, our modeling studies show that at large binding potentials, tissue penetration exhibits a threshold dependence on the rate of drug delivery. This issue is of practical significance as it suggests that the dose and duration of in vivo delivered drug cannot be solely guided by cell culture studies [77, 78] as within a tissue, distribution limitations may arise at low rates of drug delivery. In the extreme of large binding potentials, our modeling suggests that saturation of tissue receptors will display step-like

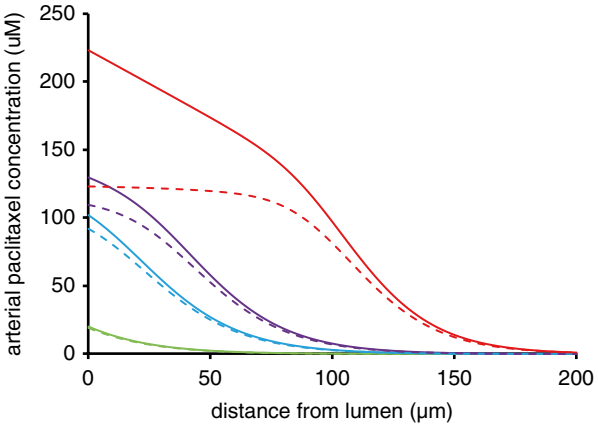

Fig. 21.14 Simulated concentration profiles of total drug (*solid lines*) and bound drug (*dashes*) within an arterial wall following luminal exposure to constant drug concentrations, $C_p/C_{th}=0.05$ (*green*), 0.5 (*blue*), 1.0 (*purple*) and 5.0 (*red*). At subthreshold surface concentrations total drug (*lines*) and bound drug (*dashes*) are synonymous and concentration profiles are graded. All simulations assume 3 min drug exposures without a pressure gradients (e.g., $v=0$). Transport parameters are based on estimates for paclitaxel in arterial tissue: $\varepsilon=1$, $D=60$ **μ**m^2 s^{-1}, $k=0$, $B_{max}=127$ **μ**M, $K_d=3.12$ **μ**M. Thus, $B_p=40$ and $C_{th}=19.9$ **μ**M

dependence on delivered dose, rate and duration, and may provide a mechanistic underpinning for a binary dependence of in vivo efficacy on these parameters of drug delivery [11, 18].

These phenomena are illustrated below for local delivery devices that release drug with diffusion controlled Higuchi-type kinetics, $dM/dt=Q/t^{1/2}$. Scaling analysis predicts the existence of a sharp binding-saturation front when the Higuchi parameter Q exceeds a threshold value [18]

$$Q_{th} = B_{max}\sqrt{D/2}\times\left(\frac{B_p}{1+\varepsilon^{-1}K_{ns}}\right)^{1/4}. \tag{21.44}$$

Here $K_{ns}\geq 0$ is the binding potential of nonspecific sites in the tissue. Figure 21.15 demonstrates the relevance of the predicted threshold rate for sirolimus eluting tents. Sirolimus (rapamycin) and its analogs are small and highly lipophilic drugs and due to their wide therapeutic window have emerged as the drugs of choice for elution for the inhibition of stent induced intimal hyperplasia. Unlike antibodies and growth factors whose cognate receptors are located on the cell's surface, sirolimus and paclitaxel belong to a class of small hydrophobic drugs that exert their effect through high affinity bimolecular binding to specific intracellular proteins, respectively, FKBP12 and assembled microtubules. For the purposes of this discussion, these specific intracellular drug targets operate as receptors and their dynamics of binding can be described by (21.31). Equation (21.44) predicts $Q_{th}=1$ ng cm^{-2} s$^{-1/2}$

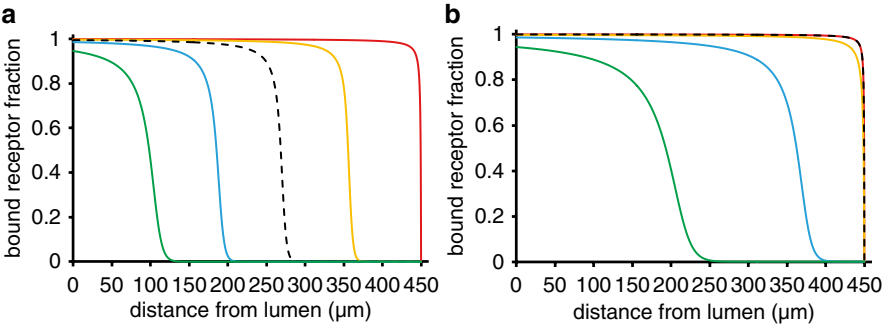

Fig. 21.15 Simulated profiles of bound receptors as a function of time and rate of drug delivery. Arterial distributions are depicted at 6 h (**a**) and 24 h (**b**) for a range of Higuchi release parameters, $Q/Q_{th}=0.2$ (*green*) 0.5 (*blue*) 1.0 (*black dashes*) 2.0 (*orange*) or 6.8 (*red*). Simulations of (21.30) and (21.31) with perfect sink outlet conditions used parameter values corresponding to the in vivo transport and binding of sirolimus in the arterial wall [11]: $\varepsilon=1$, $k=0$, $D=200$ μm^2 s^{-1}, $v=0.058$ μm s^{-1}, $B_{max}=3.3$ μM, $K_d=0.2$ nM and nonspecific binding potential of $K_{ns}=140$. $Q/Q_{th}=6.8$ approximates sirolimus the delivery rate from NEVO™

for sirolimus in arterial wall, 6.8-fold lower than the rate of delivery by the NEVO™ Sirolimus Eluting Coronary Stent [11]. Simulated distributions of sirolimus-bound receptors at 4 h post stent implantation exhibit a saturation zone that extends up to a well defined front (Fig. 21.15a). At $Q=Q_{th}$ the front extends 2/3 into the arterial wall, while sirolimus delivery with $Q=6.8\,Q_{th}$ is predicted to saturate receptors throughout the wall. By 24 h, receptors are predicted to be entirely saturated by $Q>Q_{th}$, but only 50 % saturated by $Q=0.2\,Q_{th}$ (Fig. 21.15b). Thus, when drug is delivered at saturating rates ($Q\geq Q_{th}$) sustained delivery can compensate for slow tissue distribution and saturate receptors long before the entire drug load is delivered. This no longer seems to be the case at subsaturating rates of delivery. For example, at $Q=0.2\,Q_{th}$ simulations predict that the receptor saturation zone doubles between 4 (Fig. 21.15a) and 24 h (Fig. 21.15b), but receptor-saturation across the artery wall is predicted to occur only 7 days post elution.

Rate Dependence of Drug Retention

The dynamics of drug distribution and tissue content reflect the evolving balance between transport and clearance. Drug penetration and accumulation reflect the dominance of delivery over clearance early on, whereas a decline in tissue content reflects the reverse. In the case of drugs with large binding potential where drug penetrates up to a well defined front, the transition between states of delivery dominance and states of clearance dominance is abrupt. In the example of sirolimus delivery to the arterial wall, clearance forces only appear once the receptor saturation front reaches the perivascular aspect (e.g., the outlet). Subsequently, the flux of

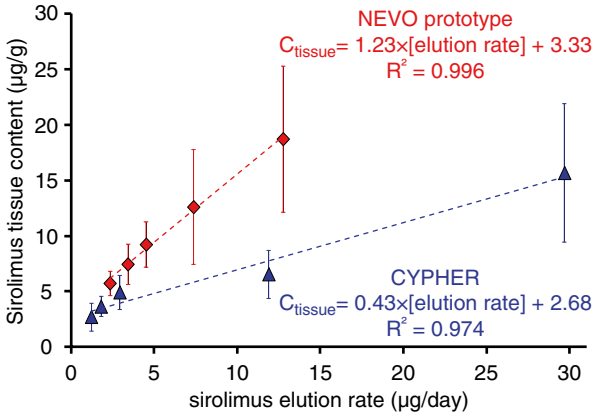

Fig. 21.16 Post peak tissue content tracks the rate of drug delivery to the artery wall. Arterial sirolimus content (*symbols*) is rendered linear when plotted against the in vivo rate of elution of two types of sirolimus eluting stents: prototype NEVO™ Stents (*red*) and CYPHER® Stent (*blue*). The difference in slope speaks to a difference in the efficiency of drug transfer to the artery that is related to the degree of stent: tissue contact, yet both curves intersect the vertical axis at the same concentration, providing the estimate $B_{max} = 3 \pm 0.3$ **µ**g g^{-1}. Reproduced with permission from Tzafriri et al. [11]

free drug equalizes throughout the tissue and is equal to the delivered flux; average concentration of free and nonspecifically bound drug in the tissue is then proportional to the rate of delivery, while average concentration of specifically bound drug is equal to the density of receptors. Thus, during the clearance phase, the average concentration of drug in the tissue C_{tissue} linearly tracks the rate of drug delivery as

$$C_{tissue} = B_{max} + \left(1 + K_{ns}\right) G\left(Pe\right) \times \left(\text{drug delivery rate}\right) \tag{21.45}$$

where $G(Pe)$ is a function of tissue density, fluid filtration, and drug diffusivity [11]. A plot of tissue content versus the rate of in vivo drug release can therefore identify the onset of the clearance phase and also be used to estimate the density of receptors (Fig. 21.16).

The existence of a clearance dominated regime during which free drug in the tissue closely tracks the rate of drug delivery reflect a fundamental balance between the rates of drug delivery into tissue and drug clearance, and transcends the specific geometry and even the specific clearance mechanisms. For example, we observed analogous dynamics in the study of microsphere-based intratumoral delivery of paclitaxel [74]. Simulations of a model of intra-tumoral drug release, transport, binding, and microvascular clearance [(21.30) and (21.31)] predicted that drug delivery dominates during an accumulation and receptor-saturation phase of 1–96 h

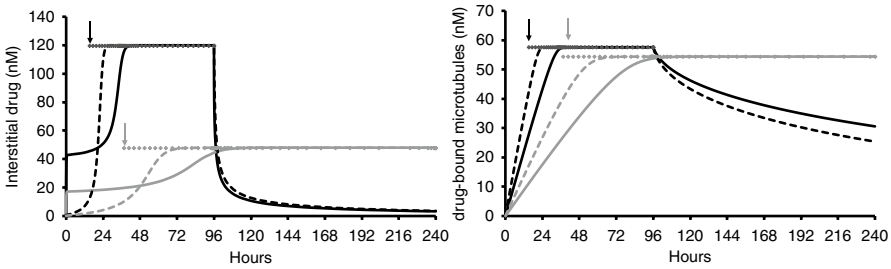

Fig. 21.17 Simulated average intratumoral concentrations of free interstitial drug (**a**) and microtubule-bound drug (**b**) as provided by homogeneously distributed microspheres that release a given paclitaxel load at a constant rate for 96 h (*black*) or 240 h (*gray*). Simulations accounted for interstitial diffusion, extracellular binding, microvascular clearance, reversible exchange of free interstitial and free intracellular drug, and intracellular binding to microtubules. The depicted results are for a representative volume element in the bulk of the tissue with negligible convection ($v=0$), $\varepsilon=1$, $D=10$ μm^2 s^{-1}, $k=36$ h^{-1}, $B_{max}=60$ μM, $K_d=4.9$ nM and cell-uptake rate constants $\alpha=64.8$ h^{-1} (*solid lines*) or 64,800 h^{-1} (*dashes*). *Diamonds* denote the steady state concentrations implied by (21.46) and *arrows* denote the time at which released drug would be bound in the absence of drug clearance and transport limitations. *Panel* (**a**) is reproduced from Tzafriri et al. [74] with permission

and that post-peak content of free drug in the tissue quasi-statically tracks the rate of drug delivery (Fig. 21.17).

For zero order intratumoral release kinetics, the average concentration of free interstitial drug during the clearance phase is equal to the ratio of zero order rate of drug release (zo) and the microvascular clearance rate constant

$$C \approx \frac{\mathrm{zo}}{k}. \tag{21.46}$$

Namely, though drug distribution dynamics during the accumulation and receptor-saturation phase are a complex function of transport, cell permeability, binding, and microvascular clearance, post peak drug dynamics are much simpler and predictable functions of the rate of late drug release. Free interstitial drug is the determinant of receptor binding, and in analogy to extracellular drug levels in cell culture is expected to be correlated with time dependent drug effects [77]. The post-peak relationship between free interstitial drug levels, release rate and the clearance rate constant (21.46) therefore provides a predictive design criterion for efficacious intratumoral drug delivery. The implication is that in vivo, there exists an optimum range of zero order drug release rates (Fig. 21.18) that are sufficiently slow to ensure that receptors are saturated within a time span that is short relative to the duration of drug release, but also sufficiently high to overcome microvascular clearance and achieve efficacious levels of free and bound drug.

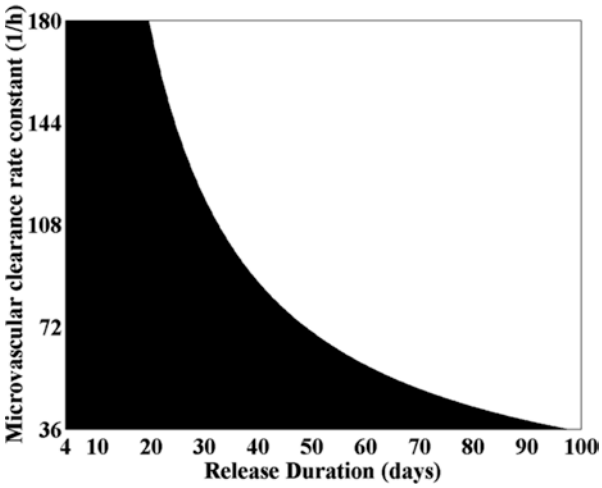

Fig. 21.18 In vivo efficacy of intratumoral drug release is determined by a balance of release duration and microvascular clearance. The *black zone* denotes the combinations of variables for which simulations predict efficacy due to the maintenance of interstitial drug concentration >100 nM for >100 h [77]. The *white zone* denotes variable combinations that are predicted to result in suboptimal efficacy due to sub-threshold interstitial drug concentrations, or sub-threshold durations of efficacious concentrations. Reproduced from Tzafriri et al. [74] with permission

Conclusions

The coupling of quantitative experiments and computational models of drug transport, binding, and clearance provides a powerful paradigm by which to understand the performance of drug therapies and develop strategies for overcoming distribution barriers in solid tissues. Though there are certainly limitations to the continuum pharmacokinetics-pharmacodynamics framework, it has provided a quantitative description in numerous drug delivery settings and has correctly captured the nature of the various barriers to drug distribution, alone or when coupled. As we have attempted to illustrate here, the coupling of convection, diffusion, sequestration, and clearance mechanisms can result in new dynamics, and the influence of coupling can be prioritized on the basis of dimensionless ratios of typical time or length scales. These criteria provide an important intuitive complement to experimental and computational probing of the complex dynamics of drug distribution in solid tissues.

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Chapter 22
Intravital Real-Time Confocal Laser Scanning Microscopy for the In Situ Evaluation of Nanocarriers

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Abstract Visualization of drug/gene nanocarriers within living tissue is essential for optimization towards clinical applications. In this regard, we have established an intravital real-time confocal laser scanning microscopy (IVRTCLSM) technique with both spatial and temporal resolution for in situ evaluation of nanocarriers.

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In this chapter, we describe the actual setup of the IVRTCLSM in detail and review several examples analyzing the behavior of nanocarriers. Our new imaging technique can elucidate mechanisms that have not been clarified by conventional methods that require tissue to be disturbed or manipulated ex vivo. IVRTCLSM can thereby ascertain critical barriers residing in a living body and facilitate the development of nanocarriers optimized for drug/gene delivery.

Introduction

Targeting tumors with long-circulating nanocarriers is a promising strategy for systemic cancer treatment. The development of therapeutic drug nanocarriers starts with polymer design and synthesis, followed by physical and structural characterization and biological evaluation. It is difficult to extrapolate the in vivo outcomes of a nanocarrier from its in vitro behavior because nanocarriers encounter numerous barriers en route to their target during the processes of blood circulation, extravasation, penetration, and cellular uptake [1, 2]. Pharmacokinetic studies are indispensable for developing and optimizing efficient nanocarriers that transport drugs specifically to the targeted tissue. Pharmacokinetic studies using animals have primarily been conducted by analyzing blood and urine samples or resected tumor tissues. These ex vivo techniques have been well established for analyzing blood circulation, target accumulation, or other pharmacological profile of the nanocarriers. However, this approach provides only static information at specific time points.

Intravital imaging has attracted significant attention in recent years because it can elucidate complex biological and pathological events within a living animal. Recent advances in imaging technologies that facilitate the in vivo validation of nanocarriers include whole-body imaging systems and in vivo confocal microscopy. Whole-body imaging systems for small animals such as positron emission tomography, single-photon emission computed tomography, computed tomography, magnetic resonance imaging, and fluorescence/luminescence imaging are commercially available and widely used. However, these whole-imaging systems cannot provide cellular/subcellular resolution.

Intravital Real-Time Confocal Laser-Scanning Microscopy

We have established IVRTCLSM with both spatial and temporal resolution for the in situ evaluation of nanocarriers [3] (Fig. 22.1). IVRTCLSM provides instant histopathology at the cellular and subcellular levels in living animals. Therefore it is ideal for investigating dynamic and complex events such as blood circulation, site-specific drug accumulation, and subcellular trafficking.

IVRTCLSM consists of the following essential features:

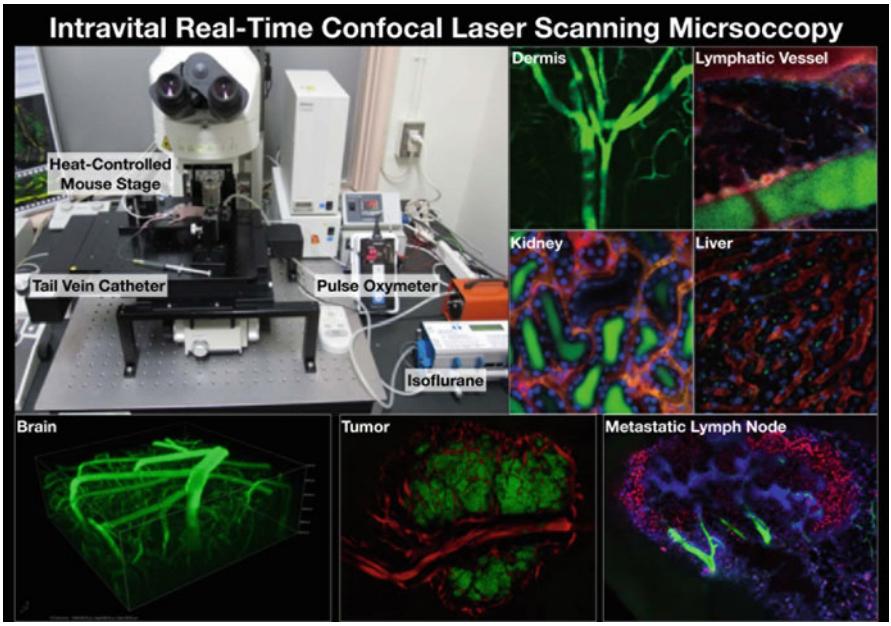

Fig. 22.1 Intravital real-time confocal laser scanning microscopy (IVRTCLSM)

High-Speed Scanning to Minimize Motion Artifacts

There is constant movement in live animals due to heartbeats, breathing, intestinal peristalsis, and other activities. Conventional galvano scanners are too slow and can only provide insufficient, blurred images. IVRTCLSM utilizes a Nikon A1R confocal laser scanning microscope system, which incorporates both a conventional galvano scanner and a high-speed resonant scanner together. The resonant scanner is capable of obtaining images in variable frame rates ranging from 30 fps at 512×512 pixels to as fast as 420 fps in the band scan mode. Several rapid scanning confocal microscopes are now commercially available (Table 22.1).

Upright Configuration for Flexible and Unrestricted Access

Although all vendors primarily recommend the use of their products with an inverted configuration (optimized for live cell imaging), the use of an upright microscope has advantages for intravital imaging. The confocal unit is attached to an upright ECLIPSE FN1 (or recently released ECLIPSE Ni).

Table 22.1 Commercially available rapid scanning confocal microscopes

Vendor	Product name	Scanning system	Maximum frame rate at 512×512 pixels	Number of simultaneously detectable channels
Nikon	A1R+	Resonant scanner	30 fps	4
Leica Microsystems	TCS SP8	Resonant scanner	40 fps	5
Carl Zeiss	LSM 7 LIVE	Linear scanner	120 fps	2
Yokogawa Electric	CSU-X1	Nipkow spinning disk	2,000 fps	3
Olympus	DSU	Spinning disk confocal	15 fps	1

Modification of the Microscope to Accommodate Small Animals Under the Objective Lens

Modification of the upright microscope is necessary for intravital imaging because it is originally designed for imaging thin-sectioned slices. The transillumination unit (halogen lamp, condenser, substage, and turret) is removed, as confocal imaging does not require transmitted light. This allows more space between the microscope stage and the objective lens. The motorized stage is set as low as possible onto a customized framework, and a custom-designed height-adjustable mouse stage is fixed onto the motorized stage. A small temperature controller pad is integrated to the mouse stage to accommodate long-term anesthesia.

Exposure Surgery with Little or No Bleeding to Facilitate Optical Access to Multiple Tissues and Organs

The earlobe is an excellent location for IVRTCLSM because blood vessels can be readily accessed through the thin dermis without surgery (Fig. 22.2a). To observe tumor tissue, we use several techniques depending on the purpose of the experiment. The simplest method is to inoculate tumor cells into the earlobe. This technique has the advantage of imaging the tumor as easily and noninvasively as possible. Additionally, observation of both earlobes (target tumor site and off-target normal skin tissue) is possible by using the motorized stage (Fig. 22.2b). Skin flap elevation is another common procedure to exteriorize a subcutaneous tumor (Fig. 22.2c). An arc-shaped incision is created around the subcutaneous tumor, and the skin flap is elevated without injuring the feeding vessels. The skin flap is everted and stretched using several bent 30-gauge needles. This technique, albeit technically demanding, provides clearer images than inoculating the earlobe with a tumor because the skin layer is eliminated. Other tissues and organs such as the brain, liver, kidneys, lymph nodes, and lymphatic vessels are exteriorized through surgery.

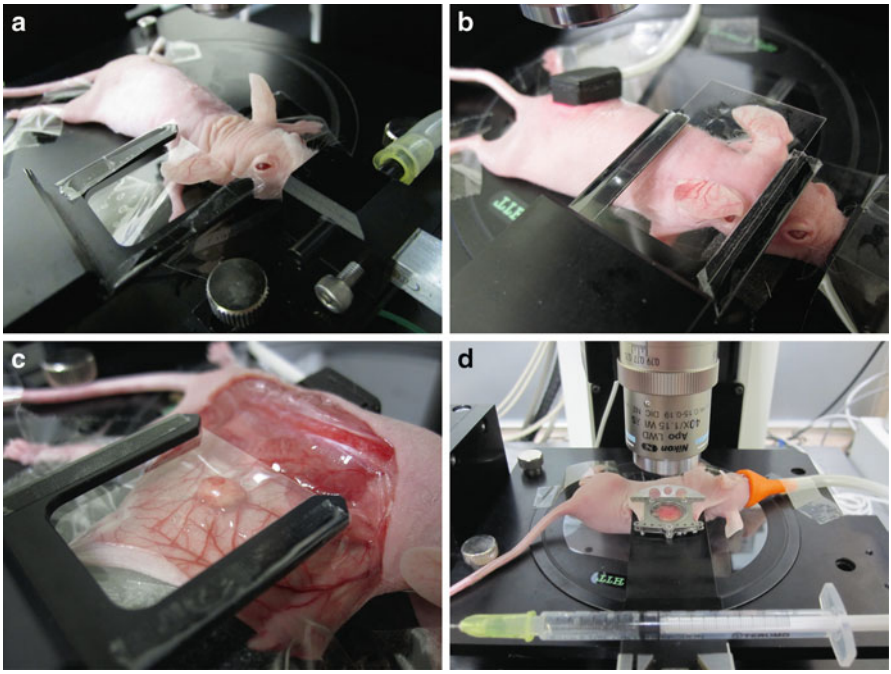

Fig. 22.2 (**a**) Setup for the earlobe dermis observation. The earlobe was attached to the coverslip with a small drop of immersion oil. (**b**) Tumor cells were inoculated in the right earlobe and both earlobes were attached to the coverslip. The motorized stage was configured to move back and forth to obtain images of the target tumor site and off-target skin tissue. (**c**) Skin flap technique. (**d**) Dorsal skinfold chamber technique

To minimize bleeding during the surgical procedure required to present tumors for imaging, a radio frequency surgical device equipped with a wire electrode is used for bloodless micro-smooth incisions with minimal tissue alteration.

Stabilization of the Sample to Isolate It from Body Movement Without Compressing the Blood Vessels

The mouse is directly placed on the mouse stage. A custom-designed, height-adjustable coverslip holder is placed onto the tissue of interest to provide a flat surface for the objective lens. The coverslip must be attached perpendicular to the objective lens with adequately adjusted pressure to flatten the tissue without suppressing the blood flow. Alternatively, the tumor can be embedded into the dorsal skinfold chamber (Fig. 22.2d) [3]. Titanium frames are surgically implanted and tightly immobilized under the objective lens. This technique is extremely useful when long-term time-lapse observation is necessary.

Tail Vein Catheterization for Timed Injection During Data Acquisition Without Moving the Subject

Lateral tail vein catheterization allows multiple and timed injections without moving the animal during data acquisition. Furthermore, the use of a syringe pump provides precise and extended drug delivery. The catheterization technique has been described elsewhere (http://imaging.bme.ucdavis.edu/files/2011/02/Mouse_Tail_Vein_Catheter_Procedure_Rev22.doc).

Examples Demonstrating the Practical Application of IVRTCLSM

Observation of Mouse Earlobes Is a Convenient Alternative to Conventional Plasma Clearance Studies

During the development of a promising drug delivery system, there is a strong need to accurately grasp the intravital behavior of the administered drugs. Stability in the blood compartment can be evaluated using IVRTCLSM [3]. Without surgery, the earlobe dermis can be observed and easily fixed beneath a coverslip with a single drop of immersion oil.

The influence of molecular weight on pharmacokinetic behavior was investigated using fluorescein (MW = 332) and fluorescein-labeled dextrans (FDs) with average molecular weights of 10, 40, and 500 kDa (Fig. 22.3). Fluorescein and FDs exhibited different pharmacokinetics. The arterial entrance was observed 10 s after injection, followed by venous migration 30 s after injection. Fluorescein diffused into the extravascular tissue concurrently with venous migration. FD, 10 kDa, gradually translocated into extravasculature tissue 10–15 min after injection, and lymphatic drainage was observed after 20 min. FD 70 and 500 kDa remained in the vasculature during the entire 60-min observation period.

This technique is superior to conventional methods used to study plasma clearance concerning the number of animals needed to generate a clearance curve and the ability to obtain more information from a single experiment. Conventional protocols used in plasma clearance studies require blood extraction at various postinjection time points and multiple animals. However, IVRTCLSM yields 30 time points/s before, during, and after the injection. Moreover, our technique provides spatial resolution, thus allowing individual investigation of multiple regions such as arteries, veins, extravascular tissue, lymphatic vessels, and even cells and nuclei if necessary. These blood circulation studies are frequently conducted in our laboratory to clarify the effects of nanocarrier modification, such as PEGylation [4], hydrophobic stabilization [5], disulfide cross-linking [6], and 2-iminothiolane modification [7].

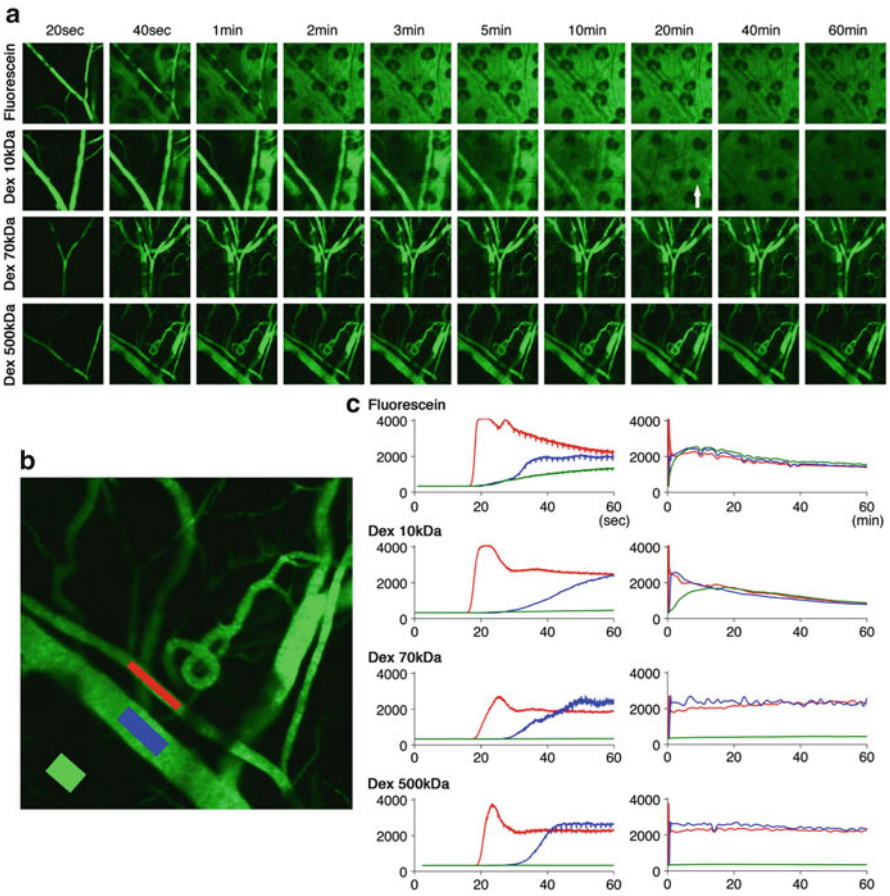

Fig. 22.3 Observation of mouse earlobes is a convenient alternative to conventional plasma clearance studies. (**a**) Fluorescein, fluorescein-labeled dextrans (FD) 10, 70, and 500 kDa were administered via a tail vein catheter 10 s after movie acquisition was initiated. Video-rate (30 fps) movies were recorded for the first min, and subsequent time-lapse images were recorded every min for an additional 60 min. The *arrow* indicates lymphatic drainage. (**b**) Three regions of interest (ROIs), an artery (*red*), vein (*blue*), and extravascular skin tissue (*green*), were selected. (**c**) The fluorescence intensities in these ROIs were plotted against time. Permission was obtained from The Optical Society © [3]

Direct Visualization of Aggregate Formation of Polyplexes

Surface modification using poly(ethylene glycol) (PEG) is a widely used strategy to improve the biocompatibility of nanocarriers. It is well known that the PEGylation of nonviral gene vectors leads to prolonged blood circulation, and this has been partly attributed to the inhibition of the nonspecific interaction between polyplexes and biological components [8, 9]. PEGylation efficacy of polyplexes in preventing

the agglomeration and interaction with platelets was demonstrated by applying IVRTCLSM to quantify their dynamic states in the bloodstream [4]. Blood flow in the earlobe blood vessels was monitored in a noninvasive manner to directly observe polyplexes or polyplex micelles in the circulation. Prior to observation, an anti-GPIbβ antibody conjugated with DyLight 488 was injected to label platelets. The polyplexes and polyplex micelles incorporating Cy5-labeled pDNA were intravenously injected 10 s after the start of the observation.

Polyplexes formed distinct aggregates immediately after intravenous injection, followed by interactions with platelets. However, polyplex micelles prepared through the self-assembly of plasmid DNA with PEG-based block catiomers had dense PEG palisades, revealing no formation of aggregates without visible interactions with platelets during circulation (Fig. 22.4a). We further developed an analytical methodology to quantify the dynamic states of nonviral gene vectors circulating in the bloodstream. For quantification of aggregates, the coefficient of variation (CV) of Cy5 fluorescence was calculated (Fig. 22.4b). The CV is a normalized measure of the dispersion of a distribution, and it is defined as the ratio of the standard deviation to the mean. The CV values of the polyplexes rapidly increased upon first entry into the vein of the earlobe immediately after intravenous injection. These values subsequently fluctuated and decreased over time. Conversely, the CV values of the micelles slightly increased upon first entry due to the admixture of micelles and blood and remained at a plateau at lower values without fluctuation. For the platelet interaction study, colocalization between DyLight and Cy5 was evaluated by Pearson's correlation coefficient (PCC) (Fig. 22.4c). PCC indicates the intensity of the correlation of two elements and ranges from −1 to +1. The PCC value of the polyplexes fluctuated and increased to approximately 0.25. However, those of the polyplex micelles were maintained at almost zero throughout the study.

This is the first report to visualize aggregate formation among polyplexes and its prevention by PEGylation. IVRTCLSM facilitated the development of a new experimental protocol that can simultaneously monitor and quantify rapidly flowing nonviral gene vectors and platelets in the bloodstream.

Evaluation of the Tumor Penetration and Distribution of Differently Sized Micelles

Liposomal and particulate carriers with diameters of 100 nm have been widely used to improve the distribution and tumor accumulation of anticancer drugs. FDA-approved drug delivery systems, such as Doxil and Abraxane (diameters of 90 nm and 130 nm, respectively), have displayed antitumor activity in hypervascular and hyperpermeable tumors such as Kaposi's sarcoma and breast cancer. However, they have also exhibited limited penetration and accumulation in tumors with hypovascular and hypopermeable characteristics [10, 11]. To overcome this barrier, drug delivery systems in the sub-100 nm range have recently been regarded as more important in the study of tumor penetration.

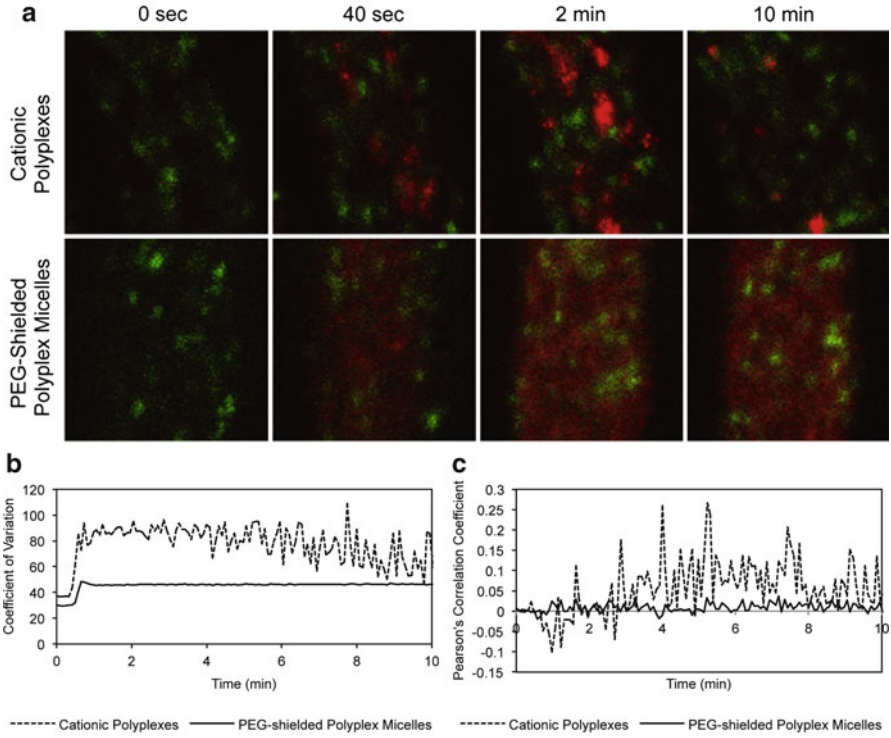

Fig. 22.4 Direct visualization of aggregate formation of the polyplexes. (**a**) Direct visualization of circulation revealed that PEGylation prevented aggregation of the polyplexes and subsequent interactions with platelets. *Green*: Platelets, *Red*: Cy5-labeled pDNA. (**b**) Quantification of aggregates of polyplexes and micelles. Aggregates of polyplexes and micelles were quantified with the CV of Cy5 fluorescence intensities in the frames extracted every 5 s from crude videos. (**c**) Quantification of colocalization between polyplexes/micelles and platelets. The colocalization was measured with PCC. PCC was calculated from the frames extracted every 5 s from crude videos. Permission was obtained from Elsevier B.V. © [4]

In both highly and poorly permeable tumors, the accumulation of different sizes of long-circulating, drug-loaded polymeric micelles was compared [12]. The 30- and 70-nm micelles were labeled with Alexa 488 and Alexa 594 fluorescent probes, respectively, and concurrently injected into tumor-bearing mice to evaluate the real-time extravasation, penetration, and microdistribution of both micelles in the same tumor.

BALB/c nude mice were inoculated subcutaneously with mouse colon cancer-derived C26 cells or with human pancreatic cancer-derived BxPC3 cells to prepare the hyperpermeable or hypopermeable tumor model, respectively. Tumors were allowed to mature until reaching a volume of 50 mm^3. In highly permeable C26 tumors, both 30- and 70-nm micelles displayed comparable extravasation and penetration. In poorly permeable BxPC3 tumors, the extravasation profiles of the micelles were clearly dissimilar.

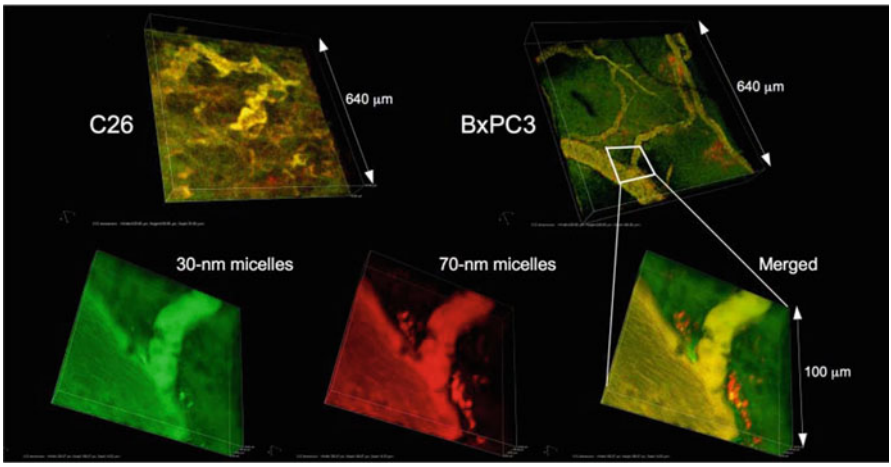

Fig. 22.5 Evaluation of the tumor penetration and distribution of different sized micelles. Simultaneous evaluation of different sized micelles revealed that smaller micelles were superior in terms of extravasation and penetration into stroma-rich, hypopermeable tumors. *Green*: 30-nm micelles, *Red*: 70-nm micelles. Permission was obtained from Nature Publishing Group© [12].

The 30-nm micelles crossed the vascular wall, whereas the 70-nm micelles extravasated at discrete sites close to the blood vessels and failed to move toward the interstitial space (Fig. 22.5).

IVRTCLSM revealed that the enhanced targeting of drugs to cancer cells within tumors by nanocarriers largely depends on size. Micellar nanocarriers with diameters of less than 50 nm might be superior in terms of extravasation and penetration into tumor tissues. Because efficient extravasation and tumor penetration are important prerequisites for targeting cancer cells, our findings are important for designing sophisticated nanocarriers that are capable of cell recognition and selective intracellular release of payloads.

Spatiotemporal Analyses of Subcellular Tumor Targeting and Drug Release

After systemic administration, nanocarriers would need to extravasate, penetrate into the interstitial tissue, undergo internalization by cancer cells, and finally dissociate and release the drug to allow it to exert its in vivo antitumor activity. To elucidate the intracellular localization and dissociation of the micelles, two fluorescent dyes, boron dipyrromethene (BODIPY) FL and BODIPY TR, were conjugated to the shell and core of the micelles, respectively [13]. In the micellar state, only BODIPY FL (green) emits fluorescence, whereas BODIPY TR (red) remains

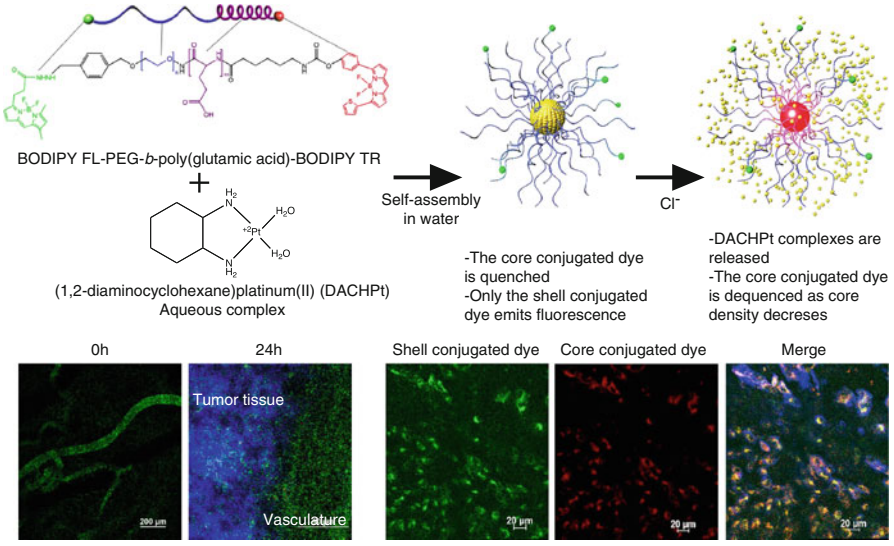

Fig. 22.6 Spatiotemporal analyses of subcellular tumor targeting and drug release. Dual fluorescent labeling of nanocarriers demonstrated that the micelles stably circulated in the bloodstream, extravasated into the cancer tissues, internalized into the cancer cells, and released the drug inside the cells. *Blue*: cell surfaces stained by CellMask, *Green*: shell-conjugated dye, *Red*: core-conjugated dye. Permission was obtained from the American Association for the Advancement of Science© [13]

quenched. As the drug is released from the core, BODIPY TR is dequenched, allowing it to emit fluorescence (Fig. 22.6).

BALB/c nude mice were inoculated subcutaneously with human colorectal cancer-derived HT29 cells and allowed to mature until the tumor reached a volume of 70 mm^3. The plasma membrane stain, CellMask Deep Red, was directly applied to the subcutaneous tumor. Immediately after intravenous injection, the fluorescence from the micelles in tumor blood vessels corresponded only to that of the shell-conjugated BODIPY FL. Even 12 h later, only BODIPY FL fluorescence was observed flowing in the blood vessels. These observations indicate that the micelles stably circulate in the bloodstream while maintaining their micellar structure, allowing them to penetrate deeply into cancerous tissues after extravasation. BODIPY TR fluorescence was clearly visible in the tumor tissue after 12 h, indicating subcellular drug release. Micelles were internalized into cancer cells distant from blood vessels where they eventually dissociated and released active drugs at the perinuclear regions of the cells after internalization.

IVRTCLSM enabled spatiotemporal analyses of the extravasation, tissue penetration, cellular internalization, and subcellular drug release of nanocarriers in living animals. Together with in vitro studies, we demonstrated that the micelles were internalized intact and disassembled permitting drug release in the late endosomes. We also confirmed that the micelles could bypass cytoplasmic detoxification and thereby improve potency and efficacy.

Investigating Effectiveness of Targeting Within Tumors and Their Surrounding Vasculature

The performance of micelles can further be improved by incorporating a short peptide on the micellar surface to enhance the cellular uptake and distribution of siRNA at the subcellular and whole-organism levels. Cyclo-arginine–glycine–glutamic acid (cRGD) peptides were particularly used because they bind to integrin receptors displayed on the surface of several types of tumors and endothelial cells associated with growing tumors [14]. cRGD peptides were conjugated to the micelle surface to achieve active targeting [7]. BALB/c nude mice were inoculated subcutaneously with genetically transformed human cervical cancer-derived HeLa-H2BGFP cells. HeLa-H2BGFP cells are HeLa cancer cells that can be identified by the green fluorescent protein signal located in the cell nucleus [15]. Hoechst dye was used to stain the nuclei of cells present in circulation and the perivascular space.

The microdistribution of cRGD(+) and cRGD(−) micelles was compared within tumors, the regions surrounding the tumors, and blood vessels distant from the tumors (Fig. 22.7). Higher fluorescence in tumor cells and enhanced accumulation within blood vessels in close proximity (several hundred microns) to the tumor mass were

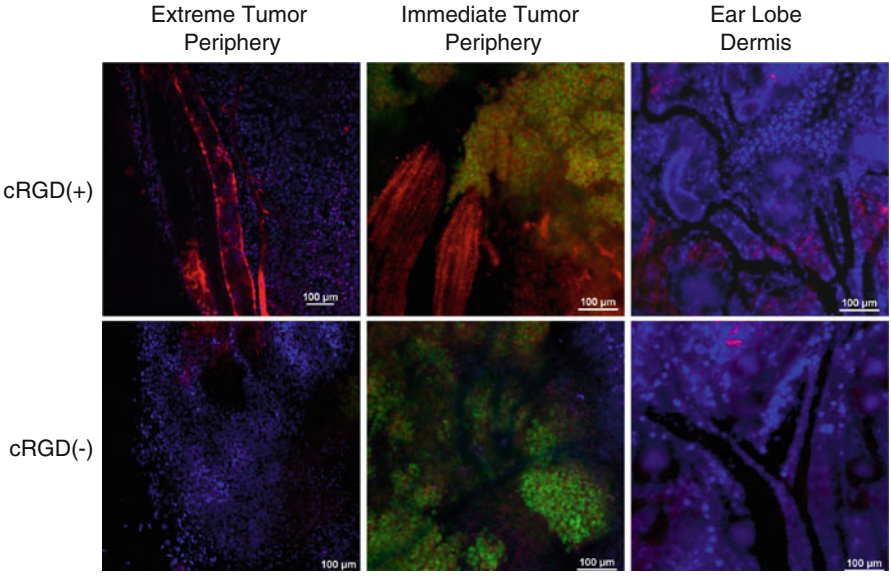

Fig. 22.7 Investigating effectiveness of targeting within tumors and their surrounding vasculature. Observation of cyclo-arginine–glycine–glutamic acid (cRGD)-functionalized targeted micelles revealed that effective targeting within tumors and their surrounding vasculature was achieved. *Green*: H2BGFP-HeLa tumor cell nuclei, *Blue*: Other nuclei stained with Hoechst, *Red*: Cy5-siRNA. Permission was obtained from the American Chemical Society© [7]

observed for cRGD(+) micelles. For cRGD(+) micelles, blood vessels containing fluorescent signals were abundant and easy to locate in the region surrounding the tumor mass and in vessels directly entering the tumor. However, blood vessels could not be located in tumors treated with micelles lacking cRGD peptides, even after extensive searching. Blood vessel targeting was specific to the tumor region because blood vessels in the earlobe dermis displayed no accumulation.

We demonstrated that the cRGD(+) micelles selectively targeted tumor cells and their surrounding vasculature while limiting off-target accumulation such as that in blood vessels in the earlobe dermis.

Conclusion

IVRTCLSM can investigate both the spatial and temporal behavior of nanocarriers in living animals, elucidating mechanisms that have not been clarified by conventional methods that require tissue to be disturbed or manipulated ex vivo. IVRTCLSM can thereby ascertain critical barriers residing in a living body and facilitate the development of nanocarriers optimized for in vivo delivery. Such information is invaluable for the logical design of new nanocarriers for drug delivery.

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Chapter 23
The EPR Effect in Cancer Therapy

Ji Young Yhee, Sejin Son, Sohee Son, Min Kyung Joo, and Ick Chan Kwon

Abstract The enhanced permeability and retention (EPR) effect is the property which small sized nanoparticles and macromolecular drugs can accumulate more in tumor than in normal tissues. The EPR effect is generally due to the larger pore size of neo-vasculatures and poor lymphatic clearance of tumors, and it is strongly influenced by the size of small molecules including nanoparticles. The EPR effect has been considered as an alternative method for delivery of conventional anticancer drugs, and favorable bio-distribution of cancer therapeutic nanoparticles in blood would be considered to achieve a high level of accumulation in solid tumors. Based on the EPR concept, a variety of drugs in nano-carrier systems have been developed for cancer therapy. In this chapter, current progress and good examples for EPR effect-utilized anticancer therapy are reviewed.

Enhanced Permeability and Retention Effect and Nanoparticles for Drug Delivery

Tumor-Targeting Strategies and EPR Effect

Conventional therapeutic strategies for cancer may include surgical removal of tumors, chemotherapy, radiation therapy, and immunological therapy. In particular, chemotherapy alone or in combination is the most common strategy for cancer treatment. However, anticancer drugs, such as doxorubicin and paclitaxel, often show poor in vivo pharmacokinetics and require high doses for effective therapy, resulting in off-target deposition of drugs leading to unpredictable systemic toxicity.

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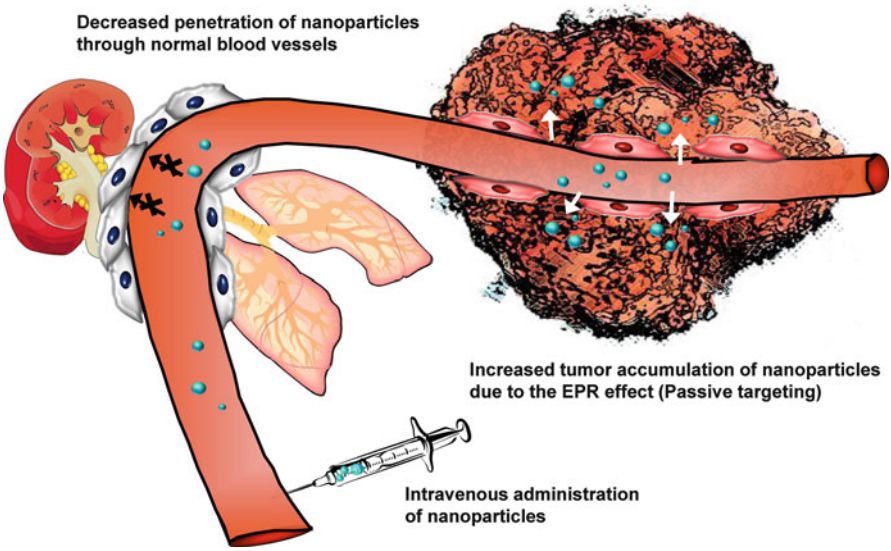

Fig. 23.1 Illustration of enhanced permeability and retention (EPR) effect and the accumulation of nanoparticles in tumor

To address these issues, multidisciplinary approaches have been facilitated with nanotechnology [1]. A variety of nanoparticles are intensively being developed for biomedical applications including molecular imaging and drug delivery for cancer. In general, nano-sized particles tend to accumulate more in tumor than in normal tissues. The tumor-accumulating properties of nanoparticles have been widely studied for biomedical applications, and the enhanced permeability and retention (EPR) effect is the most widely accepted mechanism for tumor-targeting nanoparticles.

In the 1980s, the concept of EPR mechanism in solid tumors was first demonstrated by Maeda et al. [2]. The EPR effect is the property that nanoparticles and macromolecular drugs tend to accumulate more in tumor tissues than in the normal tissues (Fig. 23.1). The EPR effect is a selective accumulation of nanoparticles and macromolecular drugs, which is due to the larger pore size of neo-vasculatures and poor lymphatic clearance of tumors. The passive accumulation of small molecules, particularly nanoparticles, in tumor is mainly due to the abnormal structure of angiogenic vessels which consists of poorly aligned defective endothelial cells [2, 3]. Spatial differences in permeability of various blood vessels could be explained by heterogeneity of the endothelium, different junctional properties, and extracellular retraction induced by functional peptides including histamine, bradykinin, and VEGF [4]. A diversity of angiogenic factors and poorly aligned endothelia of sprouting new blood vessels would provide highly permeable environment of tumor sites for nanoparticles.

A variety of physiochemical properties including size, composition, shape, surface charge, and deformability of the small molecules are involved in the EPR

effect. In particular, the EPR effect is strongly influenced by the particle size. Acharya and Sahoo had described that PLGA nanoparticles should have a size below 200 nm for successful EPR effect [5]. In addition, sub-100 nm sized particles were found to be efficiently extravasated from angiogenic vessels without the size-dependent restriction [6]. However, larger nanoparticles (~200 nm) with certain extent of deformability and flexibility can be extravasated and accumulated in tumors as well, mainly due to the EPR effect [7]. The EPR effect is also affected by molecular weight of drug or drug carrier component. Molecular weight between 40 and 70 kDa of dextran showed the greatest tumor accumulation [8] and glycol chitosan showed the highest tumor selectivity at 250 kDa [9]. In practice, molecules from 40 to 800 kDa in size are regarded as the gold standard for exhibiting an active the EPR effect [10].

After the extravasation, the biodistribution of small molecules including nanoparticles also depends on the size of the particles. Relatively large nanoparticles (>100 nm) are not distributed far from the blood vessels, while the smaller ones (<100 nm) penetrate deep into the tumor tissues [11]. It is currently an ongoing debate whether added targeting moiety could increase the accumulation of the nanoparticles in the target tissue.

EPR Effect and Drug Delivery

As described above, the EPR effect has attracted much attention from the researchers who studied targeted drug delivery. Based on the EPR concept, a variety of drugs in nano-carrier systems have been developed. For instance, glycol chitosan nanoparticles (CNPs) demonstrated excellent tumor-targeting ability in vivo [12, 13]. The hydrophilic glycol chitosan shell of CNPs enabled low protein adsorption and long circulation times in the bloodstream, which allow selective accumulation in tumor tissues due to the known the EPR effect [7]. To develop nanoparticles for enhanced drug delivery by EPR effect, multiple factors should be considered. For efficient delivery, it has been suggested that the nanoparticles have properties of valid blood half-life, minimal nonspecific delivery, and effective elimination from the body [14]. Other various studies on the nanoparticles using the EPR effect are further reviewed in section “Chemo Drug Delivery.”

EPR Effect Utilized Anticancer Therapy

The EPR effect has been considered as a general and indispensible method for anticancer therapy to overcome conventional problems associated with the chemo-drug resulted from the intrinsic genetic diversity of tumors [15]. Since the EPR effect is highly size and/or molecular weight dependent anatomical and physiological phenomenon [16], particles in the broad size spectrum which range from 10 to 800 nm have been utilized for anticancer therapy [17]. Additionally,

a long half-life and favorable bio-distribution of cancer therapeutic nanoparticles in blood also should be considered to achieve a high level of accumulation in solid tumors by the EPR effect [2, 18]. Because prolonged circulation of nanoparticles in the body allows maintaining a required concentration of nanoparticles in the blood for a long time after i.v. injection, the possibility to accumulate in target tumor will be increased where the chemo-drug can be eventually released from the cargo. Subsequently, several approaches have been attempted to maximize the EPR effect thereby establishing highly effective anticancer drug delivery cargo for clinically useful anticancer therapy [19, 20]. Attempts are mainly focused on the combination of the EPR effect with other advantageous functional traits for enhancing the EPR effect. The advantageous functionalities are as follows: (1) tumor-cell specific ligand-based targeting function, (2) stabilizing surface of nanoparticles to prolong half-life and evade immune systems, (3) facilitating intracellular uptake and/or intracellular behaviors such as endo-lysosomal escape and drug release. Hence, current progress and good examples for the EPR effect-utilized anticancer therapy are explained in a brief and concise manner.

Chemo-drug Delivery

Therapeutic agents used in cancer therapy are generally hydrophobic and poorly water-soluble which leads to poor absorption and low bioavailability [21]. To overcome these problems, the drugs can be conjugated with polymers to increase the blood circulation time and prevent renal clearance for more efficient accumulation in the solid tumor via the EPR effect [17]. Biocompatible macromolecular drugs with a size above 40 kDa or even larger than 800 kDa can be relevant for the EPR effect [22]. Polystyrene-co-maleic acid-half-butylate copolymer conjugated with neocarzinostatin (SMANCS) was developed in 1979 and has been used for the treatment of hepatoma. A small protein SMANCS (16 kDa) itself behaves like a large protein of about 80 kDa by non-covalent binding to albumin in vivo and favorably accumulates in tumor relative to normal tissues due to the EPR effect [23–25]. Hydroxypropylmethacrylate (HPMA) also has been utilized as a drug delivery carrier to cancer by attaching various anticancer drugs. Furthermore, HPMA-based macromolecular drugs could be modified by attaching certain additional functions in order to reach the cell nuclei, resulting in enhanced intracellular uptake and organelle-specific targeting [26, 27].

Another method of cancer therapy is using micelles or nanoparticles to deliver anticancer drugs. Micelles have a hydrophobic core and hydrophilic shell structure which can load hydrophobic drugs within their core. The pharmaceutical efficiency of the micelle encapsulated drug can be increased by EPR-based micelle targeting to pathological organs or tissues [28]. Paclitaxel shows a much better accumulation to tumor when loaded into PEG-b-poly(4-phenyl-1-butanone)-L-aspartamide conjugate micelles. The AUC is increased by nearly 100 times, the volume of distribution is decreased by about 15 times and a significant decrease of drug clearance is

observed, resulting in a 25-fold improvement of drug accumulation in C-26 tumors in mice and an equivalent increase in antitumor activity [29]. Genexol-PM, a Cremophor EL (CrEL)-free paclitaxel formulation using a poly(ethylene glycol)-poly(D,L-LACTIC acid) micelle system, shows a favorable toxicity profile and is under clinical trials. The CrEL-free novel taxane formulation shows a higher paclitaxel dose without additional toxicity [30]. A micelle system consisting of poly(ethylene glycol)-poly(glutamic acid) block copolymer (PEG-PGlu) is used for the delivery of SN-38, an analog of the plant alkaloid camptothecin for targeting DNA topoisomerase I. This system shows enhanced distribution and prolonged drug release and clinical studies are ongoing in patients with colorectal cancer, triple negative breast cancer, and small cell lung cancer [31].

Nanoparticle delivery systems also have attractive potentials in cancer therapy due to the high encapsulation ability and the targeting efficiency by the EPR effect. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles are the most widely investigated nanoparticles due to their nontoxic behavior, great biocompatibility and biodegradable properties [5]. As a representative example, dexamethasone loaded PLGA nanoparticles show enhanced in vivo efficacy for the local treatment of arthritis and angiogenesis, and paclitaxel loaded nanoparticles have been accessed on transplatable liver tumor in male NMRI mice and in glioblastoma tumor models. Other anticancer drugs such as vincristine, curcumin, camptothecin, doxorubicin, cisplatin, etoposide, rapamycin have been investigated in vivo and have shown improved and promising results in cancer therapy using the PLGA nanoparticles for delivery. Recently, combination therapy of anticancer drugs has been investigated. Aliphatic biodegradable copolyester (poly(butylenes succinate-co-butylene dilinoleate)) and HPMA-based copolymers (*N*-(2-hydroxypropyl)methacrylamide-based copolymers) had been designed as a carrier for hydrophobic drugs like docetaxel (DTXL) and doxorubicin (DOX).

Proteins and Antibody Delivery

The field of protein and peptide drug delivery has been validated by its potent therapeutic values and bioactivities for the past decade [32–34]. Due to the advances in biotechnology such as recombinant plasmid technology and solid-phase peptide synthesis, promising peptide and protein drug delivery systems have been developed. Currently, over 80 protein and peptide drugs are marketed, and more than 350 protein-based drugs are undergoing clinical trials in the U.S [35]. Nevertheless, the clinical use of protein and peptide drugs is severely hampered by the rapid renal clearance, low enzymatic stability, cytotoxicity and immunogenicity. It is well known that most of protein and peptide drugs lose their bio-activities due to an extremely short biological half-life, thus a high dose of protein and peptide drugs is required for achieving desired therapeutic efficacy, which in turn results in severe cytotoxicity [17]. Therefore, to accomplish successful therapeutic goal of protein and peptide drugs, various strategies have

been developed. Modification of protein using hydrophilic polymer could improve protein stability against enzymatic degradation, and increased molecular weight of modified proteins could slow down a glomerular filtration [17]. Therefore, the prolonged biological half-life allows them to increase accumulation in the tumor site through the EPR effect.

Among the feasible and popular modification method for protein and peptide drugs, PEGylation has played an important role for protein therapeutics [36, 37]. Polyethylene glycol (PEG) is a biocompatible and hydrophilic polymer. The desirable physicochemical properties of PEG facilitate to prevent sequence specific enzymatic attacks thereby increasing bio-availability. Subsequently, various PEGylated proteins and peptide drugs are in pipelines and have been successfully commercialized in the pharmaceutical industry. For example, developed by Dr. Abuchowski in 1990, PEG-adenosine deaminase (Adagen®) was the first FDA approved PEGylated drug for severe combined deficiency (SCID) treatment [35, 38]. PEG-asparaginase (Oncaspar®) has been used for acute lymphoblastic leukemia [39] and PEGylated interferon, such as PEG-interferon α2a (Pegasys®) and PEG-interferon α2b (PEG-Intron®), has been known for the treatment of hepatitis C [40, 41]. PEG-G-CSF and PEG-asparaginase (Oncaspar®) has been used clinically in combination with chemotherapeutics for acute lymphoblastic leukemia [42] and PEGylated cytokine inhibitors have also been used for protein and peptide therapy as an anticancer agent [43]. Even though there are many advantages of PEGylation technology, there still remains insurmountable limitation to overcome. It is well known that shape, numbers, length and PEGylation site of PEG chain are the main factors to establish an effective PEG-therapeutic conjugate thereby those factors should be carefully considered, because some PEGylation methods decrease biological activity of protein therapeutics [44].

On the other hand, many researchers have developed other strategies to improve therapeutic efficacy of protein-based therapeutics for anticancer therapy. Among them, utilization of nanoparticles as a cargo of protein and peptide drugs is considered as an effective way. Representatively, PLGA and its derivatives have grabbed increasing attention in biomaterials and drug delivery field. Owing to their biocompatibility and biodegradability, PLGA nano- and micro-particles have been widely accepted for protein drug delivery and is currently approved by the US FDA [45, 46]. Above all, the outstanding ability to release protein drugs in a sustained and controlled manner by gradual degradation of the matrix of PLGA nanoparticles is indispensible advantage of the biodegradable nanoparticles.

The growth hormone encapsulated PLGA microsphere (Nutropin Depot®) and leuprolide acetate encapsulated PLA microsphere (Lupron Depot®) have been marketed and they have shown ease of administration by injections [46]. However, degradation of the PLGA matrix induced release and accumulation of acidic molecules such as glycolic acids and lactic acids in the surrounding region and consequent acidic microenvironment caused aggregation and deactivation of protein and peptide drugs [47]. For this reason, Neutropin depot had been withdrawn from the market.

Due to the various pharmaceutical advantages of protein and peptide drugs, many research groups have tried to overcome their limitation including extremely short biological half-life, low molecular weight and instability. The modification technology of protein itself such as drug delivery system (nanoparticles, liposomes) and PEGylation methods have shown to be feasible approaches. However, new strategies to overcome their limitation should be persistently developed to meet the criteria for establishing the next generation of protein and peptide based anticancer therapeutics.

DNA and siRNA (Gene) Delivery

Research on gene delivery also has expanded during the past 20 years. The gene and siRNA therapy hold a great promise for the future of anticancer therapeutics due to high disease specificity and universality in therapeutic target [48]. The clinical use of genes, however, has been severely hindered by the intrinsic physicochemical properties, such as short half-life of several minutes, inefficient cellular uptake due to strong negative charge and absence of tumor-targeting ability [48]; therefore, establishment of efficient delivery system is crucial, especially for utilization of the EPR effect. Therefore, up to now, cationic polymeric systems based on polyethyleneimines (PEIs) [49–51], chitosans [51], and reducible polyamidoamines [52] have been mainly utilized to deliver genes to target tumor area. More importantly, the polymeric systems have been evolved to have desirable size and several functional traits for the efficient EPR effect, endo-lysosomal escape, and disease or intracellular targeting with minimum toxicity [53]. Among them, chitosan-based carriers are currently being utilized as one of the common cationic gene delivery system. The abundant primary amine groups existing in the chitosan can effectively interact with negatively charged phosphates of genes, forming compact genes/chitosan complexes (referred as polyplexes) for anticancer therapy. It has been reported that chitosan–siRNA polyplexes are successfully formulated by using high molecular weight deacetylated chitosan (114 and 170 kDa, deacetylation of 84 %). Chitosan-based polyplexes have shown compact and condensed structure, and they have exhibited significant gene knockdown in vitro [54]. Numerous researchers have studied chitosan as carriers of pDNA as well. They have shown that PEGylation or thiolation of chitosan improved the systemic biodistribution and enhanced the transfection efficiency of pDNA [55, 56].

Kim and Kwon groups have investigated comprehensively and constantly the remarkable tumor targeting efficiency of glycol chitosan (GC) nanoparticles, and they have insisted that deformability and size of GC nanoparticles and EPR effect were the main factors for tumor targeting [7, 9, 20, 57]. Given the promising tumor targeting efficiency of GC, the Kim and Kwon groups have developed thiolated GC (TGC) for siRNA delivery [58, 59]. As a creative approach, they have also developed polymerized siRNA to increase complexation ability with TGC and have successfully established a highly efficient GC-based siRNA delivery system for

anticancer therapy utilizing the EPR effect. Through this strategy, significantly increased tumor accumulation has been obtained up to 72 h, tumor growth inhibition has been observed up to 5 days using anti-angiogenic VEGF siRNA [58]. This research group has been consistently validating tumor targeting efficiency and therapeutic efficiency of GC based nanoparticles in various in vivo therapeutic models.

In order to obtain synergistic tumor targeting effect in addition to the EPR effect, Son et al. has presented a tumor specific cNGR ligand modified reducible PEI for gene delivery [53]. They have established one-pot synthesis method of multifunctional cationic polymer, in which low molecular weight PEI is thiolated, and mixed with PEG (5,000 kDa) and cyclic NGR peptide. In this study, it is clearly demonstrated that several advantageous functionalities, such as tumor neovasculature-specific targeting and serum stability in combination with size dependent delivery, could comprehensively affect on solid tumor accumulation.

As an another approach, a multifunctional envelope-type nano device (MEND) has been developed for the EPR utilized gene delivery system by the Harashima group [60]. A MEND consists of a gene core condensed with a polycation and a lipid envelope decorated with various functional devices, such as PEG, target ligands and cell-penetrating peptides (CPPs). They have mainly utilized the PEGylation strategy for achieving a prolonged half-life to facilitate a high level of tumor accumulation via EPR effect. They have insisted that the PEG dilemma should be addressed to obtain highly effective anticancer gene therapy utilizing the EPR effect and further suggested several helpful approaches based on installation of specific ligands, cleavable PEG or fusogenic/disruptic devices.

Conclusion

In spite of great efforts to control the cancer and cancer-related death, recent clinical cancer therapy has not been as successful as expected. Conventional cancer treatment has been mainly dependent on small molecular drugs, but most cases have exhibited adverse side effects due to poor in vivo pharmacokinetics and high dose of drug accumulation in non-tumoral organ. In this context, the EPR effect is considered to be a promising paradigm for anticancer therapy to overcome conventional adverse side effects. Although the EPR effect is applicable to a wide variety of cancers types and targeted nanoparticles dependent tumor accumulation, several caveats still remain to achieve optimal therapeutic efficacy. First, the EPR effect dependent nanoparticles may not be delivered to incipient stages of cancer and the tiny masses of micro-metastases. In general, the EPR effect is mostly active for neovascularization in solid tumors; however significant angiogenesis does not occur until a tumor reaches to a certain size. Secondly, the accumulation of nanoparticles in tumor does not always signify a successful therapeutic effect. For an effective therapy, the nanoparticles should be guaranteed with deep penetration to the central part of solid tumor tissue and good cellular uptake to release the chemo-drugs.

Nevertheless, nonselective delivery of the EPR effect is still the most important mechanism for drug delivery using nanoparticles. Rational design and engineering of nanoparticles facilitating the EPR effect will be the most intelligent and feasible choice to pave the way for eradicating cancers, considering the EPR effect is a general and universal phenomenon of tumor blood vessels. Nano-formulation equipped with suitable sizes and adjuvant functions to enhance and strengthen tumor accumulation behavior, such as serum stabilized surface passivation with PEG and ligand-based targeting function to specific cancer obviously benefit clinical cancer treatment. They potentiate a prolonged in vivo half-life and specific cancer targeting, thus allowing the maintenance of desired concentration of nanoparticles in the bloodstream. Consequently, the possibility to accumulate in target tumor will be increased where the chemo-drug can be eventually released from the cargo. Those more advanced and combinatorial approaches to go further than the EPR effect will open a new direction in clinical anticancer therapy. In fact, until now it is still a challenge to build highly sophisticated nanoparticles for drug delivery. Therefore, researchers should make a great effort to develop ideal anticancer drug delivery particles in parallel with a thorough mechanistic evaluation of their behavior in the body and tumor site, and current endeavor should be also focused on establishing effective methodology to maximize the EPR effect.

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Part VI
Preclinical Modeling

Chapter 24
In Vitro Three-Dimensional Cancer Culture Models

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Abstract The efficacy of chemotherapy drug candidates is conventionally investigated using 2D cancer cell cultures and in vivo animal models. It is crucial to determine signaling pathways, controlling cell proliferation, metabolism, differentiation, and apoptosis functions, which are not optimal to investigate in the monolayer 2D cell culture models. Further, accurate investigation of tumor growth and therapeutic drug efficacy in murine models is challenging because of technical constraints of in vivo imaging and requires euthanizing the animals. Therefore, alternative in vitro cancer models are needed to facilitate the transition of new chemotherapeutic drug candidates from bench to clinical trials. Recent technological advances in microfabrication and bioengineering have provided tools to develop in vitro 3D cancer models that mimic natural tissue microenvironment. This chapter highlights recent developments in in vitro 3D cancer models and their applications for studying the efficacy of the chemotherapeutic drug candidates. We discuss the

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methods and technologies to develop 3D cancer models including embedded and overlay cell culture, suspension culture, bioprinting, hanging drop, microgravity bioreactor, and magnetic levitation. We also discuss the extracellular matrix components and synthetic scaffolds used in vitro 3D cancer models.

Introduction

Cancer caused 7.6 million deaths worldwide in 2008 [1]. About 80 % of tumors are originated from the epithelium characterized by the uncontrolled growth of epithelial cells [2]. Epithelial cells interact with other cells like endothelial and stromal fibroblast cells, and extracellular matrix (ECM) based on microenvironmental conditions [2, 3]. Endothelial vasculature provides oxygen and nutrients to other cells present in ECM. This vasculature also provides the immune system cells (macrophages, neutrophils and mast cells) to fight against pathogens and for removal of dead cells. ECM consists of a mesh of protein fibers like elastins and collagens [4]. The fibers are further functionalized with cell adhesive proteins such as fibronectin and laminin. These cell adhesive proteins on ECM provide binding locations for the cells to get attached to ECM through cell transmembrane integrin receptors. In blood cells, the integrins also mediate cell–cell adhesion along with ECM adhesion. All of these receptors are involved in intracellular signaling pathways and regulate cell growth, shape, proliferation, migration and differentiation [4]. Occasionally cell signaling pathways are disturbed (e.g., due to injury) that results into the proliferation and movement of epithelial sheets. These abnormal conditions are reversible upon healing. If the inflammable conditions are sustained, the stromal fibroblast and macrophages continuously upregulate the matrix metalloproteinase (MMP) enzymes that remodel ECM and can promote abnormal cell proliferation [2]. Abnormal cell interactions and signaling pathways can lead to genetic mutations, and hence epithelial cells attain tumorigenic potential [5]. At this stage, the cancer cells behave as an independent organ and define their cellular responses and reorganize ECM to accommodate tumor mass formation. The tumor size cannot grow beyond 200 μm without the nutrients and oxygen supply that induce the upregulation of hypoxia-inducible factor (HIF1α) [6]. Hypoxia leads to a signaling cascade followed by upregulation of angiogenic growth factors including vascular endothelial growth factor (VEGF). New blood vessels are formed in response to these changes that can supply nutrients and oxygen to growing tumor cells. Tumor aggressiveness is further enhanced by the increased secrection of other growth factors such as basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8) [7]. As a result, the cancer grows continuously into a solid tumor along with the supporting vasculature and microenvironment.

Many chemotherapeutic drugs have been introduced to treat cancer. Most of these drugs fail during transition from murine models to clinical trials [8]. On the other hand, the number of potential drugs waiting to be tested far exceeds the number of patients available for drug testing. Therefore, alternative in vitro cancer models for verification and testing of drugs are needed [9]. Conventionally, 2D cancer cell culture and in vivo

animal models are used to determine the efficacy of chemotherapeutics. Both of these models have certain advantages and limitations. In 2D cell culture techniques, the cancer cells are cultured on the plastic substrata as a monolayer where important signaling pathways controlling cell proliferation, metabolism, differentiation, and apoptosis functions are lost; whereas in natural form the cancer cells grow into 3D tumor mass while interacting with other cells and the ECM. For instance, when breast cancer cells were cultured on 2D substrata and treated with various signaling inhibitors, there was no change in expression and activity of β1-integrin and epidermal growth factor receptors (EGFR); whereas these activities were reciprocally down regulated when cancer cells were cultured in 3D tumor model [10]. In another report, tumor cells were treated with PI3-Kinase inhibitors in both 2D and 3D tumor culture models. The inhibitors mediated the reversion of malignant phenotype only in 3D tumor model by down regulation of EGFR and β1-integrin whereas in 2D tumor culture, these phenomena were absent [11]. The 2D cancer cultures respond differently to exogenous apoptotic stimuli and chemotherapeutic agents. Tumor cells cultured in 3D spheroids attained multidrug resistance phenotype when exposed to a chemotherapeutic agent whereas cancer cells cultured on 2D substrata were sensitive to drug treatment [12, 13]. These results implied that the composition of tissue ECM and cell–ECM interactions generated resistance to apoptosis, a phenomena absent in 2D monolayer cultures [13–15]. Solid tumors such as breast and liver cancers are denser and hypoxic at the center, which cannot be modeled using the 2D culture technique that includes just a monolayer of cells. Therefore, it is obvious that 2D cancer culture models present limitations in providing a natural 3D microenvironment for cancer cells and may not be effective to study the drug efficiency.

The human tumor xenograft model is the most commonly used in vivo tumor model that can provide natural 3D tumor microenvironments. In this method, small cancerous tissue biopsies or inoculating cancer cells are placed either subcutaneously or into the other organs of immunocompromised mouse and allowed to proliferate for couple of weeks [16, 17]. Mice used in this model are immunocompromised and therefore the injected human cancer cells are not rejected. Different types of immunocompromised mice are available for xenograft models including athymic nude mice, severely compromised immunodeficient (SCID) mice and non-obese diabetic (NOD) SCID mice [17–20]. Although the xenograft mice models can induce the immune response similar to native tumor stroma, the imaging of tumor growth after specific time intervals is very challenging. The drug efficacy can only be analyzed when the mouse is sacrificed at the end. Although the modern in vivo imaging systems can be used to assess the drug efficacy during experiment, these imaging systems are costly [21–24]. Previously, xenograft mice models were used to test the efficacy of angiogenic inhibitor endostatin [25, 26]. It was concluded that tumor regressed effectively once treated with endostatin. The later clinical trials revealed that endostatin interacted differently in humans; only 20 % of the patients administrated with endostatin showed tumor regression, while no toxicity was observed in any patients [27]. Although xenograft mice models are preferable to 2D cancer culture models, they can, sometimes, lead to false interpretations. Therefore, there is an unmet need to develop in vitro 3D cancer culture models that employ human cells and which can reliably recapitulate native tissue structures.

In vitro 3D cancer models rely on encapsulating cells within hydrogels or scaffolds or seed them on a defined substrate and method where they can form 3D aggregates mimicking natural tissue microenvironments while overcoming some of the limitations of 2D and xenograft animal models. 3D cancer models can be composed of single or multiple cell types. This chapter focuses on the state-of-art technologies and processes that were developed for 3D cancer cultures and cocultures. Most of the in vitro 3D cancer models have been introduced including spheroids, hanging drop cell encapsulation and printing, and scaffold seeding. Further, the effects of dynamic fluid flow conditions and scaffold stiffness on cancer growth are discussed. In the end, future research directions are also described.

Methods and Technologies to Develop 3D Cancer Models

Recapitulating in vivo metabolic activities and spatial organization of cells within in vitro conditions hold paramount assets in cancer research. In vitro culture models mimicking features of native environments are in great importance in order to reveal cancer cell activities including self-sustained growth signal secretion, resistance and insensitivity to inhibitory cytokines, avoidance of apoptosis, continuous self-division, angiogenesis, migratory capacity, and metastasis [28]. Native tissue environment provides co-localization of different cell types in a well-defined organization enhancing cell–cell contact, exchange of secreted signaling cytokines and cell–ECM interactions [29]. Coculture of relevant cell types enhances cell–cell cross talk through secreted cytokines and growth factors. External addition of growth factors lacks in dose precision and timing. Such cross talk between cell types can be implemented simply by seeding multiple cell types together simultaneously in the same place or by introducing particular cell type on top of the pre-seeded cell layer (i.e., fibroblast cells). In such methods, cells have direct cell–cell interactions and different cell types can be plated in cell culture inserts. Such inserts have porous membranes that provide the exchange of cytokines between cells. Indirect cell–cell cross talk can also be established by obtaining a conditioned culture media from one cell type or culture time point and using it in another cell culture or time point. Classical monolayer cell cultures are able to assist needs to coculture multiple cell types and supply signaling cytokines by introducing them through culture media. However, spatial organization of cell morphology and interpretation of physical and biochemical cues from ECM are unmet.

Embedded and Overlay Cell Cultures

Efforts to mimic native microenvironment introduced basic ingredients of ECM such as collagen type I, collagen type IV, fibronectin, laminin and glycosaminoglycans, and elastin as tools to build a basement membrane in 3D culture systems

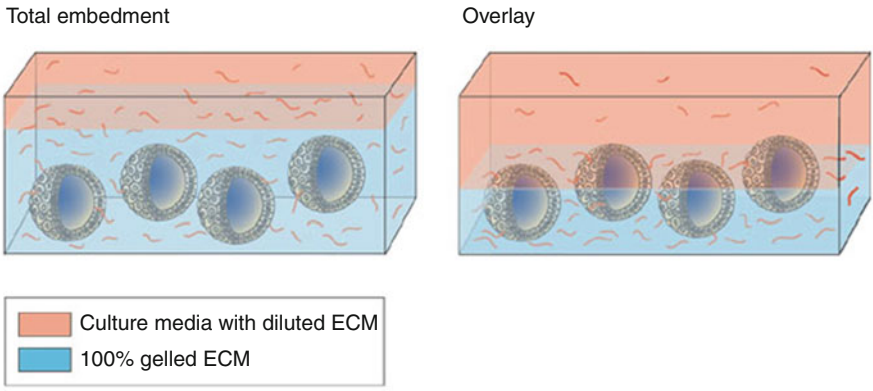

Fig. 24.1 Schematic representation of embedded and overlay culture models. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS CANCER], copyright (2005) [169]

including Matrigel™ and alginate based materials [30]. Basement membrane can be applied in two different ways to promote 3D cellular organizations: (1) embedded, and (2) overlay culture. Both methods aim to establish biologically relevant contact between cells and substratum. In the embedding method, cells are suspended into the basement membrane and applied in culture well (Fig. 24.1 left). Encapsulated cells start to adhere, reshape and secrete factors (e.g., MMP) to remodel the matrix that provide space for spreading, proliferation and migration to contact other cells [31]. In an overlay culture, basement membrane is applied to the surface of a substrate and forms a thin hydrogel coating. Later, cells are introduced with culture media on the coating (Fig. 24.1 right). Formation of the optimum 3D cell organizations depends on the type of applied cells and basement membranes. Some cell types require additional biological cues from the basement membrane such as growth factors. For instance, most of the epithelial cells form spherical hollow cysts only when embedded in Matrigel™, whereas Madin-Darby canine kidney (MDCK) epithelial cells can easily develop into polarized cell clusters with a hollow lumen when encapsulated within collagen type I hydrogels [32, 33].

Suspension Culture

3D cell cultures can also be achieved by culturing cells in suspension. In this method, cell adhesion to the substratum of culture plate is reduced or prevented. Regular cell culture plates are made of polystyrene and have treated surfaces that provide negatively charged hydrophilic properties enhancing cell adhesion. When there is no treatment applied, polystyrene surfaces are uncharged and have hydrophobic nature, which results in poor and uneven cell adhesion. Cell adhesion decreases dramatically by 99.8 % when special treatment is applied to polystyrene

surface by making it neutral and still hydrophilic (Corning Ultra-Low Attachment Surface). Alternatively, agarose coated cell culture plates also provide suspension culture conditions.

Hanging Drop Technique

In standard cell culture, cells are pressed against hard flat surfaces by gravitational force, which results in constrained cell growth in a two-dimensional plane. Hanging drop technique allows to gravitationally aggregate cells at a flexible, spherical air–liquid interface within hanging drops, which facilitates the formation of 3D cell structure (e.g., spheroids) without a scaffold [34–37]. Currently, a number of commercial 3D cell culture products are available in market based on hanging drop technique (Table 24.1). For example, InSphero Company offers GravityPLUS™ platform with 96-well and 384-well plates for 3D cell culture. To generate microtissue, 50 μL of cell suspension was seeded in each well (Fig. 24.2a, b). Spheroids were formed within 2–4 days after seeding. Long-time cell culture can finally result in cell spheroids with a dimension up to 500 μm. The spheroids can be collected using GravityTRAP™ plate with a simple media addition step. As a product validation step, formation of micro-tissues were demonstrated with a number of cancer cell lines, including human colon (HTC-166), liver (HEP-G2), prostate (DU-145), kidney (A-498) cancer cell lines. In addition, hanging drop technique also enables cocultures of multiple cell types or spheroids by various approaches (Fig. 24.2c–e), which is useful for studying cancer invasion and cell interactions. In general, hanging drop technique provides high-throughput approach for in vitro tumor researches, especially for antitumor drug screening.

Magnetic Levitation

In the magnetic levitation method, cells or cell micro-carriers (e.g., cell encapsulating droplets or hydrogel units) are labeled with paramagnetic/diamagnetic materials and then suspended cultured in cell culture medium by balancing gravitational force with magnetic force. The levitated cells can grow in 3D space, which results in the formation of spheroids. Souza et al. demonstrated a paramagnetic cell levitation method for 3D tissue culture (Fig. 24.3a–c) [38]. Cells are first adherently cultured and incubated with hydrogels containing magnetic iron oxide nanoparticles, gold nanoparticles, and filamentous bacteriophage. The cells obtained paramagnetic property by uptaking magnetic iron oxide nanoparticles. Subsequently, the mixture of the cells and hydrogels were levitated on the liquid–air interface by applying a magnetic field through a magnet. The levitated cells and hydrogels first aggregated and then self-assembled into 3D microtissue, the shape of which is related to distribution of the magnetic field. Taking human glioblastoma cells as an example,

Table 24.1 Comparison of 3D cell culture products

Product name	Liquid volume for each well (μL)[a]	Spheroid diameter (mm)[b]	Standard plate format (wells)	Cost ($)[c]	Company (Website)
GravityPLUS™	50	0.15–0.3	96; 384	140	InSphero (http://www.insphero.com)
Perfecta3D® Hanging Drop Culture Plates	25–50	0.1–0.4 [34, 36]	96; 384	206	3D Biomatrix (http://3dbiomatrix.com)
IMAPlate™	25–35	0.05–0.3	96	33	NCL New Concept Lab GmbH (http://www.nclnewconceptlab.com)
The Bio-Assembler™ System	50–75	0.6–1 [38]	1; 6 24; 96	600 (96-Well Kit)	Nano3D Biosciences (http://www.n3dbio.com/)
Rotary wall vessel	NA	Up to 10	55 mL 110 mL 250 mL 500 mL[d]	5525[e]	Synthecon, Inc. (http://www.synthecon.com)

[a]Recommended liquid volume for each well by the company. Data obtained from technical documents in corresponding websites
[b]Size of spheroid after 2- to 4-day cell culture. Data obtained from technical documents in corresponding websites or published papers
[c]Cost for 96-well plate format
[d]Rotary wall vessels are sold with different volumes
[e]Price for autoclavable vessel rotary cell culture system with single station rotator base

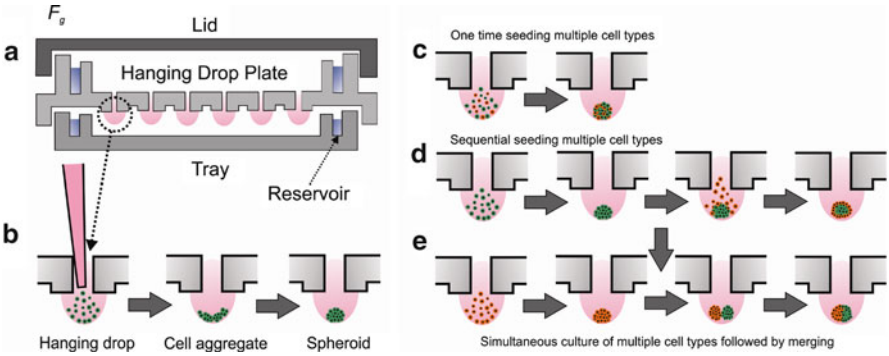

Fig. 24.2 Hanging drop technique. (**a**) Hanging drop plate; (**b**) operational procedure for hanging drop technique; (**c**–**e**) Coculture modes. (**c**) Simultaneous co culture multiple cell types; (**d**) sequential coculture multiple cell types; (**e**) fusion of multiple-cell-type spheroids by hanging drop technique

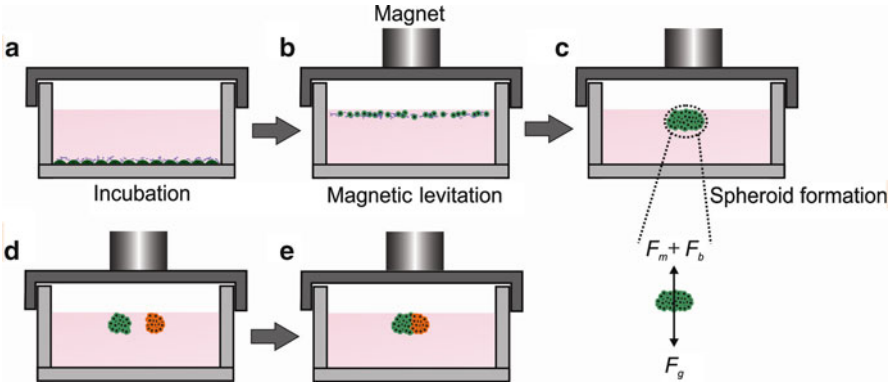

Fig. 24.3 Magnetic levitation technique. (**a**–**c**) Operational procedure for magnetic levitation technique. F_m, F_b and F_g are magnetic, buoyant and gravitational forces respectively. (**d** and **e**) Fusion of multiple-cell-type spheroids by magnetic levitation

the levitated cells self-assembled into spheroidal shape between third and eighth day and finally generated a maximum diameter of 1 mm. Morphological analysis together with molecular similarity test proved that the generated cancer spheroids were more similar to human tumor xenograft than the 2D cultured cells [38]. In addition, coculture of different cell lines was also demonstrated with human glioblastoma cells and normal astrocytes (Fig. 24.3d, e). This paramagnetic cell levitation technology has been already commercialized as a product named "The Bio-Assembler™ System" by Nano3D Biosciences (Table 24.1).

To obtain a more complex shaped microtissue, Lin et al. introduced a magnetic organoid patterning technique that allows assembly of multicellular spheroids into a complex geometry [39]. Magnetic cell spheroids were first generated using a hanging drop method by incubating cells with RGD peptide-conjugated magnetic microparticles. The spheroids were then manipulated by magnetic field and patterned into different shapes (e.g., rings, lines, and arrays). The patterned spheroids were further immobilized by thermo-responsive hydrogel and further fused together. By stacking the patterned spheroid layer by layer, complex 3D microtissues can be generated [39].

In addition, magnetic assembly of cell-encapsulating microscale hydrogels (M-gels) was developed for generation of 3D microtissues [40–42]. Both magnetic nanoparticle loaded and free radical loaded M-gels were used as building blocks for constructing various macroscopic shapes (e.g., multilayer spheroids, lines, and domes) or microscopic M-gel arrangements (e.g., square, line, and cross), respectively. Cell viability and proliferation assays indicated a good biocompatibility of this method. This method can be potentially used for modeling 3D tumor with complex structures.

Microgravity Bioreactor

Microgravity bioreactor refers to any engineered devices or systems that enable cell or tissue culture in a very small gravity/net acceleration environment compared with that at earth surface [43–46]. Comparing with standard 2D cell culture under earth-gravitational environment, cell culture in microgravity environment possesses various advantages, including no sedimentation, loss of gravity-driven convection, decreased hydrodynamic shear and hydrostatic pressure gradient, and isotropic mass transfer based molecular diffusion. These advantages facilitate a 3D cell growth with spatially uniform cell distribution, enhanced cell-to-cell interactions, large cell harvest rate, and good cell viability.

Cell Culture in International Space Station

Cancer cells have been also cultured in microgravity environment. To obtain real microgravity environment, free fall principle is widely explored. During the free fall, gravity is supposed to be the only force acting upon an object, which results in the object losing its weight. Based on the free fall principle, a number of methods are developed to create microgravity environment with a time scale ranging from seconds to days. These methods include parabolic flight by aircraft, sub-orbital (100 km above sea level) flight by rockets, low-earth-orbit (between 160–2,000 km above sea level) flight by space shuttle and international space station (ISS). However, only low-earth-orbit space shuttle and international space station (ISS) can provide long enough duration of microgravity environment for cell culture.

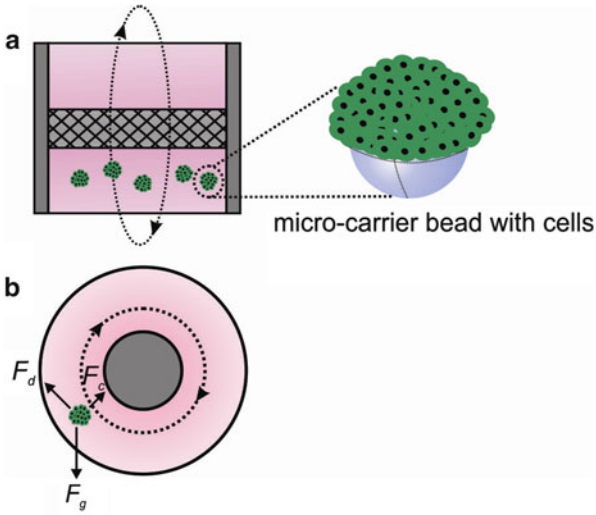

Fig. 24.4 Rotating wall vessel technique. F_d, F_c and F_g are hydrodynamic drag, centripetal and gravitational forces respectively

Since 2000, a series of cancer cell culture experiments were performed in ISS to study mechanisms of tumor formation and development. For example, culture of LN1 human ovarian tumor cells and human colorectal carcinoma cells were performed in a self-contained cell incubator separately during the period of August 2001 to December 2001 in ISS Expedition 3 [47, 48]. Both cancer cell lines grew into complex 3D structures, which were much closer in true dimension and shape to original tumors found in cancer patients. These researches imply that cancer cells cultured in microgravity environment can serve as a close in vitro model to examine cancer's behavior (e.g., growth, differentiation, maturation and death) in the body, which provide an insight into seeking potential treatments for cancer and other related diseases.

Rotating Wall Vessel (One-Axis Clinostat)

Due to high cost and limited number of ISS Expedition missions, several alternative bioreactor technologies have been developed for simulation of microgravity environment on the ground. Among these technologies, rotating wall vessel (RWV) is one of widely used bioreactors originally developed by NASA [49]. RWV works as a single-axis clinostat with two concentric cylindrical surfaces (Fig. 24.4a, b). The inner surface-made is a silicone gas exchange membrane, while the outer vessel is a rotating wall that's used to generate hydrodynamic drag force. Culture medium is completely filling the space between the inner and outer cylinders. Currently, there are three different derivatives of RWV design, i.e., the slow lateral turning vessel (STLV), the high aspect ratio vessel (HARV) and the rotating wall perfused vessel (RWPV) [50, 51], out of which HARV is commercially available in the market (Table 24.1).

Both STLV and HARV have the same operational principle with a difference in their gas exchange sources. RWPV contains additional culture medium exchange column in the central cylinder for the applications in space [45, 52]. A time-averaged low gravitational field (10^{-2} to $10^{-3}\,g$) is created on suspended cells or cell micro-carriers in the annular space by rotating the RWV along axis perpendicular to gravitational direction at rates of 15–40 rpm. RWV has advantages, such as low shear stress (0.3 dynes cm^2), good mass transfer for nutrients and wastes exchange, which promotes the formation of large-size (5–10 mm) cancer cell spheroids. These cancer cell spheroids closely resemble the natural tumor with respect to their cellular organization and specialization. The RWV facilitates generation of 3D tumor models for various cancers, such as human breast, ovarian and prostate cancers. For example, Grun and his coworkers developed 3D cancer culture models of endometrial cancers and human ovarian cancers by RWV [53]. Both immunohistochemical profiling of multiple markers (e.g., p53, BCL2, and CA125) and proteomic profiling (e.g., prohibitin, VDAC1, and annexin 4) were performed to validate the model systems. Compared to standard 2D cancer culture models, the 3D models were more similar to the natural tumors in biological and morphological characteristics. Besides RWV, random positioning machine (RPM) is also used to simulate microgravity conditions for 3D cancer cell culture [49, 54–58]. RPM is a 3D clinostat that can rotate bioreactor along three independent axes, and it potentially offers more uniform simulated microgravity environment acting on the cells. However, three-axis clinostats are rarely used in reality due to their mechanical complexity and high cost. Some representative examples for 3D cancer culture model are given in Table 24.2. Comparison of these bioreactor technologies is also given in Table 24.3.

Bioprinting for Cancer Research

In this section, we first describe use of bioprinting technologies in developing cancer models. Then, a statistical model is described to estimate probability for single target cell encapsulation. Finally, we describe a finite-difference/front-tracking model for deposition of a compound droplet composed of cell (highly viscous droplet) and an encapsulating droplet with a flat substrate.

Bioprinting can address some of the limitations in developing 3D models such as limited repeatability, poor control over cell density, low throughput, and lack of reliable control over spatial resolution between cell types (e.g., cancer and stromal cells) in the case of coculture models [59]. Recently, a high-throughput ejector platform composed of a *xyz* computerized stage and two ejectors has been introduced to micropattern a 3D coculture model using cancer cells and normal fibroblasts [59] (Fig. 24.5a). In this study, OVCAR-5 and MRC-5 cells were printed within a spatially controlled microenvironment (e.g., cell density, cell–cell distance) in a high-throughput and reproducible manner. Results showed that both OVCAR-5 and MRC-5 cells remained viable during printing and sustained proliferation capacity following patterning. Such approaches can enable: (1) miniaturization of established

Table 24.2 microgravity bioreactor technologies for cancer cells

Microgravity bioreactor	Cell type	Scaffold	Research focus	Spheroid diameter (mm)	Reference(s)
RWV	Murine melanoma cells (B16–F10)	Keratinocytes spheroids	Model of melanoma	10	Marrero et al. [172]
RWV	Glioblastoma cells (PFSK-1; KNS42; U87)	Free	Genetic, epigenetic and metabolic profiles	9 (PFSK-1); 5 (KNS42); 1–3 (U87)	Smith et al. [173]
RWV	Human hepatocellular carcinoma (MHCC97H)	Polylactic acid-co-glycolic acid (PLGA)	Model of human hepatocellular carcinoma in animal	8–10	Tang et al. [174]
RWV	Ovarian cancer cell (OV-TRL12B); endometrial cancer cell (EN-TRL 67T)	extracellular matrix gel from Engelbreth Holm-Swarm Mouse sarcoma	Model of ovarian and endometrial cancer	4	Grun et al. [53]
RWV	Primary breast carcinomas	Free	Tumor proliferation	1–3.5	Becker et al. [175]
RWV	Murine melanoma cells (B16–F10)	Free	Melanoma growth and tumorigenicity	5.8	Taga et al. [176]
RWV	Human prostate cancer cell (LNCaP)	Type I collagen-coated dextran beads	Genetic and epigenetic profiles	2	Rhee et al. [177]
RWV	Tumor epithelial (TEC)	Free	Cell interactions	0.5–5	Chopra et al. [178]
RPM	Human malignant glioma cell (D54MG; U251MG; T98G)	Free	Tumor growth and chemosensitivity	NA	Takeda et al. [54]
RPM	Human follicular thyroid Carcinoma cell (ML-1)	Free	Differentiation and apoptosis	0.3	Grimm et al. [55]
MG-6C rotating clinostat	Human breast cancer cell (MCF-7)	Free	Model of breast cancer cell	NA	Qian et. al. [179]

Table 24.3 Comparison of 3D cancer cell culture technologies

Technology	Throughput	Mass transport mechanism	Toxicity	Controllable spheroid diameter	Compatible with epifluorescence microscopy	Shear stress (dyne/cm^2)	Scaffold	Diameter	Reference(s)
Rotating wall vessel	High	Laminar flow	No	No	No	~0.8	Collagen coated porous microbeads or scaffold free	5–10 mm	[49, 51]
Magnetic levitation	Medium	Molecular diffusion	Unknown nano-toxicity and magnetic field effect on cells	Yes	Yes	0	Hydrogel consisting of gold, magnetic iron oxide nanoparticles and filamentous bacteriophage	1 mm	[38]
Hanging drop method	High	Molecular diffusion	No	Yes	Yes	0	Scaffold free	0.5 mm	[34, 36]
Bioprinting	High	n/a	No	Yes	Yes	Variable	Scaffold free	10–500 μm	[59, 62, 68, 70, 142]

macro-scale 3D culture models, (2) systematic examination into the several unidentified regulatory feedback mechanisms between stromal cells and tumor, and (3) high-throughput drug screening.

Cell encapsulation prior to bioprinting is highly probabilistic phenomena as there are several governing parameters such as number of cells that can be encapsulated, and locations of cells within a printed droplet [60, 61]. Statistical methodologies can provide an understanding of cell encapsulation process for developing 3D cancer models via bioprinting. Eventually, a reliable and repeatable control can be gained over the parameters that characterize the cell encapsulation process. Recently, a computational model of cell encapsulation process has been developed [60]. For several target cell concentrations and types of cell loading, encapsulation process was performed and captivated via a computational model (Fig. 24.5b). Probability functions, $P(X_t)$ for encapsulation of single target cells in heterogeneous cell mixture (Equation 3.6 in [60]), were plotted in Fig. 24.5c. While the percentage of target cells and homogeneity reduced in cell suspensions, each probability function, $P(X_t)$, approached a Poisson distribution (Fig. 24.5c).

Similarly, for cell printing process, computational models offer a potential to develop an understanding of how parameters, that can be adjusted experimentally, affect cell viability [62–64]. There are two critical stages during cell printing process: (1) detachment of cell encapsulating droplets from the ejector during ejection and (2) landing of cell encapsulating droplets onto receiving substrate [65, 66]. In these two stages, mechanical factors, e.g., shear stresses, hydrodynamic pressures, capillary forces, may amplify and cause deformation of droplet and cell surface. Eventually, this process may end up with cell death. However, these factors can be controlled experimentally by tuning ejection speed or by replacing encapsulating fluids with those having more suitable material properties including density, surface tension, and viscosity. Cell viability may depend on receiving surface characteristics, e.g., hydrophobicity/hydrophilicity. Prediction of cell deformation and viability via computational methods can enable researchers to successfully build 3D cancer models as well as complex viable tissue constructs [40–42, 67]. A finite-difference/front-tracking technique was described for deposition of viscous compound droplets onto a receiving surface as a model for cell printing process [68]. Inner droplet representing the cell was assumed to be a highly viscous fluid and non-wetting (not sticking to the surface) while encapsulating droplet partially wetted the substrate. A moving contact line model [69, 70] was utilized to predict the dynamic contact angle. In this study, it was also anticipated that cell viability might be correlated with deformation rate [68]. Hence, the settings that result in least cell deformation and the rate of deformation were identified. To do that, analyses were performed for a set of non-dimensional numbers, i.e., Reynolds number (Re), Weber number (We), viscosity ratio (μ_c/μ_d), surface tension ratio (σ_o/σ_i), diameter ratio (d_o/d_i), and equilibrium contact angle (θ_e). Re and We are widely used non-dimensional numbers in fluid mechanics [71] to give the ratio of inertial forces compared to viscous forces and surface tension, respectively [72, 73].

Pressure contours (left side) and pressure distribution on the surface of cell (right side) are plotted in Fig. 24.5d. Shear stresses peaked in the vicinity of the triple

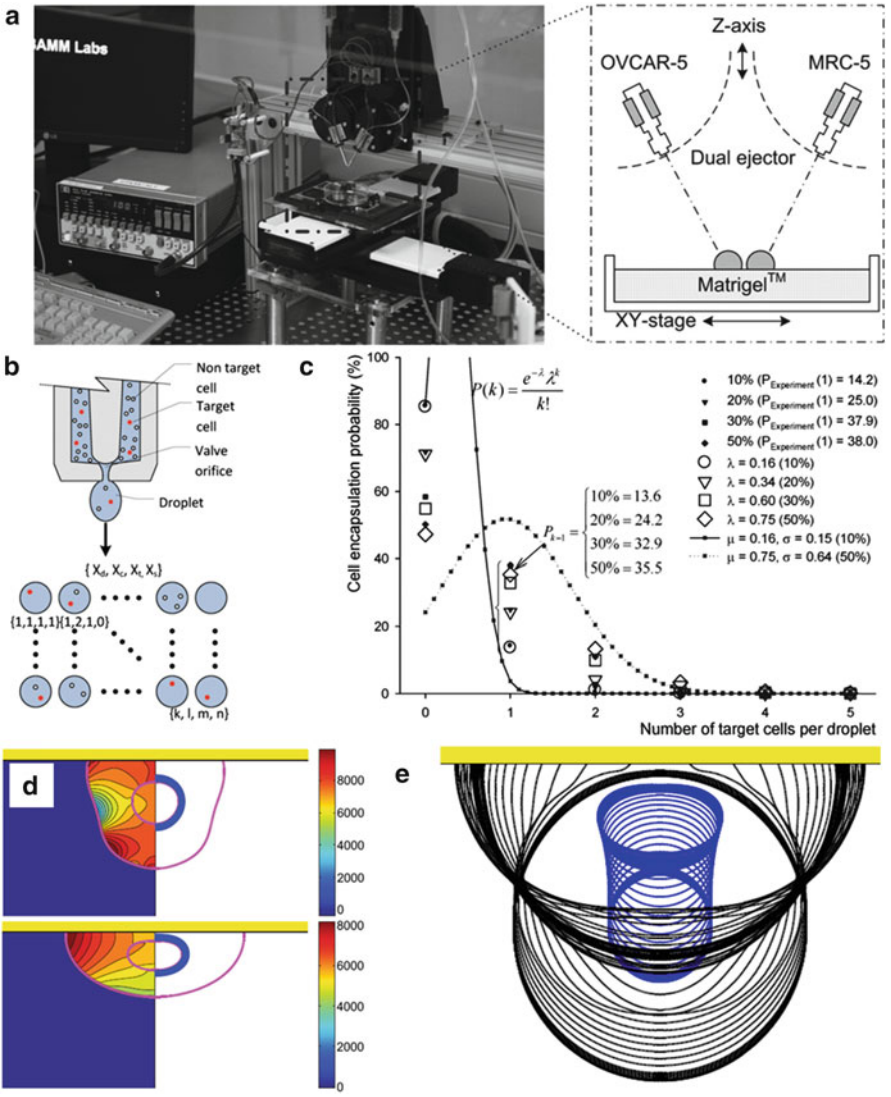

Fig. 24.5 Schematic of a high-throughput bioprinting platform composed of a *xyz* computerized stage and two ejectors diagonally aligned. Ejectors pattern cancer cells (OVCAR-5) and fibroblasts (MRC-5) simultaneously. (**b**–**e**) Statistical and computational modeling of cell encapsulation and printing process. (**b**) A droplet ejector was filled with heterogeneous mixture including target and non-target cells for random cell encapsulation process. (X_d) the number of droplets that contain cells, (X_c) number of cells per droplet, (X_t) number of target cells, and (X_s) droplets encapsulating a single target cell, were mapped onto a matrix of cell encapsulating droplets. (**c**) Cell encapsulation probability, $P(X_t)$, as a function of number of target cells per droplet for cell concentration = 1.5×10^5 cells/ml. (**d**) Pressure contours and pressure distribution on the cell were plotted at the *left half* and the *right half*, respectively. Governing non-dimensional numbers are: We = 0.5, Re = 30, $d_o/d_i = 2.85$, $\sigma_o/\sigma_i = 2541$, $\mu_c/\mu_d = 10$. (**e**) Sequential impact images of cell encapsulating droplet. (**a**) is reproduced with permission [59], (**b**) and (**c**) with permission [60], and (**d**) and (**e**) with permission [68]

point during the initial phase of droplet–surface interaction. Triple point is the point where outer droplet, receiving substrate, and ambient air coincide. Maximum pressure was located near the contact line just before recoil, and migrated to the distal end from the receiving surface where it stayed there until the recoil phase. Cell geometrical deformation was defined as, $=(W_b-H_b)/(W_b+H_b)$ [68]. The computational results demonstrated that the geometrical deformation of cell monotonically increased as: (1) Re increased; (2) d_o/d_i decreased; (3) σ_o/σ_i increased; (4) μ_c/μ_d decreased; or (5) θ_e decreased. On the other hand, a local minimum, at least, of maximum geometrical deformation was obtained at We = 2. Cell viabilities were linked to cell deformation by employing an experimental correlation of compression of cells between parallel plates [74]. Results showed that θ_e and μ_c/μ_d were highly correlated with cell viability.

To develop a better understanding of cell printing process, further computational studies have to be performed [75–78]. The described model above provided a framework to identify conditions that can increase cell viability. Next generation computational models may include non-Newtonian characteristics of flows [79–81], smaller contact angles matching better with experimental conditions, microstructured models for cells, and multiple deposition of cell encapsulating droplets.

Scaffolds (Materials/Composition Perspective)

Basement Membrane Extract

Basement membranes are sheets of ECM that form an interface between epithelial, endothelial, adipose and smooth muscle cells [82]. They contain proteins like laminin and collagen IV that play an important role in the tissue organization [82]. BD Matrigel™ and Cultrex® (Trevigne) matrixes are the examples of the reconstituted basement membrane preparations. These matrixes are extracted from a culture of Engelbreth-Holm-Swarm (EHS) mouse sarcoma. BD Matrigel™ is composed of 60 % laminin, 30 % collagen (IV), and 8 % entactin. BD Matrigel matrix also contains heparan sulfate proteoglycan (perlecan), TGF-β, EGF, insulin-like growth factor, bFG, tissue plasminogen activator, and other growth factors along with MMP enzymes which occur naturally in the EHS tumor [82]. As these matrixes resemble the structure and composition of native basement membrane, they are extensively used for tumor cell culture studies. The SEM image of Matrigel is shown in Fig. 24.6a. Cancer cells can be mixed with liquid Matrigel which forms a 3D gel at body temperature levels. The human small cell lung carcinoma cells mixed with Matrigel were subcutaneously injected into the athymic mouse [83]. It was found that Matrigel helped tumor cells to grow whereas cells did not form large tumors when injected without Matrigel. Other cancer cell lines such as transformed mouse EHS tumor cells (T-EHS), human submandibular carcinoma A253 cells, mouse melanoma B16F10 cells, human epidermoid carcinoma KB cells, and human primary renal cell carcinoma cells were also mixed in Matrigel and coinjected subcutaneously [83]. All of these cancer cell lines rapidly formed growing tumors.

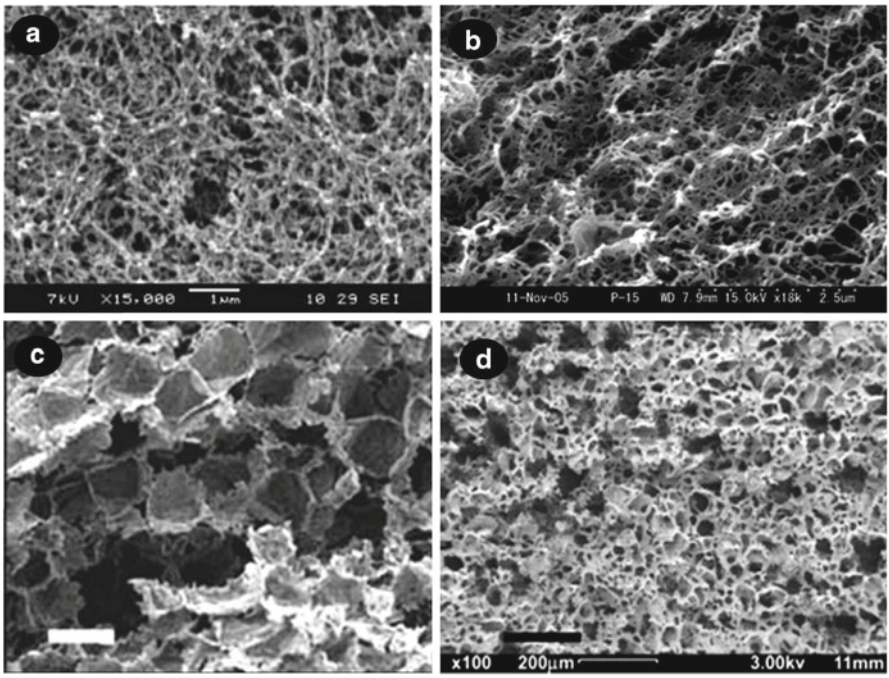

Fig. 24.6 SEM images of various scaffolds for 3D tumor models. SEM image of (**a**) Matrigel, (**b**) PuraMatrix peptide hydrogel, (**c**) PLG scaffold (Scale bar: 250 **μ**m), and (**d**) Polystyrene scaffolds. (**a**) is reproduced with permission [91], (**b**) with permission [170], (**c**) with permission [109], and (**d**) with permission [171]

The sizes of the grown tumors, in the case of A253, KB, and B16F10 cells, were five to ten times more as compared to when cells were grown without Matrigel. These findings unequivocally describe the effects of the Matrigel in improving the growth of human tumors [83, 84]. Matrigel were also employed in coculture of preneoplastic human breast epithelial cells and breast fibroblast derived from tumor tissues [85]. The presence of fibroblast cells supported tumor invasiveness by secreting MMP enzymes which disturbed the ECM architecture. Despite these advantages, Matrigel does not fully represent tumor microenvironment as it lacks collagen (I) and hyaluronan that are present in native ECM of tumors. Collagen type I protein plays an important role in maintaining tissue architecture. Absence of these proteins from Matrigel would not guarantee the fully natural response of tumor cells.

PuraMatrix™ Peptide Hydrogel

BD™ PuraMatrix™ Peptide Hydrogel (BD™ PuraMatrix™) is a biologically inspired self-assembling peptide hydrogel (RAD16-I) matrix that is used to produce 3D microenvironments for various cell cultures including cancer cells [86].

The peptides can be self-assembled into nanofibers at physiological pH by just changing the salt concentration. The diameters of the nanofibers and interconnected pores are ~10 nm and 5–200 nm respectively [87, 88] as shown in Fig. 24.6b. Composition of the PuraMatrix is similar to other natural/synthetic hydrogels as it contains 99 % water and only 1 % w/v standard amino acids. The advantage of PuraMatrix is that the researchers can control the quantity of growth factors, cytokines, ECM proteins and hormones whereas Matrigel and other hydrogels contain non-quantified substances and residual growth factors [32, 89–91]. When ovarian cancer cells (OVCAR-5) were encapsulated into PuraMatrix, they assembled into 3D acinar shapes that closely resembled the shape of metastatic nodules observed clinically [86]. In another study, human hepatocellular carcinoma cells (HepG2) were mixed with PuraMatrix hydrogel and the cell mixture was hydrodynamically focused in the middle of a poly(dimethylsiloxane) channel of a microfluidic device [92]. The cells in PuraMatrix hydrogel secreted twofold more albumin than other scaffolds; quantity of albumin secretion is related to the function of liver cells [92]. New peptides can also be designed such as RADA16 and incorporated with various functional motifs including motifs derived from laminin [93], collagen [94], fibronectin [95], and bone marrow homing peptides [96]. The incorporation of these motifs enhances cell attachment, survival, and proliferation [88, 97, 98]. In one report, motifs incorporated peptide scaffolds significantly enhanced the survival and proliferation of mouse stem cells and also helped in differentiation of stem cells into neurons cells [91]. Compared to PuraMatrix, designer peptide hydrogels significantly enhanced the proliferation of mouse pre-osteoblast MC3T3-E1 cells [99].

Synthetic Scaffolds for In Vitro 3D Cancer Models

ECM structure along with various adhesion proteins and enzymes play an important role in defining tumor aggressiveness and metastatic potential. 3D scaffolds can replicate few components of natural ECM and regulate the specific cell–cell and cell–ECM interactions [2, 100]. Scaffolds have extensively been used in various tissue engineering applications including bone and cartilage [101–103]. The 3D synthetic scaffolds have interconnected microporous structures with nanotopographical features that help cells to adhere the scaffold surface and proliferate. The cell behavior is greatly dictated by physio-mechanical and chemical properties of scaffolds. Scaffolds are composed of natural molecules (Collagen, Chitosan) or synthetic polymers such as polylactide (PLA), polyglycolide (PGA), polyethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA), and poly(lactide-co-glycolide) (PLG) [104, 105]. The stiffness of the synthetic scaffolds can be well controlled as compared to the natural scaffolds but synthetic scaffolds do not allow better cell attachment. For this purpose, either surfaces of the synthetic scaffolds need to be functionalized [106] or ECM components are premixed with scaffold solutions before synthesis [107]. In one study, PLGA and PLA polymers were used to synthesize porous microparticles using solvent evaporation method and were employed for studying 3D tumor culture [108]. Cell adhesion agents such as poly(vinyl alcohol)

and chitosan were also incorporated into the internal structure of microparticles. Different ECM components were physically adsorbed on the microparticles prior to cell seeding. The breast cancer cells (MCF-7) formed clumps on the microparticles, the morphology similar to natural tumor cells. Overall, PLA microparticles containing PVA showed better cell adhesion and growth mechanisms and gave seven times increase in cell density compared to the initial cell seeding density in 9 days [108]. PLGA and PLA are hydrophobic polymers and do not allow cell adhesion in general, but the incorporation of a hydrophilic agent such as PVA/chitosan would make scaffold's surface hydrophilic and enhances the tumor cell growth and attachment. These findings are preliminary and further clinical investigations should be done to analyze the power of microparticle based scaffold for 3D tumor modeling [108]. In another report, the PLG scaffolds were formed using the gas foaming technique and used as a 3D culture model for oral squamous cell carcinoma (OSCC-3) cells [109]. The SEM image of PLG scaffold is shown in Fig. 24.6c. Significant increase in the tumor growth was noticed when cancer cells were pre-cultured on 3D PLG scaffolds as compared to 2D culture. Angiogenic factors were also upregulated by cancer cells cultured on PLG scaffolds; 2, 23, and 98 fold increase in VEGF, bFGF, and IL-8 respectively [109]. Pre-fabricated synthetic scaffolds are also commercially available such as polystyrene scaffold (Alvetex® [110]) (Fig. 24.6d). These scaffolds are designed into the thin membranes so that they can fit into the conventional culture plates. The 3D porous geometry of the polystyrene scaffold facilitates cell growth, differentiation and migration [110]. It is reported that lymphoma cells (HBL-2 cells) showed increased proliferation in 3D Alvetex® scaffolds as compared to 2D culture plates [111]. It is evident from these reports that synthetic scaffold has great potential as 3D tumor models because their morphology and structures resemble natural ECM microenvironment. Other factors such as ECM chemical composition and proteins also influence tumor growth and aggressiveness. Synthetic scaffolds are not synthesized from natural ECM components, and therefore, getting natural response from tumor cells using synthetic scaffolds is a challenging task.

Matrix Stiffness and Fluid Flow Shear Stress for Tumor Cell Migration

Mammalian cells live in 3D microenvironments and are exposed to chemical, mechanical, and structural signals. These stimuli signals change due to disease invasion and progression through the 3D microenvironment [112–114]. Statistically nine out of ten cancer deaths is due to metastasis indicating that metastasis is the primary cause of death in cancer. Investigating the mechanical markers of single cells can help characterizing and monitoring the metastatic potential and invasiveness of cancer cells [112, 115, 116]. During metastasis, invasive cancer cells shed from the primary tumor and navigate through very tiny pores in the ECM to enter the blood vessels and circulate to create a new tumor at a remote organ [115–117]. Studying mechanical response of the invasive cancer cells to the matrix stiffness

gradient of the ECM specifically during the first stage of metastasis and intravasation can illuminate the mechanism of cancer metastasis [116]. Mak et al. explored the migration of cancer cell types in microenvironments with 3D gradients using parallel PDMS microchannels with tapered junctions that connect a large channel with a small channel. The spatially gradient tapered microchannels provide the ability to investigate the cell migration from a more confined environment to a region with greater amounts of freedom. In this case, cells were observed to migrate from wider channel to narrower channel. These results showed that MDA-MB-231 (highly metastatic) cancer cells were more invasive and therefore greater ability to migrate through the tiny high gradient microchannels where non-metastatic cancer cells (MCF-10A's) were not able to penetrate [116].

Matrix stiffness is one of the most important characteristics investigated in cellular microenvironment. Microenvironments in human body have various matrix stiffness ranging from soft such as brain with Young's modulus E ~ 250–500 Pa, to significantly more rigid matrixes including bone (E ~ GPa) and cells respond accordingly in different ECM with different mechanical stiffness. The matrix stiffness of tumors changes during the course of cancer invasion and progression [112, 118]. As an example, recent studies revealed that the breast tumor (4,000 Pa) is an order of magnitude stiffer than healthy breast tissue (200 Pa) [112]. Collagen hydrogels have been one of the most effective and widespread systems for investigating tumor cellular reaction to 3D matrixes with various stiffnesses [112, 114, 119]. This is because collagen hydrogels have physical and biochemical properties that can be altered to match the properties of tissues surrounding a tumor [119]. In a study, Casey et al. showed that the cell-scale gel microarchitecture is important in cell migration and overcome the effect of the bulk matrix density in characterizing invasive behaviors of metastatic cancer cells such as migration [119]. The tumor microenvironment matrix stiffness is a function of stromal collagen deposition and cross-linking which can alter tumor cell migration. Cancer cells employ contractile forces to change the ECM fibers surrounding tumor by aligning the fibers perpendicularly to the tumor [114, 120, 121]. Charest et al. [122] have used a 2D polyacrylamide hydrogel to develop 3D topographical features with various hydrogel stiffness. In such a system, cells are first seeded on a 2D matrix and then after spreading on the surface, cells contact the 3D features on the matrix and migrate along the 3D structures. In this investigation, cells had a higher contact length on stiffer matrixes. The traction forces produced by the cancer cells in 2D and 3D matrixes to characterize metastatic cancer cells have also been investigated [123]. These results showed that breast, lung and prostate cancer cells had metastatic ability and at the late stages of the cancer disease had significantly higher traction forces than the normal healthy tissue cells. In addition, cancer cells generated greater contractile forces on stiffer matrixes. Tumor microenvironment's mechanical properties as well as chemical properties define the degree of the traction forces [123]. Further, optical measurements and Atomic Force Microscopy (AFM) studies on the mechanical response of metastatic cancer cells have shown that these cells are more compliant compared to benign cells [123–126]. The metastatic cancer cells are then more plastic and therefore are able to easily migrate through the ECM [123].

During the first stage of metastasis, the ECM around the tumor degrades due to the action of matrix MMPs [127–129]. Fluid shear stress is one of the extracellular stimuli that modulate MMP genes [130], and therefore, shear forces could potentially alter the migratory response of cancer cells. Qazi et al. investigated shear stress as a main controller of cell migration that helped explaining the differences between the invasiveness glioma cells in vitro compared to in vivo [129]. This work explains the diverse migratory response of tumor cells and differential invasiveness of the cancer cells due to fluid flow forces and shear stress. These results show that the motility of glioma cells can be reduced due to the shear stress by changing the MMP expression [129]. Recently, the role of fluidic shear stress on the metastatic potential of epithelial ovarian cancer cells was investigated using microfluidic platform. The ovarian cancer cells showed enhanced epithelial–mesenchymal transition and metastatic potential only when cultured under continuous and controlled laminar flow [131].

Conclusions and Future Perspectives

Tumor tissues and carcinomas are comprised of a 3D spatially organized ECM [38, 132–135] with multiple cell types [136, 137]. For in vitro cancer models, presence of multiple cell types is critical as the stromal cells play a critical role in malignant progression of tumor including angiogenesis [136], metastasis [138], and invasiveness [139], and these cell types are important targets for tumor therapies [140]. Presence and spatial positioning of neighboring cells are important factors when studying the precise role of stromal cells in tumors [141, 142]. There are several technologies that can precisely position different cell types in a 3D setting, such as bioprinting [41, 42, 143–146], microfabrication [147], and microscale assembly [40–42, 148]. These methods can potentially be used to create 3D cancer models and to study the interaction between different cell types [149]. In embedded and overlay cultures, basement membranes can promote 3D cellular organizations by establishing biologically relevant contact between cells and substratum [30]. Culturing cells in 3D suspension is another way of creating 3D cell cultures by reducing the adhesion between cells and the substratum. Hanging drop is a promising method to create 3D cell culture models through aggregation of cells at a spherical air–liquid interface within hanging drops [34–37]. Cells can be magnetically labeled with paramagnetic/diamagnetic materials and suspended in culture medium using magnetic levitation and gravitational force to create 3D tissue cultures [38]. Microscale hydrogels encapsulating cells have also been assembled using magnetic assembly that can be used potentially for 3D cancer models [40–42]. Bioprinting technology is a repeatable, reliable, and high-throughput method that utilizes a programmable and automated stage with ejectors to bioprint different cell types and to create 3D coculture cancer models [59, 150]. Controlling cell culture in low gravitational forces is another way of facilitating 3D cell growth in a spatially uniform cell distribution [43–46]. Microgravity bioreactor [43–46], rotating wall vessel [49], and cell culture in international space [47, 48] are three examples of such methods as these techniques were discussed in detail in this chapter.

Cells cultured as 2D monolayers showed substantial mutations in gene expression compared to cells in 3D cultures and native tissues [151, 152]. Cancer cells cultured on 2D versus 3D microenvironments display dissimilar cell morphology [153], metabolic characteristics, and drug response [154]. Moreover, genes responsible for angiogenesis, chemokine generation, cell migration and adhesion have different expression levels between 2D and 3D settings [155, 156]. Importantly, cancer cells are known to display different behavior to chemotherapeutic drugs in 3D culture compared to 2D controls [157]. Cumulative evidence in the literature demonstrates that in vitro 3D cancer models better recapitulate in vivo conditions and response compared to 2D cultures [153, 158, 159]. There are also cancer types (e.g., Ewing's sarcoma) for which the currently available murine models and murine cells perform poorly compared to in vitro models employing human cells [160]. For these cancer types, human cells need to be used in 3D culture conditions to effectively represent the in vivo conditions.

There are many factors that need to be considered in a 3D in vitro cancer models to produce a characteristic feature of the in vivo 3D solid tumors. In embedded and overlay cell culture, 3D models provide the space for cell migration and proliferation, which mimic the 3D in vivo models [31]. RWV cancer models also provide a 3D microenvironment that were validated to produce a characteristic feature of the tumor by immunohistochemical profiling of multiple markers such as p53, BCL2, and CA125 and proteomic profiling such as prohibitin, VDAC1, and annexin 4 [53]. Multicellular spheroids mimic the native tumor microenvironment and emulate the drug-resistant hypoxia regions at the center of grown tumor mass [161]. On the other hand, these spheroids do not emulate the in vivo blood vessel barrier due to direct contact with culture media of cancer cells in spheroids [162, 163]. The spheroid culture do not fully provide in vivo host immune interactions during tumor growth [161]. In basement membrane tumor models, such as BD Matrigel matrix, the effect of multiple parameters that occur in a solid tumor including heparan sulfate proteoglycan (perlecan), TGF-β, EGF, insulin-like growth factor, bFG, tissue plasminogen activator, and MMP enzymes have been considered. However, Matrigel 3D cancer model lacks collagen-1 and hyaluronan that are essential in maintaining the architecture of tissue. The absence of these proteins affect the cancer cell response in a 3D model [82]. It was reported that mammary epithelial cells self-assembled into spherical structures with a central lumen resembling natural mammary acini, when cultured on laminin-rich basement membrane [164]. In another report, the epithelial cells have inverse polarity and did not form central lumen when cultured on 3D collagen gels alone, whereas they showed normal polarity and central lumen structure when cocultured with myoepithelial cells. The myoepithelial cells deposited the basement membrane component laminin-1 which was an important factor to determine the tumor polarity and central lumen formation. [165]. It clearly points out that ECM components have a significant effect on morphology and polarity of cancer cells during tumor cancer growth. Bioprinting is a high-throughput technology that can produce 3D cancer models repeatably with a reliable control over spatial resolution. In one report, ovarian cancer cells (OVCAR-5) and fibroblast cells (MRC-5)

were patterned on Matrigel using a bioprinting platform. The cancer cells spontaneously formed multicellular acini structures that resembled the polarity of the tumor [59]. The coculture of cancer and supporting stromal cells at various cell densities were overlaid successfully using the bioprinting technique [59]. However, more studies have to be conducted to show if such a platform mimics the aggressive and invasive characteristics of tumor cells.

In order to minimize the animal testing and cost, there is a need for 3D in vitro tissue models, which are scalable, can be produced with high-throughput methods and that mimic the tissue native microenvironment [166]. Scaffold-free 3D microtissue models are considered more organotypic and compatible with high-throughput technologies. They are currently being developed and used with automated production platform for tumor microtissues [167]. High-throughput bioscreening allows systematic and quantitative screening of chemotherapeutic drugs, supporting rapid pharmacokinetic and pharmacodynamic analyses. Since throughput is critical in cancer research, where large compound libraries are evaluated regularly with many different cell types, advanced assembly/fabrication technologies with precise compositional and spatial control over cells to establish 3D platforms are needed [149, 168]. Automation and miniaturization of these technologies would also allow rapid and effective fabrication of a large scale of 3D in vitro tissue models with patient's own cells, which then can be used to screen a palette of therapeutic candidates and to match the best fit with the patient in a personalized manner. Future 3D models of complex tissues and tumors also need to take into consideration the physiological environment, such as the mechanical microenvironment including fluid flow and mechanical forces in play.

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Chapter 25
Complex Transport Around Tumor: Need for Realistic In Vitro Tumor Transport Model

Bumsoo Han

Abstract Successful targeted drug delivery requires to overcome various transport barriers around tumors. These transport barriers are associated with dynamic biological, chemical, and mechanical conditions of tumor microenvironment. Thus, new drug delivery vehicles need to be designed considering complex transport processes around tumors. However, currently available tumor models are limited to mimic this complex and dynamic environment, or only provide the end results without allowing systematic investigation of these complex transport processes. A new tumor model system is highly desired, which can address this twofold challenge of current tumor model systems—(1) realistic simulation of in vivo tumor microenvironment, and (2) capability of systematic evaluation of drug delivery vehicles. In this chapter, the transport processes around tumors relevant to targeted delivery are reviewed and research efforts to mimic these processes to evaluate drug delivery vehicles are discussed.

Introduction

Targeted delivery of drugs to tumors is an important challenge to be addressed in order to achieve effective cancer treatment without the toxic side effects of anticancer drugs. The ultimate objective of targeted delivery is to deliver most of the administered drug to the target, while eliminating or minimizing the accumulation of the drug at any nontarget sites. Many novel therapeutic agents have been developed for cancer treatments including chemotherapeutic agents, anti-angiogenic agents, immunotoxins, and small interfering RNA (siRNA), but their in vivo efficacy is still significantly limited [1–4]. To accomplish their therapeutic purpose,

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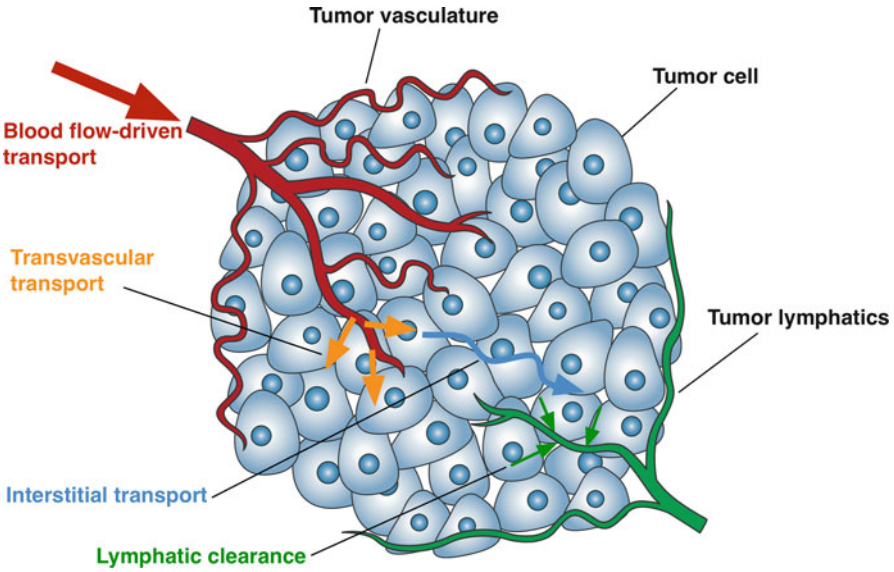

Fig. 25.1 Schematic of tumor microstructure and transport processes around tumors. Tumor vasculature has highly chaotic and nonuniform structure, and the tumor lymphatics is less developed and has few functional lymphatic vessels. When NPs are intravenously administered, the NPs reach the tumor via blood flow-driven transport along the tumor vasculature (*red arrow*), transvascular transport (*orange arrows*), interstitial transport (*blue arrow*) and cellular uptake. The excess NPs will be cleared to the lymphatics (*green arrows*)

these drugs should be efficiently delivered to tumor cells and tissues at sufficient concentrations. However, the delivery efficacy of these novel therapeutic agents is significantly limited due to complex and impaired transport processes around tumors.

The recent development of nanotechnology provides a wide variety of nanostructures as delivery vehicles, whose properties can be tailored for targeted delivery of the anticancer drugs. These nanostructures include liposomes, polymer micelles, dendrimers, drug nanocrystals, magnetic nanoparticles, gold nanoparticles/nanoshells, nanorods, nanotubes, and drug–polymer conjugates (all of which will be collectively referred to as nanoparticles, or NPs). Research aiming to precisely control the size and surface properties of these NPs responsive to tumor microenvironment is actively performed to achieve targeted delivery [5–7]. Even through the improvements of their delivery efficacy have been reported, still the majority of administered NPs does not reach to the target tumors.

After being administered to a patient's blood stream, the NPs are delivered to cancer cells via complex and multifaceted transport processes as illustrated in Fig. 25.1. These include: (1) blood flow-driven transport to tumor vasculature, (2) transvascular transport (i.e., extravasation), (3) interstitial transport, and (4) cellular uptake. Excess NPs will occupy the interstitial space or be transported through the lymphatic vessels. These transport phenomena are governed by diffusion and/or convection processes,

and the significance of each process is thought to be dependent on the size and configuration of the NPs and various physiological conditions. These physiological conditions include permeability of blood vessel wall, interstitial fluid movement, and diffusivity at tumor interstitium. These processes and physiological conditions are highly dynamic, interconnected, and spatiotemporally varying.

In order to develop NPs for successful targeted delivery, the NPs should be designed and evaluated considering these complex transport processes. However, this is extremely challenging because of limited quantitative understanding on the transport processes. Moreover, lack of versatile models makes it even more challenging, which can simulate realistic in vivo tissue microenvironment and allow systematic study of the in vivo transport characteristics. Conventional static in vitro systems including cell suspensions and cell monolayers are not adequate to study these complex in vivo transport processes because the model systems lack dynamic interactions of tumor microenvironments among the cells, extracellular matrix (ECM), interstitial fluid and NPs. Animal models can provide tumor microenvironment with all these dynamic interactions, but it is very difficult to systematically study the effects of these dynamic interactions. Thus, new model systems are highly desired to address the limitations of currently available models. In this chapter, key features of in vivo transport processes around tumor relevant to targeted delivery will be reviewed and current status and challenges of developing transport model systems will be discussed.

Transport Processes Around Tumor

The complex transport phenomena experienced by the NPs are associated with physiological characteristics of tumors. Because of the leaky vasculature of the tumor, as illustrated in Fig. 25.2, the NPs are thought to extravasate more in tumor vasculature than in normal vasculature [8–10]. At the same time, however, the increased interstitial fluid, less functional lymphatic vessels, dense ECM microstructure and high cell packing density of the tumor may result in significantly elevated interstitial fluid pressure (IFP), which can adversely affect the extravasation and interstitial transport of the NPs [11–13]. In addition to the elevated IFP, the dense ECM microstructure [14, 15] and high cell packing density [16] can also impair the interstitial transport of the NPs. These tumor microenvironmental parameters are highly dynamic, interconnected and vary spatiotemporally [17, 18]. The compounding effects of all these physiological parameters on NP transport are not yet fully understood yet.

Blood Flow-Driven Transport

After being administered intravenously, the NPs circulate in the blood stream consisting of complex cells and plasma proteins. During the blood circulation, a significant portion of the NPs are taken up by the immune cells in the blood stream

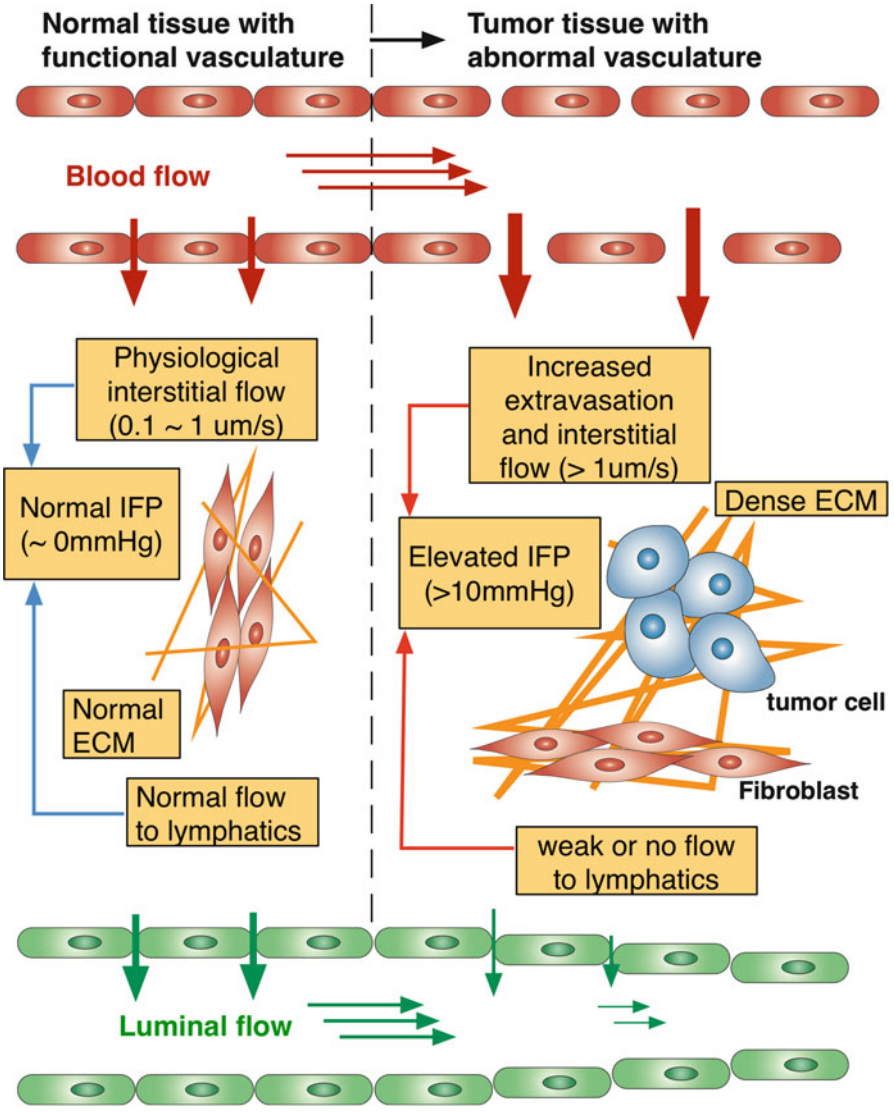

Fig. 25.2 Schematic of vascular and tissue structure relevant to fluid and NP transport of normal and tumor tissues. In normal tissue, the endothelium is tightly packed so that the cutoff pore size is small and very low interstitial fluid flow presents. This fluid flows to the lymphatics through the normal ECM, and the IFP minimally builds up. On the contrary, the endothelium of tumor tissue is leaky and has large pores, which leads to high interstitial fluid flow and more extravasation of the NPs. In conjunction with less functional lymphatics and the dense ECM, this increased interstitial fluid flow results in elevated IFP, which adversely affects the extravasation. The compounding effects of the elevated IFP, leaky vasculature, and poor vascularization of the tumor are still unknown

including monocytes, leukocytes, dendritic cells, and in tissues by resident phagocytes (i.e., by the reticuloendothelial system (RES) of the spleen, liver, and lungs) [19, 20]. Various properties of the NPs are thought to affect the NP clearance including size, surface charge, solubility, and surface functionality. This NP clearance significantly reduces the number of the NPs available in the blood stream to reach the target. Thus, the surface of many NPs is PEGylated in order to prolong the circulation of the NPs and decrease the uptake by the reticuloendothelial system [20, 21]. In addition to the size and surface properties, the effects of the NP shape on their biodistribution are also investigated [22]. Besides the uptake by the immune system, the NPs also interact with other components of the blood. These could result in hemolysis, which refers to the damage to red blood cells leading to the leakage of the iron-containing protein into the bloodstream [19], and degradation of the NP integrity, which results in premature dumping of the encapsulated drugs.

Once the NPs reach near the target site after escaping the clearance, they move close to the endothelium of the tumor vasculature to extravasate into the tumor interstitium. During this lateral transport within the blood vessel, multiple physical forces may be exerted on the NPs including hydrodynamic pressure and shear stress induced by blood flow, adhesion force from the blood cells, and other NPs and the endothelium [2, 23–26]. Moreover, the chaotic and irregular geometry of tumor vasculature including bifurcation and branching has also been thought to affect the NP transport within the blood vessel [26–28].

Transvascular Transport

Tumor vasculature is composed of a highly disorganized network of blood vessels with endothelium with enlarged intercellular gaps, known as fenestrations, heterogeneously distributed across tumor vasculature [29]. The presence of fenestrations results in increased transvascular transport of both fluid and macromolecules. The combination of enhanced transvascular permeability and poor lymphatic drainage lead to the well-known enhanced permeability and retention (EPR) effect that stands for the preferential accumulation and extended presence of therapeutics in the tumor tissue compared to the rest of the body [9, 30].

The cutoff diameters of vascular pore for different tumor have been reported to be between 300 and 700 nm, and, while in rare occasions, it can be up to 2 μm, which are significantly larger than those of normal tissue (typically smaller than 20 nm) [31–33]. Extravasation of the NPs to tumor interstitium was found to be highly heterogeneous possibly due to nonuniform vascularization as well as uneven distribution of pore size across the vasculature [34, 35]. The relationship between the pore size and transvascular permeability depends on the size of the therapeutic agent. For small macromolecules with hydrodynamic diameters that are much smaller than the pore size, transvascular permeability was observed to be quite insensitive to the pore size [33]. However, for the NPs that are larger in size, e.g.,

50–200 nm, the effect of pore cutoff diameter on transvascular permeability becomes more profound. Transvascular permeability also depends on the developmental state of cancer and organ sites, where two-fold difference between the primary site of breast cancer tumor (in mammary fat) and that in the metastatic site (in cranial window) was reported [36].

Transvascular transport in solid tumors is typically modeled as a process where the extravasation of therapeutic agents is assumed to occur continuously by convection across a pressure gradient and diffusion across a concentration gradient. The NPs that are larger than the vessel pore size are assumed to remain in the blood stream while the NPs smaller than the pore size, like most macromolecules, extravasate to the tumor interstitium. However, blood flow has pulsatile nature, which results in oscillations in the microvascular pressure (MVP), and marked changes in the vessel wall strain, pore size and permeability during the process [37, 38]. It is possible that the transvascular transport of nanoparticles with sizes equal to or larger than the pore cutoff diameter may involve accumulation of particles near pores followed by intermittent bursts of particles by blockage induced pressure buildup. Transient nature of microvascular pressure may reveal new physical processes that cannot be explained by the classical Starling's Law.

In order to improve transvascular transport, Yuan et al. [32] experimentally investigated the effect of molecular size on transvascular transport, and provided transport property database of various molecules. Monsky et al. [39] illustrated that transvascular transport of macromolecules could be enhanced using vascular endothelial growth factor. Netti et al. [40] investigated transvascular transport enhancement by modulating tumor MVP using periodic or continuous injection of angiotensin II. Recently, a strategy to normalize the tumor vasculature to achieve improved drug delivery throughout tumor tissue has been proposed [41, 42].

Interstitial Transport

After the extravasation, the NPs encounter multiple hindrances at the tumor interstitial space caused by elevated tumor IFP and abnormal ECM structure [14, 15, 43, 44]. As shown in Fig. 25.3, the IFP of a solid tumor stays at elevated level and sharply decreases at the periphery of the tumor. Due to the importance during drug delivery, physiological changes by elevated tumor IFP have been studied by many researchers [12, 45, 46]. IFPs of various tumor types were reported to vary from 4 to 50 mmHg with an approximate average of 20 mmHg, which was much higher than the IFP of normal tissues, approximately 2 mmHg [13, 47]. Drastically higher IFPs of 75–130 mmHg are reported for pancreatic tumors [48]. Boucher and Jain [49] reported that there was a strong connection between IFP and MVP, and DiResta et al. [50] experimentally illustrated the tumor IFP increased as the tumor grew. Netti et al. [51] investigated the connection between IFP and MVP using poroelastic tumor model and suggested that periodic modulation of blood pressure might lead higher drug uptake. Stohrer et al. [52] experimentally found that oncotic pressure in

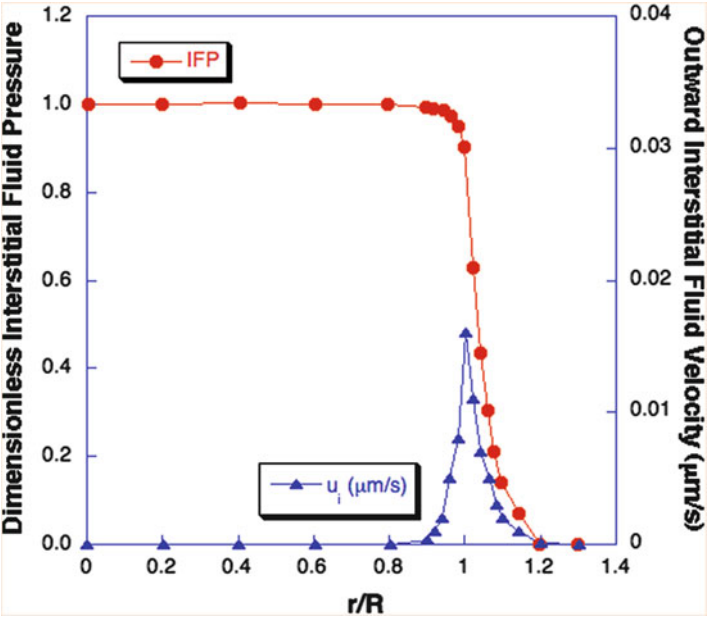

Fig. 25.3 Interstitial fluid pressure and velocity distribution of a tumor grown in subcutaneous tissue. The IFP (*filled circle*) stays at elevated level at the interior of the tumor and sharply decreases at the periphery. Due to this pressure gradient, radially outward interstitial fluid motion is induced at approximately 0.02 μm/s (*filled triangle*). This outward convection in conjunction with less extravasation due to the elevated IFP is believe to lead insufficient delivery of therapeutic agents. (Redrawn from [11])

tumors was also elevated. Milosevic et al. [53] analyzed the effects of elevated tumor IFP on blood flow to the tumor and showed a reduced blood flow due to the elevated IFP.

This elevated IFP is thought to result from anomalous characteristics of tumor vascular structure including high vascular permeability and lack of well-developed lymphatic vessels. This elevated IFP adversely affects the transport of therapeutic agents in several different levels: (1) less extravasation of the agents [54], and (2) radially outward interstitial fluid movement at the periphery of tumor (see Fig. 25.3). Consequently, the elevated IFP contributes to insufficient delivery of drugs to the interior of tumors. Moreover, high collagen content and cell packing density result in low diffusivity of NPs. Thus, transport of macromolecules and NPs is significantly limited in tumor interstitial space [14, 55–57]. A wide variety of methods have been proposed and investigated to enhance the interstitial transport, but the main underlying strategies are either lowering tumor IFP [58–60] or modulating tumor ECM structure [14, 61]. However, due to the complex interaction involving various physiological parameters, the control or manipulation of tumor IFP and ECM structure still warrants further research.

Cellular Transport

Once the NPs are transported through the tumor interstitial space, these should act on tumor cells, but their efficacy may also be limited due to complete or partial drug resistance [62–65]. Multidrug resistance (MDR) is thought to be caused by a group of membrane proteins extruding cytotoxic molecules so that the intracellular drug concentration is maintained below the effective levels. These proteins belong to the ATP binding cassette (ABC) superfamily of membrane transporters [66], most of which use the energy of ATP hydrolysis for the efflux of drugs (i.e., active transport). This family includes the well-characterized P-glycoprotein (Pgp) encoded by MDR-1 gene [67–72], the multidrug resistance protein (MRP) [73–77] and the mitoxantrone resistance protein (MXR), also known as breast cancer resistance protein (BCRP) [78–80]. Recently, a non-ABC transport protein, RLIP76, has been identified, which is also associated with MDR [81]. Numerous clinical data imply that MDR phenotypes in tumors are associated with the overexpression of these transporters. Since these transporters have wide recognition patterns of substrates, the overexpression of these proteins will result in the multidrug resistance. In addition to the over-expression of these transporter proteins, cellular drug resistance also appears to be mediated by the binding of tumor cells to the ECM [82, 83].

Since active efflux of chemotherapeutic drugs poses a serious challenge to successful cancer treatment, various strategies have been proposed to overcome MDR as reviewed elsewhere [62, 63, 84, 85]. The most extensively studied strategy is to inhibit drug efflux by modulating the activities of the MDR-associated proteins. This can be achieved by the co-application of MDR modulators with anticancer drugs. A wide variety of compounds have been identified as MDR modulators. For example, verapamil, cyclosporine, and their derivatives have been investigated in preclinical studies and resulted in increased intracellular drug concentration [86–90]. Besides these chemosensitizers, monoclonal antibodies have been studied as potential MDR modulators [91, 92]. In addition to the MDR-associated proteins, the membrane lipid has also been investigated as a target for manipulation, as reviewed elsewhere [93]. The alteration of membrane biophysical properties, including membrane fluidity and permeability, could increase or decrease cellular uptake of drugs [94–96]. Polymeric excipients [97] and transcriptional regulators [98] have also been studied. Although heat shock has been reported to induce MDR in some cancer cells [99, 100], an increase in the cellular drug uptake and cytotoxicity by ultrasound-induced hyperthermia was reported [101, 102]. Even though these various strategies have been proposed and studied, MDR in cancer is still a clinical challenge since the delivery of these modulators to the target tumor is as challenging as the drug delivery.

Current Strategies for Nanoparticle Delivery to Tumor

Current strategies for the NP delivery primarily rely on two mechanisms, extravasation and/or ligand–receptor interactions [103]. It was observed that tumor vasculature is leakier than normal vasculature [8, 43]. Since the tumor vasculature wall has

larger pores compared to the normal vasculature wall, the NPs, whose size is in between these cutoff pore sizes and surface is PEGylated for prolonged blood circulation, are believed to accumulate at the tumor more than the control non-particulate solution formulation. The drug accumulation by the difference in this vascular permeability is often called the EPR effect [8, 9] and has been a key rationale to design NPs for targeted delivery. Ligands can be attached on the surface of NPs so that the NPs, if they are near the target tumor cells, bind to the cells for increased uptake. Clearly, this process becomes effective after the NPs reach the vicinity of the target tumors. These strategies resulted in the improved accumulation of NPs at the tumor, but the in vivo efficacy of NPs and NP-mediated drugs is still significantly impaired. Only about 5 % of the administered dose ends up at the target tumors [4]. The remaining significant portion of the NPs is taken up by the RES of the spleen, liver, and lungs [20]. New design paradigm for NPs is highly desired that considers the complexity of their transport processes in vivo, in order to significantly improve the transport efficacy of the administered NPs to target tumors.

Tumor Models for Nanoparticle Evaluation

Current Tumor Models

Most widely used tumor models are two-dimensional (2D) cell monolayers, human cancer cell lines on a substrate [104, 105]. The cell monolayers provide experimental environments that are generally convenient, provide good cell viability and are quickly reproducible. However, the 2D models lack various key features of in vivo tumor microenvironments including cell–cell and cell–matrix interactions [104–107]. These architectural and environmental differences further affect the cellular difference in both gene expression [105, 108] and drug resistance [109]. In order to address these deficiencies, three-dimensional (3D) models such as spheroids and engineered tissue scaffolds have been developed [107]. The 3D microenvironments and architectural structure provided by these models induce cell morphology, signaling, and gene expression similar to in vivo tumor microenvironments [105, 107], but the effects of fluid dynamics relevant to tumor microenvironments including interstitial fluid flow and pressure are lacking [110, 111]. Thus, these models are significantly limited to characterize the NP transports around tumors in vivo.

Animal models have been valuable platforms to characterize the in vivo behavior of the NPs. Xenograft models, one of the most widely used model systems, are established by culturing human cancer cells or tumor explants into immunodeficient mice. Besides the xenograft models, autochthonous models, where cancers are spontaneously developed by chemical or genetic methods, are also being used depending on target cancer types. A recent detailed review on animal models used in preclinical drug testing can be found elsewhere [112]. The tumor microenvironment of the animal models has many key features lacking in 2D and 3D in vitro models. However, even animal models often fail to simulate human in vivo environments and to provide a mechanistic explanation of the in vivo behavior of NPs.

This is because of: (1) the unknown scaling factors to extrapolate from animal models to human subjects [2], (2) the mismatch between human cancer cells and mice matrix environments [113, 114], (3) the difficulties to simulate the heterogeneity of tumor microenvironmental parameters [48, 115], and (4) the inability to independently control these parameters in the model. Thus, a new model system is greatly desired, in which the tumor microenvironmental parameters can be systematically and independently controlled, but at the same time the dynamic interactions among the fluids, ECM, cells and NPs are maintained.

In order to address the limitation of in vitro static cell culture and mimic more in vivo-like environment, various new cancer cell cultures on microfluidic platforms have been developed as reviewed elsewhere [116, 117]. Briefly, the first generation of microfluidic cancer cell cultures were 2D cell monolayers cultured on microchannels in the presence of fluid flow exerting shear stress on the cells [118–120]. The presence of cell–fluid interaction is a significant advance from the static 2D cell monolayer models. However, several studies reported that the cell physiology of 2D models is distinctively different from that of cells cultured in 3D matrices [121–123]. Then, 3D culture environments have been created by combination of tumor spheroid and tissue engineering technology [106, 124–127]. In these models, cancer cells were cultured within polymeric matrix to mimic cell–matrix interaction in vivo, and could impose spatial gradients of growth factors and pH [128–131]. Tumor spheroid culture on a microfluidic platform was also reported [126]. More recently, 3D tumor cell cultures on a microfluidic platform with interstitial fluid flows have been reported [132]. All these 3D tumor models show great promises to mimic the in vivo tumor microenvironments and ultimately engineering tumors [133]. The most significant advantages of these microfluidic 3D models are flexibility and controllability to systematically study the effects individual tumor microenvironmental parameters, which the animal models cannot offer. However, these microfluidics models still warrant further research to create directional cell–matrix and tissue–tissue interactions [117, 134, 135]. Since cells are typically seeded within polymeric scaffold in these 3D models, their cell–matrix interactions are nondirectional and affect cell polarity differently from in vivo during cancer development [107, 136, 137]. Moreover, the NP transport in vivo is greatly affected by the interfacial phenomena at tissue–tissue interface including endothelium–blood, endothelium–interstitium, and interstitium–lymphatics endothelium. These interactions should present on the microfluidic model in order to properly simulate the NP transport in vivo.

A New Tumor Transport Model

In addition to these tumor culture models, a concept of tumor "transport" model has been recently proposed to simulate 3D in vivo tumor microenvironment relevant to the evaluation of NPs. This new model consists of three compartments to simulate in vivo tumor microenvironments—vasculature, interstitium and lymphatics.

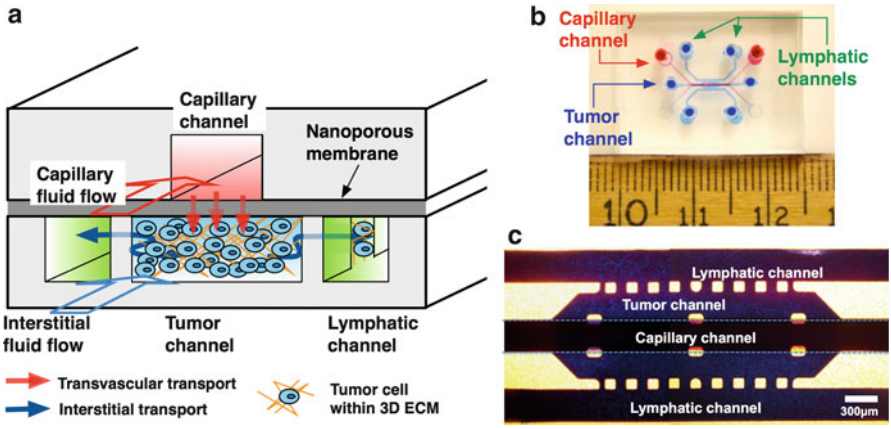

Fig. 25.4 Conceptual design and fabricated prototype of a microfluidic tumor transport model. (**a**) The model has a 3D structure. The *top* channel (*red*) simulates the capillary using nanoporous membrane. Various NPs can be introduced along this capillary channel. The *bottom* layer has a center channel (*blue*) mimicking a 3D tumor microstructure (i.e., cells in 3D matrix) and two side channels simulating the lymphatics (*green*). The tumor channel will be pressurized to establish the elevated IFP. (**b**) Prototype of the fabricated tumor transport model (the ruler marks are in millimeters; capillary channel is filled with red fluid; tumor and lymphatic channels are filled with blue fluid). (**c**) A micrograph of the top view of the channel structure. Scale bar = 300 **μ**m

The conceptual design of the proposed model and a prototype fabricated are shown in Fig. 25.4. Its fabrication and operation procedures are described in [138]. The model has a 3D structure formed by stacking two layers of polydimethylsiloxane (PDMS) microchannels with a porous membrane sandwiched between the layers. The top layer has a channel simulating the capillary of the tumor vasculature, and NP-suspended fluid will flow along the channel at a physiologically relevant velocity and pressure. The porous membrane mimics the endothelium of the capillary whose pore size determines the transvascular transport. The bottom layer has three channels, which are partitioned with periodic posts. The center channel simulates the tumor interstitium and the two side channels simulate the lymphatics. In the tumor channel, cancer cells grow within 3D collagen matrix, and the interstitial fluid flows through the matrix and exerts elevated IFP. The NPs are transported through this 3D tissue structure and reach the cancer cells. The excess NPs and interstitial fluid are collected into the two side lymphatic channels. Including the tumor channel, the pressure of all the channels is independently controlled by connecting fluid reservoirs at the desired pressures.

This model mimics several key physiological features of tumor microenvironment including—(1) fluid flow-driven transport along the tumor vasculature, (2) transvascular transport across the endothelium, (3) interstitial transport through the tumor interstitium, (4) cellular uptake of the NPs by tumor cells with cell–cell

and cell–ECM adhesion, and (5) transport of excess NPs to the lymphatic vessels. Thus, the transport processes on this model simulate better those of tumors in vivo, and can be systematically studied. Although it is promising, the model needs to be further developed to create more realistic biological, chemical and mechanical conditions of in vivo tumor microenvironment. These include the complex and heterogenous composition of tumor cells and ECM, the clearance of the NPs via the RES, the presence of stroma, and lymphatic endothelium.

Concluding Remarks

Targeted drug delivery has profound impacts on cancer treatment, and emergence of nanotechnology shows a great promise on achieving this goal. Many NP systems are designed using the concept of the EPR effect whose size is the primary design parameter. However, the NP transport to the tumor is a confounded outcome attributed to various in vivo transport mechanisms including the clearance by the RES, the extravasation, the hindered interstitial transport by the high IFP and dense ECM microstructure of the tumor, and the cellular uptake of drugs often compromised by the MDR associated protein transporters. Nanoparticles will have to transport through these highly complex tumor microenvironment, whose biological, mechanical, and chemical conditions vary in a spatiotemporal manner. Development of truly targeted drug delivery systems, thus, requires new design paradigms considering these complex biotransport processes. In addition to changes in design strategies, new experimental models and evaluation criteria for successful delivery are also required to address the limitations of currently available in vitro cell culture models and in vivo animal models.

The new models should be capable of simulating the complex in vivo tumor microenvironments, i.e., vasculature/interstitium/lymphatics, relevant to the transport processes of NPs. In addition to simulating these complex in vivo processes, the controllability enabling systematic variation of various tumor microenvironmental parameters including IFP and flow, ECM structure, and cell packing density is essential. The model should also be able to mimic the heterogeneity of cancers in terms of cell population and distribution within a tumor. Although the animal models will be still essential tools for drug development and testing, the new models will enable to answer many critical questions to establish quantitative understanding of the in vivo transport processes, which cannot be answered using conventional in vitro or small animal models. Moreover, the new models can also be useful to test various new targeting strategies based on the physiological features of the in vivo tumor microenvironments including pH gradients, overexpressed oncogenes, lowering IFP, ECM degradation, and normalizing tumor vasculature [42, 139]. Recent developments of microfluidics and tissue engineering technologies can realize new robust but versatile platforms and provide answers to this critical bottleneck of achieving targeted drug delivery to tumors. However, further research for verification and refinement of these platforms is urgently desired.

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Part VII
Prospective and Future Direction

Chapter 26
The Missing Components Today and the New Treatments Tomorrow

Kinam Park, You Han Bae, and Randall J. Mrsny

Abstract The history of drug delivery technology is only 60 years old, but various mechanisms of controlled drug delivery have been well established. While numerous controlled release formulations have been developed, only a handful of these approaches has been used successfully as anticancer treatments. Current approaches to deliver anticancer agents to tumors commonly involve the intravenous administration of submicron size formulations. These nanoparticle-based approaches frequently show impressive efficacy in small animal tumor models, but their translation to safe and efficacious clinical outcomes has been disappointing. It is our thesis that the poor success rate of these approaches is primarily due to an insufficient understanding of cancer biology and physiology; knowledge that is necessary to achieve selective and efficient targeting of these anticancer therapies. To substantially improve targeted drug delivery to treat cancers we must know more about how cancer cell heterogeneity, cancer cell drug resistance, as well as tumor properties and microenvironments play a role in cancer development, progression, and metastasis. Additionally, there is a great need to identify in vitro and in vivo models that more directly emulate specific elements of cancer cells and tumors that restrict the success of our current anticancer approaches. Although a complete cure of cancer is the ultimate goal, it may be more realistic in the near future to treat cancer as a chronic disease using improved drugs and better drug delivery systems.

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Introduction

In The Year 2525 (Exordium and Terminus)
In the year 2525
If man is still alive
If woman can survive
They may find
… (Dennis Zager and Richard Evans, 1969)

The year 2525 is 500 years from now. Since humans have survived the last 50,000 years, our survival of the next 500 years does not seem to be at a danger. This is of course assuming that humans do not blow up all of the nuclear bombs at the same time. It is easy to predict how the future will unfold 500 years from now when none of us will survive to confirm the prediction. One could only imagine the life of humankind in the year 2525 based on the futuristic stories such as Star Trek and Avatar movies. The story of Avatar is set in the year 2150, and the time line for Star Trek episodes is around the Earth Year 2200–2400. Many seemingly incurable diseases today can be treated in the future. The paralyzed will be able to walk again, and chronic diseases can be treated with a single needle-less injection of a magic drug. While treatment of various medical conditions may be available, it will be extremely costly.

It will be more challenging to predict the medical, pharmaceutical, and technological advances in the next 50 years than in the next 500 years. The reason we cannot really predict even the near future is that the scientific and engineering progresses are exponential. When a scientist develops a new technology, he has one problem to solve in his mind. When the technology is available to the public, however, there are always many others who utilize the technology to invent something never dreamed of by the inventor. This is the beauty of the exchange of ideas and information, leading to the development of unforeseen new applications. This is something that is extremely difficult to predict from the linear extrapolation of the technological advances. On the other hand, we also need to consider the possibility that a seemingly novel hypothesis which dominates a certain research topic during a given period may turn out to be a fad, preventing others from thinking outside the box and pursuing novel approaches. It takes a few decades to find out whether the current hypotheses and observations are indeed valid. Thus, the only thing that can be done is to find out today's problems, review the information available with a critical eye, and try to find the answers that stand the test of time.

Drug Delivery: Past and Present

In treatment of cancers, drug delivery systems play a significant role. Most anticancer drugs are poorly water-soluble and thus, proper formulation is necessary for administration. Moreover, targeted drug delivery becomes critical to selectively kill

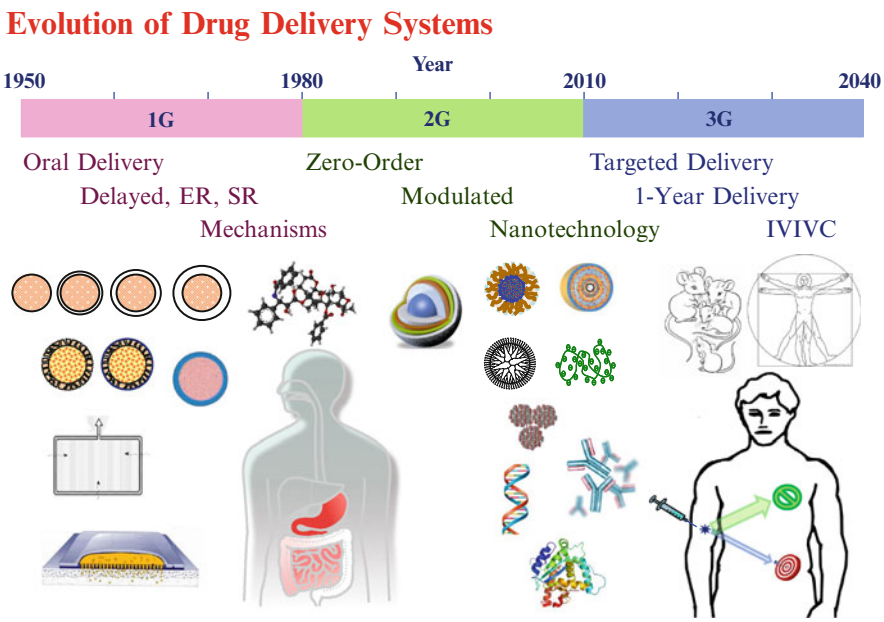

Fig. 26.1 Evolution of controlled drug delivery systems

cancer cells without harming normal cells. In cancer treatment, a "tumor-specific" biological agent is often referred to as a "tumor-targeted" biological agent in the context of the Paul Ehrlich's magic bullet [1]. The term "tumor-targeted" has different meaning in the drug delivery community where it is used to describe delivery of drugs and drug delivery systems preferentially to the target tumor even though the drug may not be tumor-specific [2]. To avoid any confusion, we will use "targeted" drug delivery to mean preferential delivery to the target tumor, rather than selective binding to tumor cells. It is necessary to clearly understand the current needs for successful treatment of cancers. Thus, it is beneficial to briefly review the history of controlled drug delivery for treating cancers.

The Short History of Drug Delivery

Predicting the future starts from understanding the past and present. Figure 26.1 shows the evolution of drug delivery systems. The first generation (1G) of controlled drug delivery technologies began with the introduction of the Spansule technology in 1952 [3]. The technology allowed 12-h drug delivery upon oral administration. It was a groundbreaking advance over oral formulations that had to be taken three or four times a day. Since then, numerous extended release (ER) and

sustained release (SR) formulations have been developed with further advances in controlled release mechanisms, such as dissolution, diffusion, osmosis and ion-exchange mechanisms [4, 5]. The 1G formulations have been extremely successful, as evidenced by the introduction of thousands of clinical products that increase patients' convenience and compliance. The success, however, has been limited largely to oral and transdermal formulations [6].

The second generation (2G) technologies were focused on developing zero-order drug release oral formulations in a hope to maintain a constant drug concentration in the blood. Even the zero-order release formulations, however, could not achieve constant blood levels due to the heterogeneous absorption ability throughout the gastrointestinal (GI) tract. The lack of constant blood levels, however, turned out to be a nonissue, because a drug becomes safe and effective as long as the blood level is above the minimum therapeutic level (C_{min}) and below the concentration causing toxic side effect (C_{max}). For most drugs the therapeutic index, i.e., the ratio of C_{max}/C_{min}, is large enough to be effective despite the decreasing blood levels of a drug after reaching the peak concentration shortly after administration. The 2G technology also focused on developing modulated drug release systems, e.g., glucose-dependent insulin release systems [7, 8]. While there have been a variety of systems developed for self-regulated insulin release, none of them have been translated into clinical products. This is mainly due to the fact that there was not a system able to maintain the glucose-sensitivity over time, and the system did not function well in the in vivo environment. A new research initiative on nanotechnology was launched during the last 10 years (i.e., 2000–2010). The entire decade was consumed by developing nanotechnology-based drug delivery systems, and the last 10 years can be described as the nanotechnology decade. Somehow, it was believed, without any proper evidence, that nanoparticle formulations would deliver a drug in a way that was never seen before. Nanoparticle formulations undoubtedly improved the efficacy of targeted drug delivery, e.g., to a tumor site, and the improvement was easily 300 % or more over the control formulations [9]. This impressive improvement, however, requires careful reexamination [10, 11].

The Big Picture

Of the many improvements to be made in controlled drug delivery systems, the targeted delivery of an anticancer agent to target cancer cells or a target tumor is probably the most urgent goal to achieve. The advances made in the nanotechnology-based drug delivery systems have been mostly focused on improved delivery of a drug to the target site. The fundamental assumption in the nanoparticle approach is that nanoparticulate formulations accumulate more at a target tumor site than the non-particulate control formulations. Once nanoparticles extravasate into surrounding tissue, they are usually not able to reenter the blood stream due to their solid nature, and thus, retained at the site for a longer period of time. This phenomenon was first observed in the 1980s and was called the "enhanced permeation and retention" (EPR) effect [12]. It has been repeatedly observed that nanoparticles

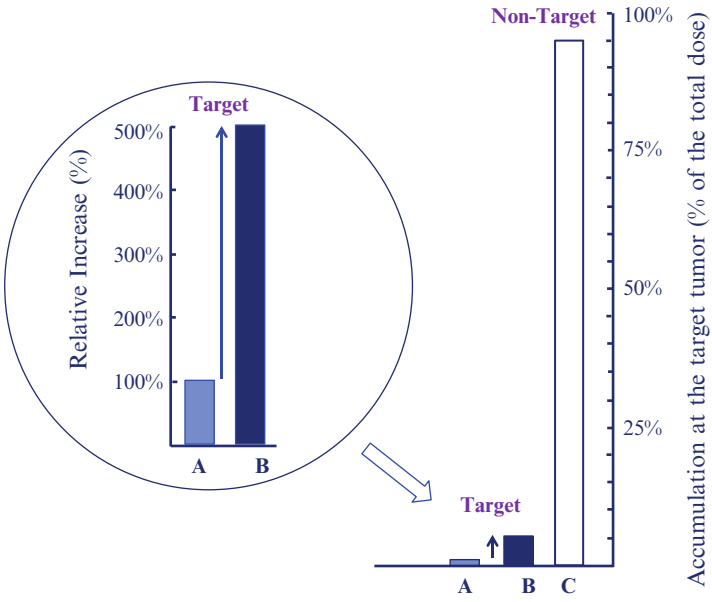

Fig. 26.2 Accumulation of a drug at a tumor site by non-particulate solution formulation (A) and nanoparticulate formulation (B). The relative increase in accumulation by nanoparticulate formulation can be 500 % or higher (*left circle*). If the amount of a drug accumulated at a target tumor is plotted as the percentage of the total intravenous (i.v.) dose, only approximately <5 % of the administered drug is found at the target tumor and >95 % of the drug is found in other tissues, sometimes causing critical side effects

accumulate more around a tumor than a solution counterpart. The increase in nanoparticle accumulation is substantial, in the range of several folds increase. This is an impressive increase by any standard. This seemingly impressive increase, however, needs to be viewed in the context of a bigger picture.

The left insert of Fig. 26.2 shows an impressive increase in relative values of a drug accumulated at a target tumor. Numerous studies have shown a substantial increase in drug accumulation as the non-particulate control formulation (A in Fig. 26.2) is replaced with nanoparticulate formulation (B in Fig. 26.2). If one examines such a large increase in the context of the total amount of a drug administered, even a 500 % increase in drug delivery by nanoparticulate formulation still accounts for only <5 % of the total dose. More than 95 % of the administered drug ends up at organs other than the target tumor. This nondiscriminatory biodistribution around the body may be the cause for serious side effects of anticancer treatment. The question is whether <5 % delivery is sufficient enough to kill tumor cells. It looks like the maximum amount of nanovehicles delivered to a target tumor is around 5 % of the total intravenously (i.v.) administered drug [2, 10]. Thus, the real success of the nanoparticle-based formulation will be based on how well we can exploit such an increase and how we can maximize the drug loading to nanoparticles.

The War Against Cancer

The cancer-related death rate has dropped significantly since the start of the war on cancer at a total cost of US$90 billion spent over the last 40 years, but still ranks at the top of the causes of death [13]. Current cancer treatment, on average, results in an extension of patients' lives for months despite the use of most advanced but expensive drugs. Even today it is possible to extend the life of cancer patients, even though only for a few months to a year, at the expense of about US$100,000/year [14, 15]. One of the goals to achieve in the near future is to extend patients' lives from months to years at affordable costs. Cancer is known to be caused by a variety of factors and they can be classified into environmental, dietary and genetic factors [16]. Smoking is a leading cause of lung cancer related deaths which can be dropped significantly by quitting smoking. Smoking is known to reduce life expectancy by decades [17]. Yet people just do not stop smoking easily. People's behavior probably will not change in the future. Considering diverse sources of cancer causing events, one can expect that eliminating cancer is rather difficult. Preventing the causes of cancer is one of the most desirable ways of dealing with cancers, and this requires early detection of cancer with simple diagnostic tools. Thus, future cancer research needs to focus on understanding the causes of cancer, improved genetic-screening tools, and personalized cancer medicine, such as prevention, early detection, and improved treatment [18].

To win the war against cancer, we need to implement a system that fosters thinking differently and creatively. There are two sides to this argument. One is to think of radically new approaches and come up with new formulations that have not been previously described. The result may be in the synthesis of new biomaterials which have not yet been tested in humans. The other is to utilize existing biomaterials to make anticancer delivery systems more efficient and effective. The latter could be desirable for faster translation from bench to bed.

The Current Missing Components in Cancer Biology

As it will be discussed later in this chapter, optimal methods to detect and treat cancer will require personalized medicines. This is because, as no two individuals are the same, no two cancers are identical. While our earlier understanding of human genetic diversity provided the basis for this issue, recent studies in the area of epigenetics have demonstrated remarkable variability in cancer cells based upon environment; both location in the body and within a tumor. Such advances have provided a significant improvement in our understanding of the biology of cancer. Unfortunately, however, they have not brought us to a point where such information can provide the definitive strategy required to completely and safely eradicate a cancer through a targeted pharmacotherapy. While recent advances in cancer management are largely based on this improved understanding of tumor biology, it appears we are still a long way from identifying the true magic bullet therapies that drive our research efforts.

Gene profiling allows identification of patients who can be spared the burden of adjuvant chemotherapy because of their excellent prognosis [19]. Our goal, however, is to use gene profiling to optimize treatments as well. To achieve this outcome, we need to acquire an improved understanding of not only how cancers are individually unique but also what they have in common. This latter element is important since the development of any new treatment method must make commercial sense for its support from a pharmaceutical company. Initial hopes that viruses might be the root cause of cancers that could be treated in a manner similar to any infection with pathogen-specific drugs have turned out not to be possible. This is because there are 100 viral-derived genes (representing 0.3 %) that are integrated into the human genome [20]. Thus, it is not so much the presence of viral-associated genes in cancer cells; it is their functional dysregulation that makes them likely targets for them to be promising targets for cancer-selective treatment. Such information reinforces the strategy to examine functional dysregulation of specific cell elements or pathways to identify potential therapeutic targets that might be common to a wide range of cancers.

The focus of this text is to examine previous and current approaches to achieve selective targeting of therapeutic agents to treat cancer—the goal being to focus therapeutic agents to cancers where essentially the entire dose, if possible at all, reaches cancer cells. This outcome would, at its surface, suggest that there would be a minimal potential systemic toxicity issue for such an approach. Cancer cells have many similarities to selective stages of rapid growth and dissemination throughout the body that occurs for normal cells during wound repair and development, for example. The reality, therefore, is that such a therapy would still have to circulate in the body for a sufficient time to reach all of the cancer cells being targeted and thus systemic exposure will remain a potential concern. For this reason, our efforts to understand tumor biology in the context of selectively targeting cancer cells will benefit from an improved understanding of how to reduce the impact on non-cancer cells that might be encountered prior to these therapies reaching their target.

How do we achieve this goal of finding cancer-specific elements that can be exploited for a number of cancers, or at least a number of cancer patients? Our current best hope for such an outcome involves the extensive efforts to understand not just the genetic modifications that have been associated with cancers, but to identify how these changes modify the functional properties of a cancer cell relative to non-cancer cells. In particular, these epigenetic studies need to look for differences under conditions that might better relate to the conditions in which they might ultimately be applied. Most of these studies compare epigenetic properties of isolated cancer cells growing in culture with noncancerous tissue samples from the same organ. Cancer cell epigenetics are affected by surrounding cells and factors within the tumor microenvironment, and noncancerous cells that are most affected by anti-cancer therapies are those that are rapidly dividing, just like cancer cells. Thus, a better way might be to examine cancer cells within the environment of the tumor and compare this to noncancerous cells that are undergoing rapid growth in response to wound repair or a step in the development process.

What would one look for in a therapeutic approach that can discriminate cancer cells ensconced within a tumor from noncancerous cells that are performing critical functions required for homeostasis? Epigenetics studies have suggested that there may be differences between the way noncancerous cells fine-tune their replicative processes compared to what frequently happens in cancer cells that allow cells to drive unregulated cell division. Such differences could lead to the rational identification of therapeutic strategies that preferentially affect cancer cells. Should we ever expect to find a strategy that is completely selective for cancer cells? The answer here is: probably not. The reason for this negativity is that cancer cells are derived from non-cancer cells and, no matter what the therapeutic strategy, at some point non-cancer and cancer cells will use similar, if not identical, elements to sustain their survival, support replication, and allow their local and/or distant migration.

The Current Missing Components in Targeted Drug Delivery

Smart Drugs and Targeted Drug Delivery

The so-called smart drugs are supposed to kill only tumor cells, while exerting no harm to normal cells. In this sense, smart drugs can be considered another name for the "magic bullet," but all current smart drugs for cancer treatment do not possess such specificity and are not really smart. One of the best known smart drugs is bevacizumab. It, however, is still a nonspecific toxin that interferes with small blood vessel growth throughout the body, resulting in side effects. It may have more exquisite activity, but is still nonspecific. The non-specificity of most anticancer drugs brings on the topic of targeted drug delivery.

Targeted Drug Delivery in Small Animal Models

Intravenous administration of a nanoparticle formulation usually results in slowing the growth of a tumor. Typical growth curves of tumor volume after treatment are shown in Fig. 26.3a. Nanoparticle formulation always shows higher efficacy than the control solution formulation in preventing tumor growth, and depending on the nature of nanoparticles, tumors sometimes do not grow and even shrink in size. The ultimate goal of administering anticancer agents is to prolong human survival time. Figure 26.3b shows typical comparative survival rates using different formulations. While many experiments show promising positive trends in treating tumors, we need to further consider a few things for the exemplary data shown in Fig. 26.3. First, most small animal experiments are limited to observing tumor growth or survival for only about a month or two. The goal of anticancer treatment is not to extend the survival for only a month. The literature data rarely show the survival time for longer than a month.

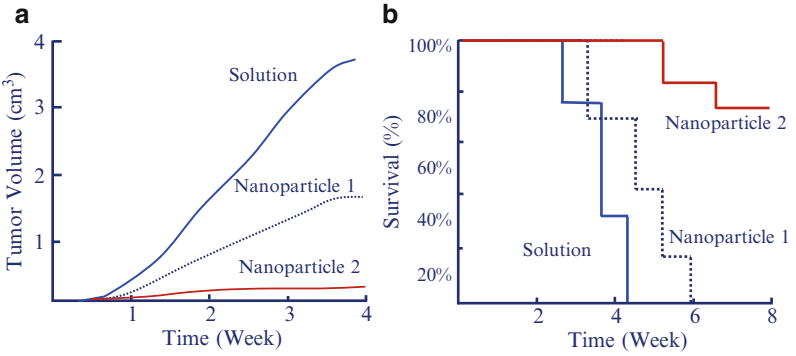

Fig. 26.3 Typical examples of tumor growth (**a**) and survival time (**b**) after i.v. administration of solution and different nanoparticle formulations

The 1 month study may be enough to show the superior efficacy of nanoparticle formulations over the control, but for development of truly life-saving anticancer formulations, it is necessary to monitor tumor growth and survival time for extended periods of time. It appears that the small animals die even if the nanoparticle formulations are administered repeatedly for more than a month. This result requires an explanation. Why would an animal die if a nanoparticle formulation is administered repeatedly when the first month of data clearly shows the superior properties of the nanoparticle formulation? Such limited short-term improvement by a nanoparticle formulation has to be reconsidered to find formulations that can allow survival for significantly extended periods of time. If a nanoparticle formulation can really accumulate at a tumor site and release a drug sufficiently enough to selectively kill only the tumor cells, repeated administration should be able to make a small animal live without side effects for more than a few months, if not cure the cancer.

The True Role of EPR Effect and Its Significance

Enhanced permeation is one of the gateways for nanosized particles to translocate from the blood compartment to tumor interstitial space. There are scanning electron microscopic images showing openings (fenestrae) in the endothelial cell lining in the blood vessels in a solid tumor in the small animal models [21, 22]. Scientific work measuring permeability of probes varying in size also supported the existence of the large pores up to ~2 μm in tumor vasculature [21]. Although the translocation of nanoparticles via transcytosis mechanisms cannot be excluded [23], the drug delivery community refers the enhanced permeability only to the transport of the nanoparticles through the openings driven by diffusional and/or convectional forces. Considering significant resistance in extracellular space for various factors, such as high cell density and fibrous collagen networks, the spreading of the nanoparticles

is limited to the area near leaky blood vessels. When the nanoparticle concentration in the blood compartment becomes low after clearance, it is possible that the nanoparticles diffuse back to the blood compartment, if the transport resistance back to blood is less than that in to the tumor space. This in part explains why the translocated nanoparticles are cleared from the tumor over time despite the poor development or collapsed lymphatic drainage, along with the phagocytic activity of macrophages. Thus, the EPR effect may support improved accumulation of the nanoparticles in the tumor compartment, although limited to the peripheral region of a tumor with heterogeneous distribution. The EPR effect, however, does not necessarily indicate better efficacy, because most functional nanoparticles have been designed to work at a cellular level and may not reach each individual cancer cell even after extravasation. The efficacy of nanoparticles treating tumors may be beyond the EPR effect, especially in clinical tumors.

Drug Delivery: Future

Consideration of Cancer Heterogeneity

Cancer is a genetic disease characterized by several hallmarks, such as sustaining proliferative signaling, inducing angiogenesis, activating invasion and metastasis, evading growth suppressor, resisting cell death [24, 25]. As pointed out above, no two cancers are identical, and in fact are extremely heterogeneous. The extreme heterogeneity of a cancer has to be considered in designing clinically useful drug delivery systems. Significant variations of a particular cancer type result from the fact that each one is genetically discrete, as each patient will have a unique genetic profile. Thus, a particular cancer patient group to be treated is composed of a heterogeneous population of cancers with some similarities and many differences. Studies have shown that cells within a specific cancer grouping can have different genetic mutations within the patient population. This variability is compounded by the fact that a specific cancer within an individual patient can continue to change genetically due to its hyper-mutable nature. Thus, a single patient can have multiple clones of a cancer over the course of their disease. This temporal difference in genetic variation is further complicated by the observation that cancer cell properties are dependent upon their microenvironment within a tumor.

Temporal and spatial differences in cancer cell characteristics within a single patient can account for the observation that many therapies can result in a significant decrease in tumor mass with the subsequent cancer recurrence in a more aggressive form. Such a phenomenon has been suggested to occur due to selective killing of sensitive cancer cells and an enhanced growth opportunity for cells that were insensitive due to their location within a tumor and/or the different stages of the cancer. In essence, most cancer therapies act to enrich populations of cancer cells that are distinct from those cells that are sensitive to the therapy that was used.

Thus, the ability to effectively clear a cancer using a specific targeted strategy can be complicated due to spatial and temporal changes that could occur within a specific cancer. This adds complexity to strategies not only to treat populations of cancer patients but also to identify optimally effective strategies for personalized medicine approaches.

Personalized Medicine

The Priorities for Personalized Medicine published by the President's Council of Advisors on Science and Technology describes "personalized medicine" as the tailoring of medical treatment to the specific characteristics of each patient [26]. It involves classifying individuals into subpopulations that are uniquely or disproportionately susceptible to a particular disease or responsive to a specific treatment. This requires identification of genome-related molecular markers for specific variants of disease that are especially responsive to particular treatments or identification of genetic alterations causing particular forms of disease.

One of the current cancer treatment goals is to maximize the benefits of combination therapy. This requires identification of patient subgroups that can benefit most from a set of chemotherapeutic agents and tumor-specific agents. While personalized medicine can be guided by merging gene-expression data and sophisticated bioinformatic tools, it still faces significant difficulties stemming from the heterogeneity of cancer cells and transitory responses to most mechanism-based tumor-specific therapy [27]. The purpose of personalized medicine is to identify the best drugs and drug combinations for individual patients. If gene therapy becomes common, then patients will be more routinely tested for gene mutations underlying their cancer to match them with a targeted treatment.

As outlined above, the temporal and spatial variations in the cancer add significant complexity to the already challenging problem of finding targeted therapies that can truly eliminate cancer from a patient. This has led to a suggested idea of treating cancer as a chronic disease where different treatment strategies can be used to allow cancer patients to maintain a reduce tumor burden and to live a normal life. Such a strategy requires chronic administration of drugs, and thus, adds another level of challenge for scientists to identify a useful sequence of targeted therapies for each patient to treat them in a personalized fashion.

Overcoming Drug Resistance

Patients who respond well in the beginning of a treatment will eventually acquire drug resistance [28]. This may be due to the mutable nature of a cancer or the fact that cancer cells that survive the initial treatment have been selected for their resistance to that particular approach. Since there are many pathways of drug resistance,

it is not always clear as to why this resistance has occurred. In some cases the cancer cell target can be changed and thus, it is beneficial to use a combination of different drugs to yield additive or synergistic effects improving outcomes [28].

It is challenging to identify a series of personalized medicines that might be used to treat cancer patients chronically. Temporal and spatial changes that can occur within a cancer could involve a change in sensitivity to a therapeutic agent or in mechanisms used by cells to efficiently eject these agents before they have their intended action. In an effort to minimize cancer cell resistance, adjuvant and neoadjuvant therapeutic strategies are typically employed [29]. While such approaches have been shown to provide a benefit in the setting of particular populations of cancer patients, the incorporation of this added complexity to personalized medicine approaches has not been examined.

Combination Therapy

Combination therapy, also known as concurrent, multiple-drug or drug cocktail therapy, is to treat many disease conditions with more than one medication. Various studies have been conducted to compare the relative efficacy of combination therapy and sequential monotherapy. Combination therapy deals with ≥2 cytotoxic agents or chemotherapeutic agents and tumor-specific biologic agents. In patients with relapsed, platinum-sensitive ovarian cancer, sequential therapy of docetaxel followed by carboplatin showed improved health-related quality of life, although the progression-free survival (PFS) was not as good as the combination therapy [30]. The trade-off between improved PFS and improved overall response rate (ORR) has to be considered based on an individual patient's preferences. In colorectal cancer, combination therapy has become the standard of care in the vast majority of patients [31].

In early-stage breast cancer patients the sequential docetaxel (a taxane)/anthracycline-based regimen was found to be better than the concurrent therapy in disease-free survival and overall survival [32]. In metastatic breast cancer patients, however, taxanes did not improve the survival rate, and this might have been due to the use of taxanes as a substitute for other active drugs, e.g., cyclophosphamide, rather than in addition to a standard regimen [33]. Even for metastatic breast cancer patients, some combinations of anticancer agents are known to produce desirable outcome with manageable side effects [34]. Clinical trials on patients with $HER2^+$ metastatic breast cancer indicate that the combination therapy of trastuzumab (Herceptin) and docetaxel is preferred over the sequential monotherapy because of the significantly improve PFS and ORR [35, 36]. The goal of treating metastatic breast cancer is essentially delaying disease progression and maximizing survival with a good quality of life [36].

The ideal combination therapy can occur when two or more drugs have different or complementary mechanisms of action. The mechanisms of action include DNA intercalation, growth factor inhibition, receptor tyrosine kinase inhibition,

angiogenesis inhibition, and microtubule stabilization [34]. Even if we choose one drug from each mechanism of action, there will be five different drug types with ten different combinations to test. Clearly the number of experiments to be done for finding the optimum combination therapy is large, and the availability of a simple in vitro system for testing efficacy of different combinations is highly desired.

Improved In Vivo Models vs. Better In Vitro Models

The success rate of experimental oncology drugs in clinical trials varies depending on the origins of the candidate drug but is generally low; approximately 5 % for those selected from screening pools and less than 10 % for molecularly designed targeted candidates [37]. The average success rate of drug candidates for other major disease categories approximately doubles that of oncology candidates [38]. These statistics suggest that current preclinical models in oncology present poor predictive power for the outcomes of costly (more than US$400 million/case for Phase I, II, and III) and often time-consuming (>10 years) clinical trials [39]. There is a strong consensus among anticancer drug developers for new preclinical animal models which better correlate and present improved predictability for clinical outcomes. Better predictive preclinical test systems with novel in vitro and in vivo models are therefore clearly required. But how can this be achieved?

To date, literature information shows that hundreds, if not thousands, of therapeutic approaches have cured cancer in a wide range of mouse models; many of these methods have involved nanoparticle-based formulations. None of the formulations, however, has shown similar efficacy in humans. As a consequence, it is critical that we reconsider the current assumptions used in the establishment and evaluation of these models. It is not that animal models cannot provide essential information for identification of successful anticancer therapies; all of the currently approved drugs have been evaluated using these models. Considerable knowledge can be gained from mouse models, but these experimental successes must be interpreted in light of the limitations of the model used, as such models have clearly fallen short of accurately representing essential elements to successfully treat cancer in a clinical setting.

There are many potential reasons for the discrepancies observed between mouse models and clinical outcomes. Most of these models are designed for rapid screening of many compounds and/or formulations. There is motivation to establish a rapid model that is relatively consistent—two properties that are inconsistent with human cancers. For this reason tumors grown in mice are often started from millions of a single clone of human cancer cells that will consistently grow into readily detectable tumors in a few weeks. Human cancers are extremely heterogeneous and are likely to develop slowly that may be started from a single cell. Solid tumors developed in mice using such a xenograft approach are therefore organized quite differently from human cancers with architectures that are enriched in human cancer cells at the expense of stromal components present in human tumors.

Stromal components play a significant role in the physical and biological properties of the tumor environment, with these properties affecting the distribution and efficacy of potential therapeutic formulations and agents [40]. One important difference is that xenograft models are typically established in immunodeficient mice to suppress immune-mediated rejection of the implanted human cancer cells. Most human solid tumors, that this approach is intended to model, have a wide range of immune-associated cells that are affected in their function and properties through a complex interplay with the cancer cells that could affect vascularity, cell density, as well as metabolism of chemotherapeutic agents and formulations. Thus, xenograft models do not typically recreate the true complement of cell type heterogeneity and architectural complexity present in human tumors.

Another challenge of using mouse models to assess chemotherapeutic agents and formulations relates to the difficulty of accurately assessing their actions. First of all, most patients present clinically with disseminated disease; only rarely is a cancer detected at an early stage through opportune examination. This means that the primary tumor has typically not become very large before it drives to metastasis. Typical heterotopic xenograft mouse models of human cancers establish a single exceptionally large primary tumor located in the subcutaneous space where it can be readily monitored and measured to assess the actions of a chemotherapeutic agent and formulation; the size of tumors used in mouse models range from a few millimeters to a centimeter—equivalent to the size of a golf ball or a baseball (or larger) for a human. Mechanisms that affect the growth of such large xenograft tumors may have little to do with what might affect a smaller, more metastatic form of cancer seen in most patients. What is needed are better methods to identify small and possibly disseminated tumors in mice that will allow for accurate monitoring to follow the actions of chemotherapeutic agents and formulations and more realistic models that emulate human clinical conditions.

Several approaches have been taken to improve the potential for animal models to accurately identify promising chemotherapeutic agents and formulations. Efforts have been made to move away from the heterotopic xenograft to at least set up orthotopic xenografts where the cancer cell being studied is allowed to establish itself in the same organ (albeit a different species) of its origin. While a little more expensive to set up and a bit more time-consuming, orthotopic xenografts do offer some improvement in reproducing human clinical disease over heterotopic xenograft, but this is not overwhelming and is different depending upon the cancer [41]. More recently, transgenic mice have been engineered to express (multiple) specific cancer-related modifications that better emulate the complex characteristics of human cancer in a model of spontaneous disease [41]. While showing a much greater reproducibility to clinical disease, transgenic mice engineered to more closely model specific human cancers are very costly to set up and run relative to heterotopic xenograft models. But even these highly complex models fail to truly emulate human disease.

A truly humanized and predictive model to emulate clinical cancer is still far from reality. Rather than waiting for such a day, several approaches are being taken using the models currently available. One of these approaches is to focus on using

an animal model to ask a question related to specific aspects of a potential cancer therapy and acknowledging that other aspects of the model do not recapitulate human disease. For example, specific studies were performed to model aspects of stromal desmoplasia and vascular dysfunction that can impair drug delivery to pancreatic cancer [42]. Others have taken the approach of including inflammatory events and/or tissue damage that are known to promote neoplasia into models of cancer [43]. Still others are trying to examine specific processes that drive metastasis [44]. Such models are bringing us closer to methods to accurately model clinical disease, but they still suffer from a limitation of tools to accurately assess the actions of a chemotherapeutic agent and formulation that follows cancer cell survival and not just tumor volume which can relate to the loss of non-cancer cells.

As a final thought about animal models, to better describe events related to optimizing approaches to treat cancers, the goal of activating an aggressive anticancer cell immune response has been validated in the clinic [45]. More recently, a vaccination protocol for prostate cancer has been approved for commercialization [46]. While such advances have led to a goal of engaging a patient's immune system as part of the anticancer therapy, we still do not know about how the immune system works and our ability to model these events in preclinical models as witnessed by the catastrophic Phase I study events recently observed for an anti-CD28 therapeutic candidate [47].

Living with Cancer

During a conventional chemotherapy, most patients experience hair loss, low blood cell counts, and GI irritation. After stopping chemotherapy, however, the patients recover from side effects with regrowing of hair, even after the complete loss. This indicates that adult stem cells are resistant to chemotherapeutics. Cancer biologists have now obtained evidence for the existence of cancer stem cells but with serious dispute of the origins of these cells. The implication of a cancer stem cell population obviously changes cancer patient management strategies with current chemotherapies being unlikely to eradicate these cells. Thus, to reduce the number of cancer cells in a tumor and to suppress metastasis may be a relatively easy task compared to completely eradicating a cancer. This challenge is further complicated by the concern that methods to target cancer stem cells may put non-cancer stem cells at risk.

If cancer stem cells exist and they have the capability of replenishing a subpopulation of cancer cells in a particular patient, it may be that we need to more closely consider the idea of living with, rather than beating, cancer for the average patient treated by an oncologist. Indeed, cancer therapies have been shifting toward the identification of agents that are less and less overtly toxic. Previous therapies employed the strategy of taking the patient to the brink of death to kill off as many of the cancer cells as possible with the hope that all of the cancer cells might be killed. In light of the possibility that cancer stem cells would likely not be affected

by this approach, chronic therapies can be used with less toxicity to suppress the growth of the cancer cells. Thus, even in the absence of a complete cure of cancer, new drugs are being developed to allow cancer patients to live almost normal life. There is also a greater interest in harnessing the immune system to treat cancer. Despite our current incomplete understanding of cancer-related immunity, it is clear that robust immune responses to a cancer can have great clinical benefit [48].

Based upon the reality that cancer will likely be viewed as a disease to be treated chronically and those ever-increasing efforts to engage a patient's immune system as a therapeutic tool, there appear to be several opportunities for effective and selective targeting strategies. One is associated with improving the focused delivery of chemotherapeutic agents and formulations. In some cases developing formulations for long-term drug delivery ranging from months to years would be desirable. In other cases where continuous drug delivery would not be desirable, such as loss of sensitivity due to receptor down-regulation on the cancer cell, formulations that can be self-administered as a chronic at-home therapy would be important. Finally, delivery vehicles and strategies to optimize anticancer immune responses could lead to a means where cancer patients can live with cancer for many, many years.

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Editor's Biography

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Professor You Han Bae received his Ph.D. in 1988 from the University of Utah. He has a background in chemical engineering and polymer science/engineering and pharmaceutical chemistry. He served at University of Utah as a postdoctoral fellow and research assistant/associate professor till 1994. He joined the Department of Materials Science and Engineering at Gwangju Institute of Science and Technology (GIST) in Korea in 1994 as an associate professor and was promoted to a full professor in 1998. After serving 7.5 years at GIST, he came back to the University of Utah as a full professor in 2002.

He did research on stimuli-sensitive polymers and drug delivery, protein drug stabilization and controlled release, and functionalized polymers for glucose sensor and biohybrid artificial pancreas. His current research interests include nanoparticulates for multidrug resistance in cancer and tumor heterogeneity, novel carrier design for the delivery of genetic materials, and preclinical cancer models. He is the author or coauthor of over 240 peer-reviewed scientific papers, book chapters, and US patents which have been cited more than 14,000 times.

He is an editorial board member of Pharmaceutical Research and Bioconjugate and is also currently serving the Journal of Controlled Release (JCR) as an America Associate Editor as well as the editor of the Concept Paper in JCR.

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Professor Randall Mrsny received his Ph.D. degree in Human Anatomy and Cell Biology from the University of California at Davis in 1981 where he was an NIH predoctoral fellow. He was awarded an NIH postdoctoral fellowship that he undertook at the Institute of Molecular Biology at the University of Oregon, where he focused on research in Membrane Biophysics. Professor Mrsny then joined ALZA Corporation where he led the Peptide Biology Group and then moved to Genentech where he led the Drug Delivery/Biology group. In 2000 he left Genentech to start

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two separated biotech companies and take a part-time Professor of Drug Delivery post at Cardiff University. In 2009 he moved to the Department of Pharmacy and Pharmacology at the University of Bath where he now holds a full-time Professor of Epithelial Biology post.

Professor Mrsny's research has focused primarily on understanding biological barriers of the body that limit or modulate the delivery of biopharmaceuticals using a multidisciplinary approach. His current research includes the identification of vesicular trafficking pathways in epithelial hijacked cells that are by bacterial toxins, mechanism epithelial cells to regulate the transmigration of neutrophils, and protein–protein contacts involving tight junction elements that control paracellular permeability properties of epithelia. He has published more than 90 peer-reviewed papers, 25 book chapters and reviews, and presented more than 100 abstracts at national and international meetings. He coauthored and coedited 5 books in the area of controlled drug delivery. He is the founder of Unity Pharmaceuticals and Applied Molecular Transport.

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Professor Kinam Park received his Ph.D. degree in pharmaceutics from University of Wisconsin in 1983. After a postdoctoral training at the Department of Chemical Engineering of the same university, he joined the faculty of the Purdue University in 1986 and promoted to Full Professor of Pharmaceutics in 1994. Since 1998, he has held a joint appointment in Department of Biomedical Engineering, and became Showalter Distinguished Professor of Biomedical Engineering in 2006.

His research has been focused on the use of various polymers and hydrogels for controlled drug delivery. His current research includes hydrogel template methods for fabrication of homogeneous microparticles for long-term drug delivery, hydrotropic polymeric micelles for increasing the water solubility of poorly soluble drugs, hydrogels for biomedical applications, and drug-eluting stents and balloons. He has published more than 230 peer-reviewed papers, 90 book chapters, 90 JCR cover stories, and presented 200 abstracts at national and international meetings. He has coauthored and coedited 11 books in the area of controlled drug delivery, and has trained more than 100 Ph.D. graduate students, postdoctoral fellows, and visiting scientists. He is the founder of Akina, Inc. specializing in drug delivery technologies in 2001. He is currently the editor-in-chief of the Journal of Controlled Release.

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