Advances in Experimental Medicine and Biology 1259

Alexander Birbrair *Editor*

Tumor Microenvironment

Molecular Players – Part A

Springer

Advances in Experimental Medicine and Biology

Volume 1259

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2018 Impact Factor: 2.126.

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-43092-4 ISBN 978-3-030-43093-1 (eBook)
https://doi.org/10.1007/978-3-030-43093-1

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of Mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life I owe to her.

My beloved mom Marina Sobolevsky of blessed memory (July 28, 1959–June 3, 2020).

Preface

This book's initial title was *Tumor Microenvironment*. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book, *Tumor Microenvironment: Molecular Players - Part A*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different key molecular players within the tumor microenvironment during cancer development. Further insights into the mechanisms will have important implications for our understanding of cancer initiation, development, and progression. We focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of tumor microenvironment in several organs using state-of-the-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different tissues. Thus, the present book is an attempt to describe the most recent developments in the area of tumor biology which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about different molecular players within the tumor microenvironment in various tissues. Nine chapters written by experts in the field summarize the present knowledge about distinct characteristics of the tumor microenvironment during cancer development.

Zahidul I. Pranjol and Jacqueline L. Whatmore from the University of Exeter Medical School discuss the role of cathepsin D in the tumor microenvironment of breast and ovarian cancers. Neus Martínez-Bosch and Pilar Navarro from the Institute of Biomedical Research of Barcelona update us with what we know about galectins in the tumor microenvironment, focusing on galectin-1. Driton Vela from the University of Prishtina describes the effects of iron in the tumor microenvironment. Edoardo Milotti and colleagues from the University of Trieste summarize current knowledge by mathematical modeling on the effects of oxygen in the tumor microenvironment. Daniela Barisano and Michael A. Frohman from Stony Brook University School of Medicine address the importance of phospholipase D1 in the tumor

microenvironment. Adriano Angelucci and colleagues from the University of L'Aquila compile our understanding of leptin in the tumor microenvironment. Mark W. Robinson and colleagues from Maynooth University focus on the immune consequences of lactate in the tumor microenvironment. James E. Talmadge and colleagues from the University of Nebraska Medical Center give an overview of fatty acid mediators in the tumor microenvironment. Finally, Crislyn D'Souza-Schorey and colleagues from the University of Notre Dame talk about extracellular vesicles in the tumor microenvironment.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife, Veranika Ushakova, and Mr. Murugesan Tamilsevan from Springer, who helped at every step of the execution of this project.

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Cathepsin D in the Tumor Microenvironment of Breast and Ovarian Cancers

1

Zahidul I. Pranjol and Jacqueline L. Whatmore

Abstract

Cancer remains a major and leading health problem worldwide. Lack of early diagnosis, chemoresistance, and recurrence of cancer means vast research and development are required in this area. The complexity of the tumor microenvironment in the biological milieu poses greater challenges in having safer, selective, and targeted therapies. Existing strategies such as chemotherapy, radiotherapy, and antiangiogenic therapies moderately improve progression-free survival; however, they come with side effects that reduce quality of life. Thus, targeting potential candidates in the microenvironment, such as extracellular cathepsin D (CathD) which has been known to play major pro-tumorigenic roles in breast and ovarian cancers, could be a breakthrough in cancer treatment, specially using novel treatment modalities such as immunotherapy and nanotechnology-based therapy. This chapter discusses CathD as a pro-cancerous, more specifically a proangiogenic factor, that acts bi-functionally in the tumor microenvironment, and possible ways of targeting the protein therapeutically.

Keywords

Ovarian cancer · Breast cancer · Tumor microenvironment · Cathepsin D · Angiogenesis · Proteolytic-dependent activity · Proteolytic-independent activity · MAP kinases · PI3 kinases · Pericytes · Immunotherapy · Graphene-based nanomedicine

1.1 Introduction

Globally, more than 2.28 million new cases of breast and ovarian cancers are diagnosed, with approximately 810,000 deaths each year [1–3]. Thus, tackling these two major cancers remains a daunting task for clinicians and researchers. By the year 2025, it is estimated that, globally, there will be a surge in the number of cancer cases (>20 million annually) – an alarming statistic that has compelled researchers to expedite research to discover newer targets and develop more potent therapeutic compounds to overcome drug resistance as well as eradicate cancer cells from the biological setting [4]. However, the disease remains a global challenge due to the lack of early

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_1

diagnosis, the inherent biological complexity, and the high demands for designing safer and selective drugs to restrict tumor growth [5].

Although researchers have a much better understanding of many characteristics of cancer [6], the complex systems that allow tumors to form remains to be solved. It is the complex crosstalk between the cellular and non-cellular components of the host organ which, under the influence of the tumor cells, help create a niche for tumors to grow uncontrollably, invade local tissue, evade local immune-mediated destruction, and stimulate angiogenesis and metastasis [7]. This newly formed niche where tumors sit and grow is known as the tumor microenvironment (Fig. 1.1). A number of cells such as cancer-associated fibroblasts (CAFs), immune cells, adipocytes, neuroendocrine cells, the blood and lymphatic vascular networks, and tumor cells help build this niche [8]. Once tumors start to grow in this hypoxic microenvironment, where the normal cell and tissue homeostasis is dysregulated, they secrete both pro- and antitumorigenic growth factors, cytokines, extracellular vesicles, extracellular matrix (ECM) proteins, and ECM-remodeling enzymes that trigger a switch to a more pro-tumorigenic response from the surrounding cells [7]. For instance, CAFs, pericytes, endothelial cells (ECs) from local microvasculature, and tumor cells secrete a wide range of enzymes that effectively degrade the surrounding ECM to allow tumor cell invasion of the host tissue and microvascular ECs to migrate, proliferate, and form a new blood supply to feed the growing tumor [7, 9]. A number of these enzymes have been discovered and characterized over the years such as metalloproteases, lysyl oxidases, and cysteine and aspartyl cathepsins [7]. Interestingly, over the last couple of decades, aspartyl cathepsins, particularly cathepsin D (CathD), have gained increased attention due to their extracellular presence in the tumor microenvironment and reported roles in tumor development and metastasis as well as their potential as therapeutic targets [10–18].

CathD is a ubiquitous, aspartic endoproteinase that is expressed in all human tissues. Physiologically, it resides in the lysosomes, proteolytically degrading unfolded or nonfunctional proteins.

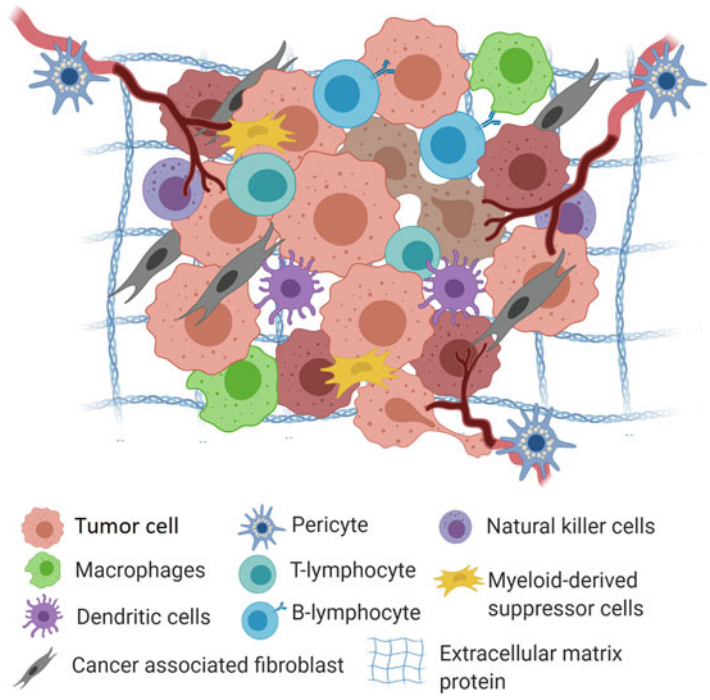

Fig. 1.1 Tumor microenvironment. An illustration of the key cellular components of the tumor microenvironment

CathD is involved in essential biological processes, such as during development and maintaining tissue homeostasis, where the enzyme is believed to act proteolytically outside its acidic milieu [19]. Thus, dysregulation of[-255pt] CathD expression and/or function is associated with pathologies such as atherosclerosis, neurological and dermatological disorders, and cancer [20]. For instance, CathD secreted from tumor cells into the extracellular space has been suggested to play an important role in invasion and metastasis of breast cancer [21, 22]. Winiarski et al. also reported an overexpression and secretion of CathD in cancerous tissue and ascites of ovarian cancer patients [23], which enhanced proangiogenic responses such as proliferation, migration, and angiogenic tube formation in local omental microvascular ECs [11]. Overexpression and hypersecretion of CathD have now been demonstrated in other cancer types including lung, prostate, endometrial, malignant glioma, and melanoma, and the protein is considered to be a prognostic biomarker in breast cancer [24] and a potential marker in predicting prognosis of endometrial adenocarcinoma [25]. These data, along with unresolved complexity of the microenvironment, which facilitates tumor cell invasion of local host tissue, highlights the importance of further research on the biological aspects and therapeutic purpose of CathD in cancer development. This chapter focuses on cancer cell-secreted CathD in the tumor microenvironment and its role in tumor invasion, angiogenesis, and metastasis and also gives a brief perspective on the possibility of targeting extracellular CathD therapeutically.

1.2 Processing of Cathepsin D

The synthesis process of CathD is regulated in the conventional endoplasmic reticulum/Golgi pathway. After synthesis in the rough endoplasmic reticulum as inactive preprocathepsin D (43 kDa), it is further cleaved and glycosylated to form 52 kDa procathepsin D (pCathD) containing two N-linked oligosaccharides modified with mannose 6-phosphate (M6P) residues at asparagine residues 70 and 199 [26, 27]. Modified pCathD is then targeted to intracellular vesicular structures such as endosomes, lysosomes, and phagosomes both by M6P receptor (M6PR)-dependent and -independent pathways [19]. The latter mechanism of targeting is not yet understood; however, the sphingolipid activator precursor protein prosaposin has been suggested to be involved [28].

Upon entry into the acidic milieu of the late endosome, M6PRs detach from pCathD and subsequently the phosphate group is removed. Low pH- and cysteine protease-induced proteolytic cleavage of propeptide (44aa) of pCathD generates an active intermediate form of the enzyme [29]. The propeptide (also known as activation peptide) plays an essential role in correctly folding, activating, and delivering the protein to lysosomes [30, 31]. This peptide, which is expressed in, and secreted from, cancer cells, has also been demonstrated to act as a growth factor for tumor cells [32]. The intermediate form of CathD is further cleaved to generate the mature form (48 kDa) containing a heavy chain (34 kDa) and a light chain (14 kDa) linked by non-covalent interactions [33]. CathD activity is tightly regulated at pH 3.5 [34]; however, it is now known that the enzyme is active both proteolytically and non-proteolytically at neutral pH in the cytosol of apoptotic cells and during neurofibrillary degeneration and cancer progression [11, 15, 35, 36].

1.3 Physiological Roles of CathD as Both an Intracellular and Extracellular Protein

Besides its lysosomal activity, CathD also plays a significant role during fetal development. There is a gradual maturation observed in the lysosomal system that correlates with increased CathD levels in all tissues [37]. Mice deficient in CathD survive during fetal development, but die around one month after birth due to significant neurodegeneration [38], indicating the protein's essential role in developmental biology. Further studies demonstrated that congenital mutations in the CathD gene lead to a reduction in expression and subsequent production of an enzymatically

inactive protein that results in neurodegenerative disease in dogs and humans [39–44]. In a recent study, an association was shown between CathD deficiency and Parkinson's disease [45]. Interestingly, increased CathD expression and activity in cardiac cells is associated with heart failure in postpartum female mice [46]. Higher CathD levels also correlate with increasing apoptosis in the cerebellum, and this has now been suggested to play a role in the pathogenesis of autism [47].

Other functions of CathD, related to its functional activity, have also been suggested. For instance, CathD-induced cleavage of metabolism-associated intracellular proteins; activation and degradation of polypeptide hormones and growth factors such as plasminogen, prolactin, endostatin, osteocalcin, thyroglobulin, insulin-like growth factor binding proteins (IGFBP), and secondary lymphoid tissue chemokine (SLC); activation of enzymatic precursors of CathL, CathB, and transglutaminase 1; and processing of the enzyme activators and inhibitors prosaposin and cystatin C (reviewed in [19]).

Although CathD mainly acts in the lysosome, in the last two decades, its role in the extracellular space has been explored extensively. CathD differentiates from other aspartic endopeptidases in its packaging and sorting process. For instance, it has been known for a while that, physiologically, pCathD is sequestered to the lysosome and not secreted extracellularly. However, now we know that under some conditions, pCathD/CathD can escape the conventional ER/Golgi-dependent targeting pathway and be secreted from cells. The most probable explanation is that overexpression of pCathD surpasses the limited number of M6PR binding sites available, and, thus, the protein accumulates in the cytosol and is subsequently secreted. The secretory mechanism, however, remains somewhat a mystery [48]. It is believed that the addition of carbohydrate groups to CathD during post-translational modification may determine its destiny [49]. For instance, tunicamycin, a glycosylation inhibitor, produced an unglycosylated form of CathD that was found to be secreted from cultured liver cells, suggesting that lysosomal enzyme-linked carbohydrate structures may play a crucial role in directing these enzymes [49]. In the case of secreted CathD, it is understood that these enzymes lack M6P residues, which is essential for sorting lysosomal enzymes. Different forms of CathD (or pCathD) are now known to be secreted in human, bovine, and rat milk and serum, and the presence of both pCathD and CathD (34 kDa) was observed in human eccrine sweat and urine [50–53]. Interestingly, CathD in human eccrine sweat was found to be proteolytically active at sweat pH 5.5 [54], which agreed with the increasing evidence in pathologies such as cancer that extracellular CathD may act via its proteolytic-dependent mechanism.

1.4 Expression of CathD in Cancer

CathD is now known to be a major secreted protein found in the cancer microenvironment. Over the last two decades, studies have shown increased overexpression and hypersecretion of CathD in numerous cancer types including ovarian cancer, breast cancer, endometrial cancer, lung cancer, malignant glioma, melanoma, and prostate cancer (Table 1.1) [25, 55–68]. In breast cancer, in particular, CathD is considered as a "marker" associated with metastasis. For instance, overexpression of CathD in breast cancer cells correlates with increased risk of clinical metastasis and short survival in breast cancer patients [56–58]. Interestingly, increased secreted levels of pCathD were also detected in the serum of patients with breast malignancy [69]. Another study revealed that the total concentration of CathD in breast cancer tissue was much higher than in other tissues including normal mammary cells [70]. Additionally, Masson and colleagues showed, for the first time, that CathD expression is gradually increased as preadipocytes differentiate into mature adipocytes in both humans and mice [71]. CathD upregulation was also reported in obese subjects and mice, indicating a significant pro-adipogenesis role of CathD. Since adipocytes play a supportive role in the growth process of the breast, and as clinical studies have reported a

Table 1.1 Involvement of CathD in the stages of tumor progression in different cancer types. Modified from [15]

Cancer type	Metastasis	Invasion	Angiogenesis	References
Breast	↑	↑	↑	[56–59]
Ovarian	ND	ND	↑	[11, 55]
Prostate	↑	↑	↓	[60–62]
Endometrial	ND	↑	ND	[68]
Melanocytic	↑	↑	ND	[63]
Glioma	↑	↑	ND	[64]
Lung	ND	↑	ND	[67]

↑ increase in effects, ↓ reduction in effects, *ND* not determined

role of obesity in the incidence of breast cancer, CathD upregulation may actually play an indirect role in breast cancer progression.

A role for CathD has now been shown in the progression of ovarian cancer metastasis. Earlier research investigating ovarian cancer suggested that the enhanced level of CathD expression was associated with increased tumor differentiation and with clinically advanced histological type [72, 73]. More recent studies have reported enhanced CathD expression as an indicator of malignancy in serous ovarian cancer [74–76]; for instance, over 70% of invasive ovarian cancers were shown to express CathD [75]. Intriguingly, this finding was contradicted by another study which showed that high expression of CathD in the ovarian tumor was associated with a favorable survival prognosis [76]. However, our previous work investigating omental metastasis of ovarian cancer revealed that a high omental mesothelial expression of CathD (close to the metastatic tumor) was associated with poor disease-specific survival (DSS) [23]. The study also found that expression of CathD was significantly higher in the omental lesion of serous ovarian carcinoma compared with omentum from patients with benign ovarian cystadenoma [23], further supporting a potential pro-cancerous role of CathD in ovarian carcinoma.

1.5 Role of CathD in Tumor Progression

1.5.1 Proteolytic-Dependent Roles

It is now becoming clear that CathD plays a role in the tumor microenvironment. However, a number of questions arise as to how this enzyme with an optimum pH of 3.5 acts proteolytically at neutral pHs. Earlier studies suggested that CathD plays an intracellular cytosolic role at neutral pH in inducing apoptosis, indicating its proteolytic capability at neutral or near-neutral pHs. The enzyme is translocated to the cytosol due to lysosomal membrane permeabilization and actively cleaves the BH3-interacting domain (Bid) to form truncated Bid (tBid) [36, 77, 78]. tBid activates the insertion of Bax into the mitochondrial membrane, leading to the release of cytochrome C from mitochondria into the cytosol [79–81]. This apoptotic response was partially delayed by pepstatin A (pepA), an inhibitor of CathD proteolytic activity [78–80], suggesting a pro-apoptotic mechanism induced by this enzyme. The role of CathD in inducing in vitro apoptosis was further validated when a pan caspase inhibitor (Z-VAD-FMK) induced a significant reduction in cell death when given in combination with pepA [82, 83]. Additionally, tau protein degradation by cytosolic (i.e., pH 7) proteolytically active CathD has been reported in Alzheimer neurofibrillary degradation [35]. These studies strongly suggest that CathD is active at pHs higher than the optimum, although it should be noted that other works suggested that mutant CathD, deprived of its catalytic activity, was indistinguishable from that of the normal enzyme [84, 85].

Although it could be argued that a pro-apoptotic role for intracellular CathD may be antitumorigenic, this is in contrast to observations that indicate that not only is CathD secreted from tumor cells but that this extracellular CathD may have key pro-tumorigenic functions. For instance, CathD was observed to be overexpressed and

hypersecreted from estrogen-positive MCF7 breast cancer cells in in vitro experiments, which resulted in enhanced tumor growth and invasion in mammary carcinogenesis [86]. Interestingly, CathD has been shown to cleave cell-secreted cystatin C, a potent endogenous inhibitor of cysteine and metalloproteinase, at a lower pH (pH 5.5–6.8), similar to the in vivo tumor microenvironment [87]. This suggests that active CathD plays a significant role in tumor progression, by preventing the inhibitory action of cystatin C on proteases that actively cleave extracellular matrix protein in the tumor stroma, allowing cancer cells to invade local tissue. Interestingly, another study demonstrated that proteolytically active CathD stimulates the activity of secreted plasminogen activators by degrading plasminogen activator inhibitor-1 at pH 6.6, i.e., similar to the tumor microenvironment. The authors suggested that this process could be a contributory factor involved in triggering a proteolytic cascade facilitating breast cancer cell invasion and metastasis [88]. Intriguingly, CathD has also been shown to selectively degrade macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and SLC (CCL21) that, in turn, may affect the generation of the antitumoral immune response, the migration of human breast cancer cells, or both processes [89].

Although secreted pCathD is generally considered to be proteolytically inactive [90–92], in the hypoxic, acidic tumor microenvironment, this precursor form of the enzyme may be converted, by an autocatalytic mechanism into the mature form capable of degrading ECM proteins, thus releasing basic fibroblast growth factor (bFGF) [10, 59, 93]. The combination of degradation of the ECM proteins and released bFGF (Fig. 1.2), a pro-proliferative growth factor, allows local tumor and ECs cells to grow and invade local host tissue, aiding tumor metastasis [94].

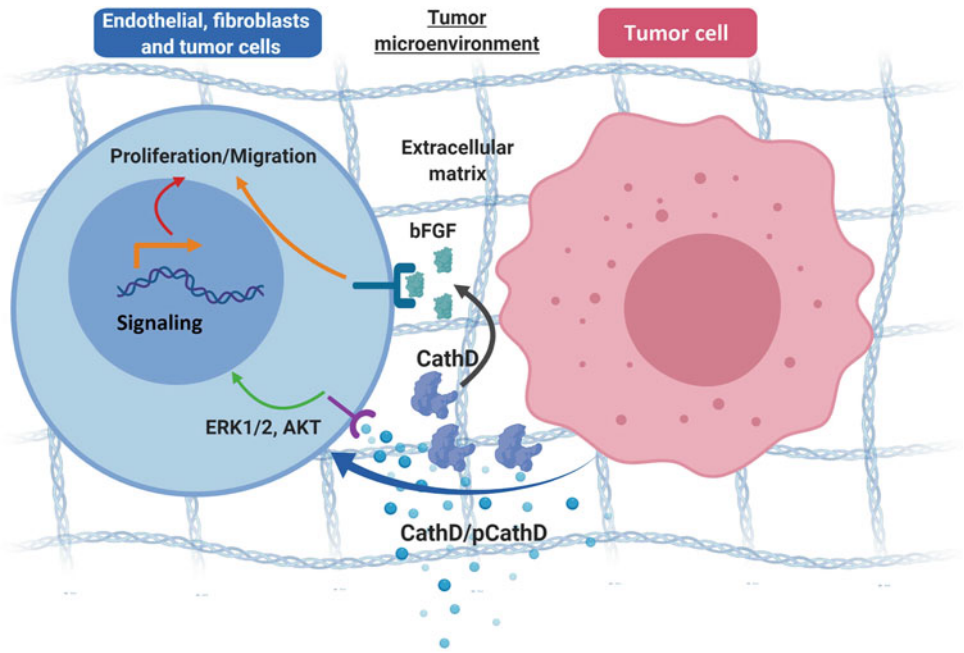

Fig. 1.2 Tumor cell-secreted CathD and its pro-cancerous role in the tumor microenvironment. Overexpression of pCathD/CathD leads to its hypersecretion into the extracellular space by tumor cells. Proteolytically active CathD cleaves ECM proteins and releases the basic fibroblast growth factor (bFGF) that induces angiogenesis. Both pCathD and CathD induce tumor cell proliferation in a proteolytic-dependent and proteolytic-independent manner, thus utilizing an autocrine mechanism. CathD also induces proliferation of fibroblasts, and both proliferation and migration of ECs via activation of the ERK1/2 and AKT pathways

A more recent study has demonstrated that both pCathD and mature CathD are involved in the migration of mesenchymal stem cells (MSCs) to tumor sites [95]. MSCs are known to secrete cytokines and chemokines that trigger both pro- and antitumorigenic responses in the tumor microenvironment. CathD-induced homing of these stem cells to the tumor microenvironment facilitates a more aggressive invasion of tumor cells into the surrounding tissue [95]. The study further revealed that pCathD acted as a potent stimulator of MSC migration which was completely reversed in the presence of pepA. Further investigation revealed an interesting phenomenon whereby pCathD in the tumor microenvironment was suggested to be uptaken/endocytosed by MSCs and converted into a proteolytically active, mature form of CathD, which then induced migration and invasion of MSCs in the cancer stroma [95]. However, CathD or pCathD had no effect on cellular proliferation in this study, contradicting the previous reports.

1.5.2 Proteolytic-Independent Roles

To answer whether CathD acts in a non-proteolytic manner in the tumor microenvironment, a number of studies have been carried out. For example, pCathD has been reported to act as a mitogen, i.e., a protein-ligand, rather than enzymatically, to stimulate MCF7 cell proliferation via an autocrine mechanism [96]. In recent years, numerous studies have emerged that suggest a non-proteolytic proangiogenic role for CathD both in vivo and in vitro. For instance, in xenografts (3Y-Ad12 cell line transfected with wild-type and/or mutated Asn 231 CathD) in an athymic mice model, overexpression of CathD correlated with increased vascular density. In these mice, a 1.5- and 1.9-fold increase in microvessel density was observed in the CathD and CathD-Asn 231 (proteolytically inactive; transfected mice) groups, respectively, suggesting that CathD induces angiogenic effects via an unknown mechanism other than its proteolytic activity [14]. Another study reported that both pCathD and CathD induced proliferation and migration of breast cancer cells, fibroblasts, and ECs in both a proteolytic-dependent and proteolytic-independent manner [97].

A similar observation was made in epithelial ovarian cancer (EOC). In an investigation on potential non-VEGF pathways in inducing tumor angiogenesis, we implicated secreted factors such as CathD, CathL, and IGFBP7 both in vitro and in vivo (Table 1.2) [23, 55]. For instance, high levels of CathD were found in the ascites of patients suffering from ovarian cancer (unpublished data), and CathD was later found to induce proangiogenic effects in disease-specific local microvascular endothelial cells [11, 55]. An increase in the secretion of

Table 1.2 Proangiogenic factors secreted by ovarian cancer cells

Activators	Function	References
Vascular endothelial growth factor (VEGF)	Stimulates angiogenesis, permeability	[99]
Cathepsin D	Stimulates EC proliferation and migration	[11]
Cathepsin L	Stimulates EC proliferation and migration	[98]
Angiopoietin-1 (Ang1) and Tie2 receptor	Ang1, stabilizes vessels by strengthening endothelial-smooth muscle interactions; Tie2R, inhibits permeability	[100]
Fibroblast growth factor (FGF)	Stimulates angiogenesis and arteriogenesis	[101]
Transforming growth factor (TGF-β1)	Stabilizes vessels by stimulating ECM production	[102]
Heparin-binding epidermal growth factor-like growth factor	Binds to epidermal growth factor receptor (EGFR) and promotes angiogenesis	[103]
IL6	Induces migration of ECs in the mesentery in EOC	[102, 104]
IL8	Stimulates VEGF expression and the autocrine activation of VEGFR2 in ECs	[102, 105]

CathD was also observed from EOC cancer cell lines (SKOV3 and A2780) [55], confirming the in vivo phenomenon. Our recent work demonstrated that exogenous CathD induces proliferation and migration of human omental microvascular ECs, suggesting a mitogenic role for this enzyme [11]. We further confirmed this proangiogenic response by showing activation of downstream signaling pathways (ERK1/2 and AKT) in response to CathD in these cells, which agreed with a study where proteolytically inactive CathD was shown to induce human skin fibroblast proliferation via activation of the MAPK/ERK1/2 pathway [13] (Fig. 1.2). Interestingly, unlike previous observations, we found that CathD was not proteolytically active at neutral pHs, but highly active at low, acidic pHs (completely inhibited by pepA), suggesting that this enzyme acts non-proteolytically in the pre-tumor microenvironment of the secondary tumor site [11]. Our theory is that EOC-secreted CathD locally induces angiogenic responses, i.e., EC proliferation and migration, during the initial stages of secondary tumor development, i.e., in a pre-hypoxic, acidic environment. However, once secondary tumor foci are established in the omentum, CathD may act proteolytically in the tumor microenvironment to further accelerate the metastatic process, as indicated in the aforementioned studies. A similar observation was also observed for the EOC-secreted cysteine protease cathepsin L, whereby the enzyme non-proteolytically induced omental microvascular EC proliferation, although in this case the enzyme remained proteolytically active at neutral pHs [98].

A proangiogenic role for CathD may be critical to its reported pro-tumorigenic importance, and this has been explored in numerous other studies. For instance, CathD was found to induce blood vessel formation in the chick chorioallantoic membrane (CAM) model [106]. A role for CathD in angiogenesis was further illustrated by the observation that migration of human umbilical vein ECs and in vitro angiogenic tube formation was increased when cells were treated with active pure CathD. The observation that pepA completely inhibited these effects manner indicated that CathD was proteolytically active in these experiments [106]. As mentioned previously, proteolytically active CathD has also been suggested to induce angiogenesis in breast cancer by cleaving and releasing ECM-bound pro-angiogenic bFGF [59]. The studies described support the suggestion that CathD can induce proangiogenic responses via both its proteolytic action and an unknown mechanism that is not dependent on its proteolytic activity.

In contrast, it has also been suggested that CathD activity may be antiangiogenic. For instance, pCathD secreted by prostate cancer cells was shown to have a possible role in generating angiostatin via proteolysis – a specific inhibitor of angiogenesis in vitro as well as in vivo [62], suggesting an opposing effect of CathD in angiogenesis.

There is ample evidence that CathD may induce mitogenic responses in the cells of the tumor microenvironment via both proteolytic-dependent and proteolytic-independent mechanisms. Vignon et al. demonstrated that the precursor of CathD, pCathD, non-proteolytically induced growth of MCF7 breast cancer cells in vitro [96]. A significant increase in human skin CCD45K fibroblast proliferation, motility, and invasive capacity was also observed to be induced by proteolytically active and inactive CathD [13]. This prompted an investigation into the target receptor molecule on these cells, and the authors observed a partial reduction in fibroblast proliferation in the presence of M6P and pCathD. Further studies investigating the effects of CathD on tumor cells reported rapid growth of human CathD cDNA-transfected 3Y1-Ad12 rat tumor cells in vitro, with an increased experimental metastatic potential in vivo [107–109]. In addition, the proliferation of 3Y1-Ad12 cells was induced in response to both wild-type and mutated (Asn 231, proteolytically inactive) CathD in vitro and in vivo [12, 14]. Based on the previous study, the authors tested whether M6P inhibited CathD-induced proliferation and concluded that M6P did not compete with CathD interacting with M6PR, indicating a novel receptor, probably LDL receptor-related protein 1 (LRP1) [110], involved in inducing a cellular

response. In the same study, the propeptide (27-44aa) of pCathD was found not to be mitogenic, contradicting studies which found otherwise [32, 70, 111–114].

1.6 Future Perspective

As discussed above, the overproduction and secretion of CathD could substantially contribute to tumor progression via directly influencing cancer cells and stromal cells such as fibroblasts and ECs non-proteolytically and indirectly by cleaving ECM proteins, cytokines, and chemokines locally. We recently showed that exogenous CathD promotes proliferation and migration in human omental microvascular ECs in ovarian cancer metastasis via inducing phosphorylation of the ERK1/2 and PI3K/AKT pathways in a proteolytic-independent manner [11], suggesting activation of a receptor tyrosine kinase. Recently, CathD was shown to induce the outgrowth of fibroblasts by binding to the LRP1 receptor which could potentially play a role in CAF proliferation in the tumor microenvironment, further aiding tumor growth [110].

Another key tumorigenic player within the TME is tumor-associated pericytes. Pericytes, through a regulated pericyte-EC crosstalk, play an important role in stabilizing vessels of the microvasculature under normal physiological conditions. In an active TME, an intimate association between EC and pericytes means that the angiogenic microvessels are more functional and stable than those lacking the support of pericytes [115, 116]. Since CathD was shown to induce EC proliferation, migration, and angiogenesis in TME, it could also play a role in destabilizing the association between EC and pericytes. For instance, a recent study has demonstrated that CathD-treated human retinal microvascular ECs and human retinal pericytes resulted in an increased vascular permeability via deregulating properties of the junctional-related proteins, reducing expression of platelet-derived growth factor receptor-β (required for pericyte survival) and increasing expression of the vessel destabilizing agent angiopoietin-2 in diabetic retinopathy [117]. Interestingly, CathD ligand bound to cell-surface cation-independent M6PR and led to activation of a downstream signaling pathway of protein kinase C-α/Ca^{2+}/calmodulin-dependent protein kinase II in both ECs and pericytes, resulting in a destabilized EC-pericyte interaction [117]. Birbrair and colleagues (2014) more specifically identified type-2 pericytes to play a major role in tumor vasculature remodeling [116]. Thus, a potential role for CathD in TME could be an induction of a dysregulation of a normal, quiescent EC-pericyte association and a deregulation of pericyte functionality, resulting in a destabilized, leaky tumor microvasculature. However, this overall hypothesis requires to be investigated in tumor- and organ-specific manner as both ECs and pericytes show vast heterogeneity in different organ systems [116].

1.6.1 Potential Anti-TME CathD Therapeutic Strategies

A number of conventional anticancer strategies such as chemotherapy, radiotherapy, and antiangiogenic therapy are available to treat advanced disease. Importantly, antiangiogenic therapies such as anti-VEGF monoclonal antibody bevacizumab (Avastin) [118, 119] have been used clinically, but many have reported side effects that limit safety in patients [120–123]. Therefore, novel therapeutic targets, such as extracellular CathD, both in its proteolytic and non-proteolytic form, are urgently required.

Ashraf et al. recently demonstrated an antitumor efficacy for anti-CathD antibody in triple-negative breast cancer (TNBC) mice models [17]. TNBC, which accounts for 15–20% of all breast cancer cases, lacks overexpression of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER-2) [124]. Thus, the only available treatments are surgery, chemotherapy, and radiotherapy. Targeting extracellular CathD, which is overexpressed in TNBC [125] and is a strong marker for poor prognosis in breast cancer patients (with potent pro-tumorigenic effects) [11, 12, 24, 96, 126], via an immunotherapy

approach could be of clinical significance. The authors in this recent study reported that two human anti-CathD antibodies efficiently bound to human and mouse CathD, even at the low pH of the TNBC microenvironment and significantly inhibited tumor growth in three different TNBC mouse models (MDA-MB-231 cell xenografts and two TNBC patient-derived xenografts) without apparent toxic effects [17]. Interestingly, the antibody prevented the recruitment of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells within the tumor, which are known to play a role in tumor immunosuppression. In peritoneal metastases, such as high-grade serous carcinoma (advanced ovarian cancer), TAMs constitute over 50% of cells in the peritoneal tumor implants and ascites [127]. CathD overexpression and hypersecretion are also observed in tumor-associated omental mesothelium and in ascites from patients [23] and ovarian tumor conditioned media [55], and CathD is now known to induce a proangiogenic effect in the tumor microenvironment [11]. Therefore, targeting CathD utilizing an immunotherapy approach may be safer and more efficacious in treating ovarian carcinoma. However, bioavailability, selective targeting, and drug-delivery pose greater challenges which would require further research.

Due to the complexity the tumor microenvironment presents, conventional drug delivery systems fail to deliver the chemotherapeutics at an effective concentration to selectively kill cancer cells and therefore can be associated with debilitating side effects. Thus, studies have been conducted to investigate alternative approaches to drug delivery such as utilizing nanotechnology. In recent years, nanomedicine and its underpinning sciences have significantly contributed to drug bioavailability and therapeutic index in cancer therapy. FDA-approved nanostructures/chemo drugs such as liposomal formulation of doxorubicin (DOX) (Doxil® or Caelyx®), daunorubicin (DaunoXome®), and albumin-bound paclitaxel (PTX) (Abraxane®) have been in use; however clinically, these formulations proved to be moderately successful due to inadequate delivery to the tumor microenvironment [128]. Therefore, in an attempt to target CathD, we developed a graphene-based compound (graphene oxide) that breaks down and adsorbs this protein [16]. Important characteristics of graphene oxide such as surface charge, large surface area, electronic features, chemical reactivity, and good bioavailability were utilized to entrap CathD in vitro [16]. Our data demonstrated that adsorption of CathD led to denaturation of the enzyme on the surface of graphene oxide. This promising outcome was also observed at low concentrations of graphene oxide, which remained nontoxic to cells in vitro. Thus, future work could address further development to integrate targeted and safe delivery of graphene oxide to the tumor sites and testing of this compound in the tumor microenvironment in vivo tumor models, with a proven clearance of disseminated CathD and extracellular enzyme-targeting specificities.

1.7 Conclusions

The complexity of the tumor microenvironment such as the crosstalk between the cellular and non-cellular components, along with the barrier to drug delivery, poses greater challenges in discovering newer targets in cancer therapy. Conventional anticancer strategies have been the strongest weapons in defeating tumor, although most of these fail to shrink tumors at secondary sites, limiting effective treatment. Current antiangiogenic therapies, in combination with chemotherapies, moderately increase progression-free survival, with side effects that could be life-threatening. Therefore, newer targets within the microenvironment, such as extracellular CathD, which has a dual functionality, may hold greater promise in reducing breast and ovarian cancer progression.

Acknowledgment The figures were created with biorender.com.

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Galectins in the Tumor Microenvironment: Focus on Galectin-1

2

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Abstract

In the last decades, the focus of cancer research has moved from epithelial cells to the tumor milieu, in an effort to better understand tumor development and progression, and with the important end goal of translating this knowledge into effective therapies. The galectin family of glycan-binding proteins displays important functions in cancer development and progression. Numerous groups have made outstanding contributions to deepen our knowledge about the role of galectins in the tumor-stroma crosstalk, defining them as key players in modulating interactions between tumor cells and the extracellular matrix, fibroblasts, endothelium, and the immune system. While several members of the family have been of particular interest until now, others are still considered as future exploding stars. This chapter provides an overview for galectin-1, the first identified and still one of the most well-studied galectins, and highlights the very important implications in its regulation of the tumor microenvironment in many different tumor types. Besides, a glimpse of the role of other galectins in the tumor milieu is also provided. Gaining a deeper understanding about the numerous roles of galectin-1 will not only help us to better understand other galectins but also is likely to result in the development of more effective cancer therapies.

Keywords

Galectins · Galectin-1 · Fibroblast activation · Tumor microenvironment · Angiogenesis · Immune evasion · Tumor-stroma crosstalk · Cancer therapy

2.1 Galectins: A Diverse Family with Important Biological Functions

Galectins were first referred as S-type lectins (thiol-dependent) by Drickamer et al. in 1988, to differentiate them from C-type lectins, which depended on Ca^{2+}. This classification proved to be inaccurate, as some members do not depend on reduced cysteine residues for carbohydrate binding [1–3]. These proteins were also called

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_2

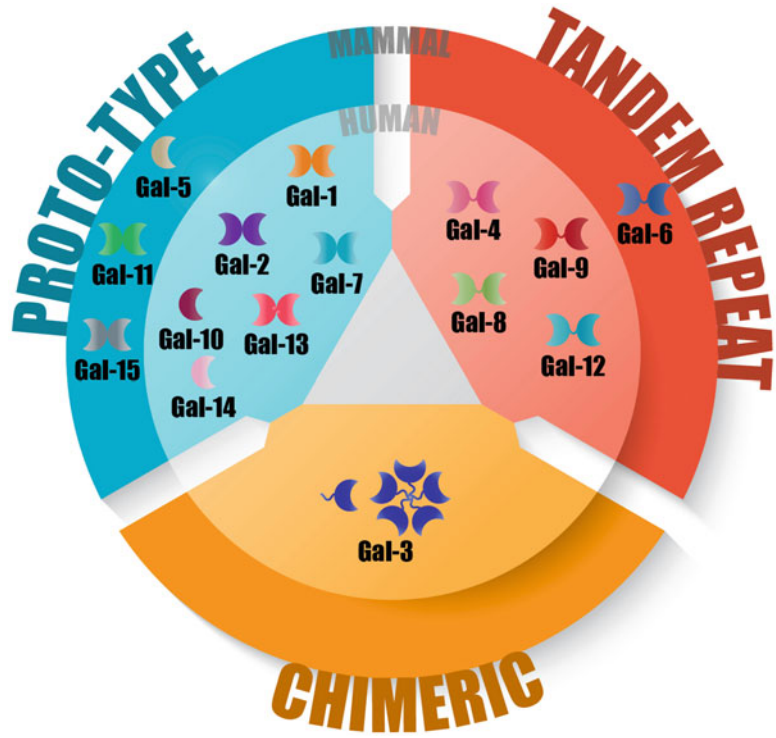

Fig. 2.1 Galectin family in mammalians. Fifteen galectins have been described in mammals, 11 of which have also been found in humans (located in the central core of the diagram). Galectins are organized in three groups: proto-type galectins that display one CRD and function as monomers or dimers; tandem-repeat galectins that have two CRDs linked; and chimeric-type galectins formed by Gal-3 with a long amino-terminal domain that allows oligomerization

S-Lac lectins (soluble-lactose binding) [4], and it was not until 1994 when the "galectin" name was given, in order to cluster all the identified β-galactoside-binding lectins and to homogenize the nomenclature of individual members [5]. The galectin family clusters a group of proteins that have a well-conserved carbohydrate recognition domain (CRD) and are classically described to bind lactosamine-containing structures found in glycoconjugates [6]. Today, galectins comprise 15 different proteins in mammals, 11 of which are found in humans (Fig. 2.1). Members are classified into three different groups according to their structural hallmarks: (1) proto-type galectins (galectin-1 [Gal-1], Gal-2, Gal-5, Gal-7, Gal-10, Gal-11, Gal-13, Gal-14, and Gal-15), which have one CRD that can dimerize; (2) tandem-repeat galectins (Gal-4, Gal-6, Gal-8, Gal-9, and Gal-12), which contain two CRDs linked by a short peptide; and (3) a chimeric-type galectin (Gal-3), which has a large amino-terminus involved in oligomerization [7, 8] (Fig. 2.1). Although they share general binding traits, galectins have a fine-tuned regulation at different levels, including at the redox environmental state, their subcellular localization, and their quaternary structure, all of which define ligand selectivity and interaction strengths.

Galectins are in both the cytoplasm and nuclei and can also be secreted outside the cell by a non-conventional mechanism [9], being deposited into the extracellular matrix (ECM) or bound to the cell membrane [10, 11]. They are expressed in numerous human tissues, including the placenta, intestine, lung, spleen, and heart [12], and by many cell types, such as epithelial, endothelial, neuronal, and immune cells [13].

The first hints that galectins play a role in cancer were observed in a tumor cell line [14], and data from the 1980s highlighted the importance of the family in mediating tumor-stroma crosstalk. Importantly, Raz and Lotan already speculated on the possible role of a galactoside-specific lectin on the surface of tumor cells in mediating metastasis through interaction of tumor cells with endothelial or immune system cells [15]. These

data were soon validated in several experimental models, and these lectins (which were still not called galectins at that moment) emerged as key players in mediating interactions between tumor cells and the host endothelial cells, immune cells, and ECM [16]. Since then, extensive data have highlighted the importance of the galectin family in the tumor microenvironment, controlling key events in cancer development and progression such as fibroblast activation, angiogenesis, and the immune response [17].

Although other members of the galectin family have shown important responses in the regulation of the tumor microenvironment (and will be briefly addressed in Sect. 2.7), this review will focus on Gal-1, as this protein stands out as one of the members with best characterized functions in stroma activation, angiogenesis, and tumor immune evasion responses.

Gal-1 was the first member of the family to be identified and consists of a 14.5 KDa protein of 135 amino acids that is encoded by the *LGALS1* gene located on chromosome 22q12 [18]. Depending on the redox conditions, Gal-1 can be found as a monomer (oxidative conditions) or as a homodimer (reducing conditions), the subunits of which are held together by an hydrophobic core [19]. The CRD in this galectin recognizes N-acetyl-lactosamine (LacNAc) residues on glycans from cell surface receptors and extracellular proteins, such as integrins, CD43, CD45, fibronectin, mucin, and laminin, but Gal-1 is also able to interact with proteins independently of their sugar-binding moieties [18]. Importantly, dimeric Gal-1 can establish cell surface microdomains or lattices with multiple glycoproteins found on cell membranes. These lattices are key to organizing membrane domains, mediating signaling at the cell surface, and determining receptor stabilization by controlling endocytosis [20]. For example, dimeric Gal-1 is able to control homotypic tumor cell adhesion by interacting with integrin receptors, or cell migration and invasion by recognizing glycans on ECM proteins, such as laminin or fibronectin [21].

2.2 Glycosylation and Gal-1 in the Tumor Microenvironment

Of the protein posttranslational modifications, glycosylation gives structural identity to the molecules by coating the cell membrane (which is known as the glycocalix). Importantly, this modification determines the cell's communication with its microenvironment, including with the ECM and other distinct cell types. Glycosylation is a finely regulated process which is able to sense and quickly react to physiological and pathological contexts [22]. Aberrant tumor glycosylation has been widely reported, and its implications for driving cancer development and progression have been partially deciphered, opening the gate to interesting applications in oncology [23]. Some of the best known specific glycan traits in cancer include a general increase in sialic acid terminal residues, enhanced N-glycan branching, and overexpression or de novo synthesis of sialyl Lewis antigens and short O-GalNac glycans [22].

Importantly, galectins can be secreted from cells and thus stand out as important code-readers of these altered glycosylation patterns found in cancer cell membranes or even in the ECM. For instance, Gal-1 recognizes aberrant glycans on mucin-1 expressed on the surface of cancer cells [24], and it can also interact with poly-N-acetyllactosamines (poly-LacNAc) on ECM proteins, such as laminin or fibronectin, to regulate cell adhesion and migration [25, 26]. Furthermore, Gal-1 also communicates with different cells within the tumor microenvironment. For instance, Gal-1 recognizes glycosylated receptors on endothelial cells (ECs) such as neuropilin-1 (NRP-1) or vascular endothelial growth factor receptor 2 (VEGFR2), triggering cell signaling pathways that induce their proliferation, migration, and activation [27, 28]. Lately, a lot of the interest in Gal-1 as a potential target in cancer therapy has focused on the ability of Gal-1 to recognize

receptors in the immune compartment (such as CD43, CD45, and CD7), which leads to specific T-helper type 1 (Th1) and T-cytotoxic cell apoptosis [29–31] as well as other immune evasion mechanisms [32]. These and other examples will be specifically addressed in the following sections.

2.3 Gal-1 Expression in the Tumor Stroma

Gal-1 overexpression has been widely reported to be present in cancer, where, in addition to being secreted by tumor cell types and deposited in the ECM, it has also been found to be expressed by diverse stromal components, including fibroblasts, ECs, neutrophils, macrophages, dendritic cells (DCs), and T lymphocytes [33]. In particular, Gal-1 has been reported in the stroma of prostate cancer, where it was identified as an independent predictor of recurrence [34]. Gal-1 has also been found overexpressed in the stroma in cervical cancer, where its levels positively correlate with pathological grade [35]. Gal-1 was also detected in the stroma of laryngeal carcinoma [36] and epithelial ovarian cancer [25], where peritumoral Gal-1 intensity positively correlates with poor progression-free survival [37] and low overall survival [38]. In breast cancer, Gal-1 levels in the stroma positively correlated with TNM stage and metastasis [58]. Gal-1 was also expressed in the stroma of colon cancer tissue, at increasing levels as the tumor progressed [39]. In hepatocellular carcinoma (HCC) samples, Gal-1 also accumulated in the stroma and positively correlated with tumor size, TNM stage, vascular invasion, poor differentiation, metastasis, high rates of tumor recurrence, and low overall survival, identifying Gal-1 as an independent marker of poor prognosis in HCC [40–42]. In pancreatic ductal adenocarcinoma (PDA), Gal-1 is highly overexpressed in tumors (localized in the abundant desmoplastic reaction) [43–48], has an increased expression in advanced precursor lesions [46] and in poorly differentiated tumors [43], and negatively correlates with longer survival times [44]. Gal-1 is also expressed in the bone marrow microenvironment of multiple myeloma patients [49]. In Hodgkin and non-Hodgkin lymphomas, Gal-1 is present in the vascular walls of lymphomas but absent from normal tissue, and it correlates with vascular density [50]. In head and neck squamous cell carcinoma (HNSCC), Gal-1 is expressed in the stroma of tumor samples but absent from normal epithelium, and it correlates with the presence of alpha smooth muscle actin (α-SMA)–positive cancer-associated fibroblasts (CAFs) as well as with increased levels of several known HNSCC poor-prognosis factors [51]. Multiple immunohistochemical studies with large patient cohorts have described Gal-1 expression in the stroma of gastric tumors. Interestingly, its expression positively correlates with tumor size [52], tumor location [53], differentiation grade [52, 53], TNM stage [52–55], histological grade [55], invasion depth [54, 55], lymph node metastasis [52–55], lymphovascular invasion [53], perineural and serosal invasion [53], lower patient survival [52–55], and with the expression of several markers, including vascular endothelial growth factor (VEGF) [52], Gli-1 ([54]), vimentin, E-cadherin ([217]), and transforming growth factor beta (TGF-β) [55] (Table 2.1).

2.4 Gal-1 and Fibroblast Activation

Several reports have addressed how Gal-1 regulates physiological fibroblast function. Gal-1 induces rodent myofibroblast differentiation, activation [59], proliferation, and migration [60], and Gal-1 knockout (KO) mice show impaired wound healing responses [61]. Mechanistically, Gal-1 promotes myofibroblast activation by triggering intracellular reactive oxygen species (ROS) production by regulating the NADPH oxidase 4 (NOX4) via the NRP-1/Smad3 signaling pathway in myofibroblasts [61]. In vitro, TGF-β1 activates fibroblasts by increasing Gal-1 expression through phosphoinositide 3-kinases (PI3K) and p38 mitogen-activated

Table 2.1 Data on Gal-1 expression in the tumor microenvironment. This table summarizes the articles in which Gal-1 has been described in the tumor stroma by immunohistochemistry, describing the amount of samples analyzed and the functions and clinical correlations observed

Tissue	Sample	Functions	References
Prostate	148 patients	Predictor of recurrence	[34]
	100 patients	Increased expression in EC from capillaries infiltrating the tumor	[56]
Cervical	20 benign cervical tissue, 40 intraepithelial lesions, and 20 invasive SCC	Increased expression according to pathologic grade	[35]
Ovarian	66 tumors	Negative correlation with progression-free survival	[37]
	156 tumors	Overall survival	[38]
	30 tumors	Increased expression compared with normal stroma	[25]
Gastric	108 tumors	Positive correlation with tumor size, differentiation grade, TNM stage, lymph node metastasis and VEGF expression	[52]
	111 tumors and adjacent normal tissue	Positive correlation with tumor invasion, lymph node metastasis, TNM stage and poor prognosis	[54]
	93 tumors	Positive correlation with tumor location, lymphovascular, perineural and serosal invasions, differentiation, stage and lymph node metastasis, and poor patient survival	[53]
	134 tumors	Positive correlation with histological grade, invasion depth, lymph node metastasis, TNM stage and TGF-β staining	[55]
	162 paired gastric cancer tissues and non cancerous tissues	Negatively associated with E-cadherin expression but positively correlated with vimentin expression	[217]
Breast	105 tumors	Positive correlation with invasion, T stage, TNM stage, lymph node metastasis	[57]
	55 tumors	Positive correlations between Gal-1 positive cells and Scarff-Bloom-Richardson scale (histological grades 2 and 3)	[58]
Colon	25 samples of mucosae, 15 adenomas, 25 carcinomas, and 11 metastases	Increased expression with tumor progression from normal mucosae to adenomas and carcinomas	[39]
HCC	386 HCC	Positive association with tumor invasive characteristics, and poor tumor recurrence and overall survival	[41]
	197 HCC	Positive correlation with metastasis	[40]
	162 HCC and 12 normal liver samples	Positive correlation with tumor size, differentiation, TNM stage and distant metastasis. Negatively with CD3	[42]
Laryngeal carcinoma	53 LSCC of different stages (I,II,IV)	Positive correlation with EC, negative correlation with CD45. Increased expression in the tumor stroma compared to normal stroma	[36]
PDA	33 normal pancreas, 21 adjacent 30 benign CP, 45 adjacent CP, 17 dysplasias, and 43 PDA	Negative correlation with very long-term survival and longer survival	[44]
	66 PDA, 18 CP, 10NP	Expression: Normal<CP<PDA	[48]

(continued)

Table 2.1 (continued)

Tissue	Sample	Functions	References
	19 NP, 13 CP, 9 PanIN, 7 IPMN, and 30 PDA	Expression: Normal<CP PDA	[45]
	28 NP and 33 PDA	Overexpression in tumors compared to normal pancreas. Increased Gal-1 in poorly differentiated versus well-differentiated tumors	[43]
	33 NP, 17 PanIN and 43 PDA	Increased expression in advanced PanIN and PDA	[46]
	6 NP, 7 CP, 6 PDA	Overexpressed in tumors compared to normal and pancreatitis tissue	[47]
Myeloma	30MM (multiple myeloma)	Gal-1 in the extracellular space	[49]
(non) Hodgkin lymphoma	25 normal lymphoid tissues, 42 non-Hodgkin, and 14 Hodgkin lymphomas	Gal-1 in blood vessels	[50]
HNSCC	31 patients with HNSCC and adjacent normal epithelium	Gal-1 overexpressed in the tumor stroma, correlating with α-SMA$^+$ fibroblasts	[51]

protein kinase (MAPK), and by retaining phosphorylated Smad2 in the nuclei, allowing cells to differentiate [62]. In liver, Gal-1 promotes hepatic stellate cell migration and invasion through its binding to NRP-1, and signaling through platelet-derived growth factor (PDGF) and TGF-β1 [63]. In experimental mouse models with liver fibrosis, Gal-1 gene silencing in hepatic stellate cells results in downregulation of connective tissue growth factor (CTGF) and α-SMA, and thus inhibition of cell proliferation and migration and concurrent promotion of apoptosis [64]. Indeed, reduced collagen and α-SMA expression were detected in liver fibrosis when studied in Gal-1 KO background [63].

As Gal-1 is overrepresented in many tumor stroma microenvironments and its expression correlates with several clinicopathological tumor features (Table 2.1), it seems likely that it has an important role in this key tumor compartment. In oral squamous cell carcinoma (OSCC)-derived fibroblasts, Gal-1 downregulation decreases α-SMA and reduces ECM deposits in vitro [65] and impairs tumor progression and metastasis in vivo [65]. In pancreatic cancer, the role of Gal-1 in activating CAFs (termed pancreatic stellate cells, PSCs) has been well established. In particular, our group has shown that genetic inhibition of Gal-1 in PSCs, or in different preclinical transgenic mouse models of pancreatic cancer, results in tumors with reduced α-SMA content and impairs tumor growth and metastasis, thereby increasing animal survival [66, 67]. In addition, we have also shown that Gal-1 modulates Hedgehog signaling in PSCs [66]. Further, Gal-1 modulates proliferation and enhances the release of monocyte chemotactic protein 1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in PSCs, thereby activating extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), activator protein 1 (AP-1), and the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [68]. Recently, in vitro approaches have also shown that Gal-1 regulates proliferation and migration of PSCs and that the expression of Gal-1 positively correlates with increased levels of fibronectin, collagen type I, α-SMA, matrix metalloproteinase 2 (MMP-2), and tissue inhibitor of metalloproteinases 1 (TIMP-1), through the TGF-β1/Smad pathway [69]. Finally, in patients with pancreatic cancer, Gal-1 staining in the tumor microenvironment has been proven by multivariate survival analysis to be an independent prognostic factor [70]. Gal-1 role in inducing fibroblast activation has been addressed in several other tumor settings, such as in human breast cancer, in which Gal-1 knockdown reduces expression of α-SMA, fibroblast activation protein (FAP), and fibronectin, while

overexpression of Gal-1 by transfection of breast normal fibroblasts induces differentiation towards a myofibroblast phenotype [71]. In HNSCC, Gal-1 overexpression correlates with the presence of α-SMA–positive activated CAFs in the tumor [51] (Fig. 2.2).

In addition to its autochthonous functions in activated fibroblasts, Gal-1 has an important paracrine effect over the cancer cell compartment. For instance, in preclinical breast cancer studies, fibroblast-derived Gal-1 promotes cancer cell migration and invasion by induction of MMP-9 [71], a pattern that has also been observed in human breast cancer patients [57]. Fibroblast-derived Gal-1 has similar pro-tumoral functions in gastric cancer, via integrin β1 binding [72]. Further, this axis is also responsible for Gli-1 upregulation in gastric cancer cells, resulting in epithelial-mesenchymal transition (EMT) [54]. In ovarian cancer, Gal-1 secreted from fibroblasts modulated cancer cell invasion through MMP-2 [37]. In pancreatic cancer, fibroblast-derived Gal-1 increases tumor proliferation and invasion by upregulating MMP-2 and MMP-9 in cancer cells [70, 73]. Besides, PSC increases the in vitro and in vivo tumorigenic capacity of pancreatic tumoral cells via paracrine secretion of Gal-1, and gene expression analysis in a pancreatic cancer cell line (RWP-1) upon addition of recombinant Gal-1 brought additional hints on the molecular mechanisms driving these effects by identifying genes involved in cell proliferation, migration, metastasis, and cell metabolism [67]. Finally, in OSCC, Gal-1 regulates cancer cell migration through MCP-1 fibroblast secretion and binding to C-C chemokine receptor type 2 (CCR2) in tumor cells [65].

Paracrine effects of fibroblast-secreted Gal-1 are not restricted to cancer cells but also affect other cells of the tumor microenvironment. In pancreatic cancer, Gal-1 from PSCs drives tumor immune privilege by promoting T-cell apoptosis

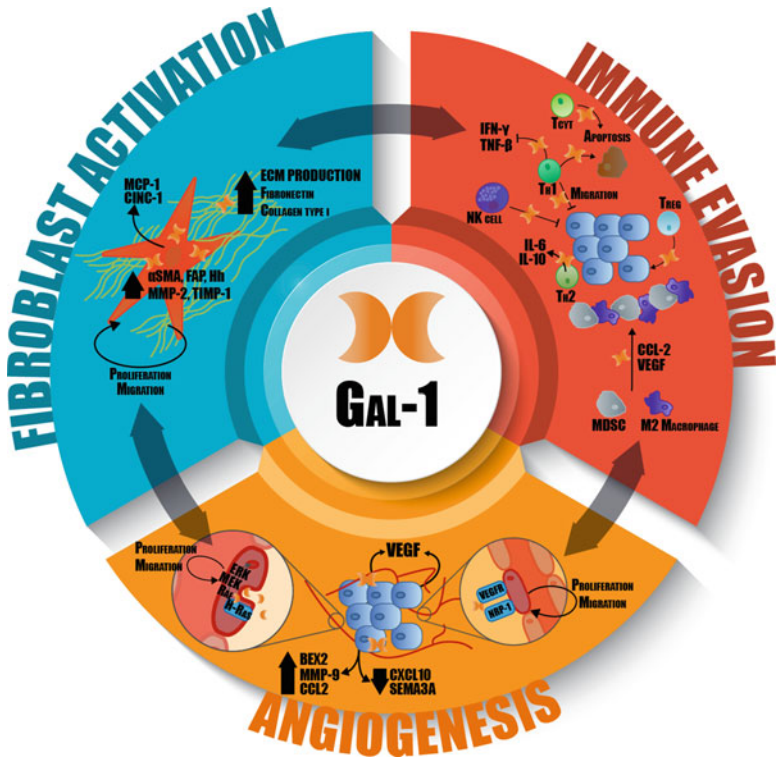

Fig. 2.2 Gal-1 functions in the tumor microenvironment. Information regarding the roles of Gal-1 in fibroblast activation, induction of angiogenesis, and tumor immune suppression is shown in this overview picture. Precise data and references are found in the main text

and Th2 cytokine secretion [32, 48, 66, 67]. In gastric cancer, Gal-1 derived from CAFs interacts with ECs to induce proliferation, migration, and tube formation, resulting in increased angiogenesis and tumor progression [74].

2.5 Gal-1 in the Tumor Endothelium

Gal-1 staining in the endothelium was already described in 1986 [75], and it has been long known to be upregulated upon EC activation [76–79], and in particular in cancer-associated capillaries in several tumors, such as prostate [56], breast, Ewing sarcoma, colon [78, 79], OSCC [28], and lung carcinomas [80]. Overexpression of Gal-1 in tumors can be explained by their typical hypoxic microenvironment, as Gal-1 has been reported to be upregulated upon hypoxia [81, 82]. For example, Gal-1 is upregulated in colorectal cancer cell lines by hypoxia inducible factor 1 (HIF-1)–dependent mechanisms [83]. Alternatively, in Kaposi's sarcoma, hypoxia-mediated increases of Gal-1 expression is driven by ROS-dependent activation of NF-κB [81].

Gal-1 modulates EC activity, inducing their proliferation, migration, and tubulogenesis in vitro [28, 74, 79, 84–88]. Indeed, ECs derived from Gal-1 KO mice show reduced tubular networks compared with wild-type mice [89]. One of the mechanisms that has been proposed for Gal-1–mediated angiogenesis is signaling through endoplasmic reticulum transmembrane kinase/ribonuclease inositol-requiring enzyme 1alpha (IRE1α), resulting in regulation of oxygen-regulated protein 150 (ORP150) [90], a chaperone which modulates VEGF maturation [91] (Fig. 2.2).

In addition to the relevance of endogenous Gal-1 in EC biology, several reports have also highlighted the importance of exogenous Gal-1 in endothelium functions, in particular in cancer angiogenesis. Indeed, Thijssen et al. [88] reported that Gal-1 secreted by tumor cells can be uptaken by ECs and signal through H-Ras and the downstream Raf/MAPK/ERK pathway to induce EC migration and proliferation [88] (Fig. 2.2). Along this line, different cancer cells knocked down for Gal-1 behave similarly, irrespective of being implanted into wild-type or Gal-1 KO mice, highlighting the importance of tumor-secreted Gal-1 for angiogenesis [27, 81, 89]. Indeed, tumor-derived Gal-1 enhances EC viability and protects them from apoptosis in the oxidative stress situations that frequently occur in the tumor microenvironment [84].

Importantly, most of the mechanisms concerning Gal-1 exerting pro-angiogenic effects in tumors have orbited around the VEGFR family of proteins. Gal-1 interaction with NRP-1, a known co-receptor for VEGF [92], results in VEGFR2 activation and downstream signaling stress-activated protein kinase-1/c-Jun NH2-terminal kinase (SAPK/JNK) (Fig. 2.2), thereby inducing migration and adhesion of ECs in a model of OSCC [28]. Importantly, Croci et al. described that Gal-1 can directly bind to VEGFR2 when displays cancer-specific glycosylation patterns, triggering its activation in the absence of VEGF, resulting in anti-VEGF–resistant tumors [27]. Extracellular Gal-1 increases vascular permeability through interactions with NRP-1 and VEGFR1, triggering Akt activation and impairing vascular endothelial-cadherin at cell/cell junctions [93]. Further, Gal-1 and Gal-3 in combination exert an enhanced pro-angiogenic effect through VEGFR1 activation, probably linked to impaired receptor endocytosis [94]. Gal-1 can also promote neovascularization by modulating platelet VEGF release through protein kinase C (PKC) and ERK signaling [95]. Besides the VEGF-VEGFR pathway, other molecular mechanisms have been related to activation of ECs by tumor-secreted Gal-1. For example, microarray analyses comparing glioblastoma cells knocked down for Gal-1 as compared to the parental cell line reveal reduced levels of microvascular differentiation gene 1 (MDG1), a marker of EC activation [96] and other hypoxia-regulated pro-angiogenic molecules [90]. In the same direction, Gal-1 inhibition in multiple myeloma cell lines decreases MMP-9 and chemokine (C-C motif) ligand 2 (CCL2) and increases semaphorin 3A (SEMA3A) and (C-X-C motif) chemokine 10 (CXCL10) [97]. Gal-1

also impairs vasculogenesis in vitro and in vivo through expression of brain-expressed X-linked 2 (BEX2) in an oligodendroglioma model [90].

Given the importance of neovascularization in tumor development and progression, much preclinical effort has been centered on deciphering the effects of Gal-1 downregulation or blockade in tumors. Further, Gal-1 has been linked to metastasis through its involvement in establishing heterotypic interactions between ECs and tumor cells [80, 98, 99]. Gal-1 triggers tumor angiogenesis in vivo in experimental models [79], and tumors grown or developed in Gal-1 KO mice show impaired angiogenesis [66, 67, 79, 100]. Further, Gal-1 knockdown in tumor cells inoculated into nude mice result in impaired tumor angiogenesis in Kaposi's sarcoma [81], prostate cancer [85], kidney cancer [101], myeloma [97], melanoma, and breast cancer xenografts [84]. Gal-1 downregulation in Lewis lung carcinoma or T-cell lymphoma cancer cell lines render them sensitive to anti-VEGF therapy by impairing Gal-1–driven angiogenesis [27]. Likewise, Gal-1 expression positively correlates with VEGF and Gal-1 knockdown in gastric CAF-impaired tumor growth and angiogenesis in vivo [74].

Altogether, information generated by these preclinical studies has laid the foundation for the development of Gal-1 pharmacological inhibitors as a novel anti-angiogenic therapeutic arsenal. Accordingly, strategies closer to clinical translation have also been assessed in preclinical setups, such as the use of Gal-1 antibodies, which in Kaposi's sarcoma result in tumor regression due to impaired angiogenesis [81], and in melanoma and Lewis lung carcinoma syngenic models restore a functional vascular network rendering tumors sensitive to anti-VEGF therapy [27]. Similarly, intratumor injections of a Gal-1 antibody impairs vascular permeability and tumor growth in OSCC xenografts [93]. In glioblastoma, introducing anti-Gal-1 siRNA into mouse brains significantly decreases angiogenesis and enhances the effects of temozolamide [86]. Anginex, an angiostatic peptide that targets Gal-1, shows impaired angiogenesis and tumor growth in a teratocarcinoma syngenic model, which of course could not be observed in a Gal-1 KO background [79]. Very recently, synthetic glycomimetic compounds (Phostine 3.1a) targeting the VEGFR2 and Gal-1 interaction have shown effectiveness in impairing angiogenesis in vitro and in vivo in a glioblastoma model [102]. Intratumor injections of thiodigalactoside (TDG) (a non-metabolized small drug that targets the amphipathic β-sheet of Gal-1) reduces EC content and tumor growth in melanoma and breast cancer models [84]. OTX008 (a calixarene compound targeting the CRD of Gal-1) restores tumor vessel normalization and impaired tumor growth in HNSCC [103] and ovarian xenografts [104, 105], where the drug potentiates cytotoxic and targeted therapies [105, 105]. Finally, several reports using different drugs that do not directly target Gal-1 have unveiled Gal-1 to be nonetheless responsible for the pharmacological effects. For instance, in a mouse model of prostate cancer, hemin treatment decreases Gal-1 tumor levels and thus impairs angiogenesis [106], and anti-VEGF and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) combined therapy in human melanoma elicits humoral Gal-1 immune responses resulting in improved outcomes and overall survival [107].

2.6 Gal-1 in Tumor Immune Evasion

Many reports have highlighted the effects of Gal-1 over a wide variety of immune cell types [8]. In fact, the role of this protein in regulating the immune system homeostasis was one of the first identified functions for Gal-1 [31]. Gal-1 recognizes different glycosylated receptors in immune cells and can trigger a wide variety of cellular processes, including polarization, maturation, activation, differentiation, and apoptosis [8, 108]. In T lymphocytes, Gal-1 binds to the CD43, CD45, CD7, and CD4 receptors on the cell membrane and regulates their function [30, 109].

Gal-1 plays a pleiotropic immunosuppressive role by directly targeting different immune cell types. For this reason, it has crucial physiological functions in immune-privileged sites, such as in

testis [110], eyes [111], and placenta, where it is key in maintaining fetomaternal tolerance in pregnancy [112]. Anti-inflammatory functions of Gal-1 are found both in innate and adaptive immune responses. In fact, Gal-1 has been associated to the regulation of most of the innate immune cells. For instance, Gal-1 has been linked to inhibition of neutrophil activation and trafficking upon inflammation [113, 114], as well as to phagocytic neutrophil removal through exposure of cell surface phosphatidylserine [115]. In macrophage polarization, Gal-1 favors an M2 profile by regulating metabolism of L-arginine, by decreased nitrogen oxide (NO) production, and/or by promoting the arginase pathway [116]. Additionally, Gal-1 impairs the expression of major histocompatibility complex II (MHC-II) as well as of Fcγ receptor (FcγR) on macrophages recruited to inflammation sites through ERK1/2 signaling pathway, thereby regulating the phagocytic potential [117]. The pro-resolving conversion of macrophages by Gal-1 has also been linked to 12/15-lipoxygenase expression [118]. In monocytes, Gal-1 induces chemotaxis and can also modulate FcγRI expression, dependent phagocytosis, and MHC-II expression and can interfere with antigen presentation [117]. Gal-1 was reported to induce migration and maturation of DCs [119–121], conferring tolerogenic potential by regulating IL-2 production and inducing IL-10–mediated T-cell tolerance [112, 122]. In the adaptive immunity, Gal-1 can modulate B-lymphocyte PI3K signaling, activation, proliferation, and differentiation [123]. However, the most important role of Gal-1 in controlling the immune system concerns T-cell function. Gal-1 can regulate T-cell effector function by inducing growth arrest and apoptosis [124–127] to specifically induce Th1, Th17, and $CD8^+$ T-cell apoptosis, highlighting an anti-inflammatory Th2 response [112, 127–131]. Further, Gal-1 is able to impair proper T-cell activation [132] and proinflammatory cytokine production [129, 133–135]. Gal-1 also regulates T-cell differentiation [136], trafficking, and transendothelial migration [134, 137, 138]. The immunosuppressive activity of forkhead box P3 (FoxP3)-positive T-regulatory (Treg) cells is also controlled by Gal-1 [139, 140], allowing expansion of T-regulatory type-1 cells secreting interleukin 10 (IL-10) [122, 131, 141]. Indeed, following intradermal parasite infection, Gal-1 KO mice fail to activate Gal-1–driven tolerogenic circuits and FoxP3+ regulatory T-cells, resulting in an increase of effector Th1 and $CD8^+$ T cell response [142].

Modulation of immune system response by Gal-1 together with its frequent overexpression in many tumors prompted numerous studies to explore Gal-1's role in tumor immune evasion. Importantly, in 2004, almost 10 years before immunotherapy was named breakthrough of the year, Rubinstein et al. published that Gal-1 was driving tumor immune escape in melanoma, as blockade of Gal-1 in vivo induced the generation of T-cell–mediated responses, including enhanced secretion of Th1 cytokines and tumor regression [143]. Since then, many articles have described that Gal-1 can induce tumor immune suppression in different tumor types, trying to decipher the mechanism by interrogating both innate and adaptive tumor immune responses (Fig. 2.2). In ovarian and lung cancer models, for instance, a major role of the lectin was reported to be to regulate DC tumor-promoting pro-inflammatory activities [144, 145]. In Kaposi's sarcoma, specific antibodies targeting Gal-1 in vivo showed enhanced tumor-infiltrating $NK1.1^+$ natural killer (NK) cells [81]. Along the same direction, in glioblastoma, Gal-1 downregulation in tumor cells impairs myeloid accumulation and tumor progression due to decreased CCL2 and VEGF expression (which are known as macrophage and myeloid-derived suppressor cells (MDSCs) chemoattractants) as well as enhanced interferon gamma (IFN-γ) production from CD8+ superindex T cells [156] (Fig. 2.2). These data were confirmed by Baker and colleagues, who have shown that Gal-1 knockdown in glioma cells leads to recruitment of $Gr\text{-}1^+$ $CD11b^+$ myeloid cells as well as $NK1.1^+$ NK cells in tumors, thereby impairing tumor growth [146] (Fig. 2.2). Recent in vitro and in vivo data also have shown that Gal-1 knockdown in glioblastoma results in diminished M2 macrophages and MDSCs as

well as decreased immunosuppressive cytokine production [147]. In mice, intranasal delivery of siRNAs against Gal-1 also reduces MDSCs and Tregs, increases $CD4^+$ and $CD8^+$ T cells, and impairs M2 polarization in macrophages [148] (Fig. 2.2).

Most articles have described the impact of Gal-1 on the T-cell population in tumors. In pancreatic cancer, for instance, co-culturing experiments of PSCs with T-cells show that Gal-1 induces T-cell apoptosis and stimulates secretion of IL-6 and IL-10 while decreasing lymphotoxin (TNF-β) and IFN-γ, thus favoring a Th2 immunosuppressive profile [149] (Fig. 2.2). These data were validated in preclinical transgenic models by our group; we reported that in c-myc–driven pancreatic tumors, Gal-1 ablation results in tumors with increased intratumoral T lymphocytes and increased neutrophil populations [66]. Similarly, in a K-Ras-driven system, Gal-1 KO tumors show enhanced infiltrating T-cells and decreased MDSCs [67]. Other descriptive tissue analyses report increased T-cells due to decreased apoptosis in low Gal-1 expressing tumors of lung syngenic models [150], a link that has been also corroborated in melanoma preclinical models [27] and in laryngeal squamous cell carcinoma patients [36]. In neuroblastoma, Gal-1 downregulation in tumor cells impairs tumor growth and metastasis due to increased accumulation of infiltrating $CD4^+$ and $CD8^+$ T-cells with enhanced IFN-γ secretion, cytotoxic T cell function, and DC maturation, and reduced T-cell apoptosis [151]. In a model of sarcoma, MDSCs were shown to promote Gal-1 secretion in γδ lymphocytes in toll-like receptor 5 (TLR5) responsive tumors, resulting in immune evasion [152]. However, Gal-1 regulation of T-cell infiltrates is not only a result of its pro-apoptotic effect. In prostate cancer, for instance, Gal-1 inhibits transendothelial migration of T-cells through CD43 clustering [137], and Gal-1 inhibition with hemin enhances $CD8^+$ T-cell proliferation and antigen-specific cytotoxicity in vivo [106]. As mentioned above, Gal-1 also affects the intratumoral Treg population (Fig. 2.2). More specifically, silencing Gal-1 in breast cancer syngenic animals reduces the presence of intratumoral and peripheral $FoxP3^+$ Tregs, impairing its function and suppressing its activity, thus resulting in impaired tumor growth and metastasis [58]. Interestingly, in hematological tumors, Gal-1 has also been reported to induce immunosuppression but is also relevant for the tumor cells themselves. For instance, in leukemia, Gal-1 derived from myeloid cells modulates B-cell receptor (BCR) signaling in tumor B-cells through Gal-1, thereby regulating tumor cell activity and favoring cancer progression [154]. Gal-1 in neoplastic Reed-Sternberg cells in Hodgkin lymphoma impairs T-cell viability and maintains expansion of $FoxP3^+$ Treg cells, to support an immunosuppressive Th2 milieu [134, 156] (Fig. 2.2).

The relevance of tumor versus host Gal-1 in immunosuppression was addressed by regulating Gal-1 expression in cell lines and injecting them into wild-type or Gal-1 KO mouse models. Interestingly, in neuroblastoma, T-cell–derived Gal-1 regulated intratumoral infiltrates without affecting tumor growth, which was however affected by tumor-derived Gal-1 [100]; this indicates important issues that need to be considered before developing therapeutic Gal-1 targeting strategies. In lung cancer and glioblastoma, for instance, tumor Gal-1 also proved to be more determinant than host-derived Gal-1 in tumor growth and metastasis [150, 155]. Although the reasons of this difference are unknown, the authors discussed that this may be due to enhanced Gal-1 expression levels from tumor cells as compared to T-cells [150]. Importantly, driving tumor immune evasion by Gal-1 seems to be one of the major functions of this protein in cancer, as the effects of reducing tumor growth and increasing survival observed after Gal-1 downregulation are not observed in immunodeficient mouse models [27, 67, 150].

Tumor-derived Gal-1 is able to not only promote an immunosuppressive tumor microenvironment but also generate a systemic immunosuppression in the animal [58]. Indeed, in lung metastases models, inhibiting Gal-1 with TDG or reducing endogenous Gal-1 levels in tumor cell lines enhances peripheral T-cell immune responses and impairs metastases. As expected based on its important role in evading the tumor immune response, blocking Gal-

1 has shown preliminary synergistic effects with immunotherapy in preclinical models. Screening The Cancer Genome Atlas (TCGA) reveals that Gal-1 levels in glioblastoma patients negatively correlates with the Th1/Treg and cytotoxic T lymphocytes (CTL)/Treg ratios and overall survival [148]. Accordingly, silencing Gal-1 improves DC vaccine and programmed cell death protein 1 (PD-1) blocking therapies in mice with glioma tumors [148, 156]. Inhibitory disaccharides targeting Gal-1 also improve vaccine immunotherapy in breast cancer preclinical models [157]. In non-Hodgkin lymphoma, a forward exome screen of primary tumors identified Gal-1 as a marker of resistance to CD20 immunotherapy; this finding was validated in an in vivo preclinical model [158].

2.7 Other Galectins in the Tumor Microenvironment

Besides Gal-1, other galectins have garnered the attention of researchers who are trying to understand the tumor microenvironment, mainly due to their role in angiogenesis and regulation of the tumor immune response [159, 160].

The role of galectins in cancer fibroblast activation appears to be monopolized by Gal-1. However, Gal-3 can also be secreted [161, 162] and is found in the stromal compartment of tumors [38, 163]. Thus, in addition to its important roles in fibroblast biology in fibrotic disease [164, 165] and arthritis [166], Gal-3 also has predominant functions in CAF activation. Indeed, in 2018, Zhao and colleagues described that recombinant Gal-3 can induce proliferation, invasion, and inflammatory cytokine secretion by PSCs, which contributes to tumor growth and metastasis in preclinical mouse models [167].

Gal-3 is also one of the best characterized members of the galectin family with respect to tumor blood vessel formation. Different reports have shown that Gal-3 induces EC morphogenesis, chemotaxis, and differentiation, leading to angiogenesis [168–172]. Several mechanistic studies have tried to elucidate how Gal-3 triggers angiogenesis in tumors; these studies have shown that Gal-3 retains VEGFR2 on the membrane of ECs [173], directly interacts with Jagged-1 (JAG1) in the EC cell membrane and activates Notch-1 [174], and induces VEGF expression from macrophages [175]. Furthermore, Gal-3 regulates tumor cell adhesion to ECs, promoting metastasis [171, 176].

Gal-8 has also been reported to play a key role in tumor angiogenesis and was classified as a pro-angiogenic molecule in 2011 by Delgado et al., who reported that this galectin controls EC migration and angiogenesis in vitro and in vivo. CD166 was identified as the receptor for Gal-8 in EC membranes [177, 178]. Regarding Gal-9 role in tumor angiogenesis, increased levels of this lectin have been found in blood vessels from lung, liver, breast, and kidney carcinomas [179]. Interestingly, ECs can express different splicing forms of Gal-9, and the expression of this protein is regulated during EC activation, although the precise role of Gal-9 in angiogenesis is not yet well understood [78]. Angiogenesis induced by these different galectin members can also be triggered in an indirect way by cytokines or soluble factors. For example, both Gal-3 and Gal-8 were shown to promote VEGF release by platelets, and Gal-8 also induces endostatin secretion, leading to angiogenesis [95]. Similarly, through its function as eosinophil chemoattractant or DC expansion, Gal-9 might induce the release of pro-angiogenic factors by these cells [179].

Moving to tumor immunity regulation, other galectin family members besides Gal-1 have raised a lot of interest in cancer, being even considered as emerging immune checkpoints. Gal-3, for example, has modulatory effects on T-cell survival and activation [109, 180, 181], NK function [182, 183], and DC expansion [184]. In particular, the axis of Gal-3/lymphocyte-activation gene 3 (LAG-3) has been postulated to be a novel cancer immune checkpoint [185]. Gal-9 gained the attention of oncologists when it was identified as a partner of the T-cell exhaustion marker T-cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3) [186]. Thus, the Gal-9 and TIM-3 interaction induces effector T-cell exhaustion or apoptosis,

leading to tumor immune evasion; therefore, Gal-9 has been also recently added to the list of cancer immune checkpoints [185]. Recent preclinical data in mouse models of liver cancer have demonstrated that therapy with antibodies against LAG-3 (i.e. targeting the Gal-3 axis) or TIM-3 (i.e., targeting the Gal-9 axis) are able to restore cancer immune surveillance and increase tumor-infiltrating T-cell number and functionality [187]. These data open new gates to assessing novel immunotherapy combinations that may result in increased percentage of responders or even allow resistance to be overcome; they further provide a rationale for studying these new immune checkpoints per se. Whether other family members have a role in promoting tumor immune suppression has not yet been elucidated, although in vitro data have already proven that Gal-2, Gal-4, and Gal-8 can also exert pro-apoptotic functions over T-cells [188–190].

Still, as we have experienced for Gal-1, special precaution and profound understanding of the context is vital, as other galectins also present apparently controversial outcomes depending on the model. For instance, breast cancer immunocompetent preclinical models show that absence of Gal-3 in the host mouse in fact boosts tumor growth and bone marrow metastasis [191].

2.8 Future Trends and Directions

In this chapter, we discuss the current knowledge about Gal-1 functions in the tumor microenvironment. These effects of Gal-1 driving fibroblast activation, angiogenesis, and tumor immune suppression are deeply entangled with one another and cannot be understood separately (Fig. 2.2). In lung cancer, for instance, Gal-1 secreted from tumor cells is important for fibroblast activation and induction of tryptophan catabolism by AKT signaling pathway and TDO2 (tryptophan 2,3-dioxygenase) and kynurenine (Kyn) upregulation, which contribute to immune evasion by impairing DC differentiation and T-cell function [192]. In fact, fibroblast activation promotes the secretion of chemokines and cytokines that are responsible for aberrant blood vessel formation in tumors and for establishing an immunosuppressive milieu [48, 68, 74]. Furthermore, tumor immune evasion driven by Gal-1 is not only a direct effect of Gal-1 over immune cell types but also a consequence of a deficient blood vessel network. Indeed, in 2014, Croci and colleagues reported that anti-Gal-1 antibody administration in immunocompetent melanoma and lung preclinical mouse models results in increased intratumoral infiltrates and tumor-draining lymph node cells, which is at least partially due to vessel normalization [27]. Tumor cells induce expression of Gal-1 by EC, which then inhibits T-cell transendothelial migration (as discussed above) [137]. Interestingly, tumor growth as well as angiogenesis are more severely impaired upon TDG treatment in immunocompetent mice as compared to immunodeficient animals [84], highlighting the strong link between Gal-1-mediated immune regulation and cancer cell proliferation and vessel formation.

The tumor microenvironment has over the years emerged as a key governor of tissue malignancy that drives tumor development and progression [194] and is often the cause of ineffective therapies. Thus, strategies targeting the tumor soil have gained the interest of both basic and clinical researchers in virtually all tumor types, with thousands of clinical trials being designed and performed [195–200]. Although clinical trials are still in their infancy, robust preclinical data suggest that galectins may represent interesting targets in this tumor compartment [33, 160, 201–203]. Among them, Gal-1 emerges as a top candidate with high potential, as this protein is a pleiotropic molecule that remodels the tumor microenvironment as a whole – including fibroblast activation, angiogenesis, and immune evasion – to allow uncontrolled tumor progression. Accordingly, development of Gal-1 inhibitors has currently a strong interest in cancer therapy and has shown promising results in several preclinical models [204, 205]. Moreover, taking into account the critical role of Gal-1 in immune evasion through its direct interaction with cell membrane receptors of effector T-cells triggering cell apoptosis, the Gal-1–CD7/CD43/CD45 axis

could be considered as a new immune checkpoint (like Gal-3/LAG-3 and Gal-9/TIM-3; [185]); therefore, Gal-1 inhibitors could enlarge the list of novel cancer immunotherapies.

However, several issues are crucial for successful and proper drug development, such as having a profound understanding of the particular tumor type and galectin repertoire, deciphering redundant versus specific roles of each member, the endogenous versus exogenous roles of the protein, drug specificity, and so on. Classical in vivo models trying to study targets at the tumor microenvironment have shown limited translational success [206, 207]. More recently, patient-derived cancer models, including organoids and patient-derived xenografts (PDXs), have emerged in cancer preclinical studies, but they still lack the autochthonous tumor microenvironment [208, 209]. With the advent of immunotherapy, many groups have joined efforts to develop more suitable and more sophisticated models to allow the tumor as a whole to be investigated, such as using humanized mice [210] and ex vivo systems that retain the whole native tumor microenvironment, such as 3D microfluidic cultures, tumor tissue explants, "tumor-on-a-chip," and multicellular tumor spheroids [198, 211]. Further experiments in systems with better recapitulation of the tumor's surrounding will for sure be necessary for completing preclinical steps before moving Gal-1 therapy into the clinics.

In addition to the emerging attention on Gal-1 inhibitors as a novel cancer therapy, Gal-1 also offers other translational applications. For example, increasing data have unveiled the potential use of detecting Gal-1 levels for cancer diagnosis [33], as Gal-1 can be detected in biological fluids and, for many of the tumors expressing Gal-1, in the stroma (Table 2.1). Further, increased levels of the protein have been detected in plasma or serum from patients with cancer of thyroid [212], colorectal [213], pancreatic cancer [45], Hodgkin lymphoma [214], glioma [215], or OSCC [216]. Moreover, in the era of personalized medicine, Gal-1 may also work for selecting patients who respond better to anti-angiogenic or immunotherapies, and it could represent a useful biomarker for therapy response. Further preclinical and clinical data will be necessary to answer whether reality meets expectations to all of us working in the galectin world.

Acknowledgments This work was supported by grants from the Spanish Ministry of Economy and Competitiveness/ISCIII-FEDER (PI17/00199), the Carmen Delgado/ Miguel Pérez-Mateo AESPANC-ACANPAN 2016 grant, and the Generalitat de Catalunya (2017-SGR-225) to P.N. We are also grateful to A. Flotats for help in graphic design and V. Raker for English proofreading and editing.

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Iron in the Tumor Microenvironment

3

Driton Vela

Abstract

Cancer metabolism is a well-known target of cancer therapeutics. Classically, cancer metabolism has been studied in terms of the dependence of cancer cells on crucial metabolites, such as glucose and glutamine. But, the accumulating data show that iron metabolism in tumor microenvironment is also an important factor in preserving the survival of cancer cells. Cancer cells have a distinct phenotype of iron metabolism, which secures the much-needed iron for these metabolically active cells. In order to use this iron efficiently, cancer cells need to increase their iron supply and decrease iron loss. As recent research suggests, this is not only done by modifying the expression of iron-related proteins in cancer cells, but also by interaction of cancer cells with other cells from the tumor milieu. Tumor microenvironment is a dynamic environment characterized with intricate relationship between cancer cells, tumor-associated macrophages, fibroblasts, and other cells. Some of the mechanistic aspects of this relationship have been elucidated, while others are yet to be identified. In any case, identifying the details of the iron phenotype of the cells in tumor microenvironment presents with a new therapeutic opportunity to treat this deadly disease.

Keywords

Iron · Tumor microenvironment · Transferrin receptor 1 · Divalent metal transporter 1 · Vacuolar ATPase · Hepcidin · Ferroportin · Tumor-associated macrophages · Cancer-associated fibroblasts · Cancer stem cells · Interleukin 6 · Iron chelation · Ferritinophagy · Ferroptosis · Nanomedicine

Introduction

Tumor cells are highly adaptive cells. They need this plasticity considering the unfavorable conditions that they are exposed to (lactic acidosis, hypoxia, and lack of sufficient nutrients) [1]. The adaptability of cancer cells is partly genetic in nature, but it also depends on the cellular origin of the tumor and its microenvironment [1]. One of the aspects of this adaptability includes specific changes in tumor metabolism; it is well known that cancer cells are able to increase the uptake of glucose and glutamine for their metabolic needs [1]. But, there are many other nutrients that are crucial for cancer cell survival. An increasing amount of research suggests that iron is one of the most fundamental metals

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_3

needed for cancer proliferation. This finding is based on a wide array of changes found in the iron metabolism of different cancers [2]. What is more, recent evidence suggests that rewiring of the iron metabolism does not include only tumor cells but other cells in the tumor microenvironment, such as cancer stem cells, neighboring normal cells, stromal cells, leukocytes, and even senescent cells [3, 4]. Also, it seems that tumor cells have the ability to affect the iron turnover in tumor microenvironment in order to facilitate their proliferation [2].

The importance of iron for cancer cells should not come as a surprise. It is one of the most abundant metals in human body, while its favorable chemical properties make it an ideal metal to be used by living cells as a transporter of electrons in crucial biochemical reactions [5]. This is why iron is involved in some of the most important biochemical reactions in the cellular milieu, namely, DNA synthesis, cellular respiration, and oxygen metabolism [2]. Experimental evidence shows that manipulating tumor iron supply can have dramatic effects in tumor's ability to proliferate [6–9].

Iron dysmetabolism in tumor microenvironment is characterized with differential regulation of cellular iron import and export proteins, changes in the activity of intracellular proteins involved in the regulation of iron metabolism, and an overall propensity towards increased accumulation of cellular iron. These changes seem to be independent from the homeostasis of systemic iron metabolism, which has implications for the treatment of cancer via iron therapeutics.

3.1 Iron homeostasis in physiological conditions

Systemic iron metabolism is mostly controlled through iron absorption in intestines due to lack of a specific excretory mechanism. In duodenum, iron is first reduced and then enters into intestinal cells via divalent metal transporter 1 (DMT1). Its export is mediated via ferroportin (FPN), which is the main target protein of hepcidin, known as the major regulator of systemic iron metabolism [2]. Hepcidin is produced in liver hepatocytes through a series of intricate pathways influenced by iron levels, hypoxia, and inflammation [10]. After being released from intestinal cells, iron is oxidized and is bound to transferrin [2]. This complex is named transferrin-bound iron (TBI). TBI travels in plasma until it binds to its target protein, which is transferrin receptor 1 (TFR1), found in different cells such as macrophages, erythrocyte precursors, and hepatocytes [2]. This binding induces endocytosis and creation of an endosome, in which iron is released from its complex with transferrin and TFR1 via proton pumps such as V-ATPase, and then reduced via metalloreductases [2, 11, 12]. Finally, iron is released in cytoplasm through DMT1 [2]. The fate of the released iron is manifold: it can be transported to ferritin, which serves as a cellular iron depot, it can be part of free cellular iron (also called labile iron pool), or it can be exported out of the cell via FPN [2]. The main regulators of intracellular iron metabolism are iron-responsive element-binding proteins (IRPs). Their activity is influenced by cellular iron availability, which means that in iron-replete conditions, IRPs increase iron import and reduce iron export and vice versa [2].

As it can be seen, most of the time iron is bound to different proteins in our body. Iron sequestration via proteins not only serves as a protective measure against the production of reactive oxygen species, but also prevents the "hijacking" of iron from microorganisms [5]. In physiological conditions, most of the iron is in TBI form, but in some cases (such as in iron overload) a significant amount of iron is in the form of non-TBI (NTBI). This type of iron enters the cells via DMT1 and zinc transporter proteins (Zip) [13].

3.2 Iron Metabolism in Cancer Cells

Iron import in cancer cells Iron import in cancer cells is directed by different proteins expressed in cell membranes. Most of the TBI enters through TFR1, while other means of iron entry including NTBI are realized through proteins such as DMT1, Zip proteins, and probably through other as yet unidentified ways

[13, 14]. In many cancers, TFR1 is upregulated, which helps cancer cells to increase their iron supply. In some tumors, there is evidence that TFR1 is not only subject of changes in its expression, but also it is differentially distributed inside cells after endocytosis compared to normal cells [15–18]. Nevertheless, blocking TFR1 action has been shown to be an effective way to suppress tumor growth [15, 16, 18, 19]. On the other hand, blocking DMT1 in colorectal cancer has been shown to have similar effects in terms of cancer progression [20]. This occurs due to DMT1 serving as the main gateway for iron entry in epithelial cells of intestines. But the release of iron inside cancer cells is an important process as well, since in many tumors one finds overexpression of metalloreductases such as six-transmembrane epithelial antigen of prostate (STEAP) proteins. The expression of STEAP proteins is in direct correlation with tumor proliferation [21, 22]. Other proteins involved in intracellular iron release from endosomes also seem to play a role in cancer cells, such as vacuolar ATPase (V-ATPase). The link between V-ATPase inhibition and cellular iron metabolism has been observed in different cancer cells [23]. An unexplained issue remains the role of cellular iron chaperons called poly(C)-binding proteins (PCBPs), which have been shown to be upregulated in different cancers [24–26], though the link between PCBPs and iron dyshomeostasis in cancer has not been elucidated. What is more, PCPBs are known to have different physiological functions independent from iron metabolism [24–26].

Cellular iron regulators in cancer cells IRPs are known as major regulators of cellular iron homeostasis [2]. They bind to iron-responsive elements present in different mRNAs of iron-related proteins [2]. In this way, IRPs influence the translation of FPN, TFR1, and DMT1 depending on the cellular iron availability [2].

IRPs have been studied in cancer cells as well, especially IRP2. It has been found upregulated in different cancers, where it is involved in tumor proliferation [27–29]. In some tumors, IRP2 expression has been observed as an early sign of iron dyshomeostasis, which correlates with tumor stage [15]. Also, IRP suppression has been used as a therapeutic modality to reduce tumor growth in cultured mediums [15, 27–29].

Iron export in cancer cells FPN and hepcidin are two major players controlling iron export in human cells. FPN is controlled translationally by IRPs, but also by transcriptional factors, and postranslationally through degradation via hepcidin binding [30]. Since FPN is the only protein involved in cellular iron export, it has been the subject of study in different cancer cells. In majority of cancers, FPN is downregulated or does not realize its function properly as a cellular iron exporter [31–34] (Table 3.1). Furthermore, FPN overactivation has been shown to reduce tumor growth and even metastasis [31–34]. Similarly to

Table 3.1 Major changes of iron metabolism in tumor microenvironment

Type of protein	Function	Expression/activity	Type of cell	References
TFR1	Iron import	↑	Cancer cells, CSCs	[15–19, 55, 57]
STEAP	Ferrireductase	↑	Cancer cells	[22]
V-ATPase	Proton pump	↑	Cancer cells	[23]
IRP1	Intracellular regulator of iron metabolism	↑	CSCs	[59]
IRP2	Intracellular regulator of iron metabolism	↑	Cancer cells	[15, 27–29]
FPN	Iron export	↓	Cancer cells	[31–34]
Hepcidin	Inhibition of iron export	↑	Cancer cells	[10]
Ferritin	Iron-binding protein	↑	Cancer cells, CSCs	[27, 28, 55–57]
Lcn2	Iron-sequestering protein	↑	M2 macrophages	[49]

Abbreviations: *CSC* cancer stem cell, *FPN* ferroportin, *IRP* iron-responsive element-binding protein, *Lcn2* lipocalin 2, *STEAP* six-transmembrane epithelial antigen of prostate, *TFR1* transferrin receptor 1, *V-ATPase* vacuolar ATPase

FPN, hepcidin expression has also been related to tumor proliferation. It has been frequently found to be overexpressed in cancer, which is mostly related with its ability to suppress iron export via FPN endocytosis [10]. In some cancers, aggressive behavior of tumors is related not only to local hepcidin expression but also to plasma (systemic) levels of hepcidin [10].

Role of mitochondria in cancer There is ample evidence through which mitochondria have been "exposed" as promoters of tumor progression [35]. These cellular powerhouses are attractive target organelles in cancer therapy. Mitochondria have their own set of iron-related proteins which regulate the iron flux in mitochondria [36]. Mitochondrial iron homeostasis is important for the proper functioning of mitochondria due to the dependence of crucial mitochondrial enzymes on using iron as a co-factor [36]. Surprisingly, there is not much research relating mitochondrial iron metabolism and cancer. The rationale exists, since some relatively specific mitochondrial iron chelators can inhibit tumor growth by disturbing mitochondrial functional parameters [37]. Some recent data even suggest an impressive potency of specific mitochondrial iron chelators compared to their classical nonspecific counterparts [38]. Still, the exact role of mitochondrial iron homeostasis in cancer awaits confirmation by future studies.

Cause of iron dyshomeostasis in cancer cells Tumors are heterogenous diseases characterized with a complex pathophysiology and a dynamic microenvironment. Changes in iron metabolism are just one piece of this puzzle, but which require further examination. Culprits for changes in iron metabolism in cancer are heterogeneous in nature. For example, mutations in oncogenes such as C-myc and BRAF are responsible for IRP2 upregulation, while adenoviral oncogene E1A is responsible for increasing labile iron pool [29, 39, 40]. Interestingly, the transcription factor p53 has opposite effects on labile iron pool [8]. p53 gene is known as a tumor suppressor gene which undergoes loss-of-function mutation in many cancers [41]. These observations suggest that the asymmetry between tumor oncogenes and suppressors found in different tumors might be an important instigator of iron dyshomeostasis in cancer cells.

Other molecules are also responsible for changes of iron metabolism in cancer. One such factor is epidermal growth factor receptor (EGFR), which is a growth factor known for its oncogenic properties. It increases cellular iron pool by promoting TFR1 activity [18]. On the other hand, reduction in cellular iron export in cancer has been shown to be mediated via sclerostin domain containing 1 (SOSTDC1), wingless and int (Wnt) pathway, bone morphogenetic protein (BMP) pathway, inflammatory cytokines such as interleukin 6 (IL6), and transcriptional factor zinc-figure protein 217 (ZNF217) [10, 42]. Another level of complexity involved in the control of iron metabolism in cancer cells is realized through epigenetic mechanisms. Epigenetic mechanisms have been shown to exert control in the activity of different iron-related proteins in cancer. In any case, the extent of epigenetic control of iron metabolism in cancer is yet to be revealed [13].

It has to be mentioned that although iron dyshomeostasis is a product of an overall cancer pathophysiology, iron may contribute directly to the initiation of the disease. Iron can cause damage to DNA structure through increases in production of reactive oxygen species (ROS) [7]. This is the reason why repeated intraperitoneal or intramuscular injections with concentrated iron cause cancer in rodents [6, 7, 43]. Many epidemiological data have examined the role of iron intake and cancer prevalence [2]. Results are contradictory and not uniform across different types of cancers. One must not forget that our cells have developed homeostatic control mechanisms to protect themselves from global changes in iron metabolism. This means that only in extreme cases of global iron load our cells will not be able to control their iron depots, in which case cancer rates will increase progressively, especially in older adults [44].

Role of tumor associated leukocytes in cancer Tumor microenvironment is abundant with different types of leukocytes, of which macrophages dominate. Their migration into

tumor environment is caused by different chemical substances such as interleukins, angiogenic factors like vascular endothelial growth factor A (VEGF-A), or chemokines like C-C motif ligand 2 (CCL2) [45]. Activated tumor-associated macrophages or TAMs are responsible for tumor growth, immunosuppression, and angiogenesis [45]. Furthermore, the density of TAMs is correlated with poor prognosis and high tumor grade [46]. These TAMs are so-called M2 phenotype macrophages compared to M1 phenotype macrophages found in the early stages of cancer, which generally have antitumor properties [45]. M1 macrophages show iron-accumulating properties, while M2 macrophages show iron-releasing properties [47]. This means that iron release from TAM M2 macrophages can potentially exacerbate iron dyshomeostasis in cancer cells. There are different strategies through which M2 macrophages achieve this effect; first by exporting high amounts of iron through FPN and second by increasing the production of iron-related proteins (Fig. 3.1). The candidate for the latter is lipocalin 2 (Lcn2). It is frequently upregulated in cancer locally but also in different body fluids [48]. Data from tumor microenvironment studies suggest that most of Lcn2 comes from tumor stroma (that is, macrophages) and not from tumor cells [49]. In cultured medium, macrophages do not change their iron-releasing phenotype even when FPN is blocked [49]. But, iron-releasing phenotype of tumor macrophages is reversed when Lcn2 is blocked [49]. Furthermore, Lcn2 suppression reverses tumor proliferation in this setting [49]. It is believed that Lcn2 production in M2 macrophages occurs from their interaction with apoptotic tumor cells via sphingosine-1-phosphate/signal transducer and activator of transcription 3 (S1P-STAT3) pathway [50].

Iron metabolism in M2 macrophages is a potential therapeutic possibility in suppressing tumor growth. Iron chelators have already been used in in vitro studies to prevent iron release from M2 macrophages by sequestering iron and by reversing their polarization from an iron-releasing phenotype to an iron-sequestering one [51]. In recent ex vivo experiments, which reflect more faithfully the in vivo tumor microenvironment, iron chelation was confirmed as a therapeutic tool which can reverse the iron-releasing phenotype of M2 macrophages [52]. In addition, iron chelation did not increase the population of M2 macrophages compared to chemotherapeutic agents, which means that iron chelation can be used with traditional chemotherapy to exert additional anticancer effects [52]. Finally, level of iron load in M2 macrophages is an important feature of the tumor microenvironment, since it is correlated with the success of iron chelation therapy in cancer [53]. In conclusion, the iron flux of M2 macrophages is a crucial therapeutic knot, further corroborated by the findings that show TAMs are some of the most abundant cells in tumor microenvironment, which can affect the metabolism of cancer cells.

Iron and cancer stem cells (CSCs) CSCs are a small fraction of cells in the tumor milieu, but their renewal ability makes them ideal cells to promote malignancy, metastasis, tumor recurrence, and resistance to chemotherapy in different cancers [54]. These highly active cells need to "fuel" their metabolism by securing the necessary nutrients. In recent years, studies have revealed that iron is important for the proper survival of CSCs [54]. This means that similar to cancer cells, CSCs interact dynamically with their microenvironment. In this respect, CSCs have evolved mechanisms to secure the proper amount of iron for their metabolic needs. Indeed, studies have shown that CSCs are much more efficient in increasing their iron uptake compared to non-CSCs and even compared to macrophages [55–57]. Strategies used by CSCs to secure the much-needed iron are manifold: they are able to secrete high amounts of TF (through which they scavenge free iron), they increase TFR expression (to increase iron import), increase ferritin depots, downregulate FPN (to decrease iron export) [55–57]. The dependence of CSCs on iron is observed when these cells are exposed to iron chelation therapy, but also when their ferritin depots are depleted, or when iron is forced out of cells; in these cases, proliferation rate and

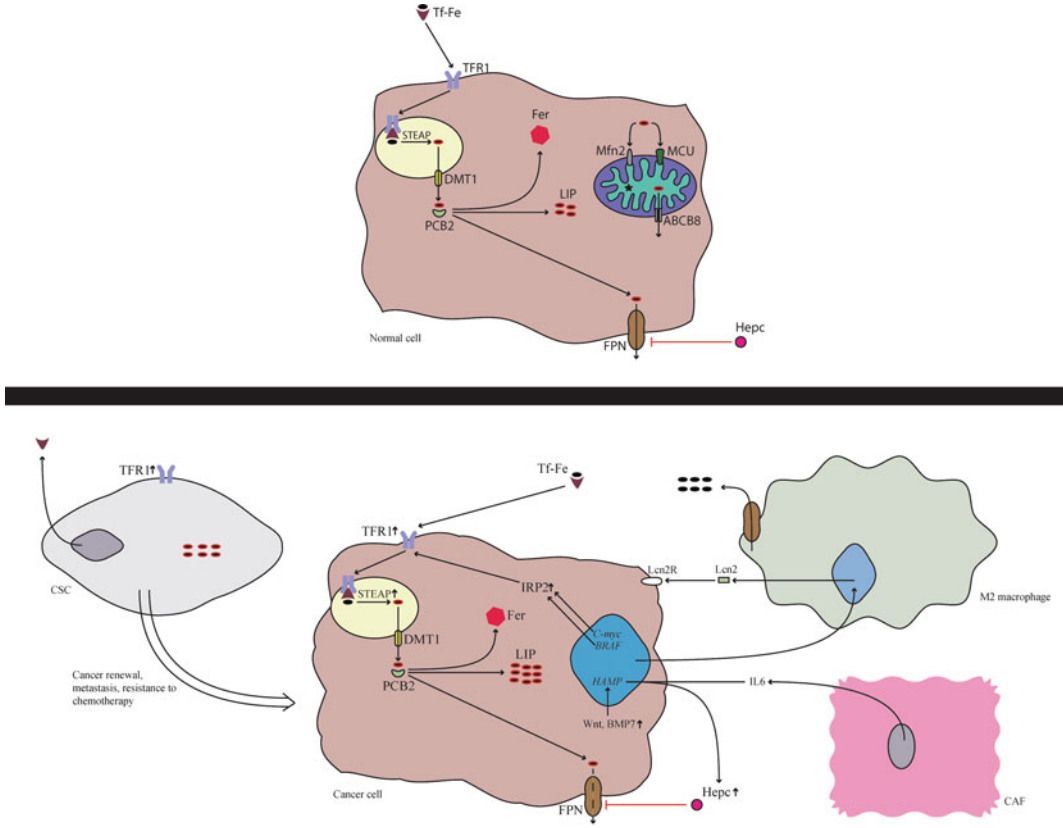

Fig. 3.1 Dysregulation of iron metabolism in tumor microenvironment. Normal cells express different iron-related proteins in the membrane, cytoplasm, and mitochondria as seen in the upper part of this figure. Iron bound with transferrin enters via TFR1 in cells. This is accompanied with endocytosis and creation of an endosome. Iron is released from endosome via DMT1 after being reduced by STEAP metalloreductases. In cytoplasm, iron is bound with PCBs, which transport iron to ferritin, FPN, or transport it to enrich LIP. Some of this iron enters mitochondria via mitochondrial iron import proteins such as MCU and Mfn2. Mitochondria have their own ferritin and their iron export proteins (such as ABCB 8). The major exporter of iron out of cells is FPN which is under negative regulatory control from hepcidin. Many of these proteins are differentially expressed in tumor microenvironment. For example, the iron import protein TFR1 is often upregulated in cancer cells, as well as STEAP proteins. Also the major regulator of cellular iron metabolism (IRP2) is overexpressed in cancer cells, which causes increased iron entry into cells. IRP2 is under regulatory control of different genes involved in tumorigenesis (such as C-myc or BRAF). Another important protein that is overexpressed in cancer is hepcidin. By increasing hepcidin production, cancer cells reduce iron export. Wnt and BMPs are some of the pathways involved in increased hepcidin secretion in cancer cells. But, hepcidin production in cancer cells is increased via IL6 produced by CAFs as well. Another strategy used by cancer cells to secure more iron for their metabolic needs is by using the iron-releasing phenotype of M2 macrophages either through increased iron supply from overactive FPN in M2 macrophages or through increased production of Lcn2. Another important player in the iron metabolism of the tumor microenvironment is CSC. They have voracious appetite for iron, which is secured through increase in TFR1 expression, but also through increased production of transferrin which serves as an iron scavenger. CSCs are important cells in this environment because they secure the replenishment of cancer cells, promote metastasis, and are responsible for resistance to chemotherapy. *Abbreviations:* ABCB8 ATP-binding cassette subfamily B member 8, BMP7 bone morphogenetic protein 7, CAF cancer-associated fibroblast, CSC cancer stem cell, DMT1 divalent metal transporter 1, Fer ferritin, FPN ferroportin, Hepc hepcidin, IL6 interleukin 6, IRP2 iron-responsive element-binding protein, LIP labile iron pool, Lcn2 lipocalin 2, Lcn2R Lcn2 receptor, Mfn2 mitoferrin 2, MCU mitochondrial calcium uniporter, PCB poly(C)-binding protein 2, STEAP six-transmembrane epithelial antigen of prostate, TFR1 transferrin receptor 1, Wnt wingless and int pathway

metastatic potential of CSCs drop significantly [55–57]. Increased intracellular iron in CSCs is linked with an overactive IL6/STAT3 pathway which promotes the invasive potential of CSCs [55, 57]. It is interesting to notice that iron chelation inhibits the expression of stemness markers in CSCs, which effect has not been observed with standard chemotherapy [58]. This is an important finding with therapeutic implications considering that stemness markers are related directly with malignant potential of CSCs [54]. Another interesting phenomenon has been observed in CSCs of breast and prostate cancer lines. IRP2 in these cells seems to be under the regulatory feedback of cellular iron depots, which is not the case with cancer cells [59]. This finding needs to be confirmed by other studies in order to evaluate its importance in tumor pathophysiology.

The activity of CSCs in cancer is one part of the complex picture that unfolds during one of the most important processes which occurs in cancer, that is, epithelial-to-mesenchymal (EMT) transition. EMT is a process which helps tumor mass to expand, proliferate, and metastasize, but also helps tumors to resist chemotherapeutic treatment [54]. Besides being responsible for transformation of CSCs, EMT enables phenotypic transformations of tumor cells, which increases their mobility and eventual metastasis [54]. This transformation is realized by making tumor cells more mesenchymal-like cells. It is interesting that many biochemical pathways involved in regulation of EMT are also involved in regulation of cellular iron metabolism such as transforming growth factor beta (TGF-β) and Wnt pathway [60, 61]. One of the most known inhibitors of EMT is N-myc downstream-regulated gene 1 (NDRG1) [60]. Its activity is dependent on iron availability, which has been used in experimental setting to reverse EMT via iron chelation [60]. NDRG1 is able to suppress Wnt pathway, which is known to be hyperactive in different tumors [62]. Wnt pathway inhibition has been shown to reduce cancer cell iron pool by reducing hepcidin production [10]. These results are in line with the observation that suppression of FPN (main target of hepcidin) via activation of TGF-β enhances markers of EMT [63].

Iron and cancer-associated fibroblasts (CAFs) in tumor microenvironment The complex tumor environment is exposed to different modes of signaling from different cells. Along with macrophages, fibroblasts are some of the most abundant cells in tumor milieu [64]. Their heterogeneous phenotype makes them a difficult cellular target for cancer therapy. Our current understanding of CAFs reveals at least three distinct populations: one group promotes tumor growth, another one retards tumor growth, while other populations are neutral in terms of their effect on tumor growth [64]. Tumor-promoting CAFs have multiple roles in promoting tumor growth. CAFs produce different chemical signals through which they affect the stemness of CSCs, transformation of TAMs into M2 macrophages, migration of tumor cells, extracellular matrix remodeling, formation of new blood vessels, and immunosuppression [64]. CAFs which express tumor-promoting markers are present in high numbers in tumor with high density of stroma and in invasive margins of the tumor [64, 65]. Furthermore, CAFs can "donate" their mitochondria for metabolic needs of cancer cells, while their secretome can promote cancer cell metabolism [64, 66]. CAFs have been shown to promote tumor growth via pathways which are known to be involved in iron dyshomeostasis in tumor microenvironment. Although the importance of iron metabolism in the activity of CAFs and tumor growth has just recently been studied, it has unveiled some interesting results. For example, in breast cancer, IL6 produced by CAFs has been shown to increase hepcidin production in cancer cells, which can be reduced significantly with anti-IL6 antibodies [67]. The elegant study done by Blanchette-Farra et al. shows that hepcidin production is affected by the spatial organization of the tumor cell milieu, which has been shown by comparing 2D with 3D models of cultured cells [67].

Senescent cells in tumor microenvironment Cellular senescence is a process through which cells suffer cell cycle arrest which cannot be reversed with mitogens [68]. The state of senescence can be induced by mutations, chemotherapy, and oxidative stress [68]. These cells are believed to contribute to the tumor microenvironment by their senescence-associated secretory phenotype (SASP) and by their ability for neoplastic transformation during chronic senescence [68]. But, senescent cells exhibit a continuum of phenotypes, from the initial tumor-suppressing one to a tumor-promoting one, which should be taken into account when trying to eliminate senescent cells as part of an antitumor strategy [68]. This means that a two-hit strategy might be the best option for dealing with these cells: the first hit turns cancer cells into senescent cells, and the second hit is used to destroy the senescent cells so they would not be able to undergo neoplastic retransformation. In fact, low dose curcumin in combination with knockdown of specific long noncoding RNAs has already been used to induce senescence and then apoptosis [69]. This is important since curcumin is known for its iron-chelating properties and its effect on iron metabolism [70]. Iron dyshomeostasis has been observed recently in senescent cells as well. It is characterized by increased cellular iron flux [4]. This occurs in response to impaired ferritinophagy, which disrupts the ability of senescent cells to detect true levels of cellular iron [4]. Iron dyshomeostasis in senescent cells can be reversed by promoting ferritinophagy [4]. On the other hand, FPN overactivation in prostate cancer is accompanied with SASP perturbations [32]. Still, the role of iron metabolism in senescent cells during cancer has not been elucidated properly and more studies are needed to address this issue.

Ferritinophagy, ferroptosis and their role in tumor microenvironment Ferroptosis is a recently observed form of programmed cellular death with distinct features. It does not involve chromatin margination or caspase activation as seen in apoptosis or non-apoptotic cell death [71]. In order for ferroptosis to occur, there are some conditions which should be met; first, there needs to be a substrate for peroxidation (phospholipids), then iron is needed as the main instigator of peroxidation, and finally these changes should be accompanied with the inability of the cell to eliminate the products of lipid peroxidation [71]. Although we still do not know the role of ferroptosis in human physiology, its importance has been observed in different pathophysiological states, including cancer. The rationale to study ferroptosis in cancer is based on the ability of cancer cells to resist classical apoptosis [71]. Also, different cells of the tumor microenvironment seem to be more sensitive to ferroptosis than normal cells [72]. This includes mesenchymal type cells, detached cells, and CSCs. But, cancer cells need activity; therefore, using iron to induce ferroptosis might be a double-edged sword in fighting cancer. For example, downregulation of TFR reduces the extent of ferroptosis in cancer cells [73]. Similarly, knockdown of FPN accelerates ferroptosis [74]. Still, there are ways to circumvent this scenario. Redistributing iron from cytosol to lysosomes is one possible approach. It causes peroxidation of liposomal membranes and resultant ferroptosis [75]. Another approach would be to induce the process of ferritinophagy in cancer cells, which is accompanied with increased levels of ROS inside the cells. This can induce ferroptosis, as it has been observed in breast cancer cells [76]. It is interesting to note that iron chelators can be used to reverse the process of EMT through induction of ferritinophagy [77].

3.3 Iron therapeutics in cancer

Iron therapy is not a recent idea in the management of cancer. Considering that excessive iron deficiency/overload is detrimental for cancer cell survival, it is expected that stimulating these conditions can affect tumor growth, especially when one takes into account that iron dysmetabolism is a feature of different cells in the tumor microenvironment.

Iron chelators have the ability to bind iron and cause cellular iron deficiency [2]. These agents are able to have synergistic effects when combined with traditional chemotherapy [78]. Thus, they have been used in experimental studies to destroy cancer cells, but they have not shown to be an effective therapeutic strategy for patients with cancer [2, 44]. This probably occurs due to their inefficient accumulation in cancer cells, their ability to cause reactive intracellular iron flux, and also because they can have side effects in normal tissue. Other options used to treat cancer by manipulating iron metabolism of cancer cells involve increasing/decreasing the activity of iron-related proteins. Genetic studies in cancer patients show that low TFR expression and high FPN/low hepcidin combination are markers of favorable outcomes in cancer [31, 79, 80]. Therefore, blocking TFR, increasing FPN activity, and downregulating hepcidin can be used as a useful strategy to prevent tumor growth and metastasis [2, 10] (Table 3.2). Other proteins involved in iron import, iron release, or control of cellular iron metabolism in tumor cells are also potential targets in cancer therapy. Up till now, these targets have been studied mostly in in vitro conditions by using antibodies, small interfering RNAs (siRNAs), or naturally occurring compounds which can modulate iron-related proteins. Hepcidin therapy is being used in clinical trials in the treatment of anemia of cancer, but there is no trial which has considered the effects of hepcidin therapy in tumor growth [10]. The problem with this approach is that blocking systemic hepcidin does not necessary translate into a clinical success in treating cancer. In many tumors, hepcidin is produced locally, while in others, systemic hepcidin also contributes in the hepcidin pool of the tumor milieu [10]. The complicated example of hepcidin shows that in order for the antitumor strategy to work, the treatment regimen should be focused in delivering compounds in specific targets in cancer cells, without affecting normal cells. Using nanotechnology is one of the options to secure this specificity of action. Nanomedicine is based on the principle of the so-called "smart delivery" of drugs [81]. It is a new field in the treatment of patients with cancer that has been evolving progressively in the last decade. Nanomedicine can be used not only to manipulate more efficiently the iron metabolism in tumor microenvironment, but also it can be useful in targeting cancer cells with different antitumor strategies by using the characteristic iron phenotype of the tumor milieu. For example, liposomal nanoparticles have been used to deliver specific genes in pancreatic cancer via TFR binding [82, 83]. Using TFR as an entry point in cancer cells is reasonable, since it is highly expressed in tumor cells compared to normal cells.

Table 3.2 Iron therapeutics in cancer

Compound	Mechanism of action	Clinical applicability	References
Iron chelators	Iron chelation	Modest results from clinical trials	[44]
Anti-TFR antibodies	Reduction of iron import	Potential use in combined regimens with iron chelators	[44]
Hepcidin inhibitors	Increase of iron export	Only tested in experimental setting	[10]
Curcumin	Iron chelation	Awaiting results from further trials	[70]
V-ATPase inhibitors	Disruption of iron uptake and cellular iron release via inhibition of endosomal acidification	Only tested in experimental setting	[23]
FPN cDNA clone	Increase of iron export	Only tested in experimental setting	[32]
IRP2 inhibitors	Cellular iron deficiency	Only tested in experimental setting	[15, 27–29]
Lp2 siRNA	Cellular iron deficiency	Only tested in experimental setting	[49]
Inductors of ferritinophagy	Ferroptosis	Only tested in experimental setting	[76, 77]

Abbreviations: *EMT* epithelial-mesenchymal transition, *FPN* ferroportin, *IRP2* iron-responsive element-binding protein, *Lp2* lipocalin 2, *siRNA* small interfering RNA, *TFR* transferrin receptor, *V-ATPase* vacuolar ATPase

3.4 Conclusion and Future Perspectives

It is evident that iron dysmetabolism is prevalent in many tumors. It results from the ability of tumor cells to secure the much-needed iron for their metabolic needs. Iron dysmetabolism can initiate tumorigenesis, enhance tumor growth, promote metastasis, and is even the main instigating factor of ferroptosis, which is a specific form of cellular death. Most of the time iron dysmetabolism is caused by local pathophysiological processes in tumor microenvironment, which means that systemic iron dyshomeostasis is not needed for these processes to occur. This does not mean that global changes of iron metabolism cannot worsen iron dyshomeostasis in tumor milieu. For example, systemic hepcidin can contribute in the total levels of this iron-related peptide in the tumor milieu. Other issues regarding the role of other proteins in cancer iron metabolism include the elucidation of the role of PCBs as intracellular iron chaperons, role of proteins involved in non-TFR iron transport, etc.

Increasing evidence suggests that iron dysmetabolism is related to the dysfunction of some of the most important genes and biochemical pathways involved in tumorigenesis. This means that iron dysmetabolism observed in tumor microenvironment is directly related to the pathophysiology of cancer. This is further corroborated by the existence of iron dysmetabolism in CSCs as well, known also as tumor-initiating cells. On the other hand, the density of CSCs is not uniform in all tumor types, which should be taken into account in the treatment of cancer.

Tumor milieu is a dynamic environment with different types of cells. Some of the most abundant ones are TAMs. A subtype of TAMs known as M2 macrophages are frequently linked with the aggressive phenotype of tumors. M2 macrophages can supply excess iron needed for cancer cells. One way which this occurs is through increased production of Lcn2, though it is still not clear if Lcn2 serves as direct iron supplying molecule for cancer cells. The ability of cancer cells to "manipulate" surrounding TAMs by using their iron flux for their metabolic needs may not be the only way how cancer cells secure increased amount of iron from their surroundings. But, most of the studies relating to TAMs are based on in vitro models which are not ideal replications of the in vivo tumor microenvironment. There is evidence that hijacking mechanisms of cancer cells might be directed towards normal cells localized in the vicinity of tumor cells, as it has been observed in PCa, though the details of this process are not known [44]. Recently, other cells, such as CAFs, have been suggested as important contributors in increasing intracellular iron in cancer cells. But whether CAFs can also be directed by cancer cells to secure more iron is still not known. Furthermore, specific markers of CAFs have still not been elucidated properly. Even less is known about the role of senescent cells in tumor microenvironment, although their SASP includes many substances which are able to modulate cancer cell metabolism.

One recent factor related to iron metabolism in cancer cells is the process of ferroptosis. This type of cellular death occurs in the presence of iron which means that iron can also be detrimental for cancer cell survival. One way to do this is to unleash the sequestered iron from intracellular proteins or from intracellular compartments where iron might be stored. Although ferroptosis is a promising therapeutic target in cancer, it is still a relatively unknown process. In some studies, ferroptosis was known to induce rather than destroy cancer. Therefore, differentiating the mechanistic peculiarities between these two types of ferroptosis is important before considering this form of cellular death as a viable anticancer therapy. On the other hand, the use of iron chelators, antibodies against TFR, or blockers of hepcidin has been met with modest therapeutic success. The problem with this approach is that these compounds are not target specific and often do not have favorable pharmacokinetic properties. The solution to this could be nanomedicine, which is a new branch of medicine with a potential to increase the specificity of anticancer drugs. Still, its actual limitations will have to be resolved

before being considered as a therapeutic solution for patients with cancer.

It must be mentioned that the specific iron phenotype of cancer cells and its microenvironment can be used for diagnostic and prognostic purposes in cancer. In many cancers, iron phenotype determines the specificity of the cells of the tumor microenvironment (such as abundance of TFRs in cancer cells or iron-releasing phenotype of M2 macrophages), their aggressiveness (e.g., hepcidin/FPN activity in cancer cells), and even their reaction to chemotherapy. Future studies should evaluate this aspect of iron metabolism in tumor microenvironment in in vivo conditions.

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4 Oxygen in the Tumor Microenvironment: Mathematical and Numerical Modeling

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Abstract

There are many reasons to try to achieve a good grasp of the distribution of oxygen in the tumor microenvironment. The lack of oxygen – hypoxia – is a main actor in the evolution of tumors and in their growth and appears to be just as important in tumor invasion and metastasis. Mathematical models of the distribution of oxygen in tumors which are based on reaction-diffusion equations provide partial but qualitatively significant descriptions of the measured oxygen concentrations in the tumor microenvironment, especially when they incorporate important elements of the blood vessel network such as the blood vessel size and spatial distribution and the pulsation of local pressure due to blood circulation. Here, we review our mathematical and numerical approaches to the distribution of oxygen that yield insights both on the role of the distribution of blood vessel density and size and on the fluctuations of blood pressure.

Keywords

Tumor hypoxia · Microcirculation · Tumor angiogenesis · Tumor growth · Tumor metabolism · Tumor hemodynamics · Tumor cords · Tumor heterogeneity · Mathematical modeling · Computer modeling · Numerical simulations · Lattice-free models · Cell-based tumor models · Radiation therapy · Darwinian evolution in tumors

4.1 Introduction

One cannot underestimate the role of oxygen in the tumor microenvironment, as it regulates both the life and death of tumor cells in many ways. Oxygen in tumors also determines the efficacy of many therapies. For instance, radiotherapy depends in a crucial way on the *oxygen effect* [1], and one of the basic aims of fractionated radiotherapy is just providing enough oxygen after each fraction to help killing tumor cells in the next fraction.

In spite of its importance for tumor biology and the clinical course of the disease, however, the current understanding of the quantitative aspects

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_4

of oxygen diffusion in tumors is not complete, and there is no doubt that this is at least partly due to the biological and biochemical complexity of the tumor microenvironment.

It is known that in addition to tumor cells, the tumor microenvironment comprises nonmalignant cells of different origin, such as stromal and immune cells. These nonmalignant cells play an active role in tumor progression by exchanging a number of molecular signals with tumor cells [2]. The mixture of different cells is mechanically supported by an extracellular matrix of polysaccharides and fibrous proteins, and all this complex tissue structure is fed by an irregular network of blood vessels [2, 3]. The network of blood vessels in tumors differs substantially from that of normal tissue. Tumor blood vessels are in general more tortuous, irregular, and leaky [4], and, importantly, the intervascular distances are larger. This means that the blood flow is irregular and that the cells that are far apart from feeding vessels receive low amounts of oxygen. Many tumors show in fact hypoxic or even anoxic inner areas [3–7]. In turn, hypoxia induces significant genomic and proteomic changes in tumor cells, and it has been shown to induce also genomic instability by increasing the mutation frequency of cells [4, 5]. The highly selective tumor microenvironment can then promote the growth of more aggressive tumor phenotypes [4–6].

In their search for nutrients, living cells wrap around blood vessels to form cords of living cells. They consume oxygen, nutrients, and eventually drugs, and since their spatial distribution in the tumor is not homogeneous, the concentration field of such molecules in the tumor microenvironment is not homogeneous as well. For example, hypoxia shows up differently in different tumors and even in different parts of individual tumors, where it is heterogeneous both in space and in time [7, 8]. Therefore the spatial distribution of cells alters the environment bringing about a complex feedback loop.

From this very short introduction to the biophysics of oxygen in the tumor microenvironment, it is clear that it displays all the complexities of biological systems. The environmental details span several hierarchical levels, from individual molecules to fully formed tumors; there is a large number of interacting elements, starting again from the individual molecules belonging to many chemical species to cells of many different types – both normal and tumor. There are structures that belong to normal tissues and their deformed counterparts in the tumor mass. Finally, all these elements are closely interacting, and the interactions are usually nonlinear. This means that mathematical and numerical approaches can only scratch the surface of this all-embracing complexity and that we must be confident in our ability of correctly separating the hierarchical levels and finding good phenomenological approximations to compensate for the shortcomings of calculations.

While here we concentrate on one single chemical species, O_2, it cannot be considered in isolation, and the equations that describe its diffusion in the microenvironment must be complemented by reaction terms that specify its interaction with the other parts of the microenvironment. This problem of the reaction-diffusion of oxygen in tissues in general and in tumors in particular has already been considered in some of its aspects by other workers in this field (see, e.g., [9, 10] and references cited therein). In the following sections, we review the two approaches that we have followed in our work: an analytical one, which brings out rather nicely the time-dependent features of oxygen diffusion in the tumor microenvironment, and a numerical approach which starts from the simulation of individual cells and capillary vessels and that recreates the hypoxic recesses with large chemical gradients that drive the Darwinian evolution of different tumor genotypes.

4.2 The Fourier Problem

Before dealing with the analytical model of oxygen in the tumor microenvironment, it is useful to introduce the methods used later on with a seemingly unrelated problem, which was considered long ago by Jean-Baptiste Joseph Fourier, that of the diffusion of the sun's heat into the ground [11].

The soil temperature is still an outstanding problem in agriculture, as it influences the growth of plants, but nowadays this is commonly monitored by measurements with temperature probes. However, for Fourier it was different, it was not just a matter of measurements, and it was something that he wanted to understand in depth, and to this end, he started from the newly discovered diffusion equation for temperature in one dimension[1]:

$$\frac{\partial T}{\partial t} = D\frac{\partial^2 T}{\partial z^2}, \tag{4.1}$$

where $T = T(t, z)$ is the temperature, a function of both time t and of space coordinate z. The equation says that the rate of change of temperature at a given position in space depends on the values of the temperature all around that given position, i.e., on the flow of heat to and from the neighborhood of that position. In the problem considered by Fourier, there is just one spatial dimension, z, because he was interested in the approximation where the ground is a uniform plane and z represents the depth.

It is important to note that the diffusion equation (4.1) is linear. This means that if we find two different solutions $T_1(t, z)$ and $T_2(t, z)$, then any linear combination $T(t, z) = a \times T_1(t, z) + b \times T_1(t, z)$, where a and b are real numbers, is again a solution of the equation. In the jargon of physics, this means that the *principle of superposition* holds.[2]

Obviously, when dealing with the Earth's ground, the source of heat is the sun, a source which is doubly modulated, daily and yearly. This double modulation can be roughly described as the sum of two sinusoidal terms

$$T(t, 0) = A_d \cos(\omega_d t + \phi_d) + A_y \cos(\omega_y t + \phi_y) \tag{4.2}$$

with $\omega_d = 2\pi/T_d$, $\omega_y = 2\pi/T_y$, where $T_d = 86{,}400\,\mathrm{s}$ is the duration of one day, and $T_y \approx 3.1558 \times 10^7\,\mathrm{s}$ is the duration of the astronomical year. A_d and A_y are the amplitudes of the sinusoidal terms and depend on the latitude (for instance, on the equator daylight always lasts 12h, there is only a weak dependence on the day of the year, and $A_y \approx 0$). The constants ϕ_d and ϕ_y are two phases that depend on the choice of the origin of the time axis and are irrelevant in the present calculation.

Thanks to the principle of superposition, we can find the solution of the diffusion equation (4.1) for each single sinusoidal term and combine them thereafter. Before proceeding further, it is also helpful to go one step further with the superposition principle. We note that a cosine is itself a weighted sum of two complex exponential functions, and therefore we can apply the superposition principle "backward." Since a given cosine function is a solution of the diffusion equation, then the two complex exponentials are themselves solutions of the same equation. This means that we can go through the whole process of solving the diffusion equation with exponential functions, use their decomposition $\mathrm{e}^{\mathrm{i}x} = \cos x + \mathrm{i}\sin x$, and use again one final time the superposition principle to just discard the "unphysical" imaginary part. This choice of the complex exponentials greatly simplifies all the calculations, and on the boundary plane (the ground), we can take the "complex" temperature $\hat{T}(t, 0) = \hat{A}\mathrm{e}^{\mathrm{i}\omega t}$, where the hat denotes complex variables, and $\hat{A} = A\mathrm{e}^{\mathrm{i}\phi}$.

[1] Formally, the diffusion equation for heat is the combined result of Fick's law applied to thermal current $\boldsymbol{J}$ and temperature, $\boldsymbol{J} = -K\nabla T$, where K is the thermal conductivity, of the conservation of energy applied to the thermal current and internal (thermal) energy U,

$$-\frac{\partial U}{\partial t} = \nabla \cdot \boldsymbol{J},$$

and of the relation between internal energy and temperature, $\Delta U = C\Delta T$, where C is the constant-volume thermal capacity, so that one finds the multidimensional diffusion equation for temperature

$$\frac{\partial T}{\partial t} = D\nabla^2 T,$$

with $D = K/C$.

[2] A "superposition" is just a linear combination as in the text, and whenever the principle holds, then any superposition of solutions is also a solution. Much of the value of the principle comes from experiment rather than theory: if one finds *experimentally* that the principle holds, then one knows that the underlying equations must be linear, just as the diffusion equation.

Because of the linear character of the diffusion equation, we know that the time dependence remains the same at all depths; however, we still have to determine the space dependence of temperature. Therefore, we write $\hat{T}(t,z) = \hat{A}(z)\mathrm{e}^{\mathrm{i}\omega t}$, we substitute in Eq. (4.1), and we find

$$\mathrm{i}\omega\hat{A}(z) = D\frac{\mathrm{d}^2\hat{A}}{\mathrm{d}z^2}. \tag{4.3}$$

Equation (4.3) is the same equation one has to solve for a harmonic oscillator, albeit with complex coefficients, and it is well-known that its solution is just a linear combination of exponential terms (again). Therefore, we can repeat the steps that we have already taken with the time-dependent part and write the space-dependent part $\hat{A}(z)$ as a linear combination of complex exponentials $\mathrm{e}^{\alpha z}$ that depend on the depth z. We can determine the constant α by substitution into Eq. (4.3), and we find the algebraic equation

$$\mathrm{i}\omega = D\alpha^2, \tag{4.4}$$

which has the solutions $\alpha = \pm\mathrm{e}^{\mathrm{i}\pi/4}\sqrt{\omega/D} = \pm(1+\mathrm{i})\sqrt{\omega/2D}$. Mathematically, this determines two spatial solutions

$$\hat{A}(z) = A_\pm \exp\left(\pm(1+\mathrm{i})\sqrt{\frac{\omega}{2D}}z\right),$$

but only the solution with the minus sign is acceptable, because the other one is unphysical, with its amplitude which increases exponentially in time.

Assembling the factors together, and equating its value at the boundary (the ground) with the known modulation $A\cos(\omega t + \phi)$, we find the complete solution

$$T(t,z) = A\exp\left(-\sqrt{\frac{\omega}{2D}}z\right)\cos\left(\sqrt{\frac{\omega}{2D}}z\right)\cos(\omega t+\phi) \tag{4.5}$$

$$= \frac{A}{2}\exp\left(-\sqrt{\frac{\omega}{2D}}z\right)\left[\cos\left(\omega t+\phi+\sqrt{\frac{\omega}{2D}}z\right)+\cos\left(\omega t+\phi-\sqrt{\frac{\omega}{2D}}z\right)\right] \tag{4.6}$$

Equation (4.6) shows that while at any given depth the oscillation has the same frequency as on the ground, it decomposes into two components with different depth-dependent and frequency-dependent phases. Moreover the amplitude itself decreases exponentially with a characteristic length λ which is again frequency dependent, $\lambda = \sqrt{2D/\omega}$, and decreases steadily for increasing frequency.

Applying the principle of superposition to each modulation, diurnal and annual, we find the solution of the original Fourier problem:

$$T(t,z) = A_d\exp\left(-\sqrt{\frac{\omega_d}{2D}}z\right)\cos\left(\sqrt{\frac{\omega_d}{2D}}z\right)\cos(\omega_d t+\phi_d) + A_y\exp\left(-\sqrt{\frac{\omega_y}{2D}}z\right)\cos\left(\sqrt{\frac{\omega_y}{2D}}z\right)\cos(\omega_y t+\phi_y). \tag{4.7}$$

This ingenious solution was the beginning of the Fourier series, as Fourier understood that it could be extended to any number of sinusoidal components. It also displays from the very start one of the strengths of the Fourier series, namely, that they can be used as a tool to solve any kind of linear differential equation – be it an ordinary differential equation or a partial differential equation – thanks to the principle of superposition.

As we shall see in the next sections, all the basic features of the solution (4.7) are carried over to the case of oxygen diffusion in the tumor microenvironment.

4.3 Linear Model of Oxygen Diffusion and Consumption

When we consider the complex tumor microenvironment, we find that the concentration of any chemical follows the same basic rules as the temperature of Fourier's problem. There is a molecular current from regions of higher concentration to regions of lower concentration which follows Fick's law $\boldsymbol{J} = -D\nabla\Phi$, where Φ is the concentration, and there is relation which is an extension of the conservation of energy in the previous section

$$\frac{\partial\Phi}{\partial t} = -\nabla\cdot\boldsymbol{J} - f\left(\Phi(\boldsymbol{r},t),\boldsymbol{r},t\right)$$

This means that the rate of change of the concentration in a given region of space around position **r** depends both on the outflow of molecules from that region – described by the current term – and from the disappearance of those molecules because of the reaction with other chemicals – described by the reaction term $f\left(\Phi(\boldsymbol{r},t),\boldsymbol{r},t\right)$.

Combining these equations together, we find the complete reaction-diffusion equation

$$\frac{\partial\Phi}{\partial t} = \nabla\cdot(D\nabla\Phi) - f\left(\Phi(\boldsymbol{r},t),\boldsymbol{r},t\right). \quad (4.8)$$

Equation (4.8) is very general: the diffusion coefficient D which parameterizes the speed of diffusion of molecules in the environment can be position- and time-dependent, $D = D(\boldsymbol{r},t)$, and the reaction term is in general a combination of one or more Michaelis-Menten (or Hill) terms.[3]

Here, we concentrate our attention on a very small spatial region, and we assume that D does not depend on $\boldsymbol{r}$. We also assume that the reaction term can be linearized in a simple way, $f\left(\Phi(\boldsymbol{r},t),\boldsymbol{r},t\right) \approx \gamma\Phi(\boldsymbol{r},t)$, which is consistent with the low-concentration approximation of a Michaelis-Menten reaction term. Then, Eq. (4.8) becomes

$$\frac{\partial\Phi}{\partial t} = D\nabla^2\Phi - \gamma\Phi. \quad (4.9)$$

In this case we are going to use this formalism to understand how the fluctuations of oxygen concentration in the blood vessels influence the concentration of oxygen in the microenvironment, and it is instructive to start with Eq. (4.9) in the one-dimensional case

$$\frac{\partial\Phi}{\partial t} = D\frac{\partial^2\Phi}{\partial z^2} - \gamma\Phi, \quad (4.10)$$

which we solve with the same methods used in Sect. 4.2. We let again $\hat{\Phi}(t,0) = \hat{A}\mathrm{e}^{\mathrm{i}\omega t}$, and we obtain the ordinary differential equation

$$(\mathrm{i}\omega+\gamma)\hat{\Phi} = D\frac{\mathrm{d}^2\hat{\Phi}}{\mathrm{d}z^2}, \quad (4.11)$$

which is nearly the same as Eq. (4.3). The solution is again an exponential function $\mathrm{e}^{\alpha z}$, where α solves the algebraic equation

$$\mathrm{i}\omega+\gamma = D\alpha^2.$$

With a little complex algebra, it can be shown that

$$\hat{\Phi}(z,t) = A\exp\left[\mathrm{i}\left(\omega t+\varphi(z)\right)\right]\mathrm{e}^{-z/\ell},$$

where the position-dependent phase is

$$\varphi(z) = \left(\frac{\omega^2+\gamma^2}{D^2}\right)^{1/4}\sin\left(\frac{1}{2}\arctan\frac{\omega}{\gamma}\right)z,$$

and the decay length is

[3] We recall that the enzymatic activity – and therefore also the individual steps of the metabolic pathways – is often described by the Michaelis-Menten (MM) equation

$$v = v_{\max}\frac{[S]}{K_m+[S]}$$

where v is the reaction rate and $[S]$ is the concentration of the substrate (in our case, oxygen). The reaction rate depends on two parameters $v_{\max}$ and K_m which characterize the specific enzymatic process. The MM equation is unable to fit some of the observed reaction rates, which are sigmoid functions of the substrate concentration, and in this case, it is common to turn to the Hill equation, a phenomenological modification of the MM equation

$$v = v_{\max}\frac{[S]^n}{K_m^n+[S]^n}$$

with one more parameter, the exponent n. For more details, see, e.g., [12].

$$\ell(\omega,\gamma)=\sqrt{\frac{2D}{\gamma+\sqrt{\omega^2+\gamma^2}}}=\ell_0(\omega)\sqrt{\frac{2}{1+\sqrt{1+\omega^2/\gamma^2}}},$$

with

$$\ell_0(\omega)=\ell(\omega,0)=\sqrt{D/\omega}$$

Except for a factor $\sqrt{2}$ – which comes from our preferred definition of the ratio ℓ/ℓ_0 – the decay length ℓ_0 is the same as in the Fourier problem in Sect. 4.2 where there is no absorption term, and here we see that the introduction of the consumption/absorption coefficient γ modifies the decay length making it somewhat smaller. It also shows that usual formulations of the reaction-diffusion problem which take into account the consumption/absorption term but ignore modulation do not properly estimate the decay length as they tend to overestimate it and therefore also the penetration of oxygen into the microenvironment. On the whole, we find that the concentration of oxygen in the tumor microenvironment must be influenced by the frequency of the fluctuations of oxygen concentration in its blood vessels.

Another interesting feature of the reaction-diffusion solution is that while the decay length $\ell_0(\omega)$ diverges for $\omega \to 0$, the presence of the consumption/absorption term limits the decay length to

$$\ell(\omega,\gamma)\le\ell(0,\gamma)=\sqrt{D/\gamma}.$$

The complete behavior of the ℓ/ℓ_0 ratio is shown in Fig. 4.1.

To conclude this section, we recall that in the solution of the Fourier problem, there were two Fourier components of the ground temperature that fluctuated about an average value of zero. When considering temperature in Celsius degrees, this may be adequate, but negative swings are certainly forbidden with chemical concentrations. It is easy to cure this problem by adding a constant component (a zero-frequency component) that restores the non-negativity of the concentration, as in Fig. 4.2.

4.4 The Near-Cylindrical Geometry of Blood Vessels

The discussion of the previous section cannot be considered complete without a proper appraisal of the role of blood vessel geometry. In this section we briefly consider the role of the cylindrical geometry which locally approximates the geometry of blood vessels. We remark that the validity of the approach is limited to blood vessels with a diameter much smaller than their length. In an environment crowded with blood vessels, the approach is useful in the linear limit of Eq. (4.9), as the overall oxygen concentration can be computed – from the principle of superposition – from the sum of the concentrations due to the individual blood vessels (see also [13]), and this holds for a fluctuating oxygen concentration as well.

We use cylindrical coordinates (r,θ,z) and take the z-axis along the axis of a blood vessel (locally approximated by a cylinder with radius R), then we find the reaction-diffusion equation

$$\frac{\partial\Phi}{\partial t}=D\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial\Phi}{\partial r}\right)-\gamma\Phi. \tag{4.12}$$

where r is the distance from the axis of the blood vessel. When we take again $\hat{\Phi}(t,0)=\hat{A}e^{i\omega t}$, Eq. (4.12) becomes

$$r\frac{\partial}{\partial r}\left(r\frac{\partial\hat{\Phi}}{\partial r}\right)-\frac{i\omega+\gamma}{D}r^2\hat{\Phi}=0, \tag{4.13}$$

which is a modified Bessel equation. The solution of Eq. (4.13) with a boundary condition which is set by the oxygen concentration on the surface of the blood vessel $\hat{\Phi}(R,t)=\hat{\Phi}_0e^{i\omega t}$ is qualitatively similar to the simpler one-dimensional case with

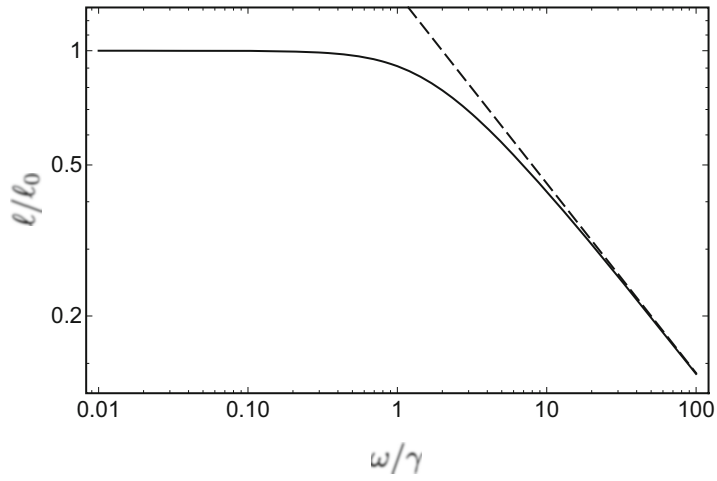

Fig. 4.1 Log-log plot of the decay length of the solution of the one-dimensional diffusion problem. Solid line: the curve is nearly flat for $\omega < \gamma$, and its value is close to 1. Dashed line: if $\omega \gg \gamma$, the ratio of decay lengths approaches the behavior of the decay length without consumption/absorption, i.e., the simple power law $\ell/\ell_0 \sim \omega^{-1/2}$. The transition between the two regimes occurs at $\omega \approx \gamma$

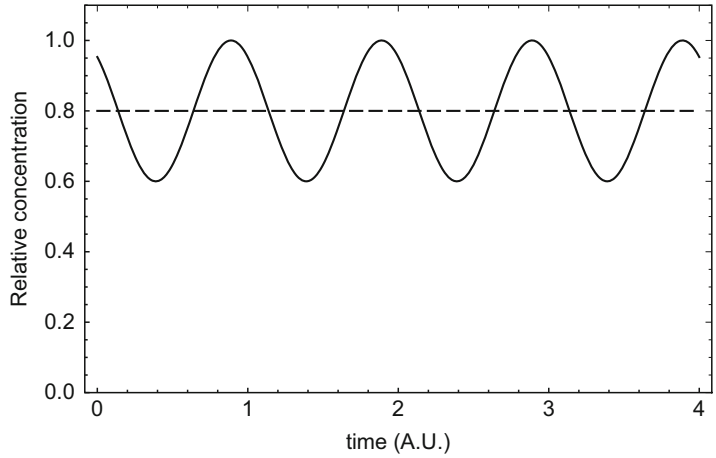

Fig. 4.2 Periodic fluctuations of the concentration about an average value (concentration normalized to the peak value vs. time in arbitrary units). In this elementary example, the relative concentration $c(t)$ is described by just two Fourier components: $c(t) = 0.8 + 0.2\cos(2\pi t + 0.7) = \Re(0.8 + 0.2e^{(2\pi t+0.7)i})$, where $\Re(x)$ is the real part of the complex number x. The solid line represents the sum of the two terms, while the dashed line represents the constant term which is essential for the consistency of the mathematical description

a plane boundary that has been considered above, though with some added mathematical complexities which are described in detail in reference [14]. The main difference with respect to the one-dimensional case is that the Bessel function that solves equation (4.13) – a modified Bessel function of the second kind with complex argument, $K_0\left(\sqrt{(\gamma + i\omega)/Dr}\right)$ – decays faster than exponentially, as shown in Fig. 4.3, where several other details are illustrated.

4.5 Dealing with Dead Cells: Tumor Cords

The solution discussed in the previous section is characterized by an extremely fast decrease of the oxygen concentration when the blood vessels are surrounded by a uniform population of live cells. In normal tissues this fast decrease is compensated by a high density of blood vessels, that are

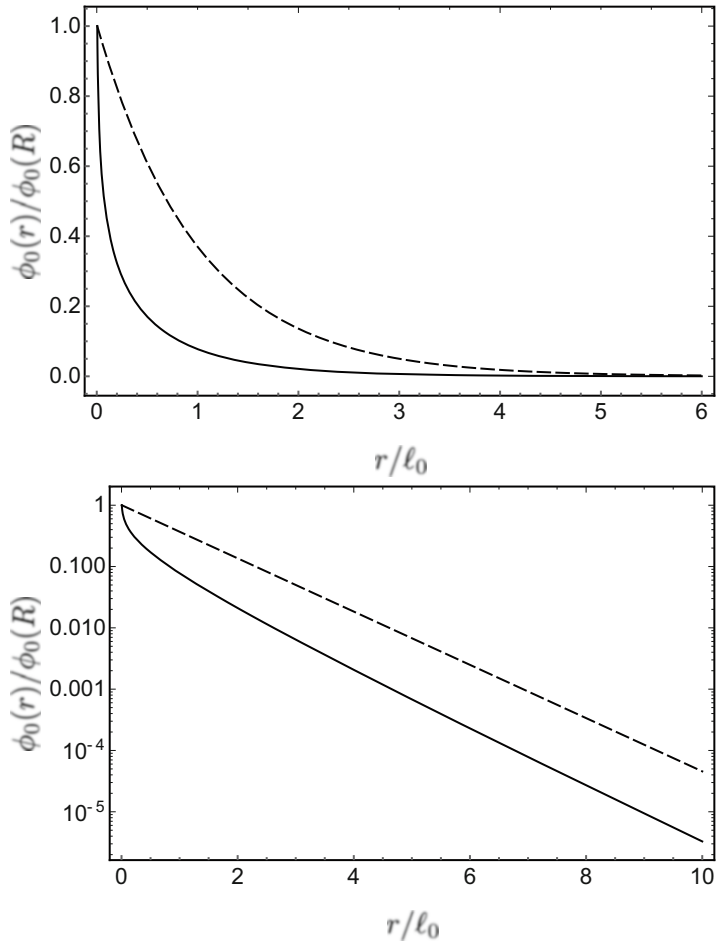

Fig. 4.3 Plots of $K_0\left(\sqrt{\gamma/D}r\right)/K_0\left(\sqrt{\gamma/D}R\right)$ (solid line) and of the exponential function e^{-r/ℓ_0} (dashed line) vs. r/ℓ_0 for $2R = 0.01\ell_0$. This shows the contribution of the constant Fourier component of the fluctuating oxygen concentration to the concentration in the surrounding environment, in the case of a blood vessel with a diameter which is 1% of the decay length ℓ_0. Since ℓ_0 is about 0.96 mm with physiological parameters ($D = 2000\,\mu\text{m}^2/\text{s}$; $\gamma \approx 2.16\times10^{-3}\,\text{s}^{-1}$, see Sect. 4.5 for further discussion about the parameters), this corresponds to a blood vessel with diameter $2R \approx 20\,\mu\text{m}$. The smallest diameter is one order of magnitude lower. Upper panel: this plot has a linear vertical scale and shows the dramatic effect of the cylindrical geometry, which leads to decay of the concentration which is much faster than in the one-dimensional, planar case (the dashed exponential). Lower panel: same plot, but with a logarithmic vertical scale, where we note that for large radius, the solution behaves again almost exponentially, but with a drops more than one order of magnitude below the one-dimensional planar case. These plots display quite starkly the effect of the cylindrical geometry of the individual blood vessels. Finally, it is important to note that the difference between the cylindrical and the plane geometry depends on the radius of the blood vessels: it is reduced for large radii, while it is enhanced for small radii

never too far apart, so that the superposition of the concentrations is always in a physiological range and cells live. However, this is not the case in the majority of tumor tissues, where blood vessels are often far apart and have a chaotic distribution and shape that lower the efficiency of oxygen transport. Hypoxic regions appear where the harsh conditions cause the death of many cells, and the resulting environment develops gradients of the concentration of live cells. Eventually, live cells are mostly concentrated around blood vessels – making up the so-called tumor cords [15] – with extended necrotic regions in between the blood vessels.

The uneven distribution of live cells means that the consumption/absorption coefficient γ is position-dependent. Here we consider a general exponential model of (radial) space dependence, motivated by our previous numerical work [16, 17]

$$\gamma(r) = \gamma_0 + \gamma_c \exp(-r/\lambda_c), \tag{4.14}$$

where γ_0 corresponds to the binding of oxygen to some environmental chemical (and we assume this to vanish in most tissues) and γ_c is the oxygen consumption associated with a population of live cells. Model (4.14) leads to the following Fourier coefficients (i.e., amplitudes of the time-dependent terms, see [14] for more details):

$$\ln \phi(r,\omega) = \ln \phi(R,\omega) + \int_R^r \sqrt{\frac{\mathrm{i}\omega + \gamma(r')}{D}} \frac{K_0'\left(\sqrt{\mathrm{i}\omega + \gamma(r')/D}\, r'\right)}{K_0\left(\sqrt{\mathrm{i}\omega + \gamma(r')/D}\, r'\right)} dr' \tag{4.15}$$

Equation (4.15) is noteworthy because it represents a decently realistic model of the oxygen concentration in a small fraction of the tumor microenvironment where the blood vessels are far apart. However, a comparison with actual data requires properly chosen parameter values. All the parameters used in the numerical evaluations that follow are extrapolated from experimental data and apply to solid tumors. We take the estimates in [16, 17] for the decay length in the definition (4.14): $\lambda_c = 120\,\mu\mathrm{m}$. The diffusion constant of oxygen as measured both in blood and tissues $D = 2 \times 10^{-9}\,\mathrm{m}^2/\mathrm{s}$ is taken from [18, 19]. The rates of oxygen consumption in different areas of in vivo tumors have been precisely measured and shown to vary in the range $1.66\ 10^{-4} - 5\ 10^{-3}\,\mathrm{s}^{-1}$ (mean value $2.16\ 10^{-3}\,\mathrm{s}^{-1}$) [20–22]. Finally, we note that measurements on melanomas [23] indicate that the average microvessel diameter is about $5\,\mu\mathrm{m}$, i.e., $R = 2.5\,\mu\mathrm{m}$, just enough to let one erythrocyte through.

The reduced consumption/absorption coefficient at larger depth means that when tumor cords form, the decay of the oxygen concentration is not as fast as in the straightforward cylindrical case. This is illustrated in Fig. 4.4 which compares the concentration decay for tumor cords with the previously examined cases (the simple exponential decay found in the one-dimensional case and the enhanced decay found in the case with cylindrical symmetry).

To conclude this section, we note that the time dependence of the boundary conditions has a very important effect on the concentration also in the case of the tumor cords. Figure 4.5 shows the concentration vs. the radial distance r for several Fourier amplitudes $\phi(\omega, r)$ computed using average parameter values for solid tumors and a blood vessel diameter close to the minimum ($6\,\mu\mathrm{m}$), and we see that fluctuations that correspond to a normal heartbeat (60 beats/minute = 1 Hz) have a very short penetration depth into the tumor tissue.

4.6 Comparison with Experimental Data

Experimental data are very hard to come by, but some high-quality data have been produced by Helmlinger et al. [24] (see Fig. 4.6), and they show a very fast decrease of the partial oxygen pressure at increasing distance from blood vessels in a colon adenocarcinoma xenograft. This is in line with the qualitative indications in the previous sections, but can we make this correspondence more robust?

Figure 4.7 shows the same data as Fig. 4.6 and some additional curves. In particular, it shows that the data can be bracketed by two curves calculated for tumor cords and that correspond to Fourier components with frequencies 10 and 100 mHz (0.6 cycles/minute and 6 cycles/minute). These frequencies define the interval of the observed frequencies with the highest amplitude for oxygen oscillations in the tumor microcirculation as observed by Braun et al. [25]. This indicates that low-frequency fluctuations in the tumor tissue may explain

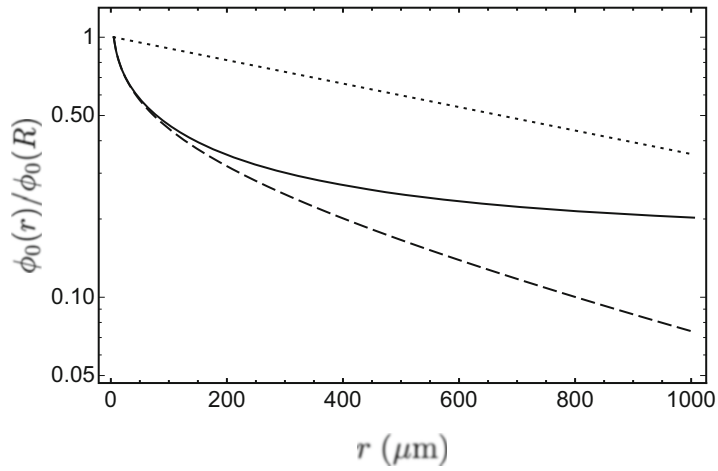

Fig. 4.4 Relative concentration in the case of tumor cords (solid line) vs. radial distance r. Similarly to Fig. 4.3, other curves show the corresponding decays in the one-dimensional case (dotted line) and in the case with cylindrical symmetry (dashed line). The parameter values used here are $D = 2 \times 10^{-9}\,\mathrm{m^2/s}$, $\gamma_0 = 0$, $\gamma_c = 2.16 \times 10^{-3}\mathrm{s}^{-1}$, and $\lambda_c = 120\,\mu\mathrm{m}$. Notice that, once again, this is the plot for the Fourier component with $\omega = 0$, and we know that the Fourier components with $\omega > 0$ decrease faster than shown here

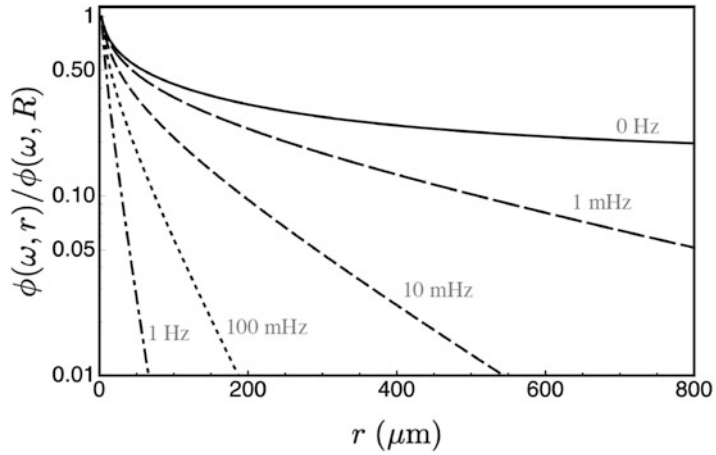

Fig. 4.5 Plots of $|\phi(\omega, r)/\phi(\omega, R)|$ vs. r (μm) in the case of a tumor cords, Eq. (4.15), for different frequencies $\nu = \omega/2\pi$. All curves have been calculated with $2R = 6\,\mu\mathrm{m}$, $D = 2000\,\mu\mathrm{m^2/s}$, $\gamma_0 = 0$, $\gamma_c = 2.16\ 10^{-3}\ \mathrm{s}^{-1}$, and $\lambda_c = 120\,\mu\mathrm{m}$. The black line is the stationary solution ($\nu = 0$); the other line shows the relative concentration for frequencies ranging from $\nu = 0.001$ Hz to $\nu = 1$ Hz

the observed decrease of the partial oxygen pressure. However, there are still too many parameter values that have been fixed to produce Fig. 4.7, and this may leave the impression that the agreement is somewhat ad hoc.

This can be remedied with a better exploration of the parameter space, which can be provided, e.g., by a Monte Carlo simulation. Consider Fig. 4.7, which has been drawn taking the median blood vessel radius $R = 22.5\,\mu\mathrm{m}$: what happens if we let this fluctuate within a reasonable range? The answer is shown in Fig. 4.8, where both the frequency (range: $\nu = 0.01\,\mathrm{Hz} - \nu = 0.1\,\mathrm{Hz}$) and the radius of the blood vessel (range: $3\,\mu\mathrm{m} - 160\,\mu\mathrm{m}$) are uniformly distributed in the respective ranges. We see that the distribution of radius does not significantly alter the band of Fig. 4.7. We can push the method further and introduce a fluctuation of the diffusion coefficient and of the consumption/absorption coefficient in addition to the fluctuation of the blood vessel diameter. The results are shown in Figs. 4.9 and 4.10, and we see once again that the resulting bands fit rather well the actual data. There are other factors that might influence these results, such as the shape of the probability density func-

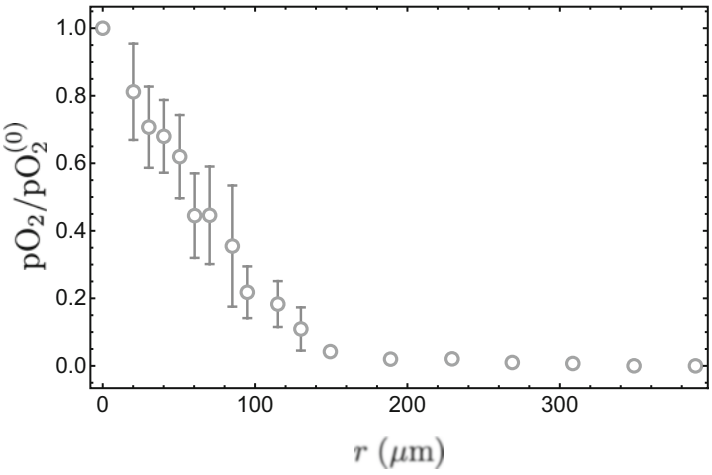

Fig. 4.6 Experimental data taken in measurements on colon adenocarcinoma xenografts, redrawn from Fig. 3 in Ref. [24]. The figure shows values of partial oxygen pressure (pO_2) in the tumor interstitium as a function of the distance from blood vessels (circles; bars represents the s.e.m. calculated from 15 samples). In this figure pO_2 has been normalized with respect to the central value in the nearest blood vessel ($pO_2^{(0)}$). The observed median radius of the blood vessels in [24] is $R = 22.5\,\mu m$

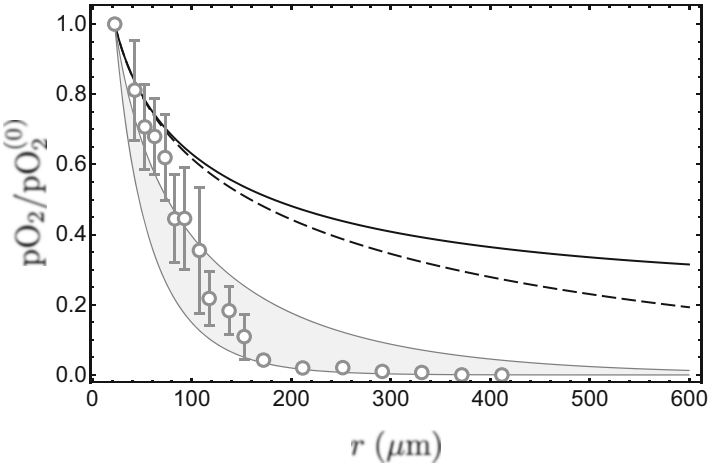

Fig. 4.7 Comparison of the observed relative oxygen pressure redrawn from [24] as in Fig. 3 vs. the distance r from axis of the nearest blood vessel. The solid line shows the decay of the relative oxygen pressure in the case of a tumor cord, while the dashed line shows the straightforward case with cylindrical symmetry, in both cases for $\nu = 0$. The grayed band delimits the region between $\nu = 0.01\,Hz$ to $\nu = 0.1\,Hz$. All curves are calculated with the values $D = 2000\,\mu m^2/s$, $\gamma_0 = 0$, $\gamma_c = 2.16\ 10^{-3}\,s^{-1}$, and $\lambda_c = 120\,\mu m$ and with the median blood vessel radius $R = 22.5\,\mu m$

tion of the blood vessel radius; a test carried out with an exponential distribution of the radius (with mean value 22.5 μm, not shown) displays very little variation with respect to Figs. 4.8, 4.9, and 4.10.

The conclusion that we can draw from this study is that the frequency of the fluctuations is the single most important factor in determining the penetration of oxygen in the microenvironment in the case of isolated blood vessels. In

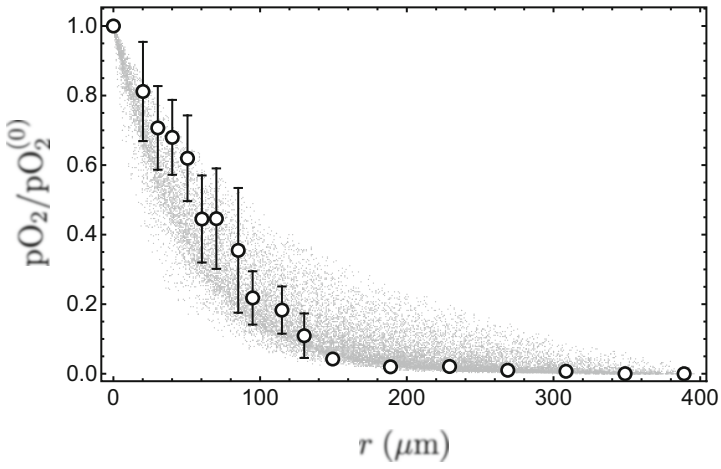

Fig. 4.8 Monte Carlo simulation of the model predictions that takes into account the fluctuation of the frequencies and of the blood vessel radii. The parameters used for the Monte Carlo are the same as those of Fig. 4.7, except that here both the radius and the frequency are allowed to fluctuate following uniform distributions in the range (0.01 and 0.1 Hz) (frequency) and (3 and 160 μm) (radius)

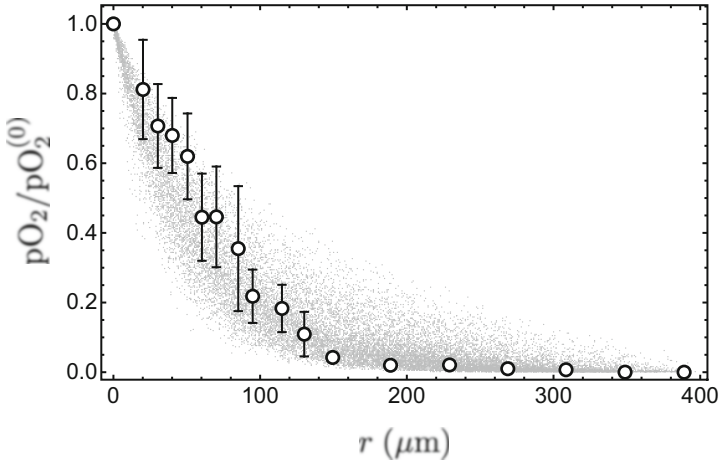

Fig. 4.9 Monte Carlo simulation of the model predictions that takes into account the fluctuation of the diffusion coefficient as well as that of the frequencies and of the blood vessel radii. The parameters used for the Monte Carlo are the same as those of Fig. 4.8, except that here the diffusion coefficient fluctuates as well, following a uniform distribution in the range (1000 and 3000 $\mu m^2/s$)

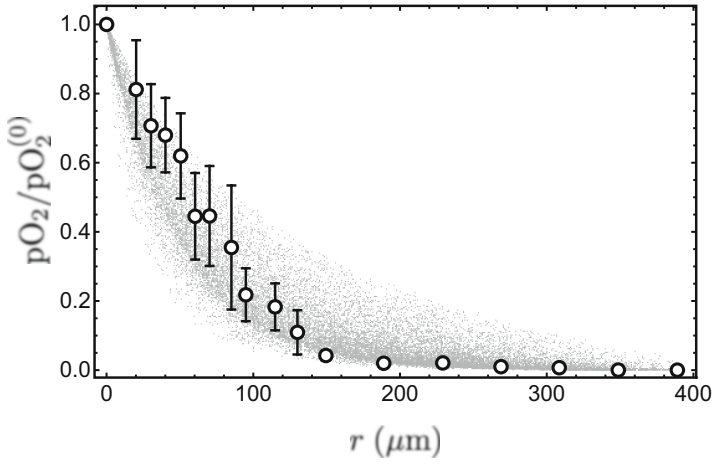

Fig. 4.10 Monte Carlo simulation of the model predictions that takes into account the fluctuation of the consumption/absorption coefficient as well as that of the frequencies and of the blood vessel radii. The parameters used for the Monte Carlo are the same as those of Fig. 4.8, except that here the consumption/absorption coefficient fluctuates as well, following a uniform distribution in the range ($0.5 \times 2.16\,10^{-3}\ s^{-1}$, $2 \times 2.16\,10^{-3}\ s^{-1}$)

the next sections, we move on to consider blood vessel geometry, the importance of its remodeling in cancer tissues, and more.

4.7 Into the Future: Numerical Simulations of Vascularized Tumors

While the considerations in the preceding sections are very useful to understand the general behavior of oxygen, they are not sufficient to describe the spatial complexity of the tumor microenvironment. A detailed calculation of the oxygen concentration due to the blood vessels in a tumor and in the surrounding healthy tissue defies the analytical methods, and we must turn instead to numerical tools. These tools are important both to gauge the importance of nonlinear effects and to unveil those aspects of the complexity that escape a simple description as the one given in the previous sections. In particular, we note that

- We neglected that the tumor microenvironment comprises both healthy and cancerous vessels/cells at the same time.
- In Sect. 4.3, we noted that the Michaelis-Menten and the Hill equations are biologically reasonable models of the reaction terms in the reaction-diffusion equation. However, the linearization of these nonlinear model works only for low concentrations, and the linear hypothesis may not be adequate in many circumstances (for instance, it may lead to an overestimate of the oxygen consumption in well-oxygenated regions).
- Finally, the most severe simplification is the small system size comprising a single blood vessel only. Realistic tissues feature a vasculature consisting of many blood vessels of different radii and surface characteristics. Since blood vessels are the source of the oxygen inside tissue, the exact form of the oxygen field depends on the strength of the sources and on the arrangement of the sources in space, i.e., the oxygen concentration field depends crucially on vascular morphology. During the growth progression of solid tumors, both the source strength and the arrangement of the vessels are altered. The vasculature becomes irregular, tortuous, and leaky with corresponding consequences for the oxygen transport.

All of these limitations can be overcome by computer simulations, which are a comprehensive tool to study tumor growth and the tumor microenvironment in a much more realistic way, although at the non-negligible cost of a large coding and computational effort [26].

4.7.1 The Oxygen Concentration Field of Simulated Vascularized Tumors Embedded in Normal Tissue

To address the problem of vessel geometry, we explicitly model each blood vessel as a cylinder with length l, radius r, and thickness w. The problem of creating realistic arteriovenous blood vessel networks in 3D was solved in [27]. Upon the construction of the initial vasculature, tumor growth starts to remodel the vasculature by vessel dilation and collapse, wall degeneration, and angiogenesis. While the first three processes require the modification of existing blood vessels, sprouting angiogenesis refers to the establishment of new connections. Blood vessels are surrounded by a layer of endothelial cells, and tumor cells secrete numerous chemicals. One family of chemical messengers, the vascular endothelial growth factor (VEGF), triggers the proliferation of endothelial cells and their migration toward the formation of new vessels. In our simulation program (Tumorcode), angiogenesis is turned on when the concentration of VEGF exceeds a specific threshold. Once this happens, angiogenesis proceeds as a two-step random process: first, a sprout is initialized, and second, a present sprout may be extended.

Tumor vasculature cannot be considered in isolation because it feeds the tumor. And just as the tumor grows and changes, so does its vasculature, and these two entities influence each other in a complex feedback loop that involves oxygen, nutrients, and messenger molecules. This means

that the computational model of the vasculature must be coupled with a properly chosen model of tumor tissue.

In our approach we have adopted two different models of tumor tissue: one of them is a continuum description, which is computationally efficient and is well suited for the description of tumors of clinical interest but lacks the ability to describe processes at the individual cell level, such as the evolutionary processes that produce the heterogeneity of the tumor microenvironment. The other one is based on a model of individual cells, and excels in the description of the single-cell events, but is much more computationally demanding.

4.7.2 Continuum Description of Tumor Tissue

A description of tumor development and growth based on continuum mechanics must deal with the fact that cells proliferate and die, in addition to the conditions of mass and momentum conservation that must normally be met. Just like the equation that expresses the conservation of thermal energy in Sect. 4.2, we can express the conservation of the number of cells (which corresponds to the conservation of mass) by means of the equation

$$-\frac{\partial \Phi}{\partial t} = \nabla \cdot (\Phi \boldsymbol{u}) \tag{4.16}$$

where the tumor cell density $\Phi(\boldsymbol{x}, t)$ depends on the space and time coordinates $\boldsymbol{x}$ and t, $\boldsymbol{u}$ is the local velocity field, and $\Phi \boldsymbol{u}$ is the current of cells that enter and leave a small volume centered at position $\boldsymbol{x}$, at time t.

Since the number of cells is not actually conserved because cells proliferate and die, we must add a term f that modifies the time derivative as follows

$$-\frac{\partial \Phi}{\partial t} = \nabla \cdot (\Phi \boldsymbol{u}) - f \tag{4.17}$$

Using the identities

$$\nabla(\Phi \boldsymbol{u}) = \Phi \nabla \cdot \boldsymbol{u} + \boldsymbol{u} \cdot \nabla \Phi$$

and

$$\frac{\mathrm{d}\Phi}{\mathrm{d}t} = \frac{\partial \Phi}{\partial t} + \boldsymbol{u} \cdot \nabla \Phi$$

we can rearrange equation (4.17) in the form

$$\frac{\mathrm{d}\Phi}{\mathrm{d}t} = -\Phi \nabla \cdot \boldsymbol{u} + f \tag{4.18}$$

which is the standard way in which this equation is usually presented.

In turn, cell death and proliferation cause shape changes that produce mechanical forces. To model this aspect, it is necessary to turn to the theory of plastic solids and introduce an equation that corresponds to the conservation of momentum. The general form of the equation of motion is

$$\frac{\mathrm{d}(\Phi \boldsymbol{u})}{\mathrm{d}t} = \nabla \cdot \sigma + \boldsymbol{F} \tag{4.19}$$

where σ is the Cauchy stress tensor and $\boldsymbol{F}$ is the total body force accounting for gravity and other external forces (see, e.g., [28] for a general derivation of the equation).

There are several other details that can be taken into account and in the bulk-tissue simulation of Tumorcode, and we follow the continuum-based model described in [29]. This is a state-of-the-art multiphase or mixture model [30]. In such models, the mass, the momentum, and the stress are given by summation over the contributions from all constituents. We take into account solid-like contributions from tumor cells (Φ_T), normal cells (Φ_N), necrotic cells (Φ_D), and fluid-like contributions from interstitial fluid (l). For each constituent, the velocity field, the mass conservation equation, and the momentum balance are explicitly formulated in [31]. The cells (solid-like contributions) are modeled as viscous liquid (including an isotropic pressure, friction, and adhesion) neglecting inertial force because tissue growth and cell migration happen at very low Reynolds numbers ($\mathrm{Re} \ll 1$). The liquid (fluid-like contribution) is modeled as a liquid within a porous medium resulting in a variant of Darcy's law. In our model, the liquid part of the blood (plasma) is allowed to extravasate from the vessels into the interstitium. Therefore we consider additional source terms for the liquid proportional

to the local volume vessel density. Finally we solve an elliptic equation for the pressure of the liquid.

To mimic a tumor mass, we impose that tumor cells and normal cells are immiscibly separated by an interface. This is defined via an auxiliary function in the context of the *level set method* [32–34]. This method allows to perform numerical computations involving surfaces without parameterization; therefore it is suitable for fast modeling of time-varying objects that include shape changes such as solid tumors.

We solved this coupled set of continuum equations numerically with the method of finite differences (FD). The FD methods applied to the elliptic equations of our model result in sparse matrix systems. Since sparse matrices are a specialized field within mathematical numerical research, a lot of tools are available to solve systems with sparse matrices (direct factorization, fast Fourier transform, multigrid and iterative preconditioned Krylov subspace methods). Because of the high portability, we decided to use the implementation of the numerical library Trilinos [35].

To facilitate computations and enable tumor sizes of clinical relevance, we have simplified the bulk-tissue tumor model [29]. In our "fake tumor simulation," the tumor is described as a growing sphere with constant radial expansion speed v_{tum}. Moreover, even though the tissue and liquid dynamics are neglected, the growing tumor is still a source of VEGF, and the remodeling of the vasculature takes place accordingly.

In our comprehensive numerical approach, we compute the oxygen saturation within tissue for arteriovenous blood vessel networks both before and after they are subject to the modifications of solid tumors [36]. Because oxygen diffusion happens much faster than vascular remodeling, it is not necessary to consider diffusion as an out-of-equilibrium process; rather it is sufficient to stop the vascular remodeling, calculate the oxygen distribution for a fixed vessel network configuration, and continue the vascular remodeling. Since the oxygen diffuses across the blood-tissue interface, the net oxygen flux depends not only on tissue microenvironment but also on the blood pressure inside the vessel [37]. Therefore the local blood pressure is an important input for the calculation of the oxygen field. Moreover, intravascular oxygen transport takes place by free diffusion, and since oxygen is bound to red blood cells (RBCs) in blood vessels, the consideration of RBCs is also crucial for a realistic calculation of the oxygen source strength.

We refer the reader to the original papers for all model details. Here we focus briefly on the part of model that deals with the oxygen calculation. The calculation of intravascular pO2 is a demanding task [38]. Since our focus is on the oxygen field in the tissue, we treat the vessels as one-dimensional line segments neglecting intravascular pO2 variations in radial direction. The net transvascular oxygen flux per blood-tissue interface surface area j_{tv} is proportional to the oxygen pressure gradient from inside the vessel (P) to the outside in the tissue (P_t)

$$j_{\text{tv}} = \gamma\,(P - P_t)\,. \tag{4.20}$$

Equation 4.20 is a phenomenological assumption with an effective (tissue dependent) proportionality factor γ that comprises information about the vessel wall and the tissue/tumor microenvironment (see the supplemental material "S1Appendix.PDF" of [36] for details on the determination of γ). Together with Michaelis-Menten-like uptake of oxygen by the tissue/tumor

$$M(P) = M_0 \frac{P}{P + P_{50}} \tag{4.21}$$

Eqs. (4.20) and (4.21) build the reaction part used in our implementation of the oxygen transport. To obtain the partial oxygen pressure inside the tissue, we solve numerically the following equation:

$$0 = \alpha_t D \nabla^2 P_t - M(P_t) + J_{tv} \tag{4.22}$$

with the solubility of oxygen in tissue α_t and diffusion constant of oxygen in tissue D. Note that unlike the previous sections, Eq. (4.22) considers the stationary equilibrium case only.

As an additional complication, in the physiological coupling of intravascular and extravascular oxygen transport, we must also match different discretization grids. To solve Eq. (4.22), the tissue

domain is discretized on a regular cubic grid, but the vessel network is not defined on the same grid. To interpolate P from an arbitrary point along the one-dimensional vessel line to the tissue grid point (where P_t is defined), we follow standard finite elements methods (FEM) using three-dimensional piecewise trilinear functions.

In summary, the oxygen transport across the vessel wall and inside the microenvironment of the solid tumors is complicated because of many reasons. Numerical simulations are not able to solve all problems, but at least in this approach, we can overcome the simplifications mentioned at the beginning of this section and in particular:

(a) we find realistic values for the source strength of oxygen by fitting the transvascular oxygen mass transfer coefficient γ to available literature values;
(b) we use the full nonlinearity of the Michaelis-Menten and Hill equations in the reaction part;
(c) we do take into account the chaotic and inhomogeneous architecture of blood vessel networks in tumors for the calculation of the oxygen field.

Figure 4.11 shows the result of one of our continuum-based simulations, as reported in [36]: it is interesting to observe the strong correlation between the pO2 in the local microenvironment and the blood vessel distribution. For further details, we refer the interested reader to reference [39].

4.7.3 Simulation of Individual Tumor Cells

A computational description based on individual cells provides an even finer view of the microenvironmental pO2: in our case it is based on another piece of software (VBL, Virtual Biophysics Lab) that has been used in the past to simulate small avascular solid tumors [16, 40, 41].

The VBL computational model is based on a lattice-free representation where cells are free to move and to exert both attractive and repulsive biomechanical forces on neighboring cells. The resulting motions of individual cells in the disordered tumor tissue can be followed in time, while the overall tumor structure takes its shape. On the whole the growth dynamics of the simulated avascular tumors is determined by this collective behavior of cells. At the same time, cells live, proliferate, and die, thanks to an embedded model of the cell cycle. The cycle is set in motion by a phenomenological model of the biochemical networks that describes nutrient uptake and utilization by cells. Nutrients can either be directly converted to ATP or energy-storing molecules in cells. The modeled pathways also include metabolites and waste products. The waste products are secreted into the surrounding environment, while both ATP and metabolites are used to build up proteins, DNA, and cellular structures. The model features a limited description of protein synthesis, with some specific named proteins such as cyclins and kinases that regulate the timing and the fate of the cell cycle [42, 43].

The pathways included in the model have been studied independently to fix model parameters and to reduce their known complexity to a few basic simplified reaction schemes. In this way we have reduced the computational cost of the model and, at the same time, preserved its quantitative predictability. All the pathways have been connected together to obtain a basic metabolic model of tumor cells [41–43]. When needed, the overall biochemical description can be modified in an incremental way to include additional pathways to address specific aspects of tumor biology.

In VBL, cells are complex objects, while the basic actors are nutrient and waste molecules which interact in the intertwined biochemical pathways that regulate a cell's life. The model assumes that tumor cells are never quiescent so that cells always grow and proliferate (or die). On an individual basis, the cell volume increases, while the cell's components – such as proteins, DNA, and organelles – are built, and the growth process proceeds in parallel with the phases of the cell cycle up to mitosis. Just as in real cells, mitosis is uneven, and the mother cell material is subdivided randomly between the two daughter cells [41–43]. The individual variability in cell

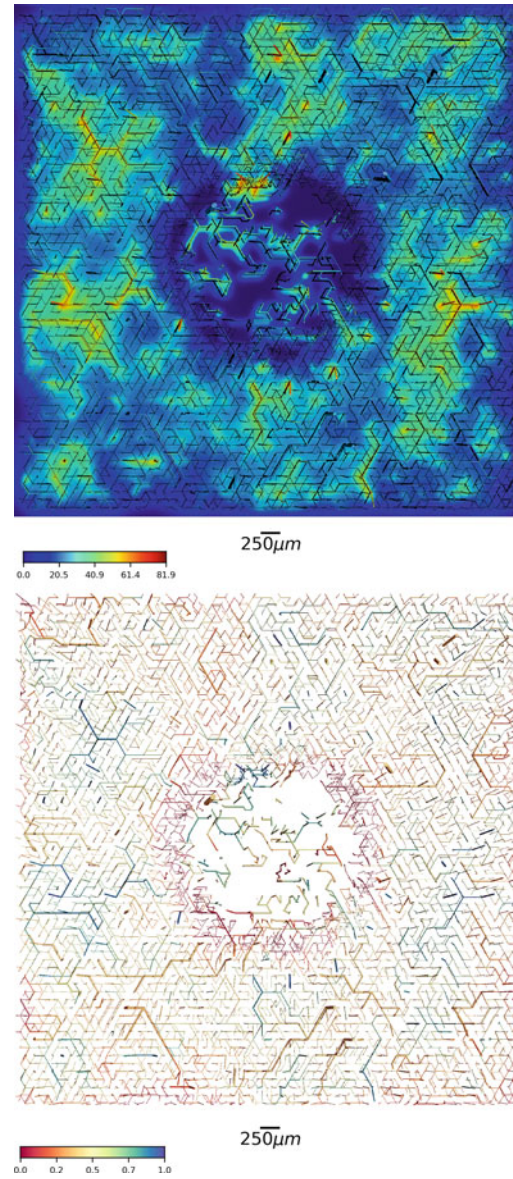

Fig. 4.11 Oxygen in a growing solid tumor. This simulation assumes a spherical expanding tumor mass including the full vessel remodeling dynamics of Tumorcode ("fake tumor"). The simulation setup is identical to the one described in [36] and comprises a volume of 8 mm^3 containing about 340k vessel segments. Both panels show a slice through the center of the simulation domain at simulation time $t = 600$ h. The vessels shown are in a 200 μm thick slice above the central plane. Upper panel: tissue pO2 in a central slice overlain with vessels (color-coded by their pO2 value). The color bar is in mmHg. Lower panel: vessels color-coded by oxygen saturation level

division propagates to the cell population, and it is one of the random factors that determine the chaotic movements of cells in the simulated tumor.

The computer code contains a mixture of deterministic steps – those related to the numerical solution of the reaction diffusion equations, and the mechanical movements – and random steps, such as the division of the cell's materials at mitosis or the protein synthesis, which is related to the availability of nutrients in a chaotic environment. The deterministic steps describe the dynamics of structures that span at least 3 orders of magnitude in space (from the few μm of cell radius up to a few mm of diameter of an avascular tumor) and 12 orders of magnitude in time (from a few tens of μs that are typical diffusion times of the molecular species up to $\sim 10^7$ s for the full development of an avascular tumor) [44]. Thus, our computer code is a true multiscale model of small avascular tumors, and it requires the use of appropriate algorithms to manage the stiff set of differential

equations for reaction-diffusion and mechanical movements [45].

On the whole, the behavior of each individual cell is controlled by ∼100 parameters, and this lends great flexibility to the computer code, which can mix in the same simulation several kinds of tumor cells. Comparisons of the results of simulation runs with experimental data have shown that our simulation program is a reliable model of the growth of avascular tumors [41, 43]. Usually we assume that cells are nonpolar and spherical and that they are immersed in a uniform environment with which they exchange oxygen, nutrients, and metabolites: in such conditions we obtain cell clusters that are invariably nearly spherical and that reproduce in good detail the structure and the chemical gradients found in tumor spheroids [46].

The computer code can also handle more complex situations, like the growth of cells around a single blood vessel that acts as the only source of oxygen and nutrients, with a surrounding environment that is oxygen- and nutrient-poor and filled with metabolites such as lactic acid that make it acidic. This case is illustrated in Fig. 4.12, which shows two different views of the same object, a simulated tumor cord about 480 μm long. The first view shows the O_2 concentration. The second view shows the distribution of cell phases, which displays the fine-grained level of detail that is reached in the simulation. Given the conditions of the simulation, the O_2 concentration is highest close to the blood vessel, and it decreases sharply further away from the vessel. Correspondingly, cells are distributed in various phases in the vicinity of the blood vessel, while they are mostly dead further away from it.

4.7.4 Oxygen in a Fine-Grained Simulation of the Tumor Microenvironment

As noted above, the continuum model is computationally efficient and capable of simulating tumors of clinical relevance. On the other hand, the less efficient cell-based simulation is extremely fine-grained and offers a different view of the tumor, one that has the potential to give a glimpse of the transformative, evolutionary events that produce the heterogeneity that is observed in real tumors. We have recently taken steps in this direction, and we have obtained the very first computational snapshots of the tumor microenvironment at the cellular level [47]. While our simulation is not yet sufficiently detailed to actually deliver the promised results on tumor heterogeneity – they still lack the plurality of cell types that populate a real tumor – they already display such basic features of the microenvironment as the large gradients that lead to the formation of ecological niches and ultimately drive the Darwinian evolution of tumor cells [48]. Obviously, one of these important gradients is associated with the local oxygen concentration.

In the combined simulation of Tumorcode and VBL [47], we improved two mean field assumptions of the standalone version of Tumorcode – the VEGF field and the oxygen uptake field – thanks to the availability of the detailed representation of individual cells. The VEGF field was previously extracted from the bulk-tissue tumor or was assumed to be spherically symmetric in the case of the fake tumor. In the combined program, we assume that each cell is a point source for the VEGF and constructs the VEGF field as a superposition of single-cell contributions. The tumor models already integrated in Tumorcode comprise three kinds of tissue: normal, tumor, and necrotic tissue, and each of them has its own oxygen consumption parameters in the Michaelis-Menten equation for the oxygen uptake. In contrast, VBL calculates the oxygen uptake for each cell, and we use this fine-grained information to interpolate a continuous oxygen uptake field which is a more realistic input for the oxygen computation in Tumorcode.

Recently, we used the combined program to study the tumor microenvironment at the angiogenic switch. The angiogenic switch is an important step toward malignancy, since it marks the onset of tumor vascularization [49]. After the angiogenic switch, malignant tumors can invade vessels, spread throughout the body via the blood stream, and metastasize at different locations. This step of the progression is particularly impor-

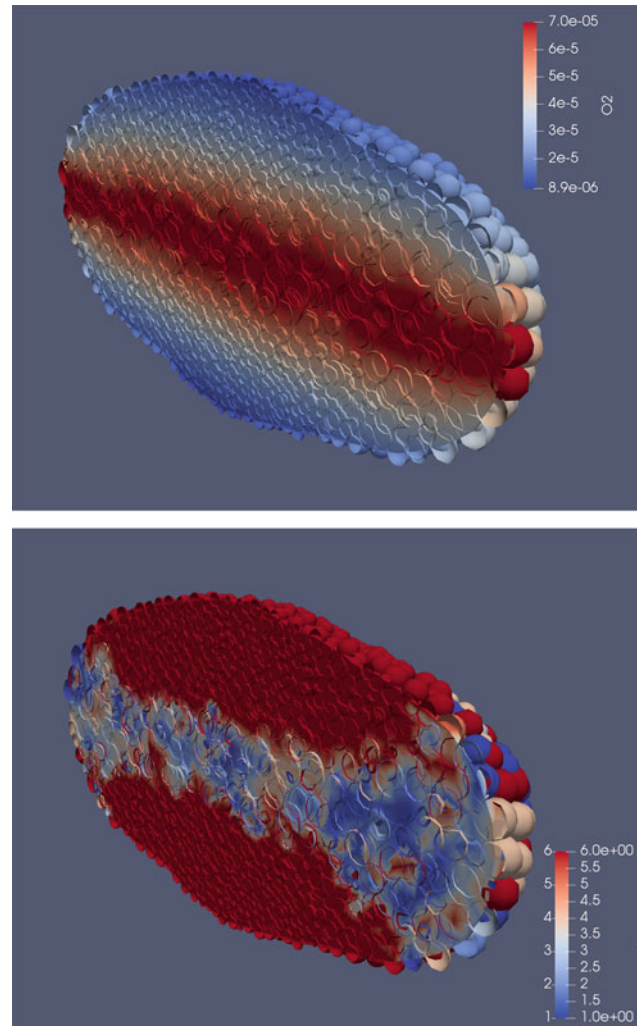

Fig. 4.12 Longitudinal section of a simulated tumor cord about 480 μm long (the blood vessel runs along the axis of the cord and is not shown). Top panel: O_2 concentration; the highest value in the color scale corresponds to O_2 in equilibrium with atmospheric oxygen. Bottom panel: cell phases. The cell phases are labeled with the numbers 1–5 (1 = early G1 phase; 2 = late G1 phase; 3 = S phase; 4 = G2 phase; 5 = M phase), while 6 indicates the dead cells

tant in the development of cancer and therefore of special interest for the prognosis and therapy of the disease. We performed two experiments: one in analogy with the experimental setup used by Helminger et al. [24] and one that nearly matches the maximum problem size on our current hardware.

Helminger et al. measured the pO2 and pH in human tumor xenografts, utilizing phosphorescence quenching for pO2 and fluorescence ratio imaging for pH. The measurements were done for different blood vessel arrangements. We focused on the topology used for panels c and d of Fig. 2 in [24], which is the measurement along a straight line between the bifurcation of two blood vessels, as illustrated in Fig. 4.13. To recreate this setup in our simulation, we used Tumorcode to build an arteriovenous blood vessel network, searched for a similar bifurcation, seeded the VBL spheroid in between the bifurcation, and started the simulation run. The resulting pO2 gradients along the line between the blood vessels are quantitatively quite similar to the measured gradients of Helminger et al. (see Fig. 4.14 and compare with the corresponding figures in [24]).

In the second experiment, we followed the tumor growth dynamics up to a wall time[4] of about

[4] In the jargon of High Performance Computing, this is the experimenter's actual waiting time for the completion of the simulation.

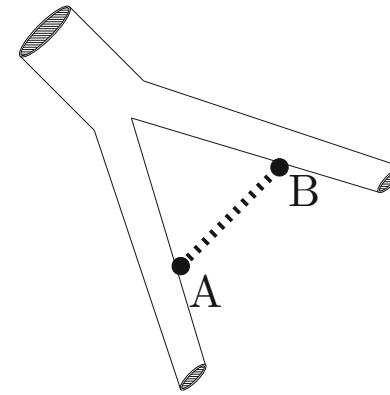

Fig. 4.13 Geometry of the measurements of Helminger et al. [24]. The measurements were carried out along an ideal line connecting two points on different branches of a bifurcation. The pO2 was reported as a function of the distance traveled along this line from A to B

1 month, resulting in simulated time of 580 h past the initial seeding of the tumor. Because oxygen and other nutrients are released by blood vessels, the distance of a cell to its nearest vessel is certainly an interesting quantity to look at. Experimentally it would be impossible (or at least very tedious) to quantify the distance of each cell in a tumor to its nearest blood vessel. For computer simulations, this can be made automatic, and it provides us with some intriguing data.

We histogrammed the pO2 for every cell according to the distance to the nearest blood vessel, thus producing a set of empirical probability distributions of pO2 for a set selected distances to the nearest blood vessel. For early time points (380 h past the initial seeding), we find that the median of these distributions decays exponentially vs. distance (see Fig. 4.15), as expected for a spheroid embedded in a homogenous tissue. However, for increasing simulation time (480 h and 580 h past the initial seeding), the medians change, up to a point where they start to increase again with distance. This happens because of the modifications of the blood vessel network and because of the death of many cells which leads to a reduction of the oxygen consumption. In the vascularized tumor mass, we observe cells that are more than 100 μm away from the nearest blood vessel and still are sufficiently oxygenated.

In [47], we have shown that such computer simulations of vascularized multicellular tumor spheroids are in good qualitative agreement with measurements of human tumor xenograft, and we showed that in our particular *in silico* model, the transport of vascular oxygen results in a continually changing and rugged microenvironment. This means that the niche diversity is large and consequently that the evolutionary pressure is high and leads to a very effective selection of different tumor clones even in small tumors.

4.8 Conclusions

As we have already noted, the numerical simulations do not take everything into account, and yet, it is interesting to observe that on average, they are in quite good agreement with the measurements in [24]. The simulations are driven by model parameters that have been taken from the literature and which have been obtained with different experimental systems and tumor cell types. Whenever experimental measurements were missing, we estimated parameter values from independent biophysical modeling of available data, once again collected in experiments with different tumor systems [44]. Therefore, model parameters have not been tuned to describe the behavior of any specific tumor, and yet we obtain a good agreement of the oxygen concentration with the actual measurements. But shouldn't there be a measurable specificity of tumor cells and tissue that shows up in the simulations? How can we explain the observed agreement?

One simple answer may be that for all their differences, tumor cells are similar in their average metabolic behavior, as we noted in our derivation of the metabolic law in reference [17] (see Fig. 4.2 in that paper). There, we found that the mean val-

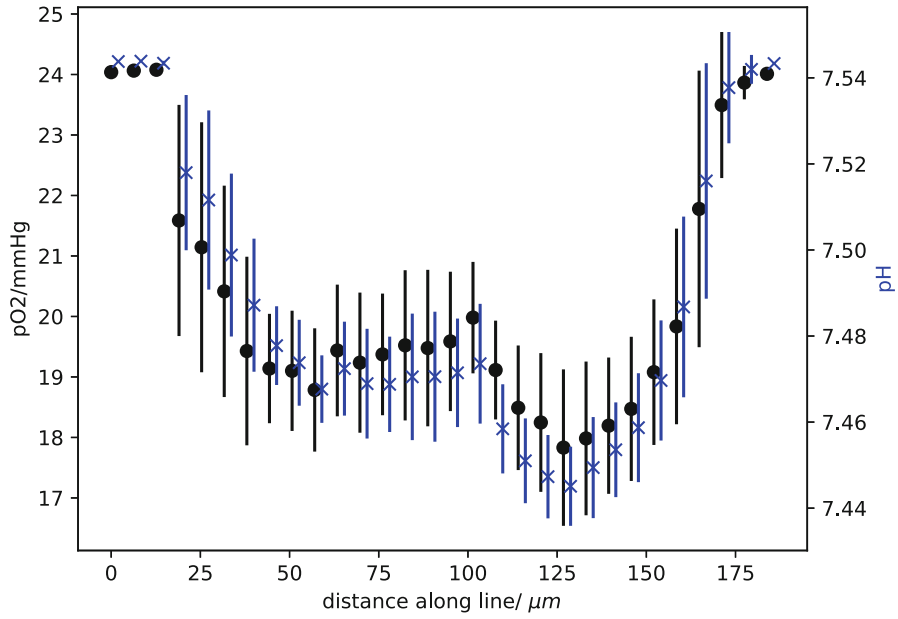

Fig. 4.14 pO2 and pH vs. distance traveled along a segment joining two branches of a bifurcation, as in reference [24], at simulated time $t = 350$ h

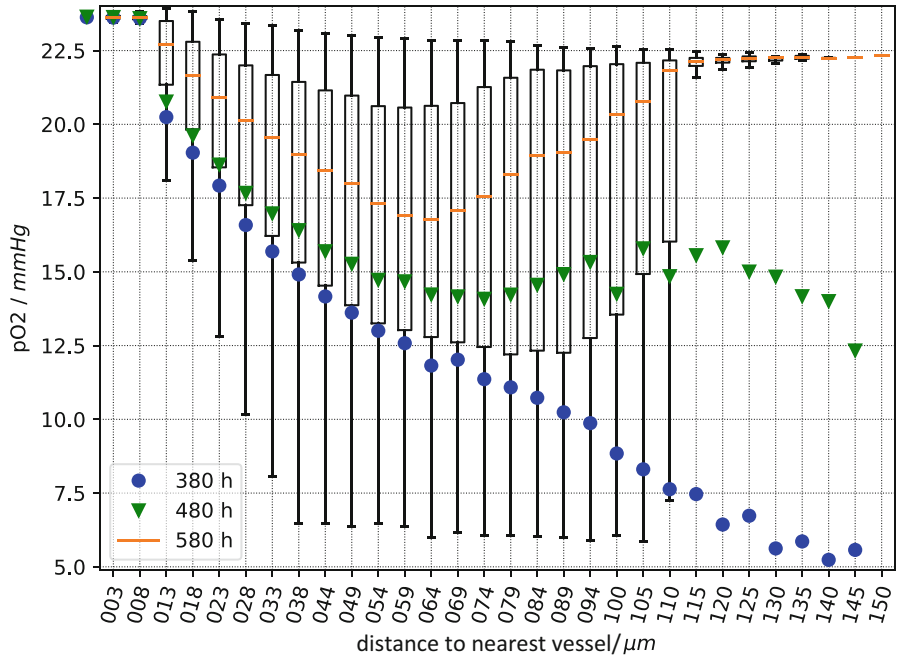

Fig. 4.15 Combined representation of the pO2 values of cells at given distances from nearest blood vessels, for three different time points past the angiogenic switch: blue 380 h, green 480 h, and orange 580 h. For the two earlier timepoints (380 and 480 h), the blue bullets and the green triangles show the median value. For the last time point, the median is shown by an orange line inside a box that marks the positions of the 25th and 75th percentiles. The whiskers represent the 10th and 90th percentiles. It is interesting to note the large widths of the distance distributions

ues of metabolic consumption are very effective descriptors of an average metabolic consumption, even though the figure summarizes the behavior of different tumor types. Incidentally, this would also mean that a model of metabolism of cells is all we need to describe the oxygen concentration.

This answer highlights that the agreement between our simulations and observations has been established for the average behavior of the simulations (we have compared the median values to the observations), but a careful observation of Fig. 4.15 shows that the empirical distributions of the distance to the closest vessel are quite wide. These spatial fluctuations are extremely important, actually they are one of the main results of our simulation effort. Indeed, they produce a large niche diversity even in small vascularized solid tumors. This means that already at the early stages of tumor progression, i.e., close to the angiogenic switch, the microenvironment can exert a high evolutionary pressure that drives the Darwinian selection of different clones. Here we stress that while the molecular mechanisms that promote genotypic changes in tumor clones are well-known and characterized, genotypic variation is but one ingredient of tumor evolution, the other important feature being the variability of the environment that supervenes the genome and sets the stage for the evolutionary process. With our computational tool, we can start to explore this Darwinian dynamics in tumors and grasp the role of evolutionary forces in the establishment of more or less aggressive tumor phenotypes.

In the first part of this chapter, we also examined the importance of time fluctuations, whose existence is supported by several experiments; see [8, 25, 50, 51]. Together with the oxygen consumption by cells, they conspire to further limit the penetration of oxygen in the tumor tissue. This may have an important clinical impact, because tumor hypoxia is known to negatively affect radiotherapy [1, 52, 53]. Our model predicts that by blocking the pathophysiological oscillations of oxygen observed in the tumor microcirculation, the oxygen concentrations in the tumor tissue should increase. It is known that the low-frequency rhythms of arterial circulation can be strongly attenuated, or even abolished, after alpha-adrenoreceptor blockade [54, 55]. Alpha blockers are well-tolerated drugs, and they are already used in the clinical setting to treat a variety of disorders such as anxiety, panic, and post-traumatic stress disorders [56–58]. Thus, this class of drugs could be used in combination with radiotherapy to transiently improve the oxygenation of the tumor microenvironment and increase the efficacy of radiation treatments.

Such considerations prove that mathematical and numerical approaches to the distribution of oxygen in the tumor microenvironment are more than mere mathematical exercises; they yield new and useful insights on the role of the distribution of blood vessel density and size and on the fluctuations of blood oxygenation and pressure, with implications on both tumor biology and radiotherapy. More generally, these models are not affected by the practical limitations that hamper the experimental collection of quantitative data at appropriate spatial and temporal resolution. For example, with current technologies it is almost impossible to follow the evolution kinetics of individual clones in solid tumors and carry out experiments to explore the space of parameters. Analytic and numerical models can illuminate the basic features of complex biological systems and can uncover novel ones.

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Roles for Phospholipase D1 in the Tumor Microenvironment

5

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Abstract

The lipid-modifying signal transduction enzyme phospholipase D (PLD) has been proposed to have roles in oncogenic processes for well-on 30 years, with most of the early literature focused on potential functions for PLD in the biology of the tumor cells themselves. While such roles remain under investigation, evidence has also now been generated to support additional roles for PLD, in particular PLD1, in the tumor microenvironment, including effects on neoangiogenesis, the supply of nutrients, interactions of platelets with circulating cancer cells, the response of the immune system, and exosome biology. Here, we review these lines of investigation, accompanied by a discussion of the limitations of the existing studies and some cautionary notes regarding the study and interpretation of PLD function using model systems.

Keywords

Phospholipase D · PLD1 · PLD2 · Lipid signaling · Tumor microenvironment · Neoangiogenesis · Inhibitor · Platelets · Exosomes · Autophagy · VEGF · Metastasis · Breast cancer · Immune responses · Cardiovascular

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5.1 Introduction

The mammalian phospholipase D (PLD) superfamily of enzymes consists of six isoforms. PLD1 [1] and PLD2 [2] encode the classic, canonical activity of hydrolyzing phosphatidylcholine (PC), the most abundant cellular phospholipid, to generate choline and a second-messenger signaling lipid, phosphatidic acid (PA), in response to activation of receptor tyrosine kinases and G-protein-coupled receptors [3]. PLD3 and PLD4 are 5′ exonucleases that remove circulating single-stranded DNA that would otherwise activate the microbial genetic sensor toll-like receptor 9; in their absence, TLR9 overstimulation leads to autoimmune disease [4]. PLD5 has no known activity or role at present [5], and PLD6, which is located on the external surface of mitochondria, both hydrolyzes cardiolipin to generate phosphatidic acid [6] and functions as a 3' RNA endonuclease to generate piRNAs [7], a form of endogenous RNAi that is critical for spermatogenesis [8].

Canonical PLD activity, i.e., PLD1- and PLD2-mediated PC hydrolysis, was defined in

A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_5

plants in the 1950s [9] and then began to be described in mammalian cells and tissues in the early 1980s [10], although it remains unclear as to exactly what combination of PLD activities was present in those early preparations. By the late 1980s, it had become appreciated that PLD was activated by signal transduction events, e.g., subsequent to chemotactic stimulation of granulocytes [11], which implicated G-protein-receptor coupling and created widespread interest in a potential role of PLD in regulated exocytosis. PLD was also noted to be activated subsequent to protein kinase C (PKC) stimulation [12]. Finally, in 1990, it was first suggested that PLD action might be involved in K-ras-driven oncogenic proliferation [13], followed by linkage to epidermal growth factor (EGF) [14] and platelet-derived growth factor (PDGF) [15] - promoted proliferation in cancer cells. Much of the excitement at this time derived from the awareness that the PA produced by PLD could be dephosphorylated to generate the signaling lipid diacylglycerol (DAG) [16]. DAG activates PKC and had been linked to cellular proliferation, providing a rationale for the proposal that PLD activity would be pro-oncogenic, although it is now well-known that there are many other downstream effector roles for PA as well [5, 17, 18]. A wave of subsequent reports that included some high-impact ones directly linking the ARF [19, 20] and Rho [21] small GTPases to PLD activation were accompanied by demonstration that PLD activation in turn stimulated remodeling of the actin cytoskeleton, an event important in cancer cell mobility [22], as well as the secretion of matrix metalloproteinases [23], which is important for metastatic invasion.

PLD activity was first reported to be increased in tumors in the setting of human breast cancer [24], followed by similar reports for a number of other types of cancer [18]. Throughout this period, the preponderant interest was in PLD's role in facilitating transformation and tumorigenesis through direct effects on the cancer cells [25, 26], which came to include regulation of mTOR signaling [27], apoptosis [28], HIF1-α expression [29], and integrin-mediated cell spreading and migration [30]. In 2012, however, a report from our group provided direct evidence for important roles for PLD1 in tumorigenesis independent of the tumor cells [31].

5.2 Roles for PLD, and in Particular PLD1, in the Tumor Microenvironment

5.2.1 Neoangiogenesis and Platelet-Facilitated Metastasis

In the run-up to our publication, we had encountered difficulties in attempting to validate a number of earlier findings on the roles of PLD in cancer cells, using a newly characterized small-molecule dual PLD1/PLD2 inhibitor [32]. In retrospect, many of the earlier studies had been conducted using the primary alcohol 1-butanol to inhibit PLD generation of PA, based on the propensity of the PLD enzymes to perform a transphosphatidylation reaction to generate phosphatidylalcohol rather than use water as the nucleophile to generate PA when given the choice. Phosphatidylalcohols are in general biologically inert, creating in theory a PLD loss-of-function phenotype when primary alcohols are present. In practice, however, it is not practical to use the alcohols at a high enough concentration to block all PA generation, and both the alcohols and the generated phosphatidylalcohol do have biological effects; so in the end, many of the phenomena reported were false-positive findings [32, 33].

A striking outcome was observed, however, when (wild-type) lung cancer or melanoma tumor cell lines were implanted ectopically into the flanks of wild-type mice or mice lacking the PLD1 gene (PLD1$^{-/-}$ mice): The tumors implanted into the PLD1$^{-/-}$ mice grew more slowly and exhibited a profound decrease in vascularization [31]. The vascularization defect was then shown to be tumor independent; a similar phenomenon was observed with implantation of matrigel plugs containing vascular endothelial growth factor (VEGF), suggesting that the primary defect lay with the vascular endothelial cells. This was confirmed

using several approaches. First, slices of the aorta (aortic rings) were cultured in media containing VEGF. The endothelial cells in wild-type aortic rings responded as expected by generating outgrowths of endothelial cells that extended as microvessels from the ring; however, very little microvessel extension was observed for PLD1$^{-/-}$ aortic rings. Second, endothelial cells purified from wild-type and from PLD1$^{-/-}$ lungs grew equally well under standard culture conditions, but when then plated onto coverslips coated with extracellular matrix, the wild-type cells self-organized into capillary-like microvessels whereas the PLD1$^{-/-}$ cells remained disorganized and underwent anoikis. Finally, VEGF signaling was shown to be blunted in magnitude for the PLD1$^{-/-}$ endothelial cells, suggesting a signaling defect at the level of the receptor or associated proteins. Related observations concerning the endothelial role of PLD1 in pathological neoangiogenesis have been reported in a non-cancer setting, along with a detailed delineation of the downstream signaling pathways involved [34].

Independently, a defect was also observed in metastasis. Tumor cells injected into the tail veins of mice metastasize to the lung in wild-type mice. A marked reduction in metastatic seeding was observed when wild-type tumor cells were injected into PLD1$^{-/-}$ mice [31]. This phenomenon was linked to the interaction of the circulating tumor cells with platelets. We had earlier shown that platelets are resistant to activation in PLD1$^{-/-}$ mice [35]. Metastasis efficiency is dependent on interaction of the tumor cells with activated platelets, which shield them from damage while traveling through the vasculature and facilitate their anchoring to metastatic sites and intravasation into the adjacent interstitial tissue. The tumor cells exhibited decreased binding to PLD1$^{-/-}$ platelets, suggesting at least a partial explanation for the reduction in metastatic seeding [31]. Moreover, a recent study has reported that PLD1$^{-/-}$ platelets exhibit strongly reduced adhesion to endothelial cells [36], which could also serve to decrease metastatic seeding. Finally, PLD1 has also been shown to be important for human platelet activation [37], suggesting that PLD1 inhibition should also decrease metastasis in people.

5.2.2 Tumor Cell Energetics

As tumors grow in size, the cells in the core of the tumor begin to experience hypoxia and nutrient deprivation, which is made worse by the above neoangiogenesis defect in PLD1$^{-/-}$ mice. One mechanism through which tumor cells address metabolic stress is to activate autophagy. Studies from several groups have shown that PLD1 positively regulates autophagy in the non-cancer setting [38–40]. These findings were then extended to cancer cell energetics by using PLD1 small molecule inhibitors in combination with culturing the tumor cells in nutrient-depleted media, which led to the observation that wild-type tumor cells can use autophagy to mobilize endomembranes to produce free fatty acids that can be used by mitochondria to generate energy, whereas tumor cells in which PLD1 activity is inhibited cannot do so and reduce their proliferation and/or undergo apoptosis [41]. In this setting, i.e., both nutrient deprivation and PLD1 inhibition, the tumor cells can be rescued only by exogenous provision of free fatty acids. Accordingly, this becomes an important role for the tumor microenvironment when tumor cells lack PLD1 activity and represents a potential avenue for therapeutic opportunities.

5.2.3 Secretion of Chemokines and Factors That Promote Tumor Growth

As described in the Introduction, some of the earliest reports on mammalian PLD activity suggested that PLD might play a role in regulated exocytosis based on elevated expression in professional secretory cells. While early reports on function used 1-butanol to inhibit PLD-mediated PA production and are now considered techni-

cally inconclusive, the proposal was revisited after cloning of PLD1 [1] and the generation of mutant inactive alleles [42] that could be used to compare gain- and loss-of-function in established models of regulated exocytosis. The initial study on this topic showed definitively that PLD1 activity promoted regulated exocytosis in neuroendocrine cells [43] and has since been followed by many reports that have made used of RNAi, small molecule PLD1 inhibitors, and PLD1$^{-/-}$ cells to demonstrate varied types of secretion defects for factors released by platelets [44], endothelial cells [44, 45], and dendritic cells [46], as well as neurons, mast cells, pancreatic beta cells, and epithelial cells [47–50]. Tumor progression is enhanced by factors released by stromal and immune cells in the tumor microenvironment such as IL-13, which has been reported to be regulated by PLD1 in the setting of allergies [51]. Although this role of PLD1 in the context of cancer has not been well studied, it is likely that PLD1 deficiency or inhibition will result in secretion defects for cells in the tumor environment that will inhibit growth and/or metastasis of the tumor cells.

5.2.4 Interaction of Tumors with the Immune System

The relationship of the immune system to tumorigenesis is complex. The immune system clearly has a key role in anti-oncogenic surveillance, as individuals with immunosuppression are considerably more prone to developing cancer [52]. However, tumors can also coopt the tumor microenvironment and key components of the immune-responding cells to create an immunosuppressive milieu that favors unchecked tumor growth [53]. Inhibition or ablation of PLD1 has been reported to decrease macrophage function and neutrophil migration into tumor sites [54]. Since tumor-associated microphages are often immunosuppressive, this could be anti-oncogenic in effect. Conversely, however, PLD1 has also been reported to be important for multiple aspects of T cell function, including TCR-mediated signaling [55] and lymphocyte adhesion and migration [56, 57]. Functional deficiencies in this aspect of the immune system would be expected to be pro-oncogenic. Taken together though, the benefits of PLD1 deficiency in terms of effects on neoangiogenesis and metastasis appear to outweigh the competing elements of the immune system, since mice lacking PLD1 in the tumor microenvironment have a lower tumor burden and reduced metastasis in comparison to mice with wild-type PLD1 activity [31].

It should be noted that the outcome is less clear for PLD2. While Ryu and colleagues reported that endothelial cell deletion of PLD2 also inhibited neoangiogenesis and tumor growth for tumors implanted into the flanks of mice [58], Kanaho and colleagues found that tumors implanted similarly were more aggressive in whole-body knockouts of PLD2 [59], which they then traced to defective signaling and proliferation of CD8(+) T cells, which are key effectors in the anti-oncogenic immune response. It will be important to assess the global benefit outcome for specific types of tumors, which may depend on the degree to which they depend on neoangiogenesis and the nature of the therapeutic approach being used to target them.

5.2.5 Exosomes and Phospholipase D1

A relatively new and growing field is focused on exosomes released by tumor cells that can alter the tumor microenvironment and make it more hospitable to metastatic growth [60]. An association between PLD function and microvesicles released by tumors was noted a decade ago [61], as mediated by a mechanism involving the ARF6 small GTPase and PLD1. ARF6 is an activator of PLD1 and has long been linked to it in the context of regulated exocytosis [62]. PLD2 has been reported to be secreted in exosomes and to elicit a tumor microenvironment that promotes increased tumor stemness [63]. PLD2 may also control the production of exosomes [64, 65]. Taken together, it appears likely that PLD activity is involved in this process, although it is not clear that only PLD2 is involved in the process. PLD1 and PLD2

often exhibit partially overlapping roles (redundancy), with a stronger phenotype being observed when both isoforms are inhibited or ablated [66, 67]; thus, clarification of the role of each isoform in exosome biogenesis and function awaits further investigation.

5.2.6 Cautionary Tales

5.2.6.1 Inhibitors

The PLD literature is extensive (>5000 articles at present), with more than 1000 articles also including the keyword "inhibitor." All of the current small molecule inhibitors in use for PLD study were derived from a psychiatric drug, halopemide, first identified as a PLD2 inhibitor in a screen by Steed and co-workers [68]. Among the improved analogs they generated was one later denoted as FIPI [32], which was shown to be a dual PLD1-PLD2 inhibitor with high potency (IC_{50} in the low nM range) and acceptable PK/PD for in vivo studies [31, 66, 69]. In parallel, Brown and Lindsley and colleagues generated an extensive series of analogs that offered isoform selectivity and potentially improved side-effect profiles [17]. Among the numerous compounds generated, some had high potency (low nM IC_{50}s) and some were 10–100-fold less potent, thus requiring high concentrations to achieve full inhibition. Unfortunately, it has become common practice in studies to use all of the PLD inhibitors at extraordinarily high concentrations. For example, multiple reports use the compound VU0155069, developed as a PLD1-selective inhibitor by Brown and colleagues [70], at 10 μM in culture. However, VU0155069 has an IC_{50} of 11 nM for PLD1 and an IC_{50} of 1.8 μM for PLD2, meaning that a concentration of 10 μM is about 30 times higher than needed to achieve full inhibition of PLD1, and is high enough to inhibit 80% of PLD2 activity, negating its utility as a "PLD1" inhibitor. Rather, in this setting, it's an incomplete dual-isoform inhibitor. As well, there are multiple studies for which the phenomenon of interest is inhibited only at the highest concentrations used (10–30 μM), for which the correct interpretation would be that it is an off-target action of the inhibitor that is causing the change in biological behavior, not inhibition of PLD1, which had already been achieved at a much lower concentration.

This situation is well highlighted by a recent report, "VU0155069 inhibits inflammasome activation independent of phospholipase D1 activity," by Bae and colleagues, in which they observed blockade of macrophage inflammasome activation in the presence of 10 μM VU0155069, but also found that inflammasome activation was completely normal in macrophages prepared from $PLD1^{-/-}$ mice [71]. Activation of compensatory mechanisms in the macrophages from the knockout mice that had experienced a long-term loss of PLD1 activity did not appear to underlie the finding, since VU0155069 also suppressed inflammasome activation for the $PLD1^{-/-}$ macrophages. Accordingly, the most likely explanation would be that VU0155069 has off-target effects at 10 μM. Other experiments in the report documented that the inflammasome activation blockade could only be achieved with very high concentrations of VU0155069, again suggesting that it was mediating its effects through an off-target mechanism. All pharmaceutical agents have "therapeutic windows" that define both their minimal effective concentration and the maximum concentration that they can be used at for any of several reasons. Reports such as this one provide a good example of the need to consider therapeutic windows in the design and interpretation of experiments. While VU0155069 appears to have off-target effects at 10 μM, it might well be highly specific in action at the highest concentration required for full PLD1 inhibition (300 nM). As also demonstrated in this report, the parallel use of $PLD1^{-/-}$ cells to validate the inhibitor findings is a very important control. Care should be taken in the interpretation of reports that neither establish a dose-response relationship for the inhibition findings nor use an independent approach to validate them.

A less common but equally serious issue relates to the in vivo usage of the inhibitors. FIPI, which has been used in vivo in multiple studies, has a $t_{1/2}$ of 5.5 hours [68], making it possible to dose animals twice daily while achieving

full and continuous inhibition [31, 66]. Many of the early isoform-selective compounds developed though, such as VU0155069, have very short half-lives (<0.15 hours) [72], making their in vivo use impractical. More recent work has resulted in PLD2-specific inhibitors with longer half-lives (>3 hours) [72]. Nonetheless, some investigators have used the earlier compounds to inject mice with them once or twice daily, which would result in only minutes of inhibition.

Taken together, there is substantial misuse of the inhibitors in the literature, and readers should examine these details carefully in assessing the rigor of published studies.

5.2.6.2 Generalizability of Studies

The preceding section on the immune system alluded to conflicting outcomes in the context of roles for PLD2 in cancer progression. Taking two other relevant reports into consideration, a summary of the findings is presented in Table 5.1. In brief, different outcomes were reported by each group, raising the issue that the interpretations are model specific, and hence caution should be employed in attempting to extrapolate these findings to human cancer settings. Whether the anti-oncogenic findings reported by some of the groups outweigh the pro-oncogenic findings reported by others may depend on the specific signaling pathways used by each human tumor type.

Similarly-conflicting roles for PLD1 in cancer progression have not been reported thus far, but a cautionary note should be considered based on findings in a different setting. Mice lacking PLD1 were first reported in 2010 [35] and studied in the context of blunted platelet activation. The mice were reported to be overtly grossly normal, i.e., had viability similar to wild-type mice and appeared ostensibly normal in the controlled setting of an animal facility. Subsequent studies on PLD1 roles in platelet function [66, 73], neutrophil migration [54], colon cancer [74], metabolic homeostasis [75], neurite formation [76], cancer progression [31], and cardiac function [77] using adult mice lacking PLD1 (and in some cases, also PLD2) were reported. Accordingly, it was highly surprising when whole genome sequencing of two families with congenital heart disease revealed inactivation of PLD1 as the cause of severe cardiovascular disease involving the pulmonic, tricuspid, and mitral valves that resulted in pre- and postnatal death or survival only with surgical repair and/or and pharmacological support [78]. Retrospective study of the PLD1$^{-/-}$ mice revealed cardiac abnormalities that were significant but yet mild enough to not cause issues with viability or general fitness, or even to be apparent to groups studying cardiac function in the setting of ischemia/reperfusion [77]. The conclusion from these findings is that caution should be drawn in extrapolating exact roles for PLD1 in signaling pathways when considering human disease entities as modeled using mice; many roles for PLD1 may be conserved between humans and mice, but none are guaranteed to be, and proposed roles will need to be validated to the best extent possible using available assays based on human tissues.

Table 5.1 Role of PLD2 in cancer progression

	Angiogenesis	Tumor growth
Ghim et al. [58]	Decreased	Decreased
Ngo Thai Bich et al. [59]	No change	Increased
Wang et al. [79]	No change	No change
Henkels et al. [80]	–	Decreased

Legend: Four mouse cancer models were used to explore the role of PLD2 in cancer progression. Ghim et al. used a model based on endothelial deletion of PLD2; Ngo Thai Bich et al. and Wang et al. used mice with full deletion of PLD2; and Henkels et al. used the PLD inhibitor FIPI in combination with human tumor lines implanted into SCID mice

5.3 Summary

PLD1 activity is well documented to play important roles in the tumor microenvironment (Fig. 5.1), and on balance, PLD1 inhibition appears to promote an anti-oncogenic outcome. More study is needed to generalize these findings in the settings of different types of cancer and to extend them to study human cancer, and ultimately clinical trials with improved inhibitors, to determine the utility of inhibiting PLD1 as a therapeutic approach.

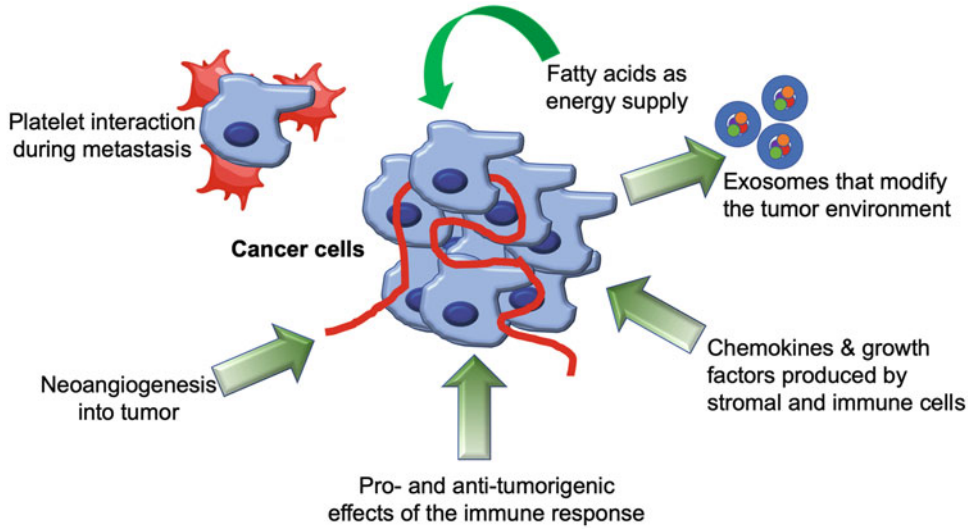

Fig. 5.1 Tumor microenvironmental roles for phospholipase D1. See text for details. PLD1 has been shown in model systems to be required for (i) vascular invasion into the growing tumor; (ii) utilization of endomembranes under conditions of nutrient starvation, making the tumor cells dependent on exogenous provision of fatty acids; (iii) generation of growth factors and chemokines such as IL-13 that promote tumor cell proliferation; (iv) monocyte invasion of the tumor and T cell function; and (v) interaction of platelets with circulating tumor cells. PLD2 and possibly PLD1 also facilitate production and release of exosomes from the tumor cells that prepare the tumor microenvironment for metastatic tumor cell seeding

Acknowledgments This work was supported by a Carol Baldwin Breast Cancer award to MAF.

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Leptin in Tumor Microenvironment

6

Adriano Angelucci, Letizia Clementi, and Edoardo Alesse

Abstract

Leptin is a hormone that plays a major role as mediator of long-term regulation of energy balance, suppressing food intake, and stimulating weight loss. More recently, important physiological roles other than controlling appetite and energy expenditure have been suggested for leptin, including neuroendocrine, reparative, reproductive, and immune functions. These emerging peripheral roles let hypothesize that leptin can modulate also cancer progression. Indeed, many studies have demonstrated that elevated chronic serum concentrations of leptin, frequently seen in obese subjects, represent a stimulatory signal for tumor growth. Current knowledge indicates that also different non-tumoral cells resident in tumor microenvironment may respond to leptin creating a favorable soil for cancer cells. In addition, leptin is produced also within the tumor microenvironment creating the possibility for paracrine and autocrine action. In this review, we describe the main mechanisms that regulate peripheral leptin availability and how leptin can shape tumor microenvironment.

Keywords

Leptin · Leptin receptor · Obesity · Adipocytes · Signal transduction · Cancer progression · Angiogenesis · Fibrosis · Inflammation · Tumor microenvironment · Breast cancer · Estrogen · Cancer-associated fibroblasts · Endothelial cells · Mesenchymal stem cells

6.1 Introduction: Biology of Leptin

6.1.1 LEP Gene and Structure of Leptin Protein

Leptin gene (*ob*) is located on 7q32.1 and consists of three exons separated by two introns. The sequence of the LEP gene is preserved in all mammalians: human leptin and mouse leptin share 84 percent sequence identity [133]. Single-nucleotide mutation in the mouse LEP gene resulting in a truncated protein (stop codon) is responsible for the mutated phenotype expressed by the well-known mouse model of obesity *ob/ob* [203]. An equivalent situation does not appear to exist in humans, with sporadic records of biologically active variations within the protein-coding regions: homozygous frameshift mutation in codon 133 [133], rare missense muta-

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_6

tions F17L, and V110M [56]. Otherwise, common variants have been reported in the 5′ flanking region and the promoter region, including rs7799039 (G-2548A), rs2167270 (A19G), and rs791620 (C-188A) [115, 121, 138]. In particular, the variant form G-2548A is associated with increased levels of serum leptin and significant intensification in its peripheral biological activity [82, 157, 196].

LEP gene encodes in humans a 167-amino acid polypeptide with a molecular weight of 16 kDa [92]. Cleavage of the signal peptide (AA 1–21) yields a non-glycosylated, mature 146-amino acid protein that is secreted as hormone. In the second half of '900, studies using two different obese mutant mouse models (the *ob/ob* and the *db/db*) permitted to suggest the existence of a blood factor involved in the maintenance of energy homeostasis and body weight. Only in 1994 and 1995, the *ob* and the *db* genes were cloned, respectively, opening a very productive period of studies that clarified the association between leptin and obesity [169, 203]. Structurally leptin belongs to long-chain class I cytokine superfamily, the same category that includes somatotropin (GH), prolactin (PRL), erythropoietin (EPO), and different interleukins [119]. Protein crystallography has demonstrated that leptin has a compact tertiary structure characterized by a double helix structure formed by two pairs of antiparallel α-helices. The correct three-dimensional structure is maintained by a disulfide bridge which involves two cysteine residues (Cys96 and Cys146) that are fundamental for receptor binding [118]. The numerous exposed hydrophobic residues increase the tendency for self-association and aggregation of the molecule, and they play an important role in receptor binding. Early studies have indicated that circulating leptin is bound to different proteins, frequently of unknown identity and biological function [51, 87]. A more recent study has confirmed the presence in human blood of several serum leptin-interacting proteins, including C-reactive protein for which has been proposed an important role in leptin resistance [38]. However, the best characterized binding protein is the soluble leptin receptor that binds leptin in a 1:1 ratio [49]. It is to be considered that blood leptin measured by common laboratory techniques includes a variable fraction between 10 and 40% bound to soluble receptor, which may render this fraction biologically inactive [98].

6.1.2 Leptin Receptors

Leptin transmits the signal through the interaction with its transmembrane receptor (LepR), which shows a remarkable structural similarity with the class I cytokine receptors [169]. This class includes the receptors for IL-12, IL-6, and IL-11, in addition to those for prolactin and erythropoietin. Three common exonic polymorphisms in the LEPR gene, namely, the polymorphisms K109R, Q223R, and K656N, are associated with amino acid substitutions in the extracellular region of LEPR and have functional consequences [40]. In particular, the Q223R substitution in exon 6 is located in leptin-binding site, and it influences receptor function, with a significant reduction in receptor cell surface expression and changes in signal transduction [39]. Other allelic variations in the coding and noncoding sequences of the LEPR gene have also been reported, some of which cause silent changes or represent rare mutations [36, 40, 57, 75].

Human and mouse LEPR share 78 percent homology [133]. The LepR is encoded by the LEPR gene located on chromosome 1p31.3 and is produced in different variant forms, all due to alternative splicing phenomena. These variants defined LepRa, LepRb, LepRc, LepRd, LepRe, and LepRf share the extracellular domain of 805 amino acids, and thus, they have a similar binding affinity for leptin [110, 183]. As expected, LEPRb-deficient *db/db* mice, and *db3J/db3J* mice, which are deficient of all forms of leptin receptors, display the obesity phenotype similar to that in leptin-deficient *ob/ob* mice [14]. Also, the transmembrane domain of 34 amino acids is common to all isoforms, except for the LepRe, while the intracellular domain is variable and characteristic for each of the isoforms. LepRe that lacks the transmembrane and intracellular domains is considered the secreted isoform, and

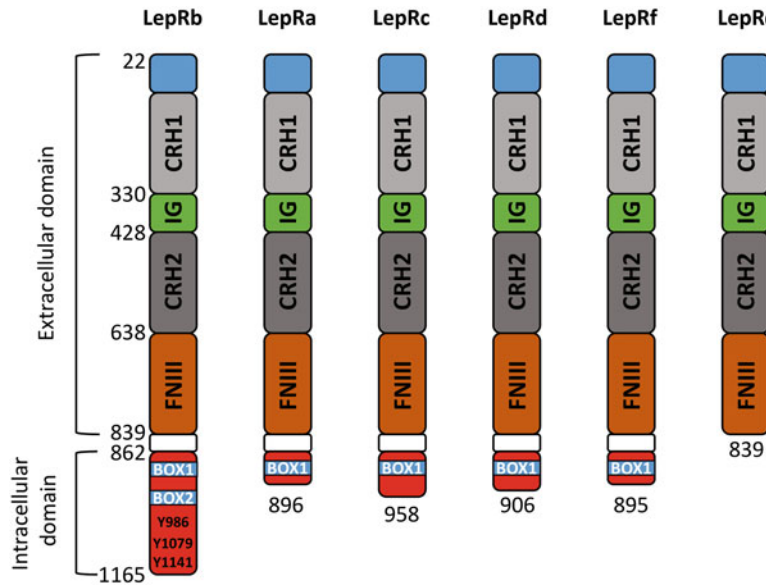

Fig. 6.1 Secondary structures of leptin receptor family with the indication of the main domains. The total number of amino acids and the length of selected regions are shown for each isoform. The intracellular domain of LepRb contains two proline-rich motifs (BOX) and three phosphorylated tyrosine (Y) residues necessary for signaling. CRH, cytokine receptor homology; IG, Ig-like domain; FNIII, fibronectin III domain; BOX, proline-rich motif

it is produced at a sufficiently high level to be an effective transporter of free circulating leptin, modulating its half-life and availability for tissues [113]. The receptor LepRe is present in tissues secreting leptin, such as white adipose tissue (WAT) [24]. Using the length of the intracellular domain as a parameter, the transmembrane receptor isoforms can be classified into two classes: long (LepRb) and short receptors (LepRa, LepRc, LepRd, and LepRf) with these latter that have only 30–40 cytoplasmic residues (Fig. 6.1). Several experimental data suggest that LepRs are present at the membrane level as inactive dimers or oligomers [18]. The interaction with leptin is necessary for the further aggregation of functional complexes Lep/LepR and activation of the different transduction pathways of the signal [49]. The intracellular sequence of the isoforms has a different amino acid composition in consequence of differential splicing downstream of exon 17, except for the first part that contains for all isoforms a proline-rich motif, known as box 1, that is necessary for the interaction and activation of Janus Kinases (JAKs). The long isoform LepRb, expressed at high levels in the hypothalamus, has been initially considered the only functional variant, and it is the only isoform that contains in its intracellular sequence a second interaction motif (box 2) and several phosphorylation sites (Fig. 6.1). The presence of box 1 and box 2 binding domains permits to recruit and bind JAK kinases and to fully activate signal transducers and transcriptional (STAT) activator axis. Short LepR isoforms, although lacking the long cytoplasmic domain, are able to mediate signal transduction via JAK; however, this capacity is considered weaker with respect to LepRb [21]. As predictable by the similar extracellular domain, experimental data suggest that lepR may form heteromers at the cell surface, and thus, the presence of lepRb in the heteromers may expand the signaling capacities of short LepR isoforms [6].

In addition to intracellular signaling, leptin receptors are involved in a complex and only partially elucidated, role in transporting leptin. In fact, it is well known that the common intracellular domain is involved in mediating endocytosis of leptin by a coated pit-dependent mechanism [175]. As suggested by the high levels of leptin that have been detected in the plasma of *db/db* mice as compared with wild-type mice, these gatekeeper functions could be mainly exerted by short leptin receptors [60]. This hypothesis is supported by the fact that only a minor fraction of LepRs is expressed at the cell surface, while the majority is found in intracellular compartments

[16]. LepRa and LepRc are highly expressed in the choroid and microvessels, where they play an important role in the uptake of leptin and its transport through the blood-brain barrier [84]. It was also proposed that LepRs are involved in the exocrine metabolism of leptin. In fact, gastric mucosa cells secrete leptin bound to soluble form of LepRb generated by convertase 7, and this complex is resistant also to degradation by gastric juice [30].

6.1.3 Leptin Signaling

The homology of LepRs with class I cytokine receptors is confirmed by the activation of canonical signal transduction pathways, including the JAK/STAT axis and mitogen-activated protein kinases (MAPKs) axis [15]. However, with respect to other cytokine receptors, the activation of LepRs has peculiar aspects. In fact, the current model suggests that leptin first binds with high affinity the cytokine receptor homology domain (CRH2) and then engages a second receptor through the Ig-like domain of LepR. The presence of a receptor-receptor binding site, FN III domain, can lead to additional interactions and the formation of Lep-LepR complexes [130]. The JAK/STAT transduction pathway has been identified as one of the main signaling pathways activated by leptin [15]. Importantly, only the long isoform, LepRb, contains the intracellular motifs required for the full activation of the JAK/STAT system [15]. Leptin-dependent LepR oligomerization promotes binding and activation of JAK2 (Fig. 6.2). Both box 1 and box 2 motifs in the membrane-proximal portion of LepRb are necessary for JAK2 binding, and thus, short receptors cannot activate JAK-STAT signaling with the same modalities [70, 111]. JAK2-LepRb complex, in turn, can phosphorylate the three tyrosine residues Tyr986, Tyr1076, and Tyr1141 [188] (Figs. 6.1 and 6.2). Each of these phosphorylated tyrosine (pY) residues represents a Src homology 2 (SH2) binding motif that recruits specific SH2-containing effector proteins to the receptor. The short forms of the LepR are incapable to generate the full transduction signaling described for LepRb. However, they are not completely devoid of signaling activity and preferentially activate ERK independently of Y985, likely via direct binding of growth factor receptor-bound protein 2 (Grb2) to JAK2 [21, 205].

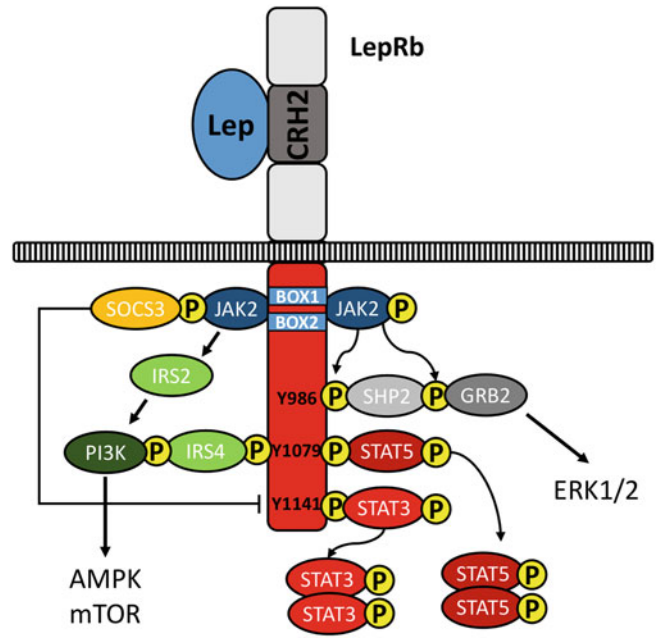

Fig. 6.2 Schematic of possible signaling pathways activated upon leptin (Lep) binding to LepRb. For clarity, only one receptor is shown; however, the activation of the downstream signaling is associated with the formation of multimeric complex receptor/ligand on the plasma membrane. Yellow circle with P indicates the phosphorylation needed for the activation of signaling cascades. The protein Socs3 acts as inhibitor blocking phosphorylation of Y1141. The signaling pathways are described in the text

Phosphorylation of Tyr986 is necessary for binding of SH2 domain of protein tyrosine phosphatase-2 (SHP2) [19], which upon JAK phosphorylation mediates the leptin-stimulated activation of the extracellular signal-regulated kinase (ERK) pathway [144]. Phosphorylated SHP2 recruits the adaptor protein Grb2 which then leads to the activation of ERK1/2 [144].

SHP2 may downregulate the JAK2/STAT3 pathway under some conditions [104]. As deletion of the SH2 gene in the brain causes early-onset obesity in mice, this event suggests that the SHP2 pathway is important in mediating the anti-obesity effects of leptin [83]. Pharmacological inhibition of the ERK pathway is also known to reduce food intake in mice and thermogenesis in mouse brown adipose tissue, further indicating that the SHP2/ERK pathway is related to thermogenesis and anti-obesity action of leptin [144].

An alternative pathway involves leptin-mediated phosphorylation of Tyr1076 via JAK2 activity, binding of STAT5, and its phosphorylation [136]. Phosphorylated Tyr1138 also contributes in part to the activation of STAT5 [73]. Deletion of STAT5 in the central nervous system (CNS) causes hyperphagia and obesity, whereas the activation of STAT5 in hypothalamic neurons reduces food intake in mice [101]. In response to the presence of leptin, the JAK2-LepRb complex phosphorylates Tyr1141 which then binds STAT3 [185]. Subsequent phosphorylation of STAT3 by JAK2 allows the formation of STAT3 dimers and their nuclear translocation [177] (Fig. 6.2). In the cell nucleus, STAT3 dimer stimulates the transcription of target genes, including suppressor of cytokine signaling 3 (SOCS-3) that represents a negative feedback on LepR activation by inhibiting the phosphorylation of tyrosine residues by JAK2 [34, 193]. SOCS-3 binds to LepRb Tyr985 and mediates negative feedback by directly inhibiting JAK2 activity and/or targeting the receptor-JAK2 complex for proteasomal degradation [20, 22].

Although phosphatidylinositol 3-OH kinase (PI3K) activation is not necessary for leptin-mediated regulation of body weight, PI3K pathways mediate some leptin effects on proopiomelanocortin (POMC)-expressing neurons [85]. The activation of PI3K is mediated by insulin receptor substrate (IRS) family, with different modalities. The activation of JAK2 permits the recruitment of IRS2 via SH2B1 without a direct involvement of phosphotyrosine sites [55]. On the contrary, the binding of IRS4 is dependent on the phosphorylation of the Y1079 motif of the LepRb, in line with its function as an adaptor protein [187]. Downstream of PI3K/Akt, there is an important nutrient sensor that has been associated with leptin activity, the mammalian target of rapamycin (mTOR), and its activation has been described as mediator of anorexigenic effects of leptin [23].

An association between leptin and AMP-activated protein kinase (AMPK) also exists. However, data suggest a dual potential effect of leptin on AMPK. On one side, leptin decreases hypothalamic AMPK activity contributing to reduce appetite and body weight [127]; on the other side, leptin stimulates AMPK activity on the skeletal muscle [128]. Activation of STAT3 and PI3K is required for the inhibition of AMPK by leptin [127], while SOCS-3 inhibits leptin activation of AMPK in muscle cells [163]. The activation of AMPK in muscle is associated with acetyl coenzyme A carboxylase (ACC) and increased lipid oxidation [128].

6.2 Source of Leptin in Tumor Microenvironment

6.2.1 Leptin from Blood

In addition to storing energy, WAT represents an endocrine organ, secreting more than 50 different adipokines, a heterogeneous family of cytokines including leptin. Leptin is produced by adipocytes within WAT, and this phenomenon is strategically justified by the role of leptin as energy metabolism controller, with a prevalent function of negative feedback signal in the regulation of the body weight. Circulating leptin concentrations are in proportion to body fat mass, and in particular, leptin is produced in adipose tissue in proportion to triglyceride stores. The

role of leptin as critical indicator of an organism's long-term energy status is evidenced also in its name that was taken from the Greek word "leptos," which means "thin" [60]. In humans, the secretion of leptin by fat cells is a signal to the hypothalamus of the presence of sufficient energy reserves [123]. Thus, circulating leptin levels fluctuate in accord to changes in nutritional states, and plasma leptin levels are decreased by fasting before fat depletion [1]. However, now we know that the control of leptin secretion by adipocytes is more complex. In fact, leptin is secreted in a pulsatile fashion and also displays a circadian rhythm. Adipocytes release leptin monitoring both exogenous glycaemic and lipid status and also intracellular energy signals, including cAMP [29, 158, 167]. In addition, it is possible that other tissues than adipose tissue participate in rapidly increasing plasma leptin levels. In fact, on fasting and refeeding, gastric endocrine cells secrete leptin, which may account for the rapid fluctuations in plasma leptin [7]. Subcutaneous adipose tissue, contained in the abdominal, gluteal, and femoral depots, was demonstrated to be a more important contributor for serum leptin with respect to visceral adipose tissue, because of both its predominant mass and its higher releasing activity [126, 178]. However, some studies failed in finding significant relationship between leptin and fat distribution [17]. The serum leptin concentration is below 20 ng/mL in normal weight subjects, but it is up to four times higher in obese subjects [41]. Although leptin concentration is strongly regulated by fat depots, age and sex have been described as significant co-variables. Age is associated with decline of serum leptin [140]. Interestingly, 17beta-estradiol is able to increase leptin release from adipose tissue in women, an effect that is antagonized by antiestrogen drugs, and that could explain the sexual dimorphism of leptinemia in humans, with leptin levels that are 40% higher in women than men [117]. However, in older adult women, who have higher mean values with respect to men (16.5 vs. 5.7 ng/mL), serum leptin concentrations could be explained also by higher percentage body fat. In addition, higher leptin concentrations are usually measured in blacks than in whites (from 1 to 5 ng/mL higher) [148].

Leptin crosses the blood-brain barrier (BBB) through a partially saturable mechanism, and reduced brain permeability may contribute to leptin resistance in obese subjects. While leptin has a direct access to mediobasal hypothalamus, central brain permeability is mediated by leptin receptors expressed by brain endothelial cells [50]. The direct contribution of tissues other than adipose tissue to prolonged serum leptin is marginal; however, pharmacokinetics studies provided evidence on the high abundance of leptin distributed throughout the peripheral tissues with small intestine containing the highest concentration of leptin, and skin, muscle, heart, caecum, and brain with the lowest [86]. These studies also demonstrated that leptin has a prolonged half-life in human blood and that peripheral tissues could represent an additional pool of tissue binding sites. Indeed, early studies have demonstrated, although in absence of strong evidence about a direct production of leptin in the brain, that women and obese men have higher concentrations of leptin in the internal jugular vein than in arterial blood, suggesting an important cerebral release of leptin after prior central nervous system uptake [189].

6.2.2 Autocrine Leptin in Peripheral Tissues

Leptin concentration in tumor microenvironment could be higher than concentration of leptin found in the blood, and this is the reason for a local production of the hormone or the uptake of leptin from the blood. Indeed, complex and only partially explored peripheral functions are described for different tissues suggesting for leptin the possibility for an autocrine role in cancer tissue. Paracrine and autocrine leptin signaling may be an underestimated event in tumor microenvironment. Breast cancer is one of the most studied cancer models for its effective responsiveness to leptin, and the overexpression of leptin and its receptor in the healthy tissue surrounding breast cancer is associated with a high propensity for metastasis [90]. Adipocytes remain the first source of leptin, and although subcutaneous adipose tissue is thought to be the main systemic

modulator of leptin levels, a variety of adipose depots can exert regional control on energy signaling. Visceral adipose tissue surrounds vital organs while numerous smaller fat tissue accumulations are present throughout the body. Adipose tissues surrounding organs seem equally able to produce leptin than subcutaneous adipose tissue [202].

Periorgan adipose tissue. Carcinomas can engage in a direct interaction with adjacent adipose tissue after the invasive phase, while liposarcomas develop just in the fat tissue. When cancer cells invade periorgan adipose tissue, this latter contributes to creating the tumor microenvironment, participating in tumor progression and metastasis. Both mature adipocytes and lipoblasts express leptin in well-differentiated liposarcoma [139]. The prostate, primary site of one of the most common cancers in men, is surrounded by periprostatic adipose tissue that is invaded by cancer cells in the stage of extraprostatic extension [166]. Adipocytes in the peripheral zone of the prostate through the release of soluble factors support the directed migration of prostate cancer cells facilitating the extraprostatic extension [100]. In a rat model, adipocytes from perirenal adipose tissue secrete physiological relevant amount of leptin [105]. In addition, leptin has been associated with the ability of conditioned media of human perirenal adipose tissue to stimulate proliferation and migration of tumor renal cell lines [31].

Mammary tissue. Leptin mRNA is detectable in different cells resident in mammary tissue, including human epithelial cells [161]. Adipokines can be found in human breast milk, and milk leptin was proposed as an important modulator of weight gain in breast-fed infants [129]. Leptin concentration in human milk is directly correlated with maternal serum leptin and adiposity; however, other local sources could not be excluded. In fact, adult female breast tissue is composed mainly of mature adipocytes and preadipocytes (>50% volume), and during development, the highest concentration of tissue leptin in mouse is associated with the highest extension of fat pad [109]. The intimate interactions between epithelial and adipose cells could regulate homeostasis of the tissue also by involving the release of adipokines from adipose cells. In particular, leptin plays an important role in stimulating the proliferation of normal breast epithelial cells by activating LepRb, acting as a promoter of gland development and eventually of malignant transformation [88, 109].

Perivascular adipose tissue. The functional properties of perivascular adipocytes remain largely unexplored. However, data suggest that periadventitial adipose tissue is an active component of the vasculature, and it can control arterial tone by modulating the activation in vascular smooth muscle cells [180]. Perivascular adipose tissue expresses biologically active substances that can work in both paracrine and autocrine manner. Among these secreted factors, there are different adipokines, including leptin. Rat periaortic adipose tissue expresses about 30 ng of leptin per mg of proteins [63].

Cancer-associated adipocytes. Adipocytes in tumor microenvironment are able to actively interact with breast cancer cells; in fact, cancer cells induce phenotypic changes in fat cells, and for these adipocytes, the term "cancer-associated adipocytes" (CAAs) was proposed [54, 186]. Markers of lipid content and differentiation were reduced when CAAs were co-cultured with human breast tumor cells. Conversely, the expression of adipocyte-inflammatory cytokines and proteases was increased [54, 168]. Phenotypic characterization of CAAs is controversial, and for example, in breast cancer, there are evidence of both greater differentiation of preadipocytes into adipocytes [47] and inhibition of the differentiation of preadipocytes into mature adipocytes [150]. Leptin mRNA is absent in preadipocytes, and it becomes detectable only in terminally differentiated adipocyte [103]. However, also preadipocytes can sustain cancer progression through the release of pro-inflammatory cytokine and de novo expression of leptin [160]. This effect could be particularly relevant in tumor microenvironment considering that high concentrations of leptin in

vitro stimulate proliferation of preadipocytes, and thus sustaining a positive feedback [116].

Bone marrow. Adipose tissue is abundant in bone marrow, and it increases from 40% at age 30 to 68% at 100 years [94]. Adipocytes in bone marrow are derived from mesenchymal stem cells (MSCs) depending on the activation of phenotype-specific transcription factors; however, there is much uncertainty about the microenvironment events that favor this type of differentiation and also about the functional role of adipocytes in bone marrow. A recent hypothesis suggests that adipocytes can play an important local role as the source of energy metabolites and paracrine factors, for example, modulating hematopoiesis [197]. Bone marrow adipocytes are able to secrete leptin [147] that could locally promote osteoblast formation and hematopoiesis [175]. Systemic changes in energy metabolism can affect also bone fat mass; however in conditions of decreased calorie intake, the content of fat in bone is increased, in opposition to a decrease in the amount of peripheral fat [48]. Bone marrow is a common metastatic site of breast and prostate cancers, and osteotropic tumor cells have been shown to be attracted to and to take a direct contact with fat cells within the bone marrow [25].

Mesenchymal stem cells. MSCs are multipotent stromal cells that reside in various tissues, mainly in bone marrow and fat. MSCs can differentiate in vitro into various cell lineages, including osteocytes, chondrocytes, and adipocytes. Data available suggest that leptin is an important modulator of MSCs phenotype and fate. It was proposed a model in which leptin effect is dependent on the differentiation state of MSCs. LepR represents a marker of bone marrow MSCs, and LepR-positive cells give rise to most bone and adipocytes in adult bone marrows, including bone regenerated after injury [204]. Leptin treatment of bone marrow MSCs in vitro is associated with increased mineralization and osteoblast differentiation [151]. Current in vivo data support a role of leptin in both bone and adipocyte formation from MSCs. In fact, other authors demonstrated that leptin was a physiologic effector of MSCs that decreases osteoblastogenesis while increasing marrow adipogenesis [199]. In addition, in some situations, leptin contributes to maintaining MSCs in an undifferentiated state. More recently, MSCs have become an important focus in oncology because they have been found to migrate toward and incorporate into the tumor from both bone marrow and remote fat depots. MSCs have an active role in cancer progression, promoting epithelial-mesenchymal transition and metastasis through paracrine signaling [97]. Numerous growth factors and cytokines secreted by MSCs that are essential for regulating the homeostasis within the stem niche for hematopoietic stem cells can sustain cancer progression. Perivascular MSCs are positive for LepR, and they are important in creating a specific stem niche releasing stem cell factor (SCF) and CXCL12 [52, 53]. MSCs isolated from adipose tissue express leptin, mainly when MSCs were isolated from obese patients. In the same study, it was demonstrated that leptin was the main determinant of a more aggressive phenotype associated with metastasis, in estrogen-positive breast cancer cell lines [164]. Also in another study, obesity has been indicated as modulator of gene expression profile in adipose MSCs, upregulating leptin expression [165]. Interestingly, bone marrow LepR-positive MSCs were identified also as precursors of myofibroblasts [45]. Indeed, the differentiation toward myofibroblasts and secretion of proangiogenic and fibrotic cytokines is one of the promotion mechanisms proposed for MSCs homed in tumor microenvironment.

Cancer-associated fibroblasts. Fibroblasts secrete low levels of leptin in physiologic conditions. Significantly, leptin secretion by fibroblasts is modulated by several stimuli. Insulin stimulates leptin synthesis in human skin fibroblasts in a dose-dependent manner [72]. In a rat model of renal interstitial fibrosis, activated fibroblasts secrete about tenfold more leptin with respect to control fibroblasts [108]. Fibroblasts are the most abundant component of tumor stroma, where they acquire a new phenotype associated with the secretion of various growth factors. Also, cancer

environment is able to stimulate leptin secretion in associated fibroblasts. ELISA measurement in conditioned medium from cancer-associated fibroblasts isolated from biopsies of primary breast tumors showed that leptin levels were about 2 ng/mg protein [71] or 10 ng per $2^{*}10^{5}$ fibroblasts [13]. Thus, leptin was proposed as one of the growth factors able to mediate tumor-stromal interactions in different cancers, such as breast cancer [13].

Cancer cells. Leptin is expressed also by cancer cells, and the highest degree of expression was associated with poorly differentiated, metastatic cancers. The analysis of the relative mRNA expression in pan-cancer panel demonstrated that leptin expression is highly found in bladder cancer, breast cancer, large B cell lymphoma, lung cancer, ovarian cancer, pancreatic cancer, and testicular cancer. In addition, in breast carcinoma, colorectal adenocarcinoma, gastric cancer, ovarian cancer, and brain cancer, the expression of leptin mRNA is correlated with poor patient survival [107]. Garofalo demonstrated by immunohistochemistry the overexpression of leptin in malignant breast epithelial cells when compared with normal breast tissues [65]. Leptin was expressed in a diffuse cytoplasmic pattern in all subtypes of liposarcoma [139]. Leptin mRNA expression was detected also in prostate cancer tissue, and it was higher than in subcutaneous adipose tissue [202]. Other malignant cell types express leptin, including endometrial [74], pancreatic [81], gastroesophageal [9], and colorectal carcinoma [142].

6.2.3 Regulation of Leptin in Tumor Microenvironment

Different pathologic adaptations in tumor microenvironment can enhance local leptin secretion. A pro-inflammatory state, frequently associated with tumor progression, is considered a stimulus sufficient to upregulate the secretion of leptin from adipocytes and to increase its serum levels [58, 79, 99]. Leptin is considered an inflammation mediator, and its regulation is under the control of pro-inflammatory cytokines, including TNF-α and IL-1β. Thus, leptin may participate in maintaining a chronic inflammatory state in tumor microenvironment. Leptin has an important peripheral role as immunomodulatory factor, and indeed, several inflammatory cytokines provide a positive feedback mechanism, modulating the synthesis and secretion of leptin. Surprisingly, also preadipocytes, which do not express leptin, are able to mount an effective inflammatory response, releasing leptin following stimulation with pro-inflammatory cytokines [160]. IL-6 increased leptin production in both omental and subcutaneous adipose tissue, and because IL-6 is produced in a paracrine manner from stromal/-vascular fraction of adipose tissue, this mechanism may contribute to hyperleptinemia associated with obesity. In fact, the local concentration of IL-6 in the adipose tissue was estimated to exceed plasma levels [162, 172].

Leptin expression is regulated by several hormones, including insulin, glucocorticoid, and leptin itself. Women have higher leptin levels than men because of an increase in leptin expression in subcutaneous adipose tissue, stimulation of leptin synthesis by estrogen, and inhibition of leptin synthesis by testosterone [91]. Estrogen stimulates directly leptin expression, as demonstrated in mammary tissue by increased leptin concentration after estrogen administration [135]. A tangled relationship between leptin and insulin exists. Obesity and insulin resistances frequently coexist in diabetes, and leptin has been proposed as critical factor in controlling peripheral glucose and insulin responsiveness [3]. In parallel, in human adipocytes, insulin stimulates in a dose-dependent and reversible manner, the rise in leptin protein [181]. Leptin secretion is stimulated by insulin through a transcriptional mechanism mediated by Egr1 [131] and posttranscriptional mechanism mainly mediated by PI3K/mTOR pathway [201]. Thus, hyperinsulinemia may be an important determinant in the control of leptin production also in CAAs, determining an enrichment of local leptin concentration.

Hypoxia is an important regulator of leptin expression in adipose tissue, inducing the

transcription of LEP gene in mature adipocytes. Interestingly, incubation under hypoxia resulted in marked de novo synthesis of leptin also in the preadipocytes that do not normally produce the hormone [182]. This evidence was repeated in other cell lines, including trophoblast-derived cells [77] and breast cancer cells [35]. Sequence analysis revealed the presence of several putative hypoxia-responsive elements (HREs) within the first sequence of the human LEP promoter [76]. Cultured adventitial pericytes release high amounts of leptin following exposure to hypoxia, suggesting the existence of an underestimated role of leptin in vascular repair [146]. In addition, the release of leptin under hypoxia may contribute to the long-term stabilization of HIF-1α in cancer cells [28].

Angiotensin is implicated in systemic cardiovascular function and electrolyte balance, but it is involved locally in many tissues in mediating cellular homeostasis and proliferation. There are many local renin-angiotensin systems in tissues that permit the secretion of autocrine and paracrine angiotensin II [64]. A paracrine renin-angiotensin system mechanism has been identified also in many types of cancer [68]. Physiologic doses of angiotensin II significantly increase leptin secretion in human adipocytes [96]. This effect is mediated through ERK1/2-dependent pathway and includes the angiotensin II type 1 receptor subtype [68]. Angiotensin II increases leptin synthesis in both vascular smooth muscle cells and rat portal vein [69]. In a rat model, the treatment with angiopoietin II stimulated leptin release from differentiated adipocytes [105].

6.3 Role of Leptin in Tumor Microenvironment

6.3.1 Cell Targets of Leptin

Different studies reported significant dissimilarities in the biological peripheral effects of leptin. Concentration and source of leptin but also the species from which the cells were derived have contributed to these discrepancies. An important aspect is represented by the amount of leptin used in the experiments. Leptin is utilized at concentration that varies of 1000-fold, and sometimes, biological effects have been described as opposite for different concentrations. It should be considered also that some studies have been conducted with leptin concentrations that could not be reached in human serum.

Leptin receptor, mainly the long isoform, is expressed at high concentrations in the arcuate nucleus of the hypothalamus [155] where its activation suppresses food intake and stimulates energy expenditure. The CNS, particularly the hypothalamus, is believed to be the main leptin target and mediates leptin's anti-obesity action [61]. Hypothalamic leptin action promotes negative energy balance and modulates glucose homeostasis, as well as serving as a permissive signal to the neuroendocrine axes that control growth and reproduction. Body weight is determined by the amount of energy taken in relative to energy expenditure [154]. Leptin acts via LepRb to stimulate the synthesis of POMC neurons that generate α-melanocyte-stimulating hormone (α-MSH) [42]. α-MSH reduces body weight by binding to and activating melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) [154]. MC4R knockout mice develop leptin resistance and obesity, and MC3R null mice also exhibit the obesity phenotypes, but to a lesser degree [27].

The functions performed by leptin are not limited to the hypothalamic level and do not exclusively concern the maintenance of energy homeostasis [11]. Leptin, in fact, is involved in various ways in interfering in numerous physiological processes such as regulation of metabolism, growth, development, modulation of endocrinological and immunological processes, reproduction, cardiovascular pathophysiology, and maintenance of respiratory function. The pleiotropic role of leptin hormone is demonstrated by the expression of LepR on many cells that actively participate in tissue homeostasis: endothelial and vascular smooth muscle (VSM) cells [152, 159]; stem cells including embryonic stem cells, in-

duced pluripotent stem cells, bone marrow MSCs, and pericytes [53, 59, 146, 204]; and immune cells [200]. In addition, the expression of LepR was associated with the physiology of various organs such as the kidney [156], liver [89], and lung [173]. Among the short isoforms, LepRa is highly expressed in the peripheral tissues [48]; however, weak staining also for LepRb was detected in the same tissues [44]. The long isoform is present in immune cells [200], in the medulla of the adrenal gland, and in the inner zone of the medulla of the kidney. However, there are numerous reports about the expression of the short isoforms of leptin receptors in peripheral tissues, including the kidney, liver, and lung [89]. Thy-1 positive human skin fibroblasts express both leptin and leptin receptor [72]. Cancer-associated fibroblasts also express LepRb, but they do not express LepR short isoforms [13]. LepRb is expressed on human endothelial cells both in vivo and in primary cultures [159]. Interestingly, LepRb is overexpressed in hepatic stellate cells in consequence of several types of different liver stressors suggesting that leptin is a direct modulator of energy metabolism during stress [28, 89]. Indeed, also adipocytes express both forms of leptin receptors, and leptin has been shown to directly control lipid metabolism in these cells [95]. In fact, physiological concentrations of leptin rapidly suppress fatty acid synthase expression in both adipocytes and preadipocytes [8, 184]. In parallel, there is an increased fatty acid oxidation [184]. These enzymatic modulations that have been described in isolated cells to short-time exposure to leptin are not associated with the release of fatty acids, confirming that leptin could control basal lipid turnover.

6.3.2 Vascular Effects of Leptin

One of the best known and effective peripheral effects of leptin is modulation of vasculature physiology. New vessels are a major component of tumor microenvironment. Cancer growth determines endothelial cell recruitment and formation of new vasculature, primarily through the activation of angiogenesis. Novel vessels fuel cancer cells, and they represent a new potential way for cancer spreading. However, endothelial cells can contribute to tumor progression also by other mechanisms, including extracellular matrix remodeling and inflammation. Indeed, leptin is highly vasoactive, and thus, it can play an indirect key role in tumor progression. First, physiologic concentrations of leptin stimulate endothelial cell growth and angiogenesis [159]. Proangiogenic effect of leptin is synergic with fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) [33]. The proangiogenic role of leptin is realized through mechanisms that depend on LepRb, p38MAPK, and PI3K. However, blockade of cyclooxygenase-2 (COX-2) and VEGFR2 activities abolished the leptin effect on endothelial cells [67]. The secretion of extracellular matrix proteases by endothelial cells is necessary for the formation of new vessels, and the release of gelatinases metalloproteinase-2 (MMP2) and MMP9 is stimulated in endothelial cells by leptin [143]. These gelatinases have been associated with matrix remodeling and invasion ability in different carcinomas.

In addition, leptin is able to modify the physiology of formed vasculature through both acute and chronic effects. In order to understand these effects, we have to consider also the action of leptin on cells annexed to vessel, and in particular smooth muscle cells. As short-time mediator, leptin is able to induce vasodilation and increased vascular permeability. In fact, leptin increases the levels of endothelial nitric oxide (NO) synthase (NOS) and NO, which is a major vasodilator [102]. Interestingly, stimulation of NO by leptin was reported also for circulating endothelial cells [153]. NO production was mediated by Akt-eNOS phosphorylation pathway [178]. In addition, the upregulation of COX-2 in endothelial cells by leptin is associated with the release of prostaglandins, PGI2 and PGE2, and contributes to vasodilation, but also to sustain the inflammation in tumor microenvironment [67]. COX-2 induction is a common effect of leptin and is described also in cancer cells and in immune cells. A vasorelaxive effect of leptin mediated by smooth muscle cells was also observed [132]. Leptin seems to have a role similar to VEGF

in the stimulation of vascular permeability with the increase of endothelial fenestrations [33]. The stimulation of vascular permeability is compatible with the physiologic role of secreted leptin in adipose tissue, where this process facilitates the release of adipokines in the circulation. The high active remodeling needs of the adipose tissue are also compatible with a role of leptin in increasing mobilization of vascular progenitor cells from bone marrow [153]. This latter effect has been demonstrated to play a critical role in melanoma growth [4].

Leptin seems to realize also long-term effect on vasculature, determining a prolonged action on endothelial function. Elevated concentrations of leptin stimulate the persistence in vitro of endothelial tubules through SIRT1 activation and HIF-2α stabilization [46]. In addition, leptin has been shown to induce oxidative stress in endothelial cells by increasing the formation of reactive oxygen species [194]. In parallel, leptin upregulates various mediators of vascular inflammation, including TNF-α, IL-2, TGF-β, and MCP1 [190, 194].

6.3.3 Leptin Modulates Sex Hormone Metabolism

Leptin represents a necessary signal to properly regulate female reproduction; in fact, *db/db* mice and specific LepR mutants mice show an infertile female phenotype [173]. The actions of LepR in the control of reproductive function are thought to be exerted mainly via the hypothalamic-pituitary-gonadal axis. However, several data indicate that leptin has also a role in peripheral sex hormone synthesis. Indeed, leptin has a direct complex effect on ovarian functions. In fact, it has been associated with both inhibition of steroidogenesis and stimulatory effect on the ovary [12, 174]. These discrepancies may derive from differences in the doses of leptin, species, and age. Further evidence for the involvement of leptin in the estrogen paracrine action is provided by its capacity to induce CYP19A1 synthesis in stromal cells isolated from the subcutaneous fat and breast adipose tissue [198]. In addition, there are several data about a direct role of leptin in sex hormone metabolism of cancer cells. In fact, leptin stimulates aromatase expression in human breast adipose stromal cells through the regulation of liver kinase B1(LKB1)/AMPK pathway [120]. There is a bidirectional interplay between LepR and estrogen receptor alpha (ERα), in which the two signaling axes can potentiate each other [62]. Thus, it was hypothesized that systemic/local upregulated levels of leptin may contribute to antiestrogen resistance in breast cancer [66]. On the other hand, the crosstalk between the two signaling pathways may explain the increased sensitivity of estrogen-positive breast cancer cells to leptin with respect to estrogen-resistant cells.

6.3.4 Leptin Modulates Innate and Adaptive Immunity

Immune cells are active characters in cancer tissues. Innate and adaptive immunity are involved in the physiological protection against carcinogenesis, and many current anticancer therapies aim to reactivate immune response against cancer cells. The immune cells more frequently found in tumor microenvironment are macrophages, neutrophils, myeloid-derived suppressor cells, and CD4+ regulatory T cells. Many of these immune cells actively secrete tissue remodeling cytokines, including TNF-α, TGF-β, IL1, and IL-6 [122]. Data from early studies in mice defective for leptin axis have already suggested its immunoregulatory role. Leptin has a general immunostimulatory and pro-inflammatory activity, and leptin deficiency is associated with impaired phagocytosis and downmodulation of inflammatory immune response [112]. Leptin has a widespread stimulatory effect on immune cells, activating monocytes, polymorphonuclear cells, and lymphocytes. Short-stimulation of human NK cells with leptin increased the expression of the activation marker CD69, IFN-γ, and IL-2 secretion [171]. Considering leptin as a signal of starvation, in that a low serum leptin concentration leads to preserving energy reserves, leptin deficiency is associated with immune suppression and enhanced susceptibility to several infections

[2, 114]. Indeed, leptin contributes to other pro-inflammatory pathologies such as arthritis and nephrotoxic nephritis [26, 170]. Thus, a potential cancer promotion role of leptin through immune cells is represented by the secretion of cytokines and the induction of chronic inflammation. Leptin promotes activation of macrophages potentiating COX-2 and NOS activity [145] and stimulates the release of major pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) [149]. This mechanism is particularly relevant if we consider that inflammatory stimuli, such as TNF-α and IL-1, can increase leptin secretion, inducing a positive feedback that propagates inflammation [125]. IL-1, IL-6, and TNF-α are pleiotropic cytokines that primarily affect the inflammatory and immune response, and increased levels of these cytokines have been regularly implicated in the pathogenesis of acute and chronic inflammatory diseases. Although inflammation represents a hallmark of cancer, these cytokines have numerous, and potentially opposing, biological activities in cancer tissue. However, consolidated evidence links their pro-inflammatory activity to tumorigenesis in numerous experimental models. In addition, IL-6 is considered as the major player in neoplastic progression by supporting angiogenesis and evasion of immune surveillance. TNF-α is involved in many aspects of carcinogenesis and cancer progression, including angiogenesis, invasion, and metastasis [10]. Both TNF-α and IL-6 are frequently detected in biopsies from human cancers, produced at higher levels also by infiltrating cells [195]. Also, IL-1 is frequently detected in tumor tissue, and its upregulation was prevalently correlated with tumor invasiveness and a bad prognosis [5]. Leptin rapidly induces the production of TNF-α and IL-6 in blood mononuclear cells, and this effect is additive to the action of endotoxin [200]. Significantly, leptin stimulation seems independent of the activation status of leukocytes, and biological activity was observed at doses as low as 1 ng/ml [200]. Human leptin stimulates proliferation in a dose-dependent manner and functionally activates human circulating monocytes in vitro, by inducing the production of pro-inflammatory cytokines such as TNF-α and IL-6 [149]. Other sources of IL-6 and TNF-α are dendritic cells [124]. Besides a direct effect on eosinophils, leptin cannot modulate the secretion of IL-1 in immune cells [192]. However, several studies indicate that leptin acts in synergy with IL-1, not only in the modulation of immune response but also in tissue homeostasis [141, 190].

In addition to represent a potent pro-inflammatory mediator, leptin released from tumor tissue represents a chemoattractant for different immune cells, including monocyte/macrophage [78], eosinophils [193], and neutrophil [134], recruiting the inflammatory infiltrate [43]. Leptin-mediated chemotaxis requires the presence of full-length leptin receptor, and it is associated with the activation of several downstream pathways including JAK/STAT, MAPK, and PI3K [78, 134].

6.3.5 Leptin Promotes Fibrosis

Accumulating data suggest that leptin has a critical role in the fibrosis process in multiple organ systems. This was described in the liver, kidney, and lung through different molecular mechanisms. Liver fibrosis is connected with obesity, and leptin was proposed as key profibrogenic mediator. The main cellular mediators of fibrosis are fibroblasts, MSCs, fibrocytes, and stellate cells. In addition, also in the bone marrow, LepR-positive stromal cells can generate fibrogenic cells responsible for pathologic matrix deposition [45]. Fibrosis is associated with increased rigidity of extracellular matrix, and it influences cancer cell morphology, migration, and growth [137]. At the same time, the impact of fibrosis on carcinogenesis, cancer progression, and metastasis is debated with evidence that supports a role as both tumor promoter and suppressor [37]. However, a key mediator of the fibrogenesis program is TGF-β1, a cytokine frequently involved in cancer progression. In particular, TGF-β1 is a major player in epithelial-mesenchymal transition and cancer metastasis. In addition, TGF-β1 can also directly induce immune suppression by inhibiting the T cell response [106]. Local production of TGF-β1 leads to an increase

of collagen and fibronectin production by fibroblasts and the transition of fibroblasts in myofibroblasts. Leptin through LepRb receptor increases the expression of TGF-β1 and TGF-β1-regulated profibrotic genes, in hepatic stellate cells and lung fibroblasts [32, 93]. In stellate cells, leptin promotes liver fibrosis also through the upregulation of procollagen and tissue inhibitor of metalloproteinase-1 [32]. Higher levels of leptin are correlated with the severity of idiopathic pulmonary fibrosis, inhibiting autophagy-dependent collagen degradation. The activation of PI3K/Akt/mTOR pathway by leptin was necessary to inhibit autophagosome formation in human lung cell lines [80].

6.4 Conclusions

Leptin is a central hormone in the control of energy expenditure and food intake. Many tissues, with active energy metabolism, must consider the serum levels of leptin in order to take the right metabolic decisions. This control mechanism is particularly relevant for the immune system for which the leptin constitutes a boost signal for an effective response. New suggestions derive also by a potential role of leptin in regeneration of tissues, with strong evidence about a role in promoting angiogenesis and extracellular matrix remodeling. Also in these contexts, leptin may play the role of sensor of energy state. In cancer tissue, which could be considered a nonhealing wound, leptin can actively fuel the never-ending inflammatory/reparative processes, sustaining cancer progression (Fig. 6.3).

Another important aspect is the local production of leptin. The current knowledge indicates that adipocytes, the primary source of leptin, play an important promoting role also in the cancer microenvironment. Particularly important could be the presence of fat depots in the primary site, e.g., mammary gland, or in the metastatic site, e.g., bone marrow. At the same time, other cancer-associated cells may contribute to the local leptin enrichment, such as activated fibroblasts and MSCs. However, the mechanisms underlying the peripheral expression and action of leptin are largely unexplored. Many data are present in literature about the expression of leptin in different cancer-associated cells, but frequently, the effective in vivo contribution of these cells to local leptin concentration is unknown. The expression of the short isoforms of LepR, mainly individuated in peripheral tissues and in cancer-associated cells, through a function of gatekeeper of leptin, could further contribute to modulate local levels of the hormone.

In conclusion, the new concept of energy sensor for peripheral tissues renders leptin an important modulator of tissue homeostasis. Cancer microenvironment is shaped by elevated levels of leptin in the blood or locally produced, contributing to form an inflammatory and proliferative environment that may promote cancer progression. However, many aspects of peripheral

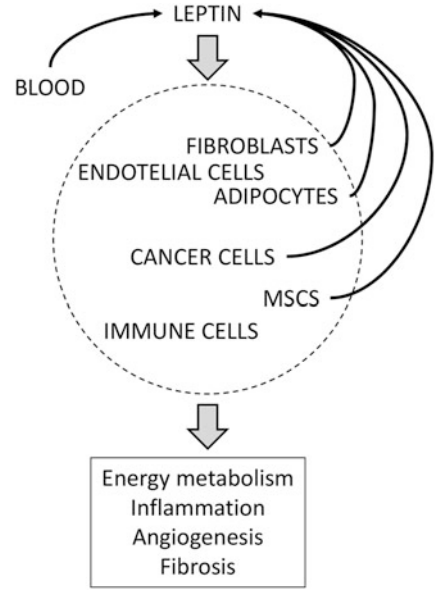

Fig. 6.3 Exemplificative diagram of the role of leptin in tumor microenvironment (circle). Leptin in cancer tissue derives from blood (endocrine) or from resident cells (paracrine, autocrine), including cancer cells, fibroblasts, adipocytes, and mesenchymal stem cells (MSCs). Leptin potentially influences the metabolism of the indicated cells determining several responses, including modulation of energy metabolism, activation of inflammation, stimulation of angiogenesis, and remodeling of extracellular matrix

signaling network regulated by leptin remain to be explored.

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The Immune Consequences of Lactate in the Tumor Microenvironment

7

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Abstract

The tumor microenvironment consists of complex and dynamic networks of cytokines, growth factors, and metabolic products. These contribute to significant alterations in tissue architecture, cell growth, immune cell phenotype, and function. Increased glycolytic flux is commonly observed in solid tumors and is associated with significant changes in metabolites, generating high levels of lactate. While elevated glycolytic flux is a characteristic metabolic adaption of tumor cells, glycolysis is also a key metabolic program utilized by a variety of inflammatory immune cells. As such lactate and the pH changes associated with lactate transport affect not only tumor cells but also immune cells. Here we provide an overview of lactate metabolic pathways and the effects lactate has on tumor growth and immune cell function. This knowledge provides opportunities for synergistic therapeutic approaches that combine metabolic drugs, which limit tumor growth and support immune cell function, together with immunotherapies to enhance tumor eradication.

Keywords

Lactate · Lactic acid · pH · Tumor microenvironment · Anti-tumor immunity · Metabolism · Glycolysis · Immunotherapy · Biosynthesis · Warburg · NK cells · T cells · Macrophages · MCT · Metabolic therapies

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7.1 An Overview of the Tumor Microenvironment and Tumor Metabolism

Human tissues are a complex mixture of parenchymal cells, immune cells, stromal cells, extracellular matrix, and soluble factors cooperating, as components of a healthy microenvironment, to perform the necessary

A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_7

physiological and structural functions of that specific organ. Tumor cells are derived from these healthy cells through accumulation of genetic and epigenetic alterations, which lead to disruption of this finely tuned microenvironment. As a tumor develops, it constantly interacts, physically and through secreted factors, with its neighboring cells, often altering their phenotype and function [1, 2]. The interaction between malignant and non-malignant cells creates a dysregulated microenvironment that promotes tumor growth through a variety of mechanisms. A dynamic network of cytokines, growth factors, and extracellular matrix-degrading enzymes develops, which collectively result in significant alterations in the tissue architecture, dysregulated proliferation, and immune dysfunction [3, 4].

Proliferating cells require a constant supply of biomolecules to replicate cell structures and divide; these include cholesterol, glucose, glutamine, fatty acids, nucleotides, and non-essential amino acids [5]. To meet the metabolic demands of relentless cell division, tumor clones dramatically alter their metabolic activity. Biosynthesis of cellular components during cell division requires a range of carbon intermediates, which are provided primarily by the catabolism of glucose, via glycolysis (Fig. 7.1). The TCA cycle (or Kreb's cycle) and oxidative phosphorylation are the primary sources of cellular energy in quiescent, regulatory, and non-proliferative cells. Tumor cells switch from TCA, which can efficiently generate 28 molecules of ATP per molecule of glucose, to glycolysis, which is far less efficient, but produces key carbon intermediates as by–products. By converting pyruvate to lactate, tumor cells can prevent negative feedback signals and the consumption of NAD^+ during mitochondrial respiration, thereby maintaining constant biosynthesis through glycolysis intermediates [6, 7]. This phenomenon, termed the Warburg effect, was first observed in tumor cells 90 years ago by Otto Warburg [8]. Due to a large amount of glucose consumed by tumor cells during glycolysis, metabolic by-products, in particular lactate, are produced in significant quantities within tumors and released into the extracellular space (Fig. 7.1).

In the intervening years, additional metabolic changes in tumor cells have been identified beyond their requirement for glucose. This includes increased reliance on glutamine, which provides the building blocks of nitrogen-based compounds such as nucleotides and non-essential amino acids [9], and the ability to harvest free fatty acids from the environment [10]. In cases of extreme nutrient deprivation, tumor cells can even catabolize their proteins and lipoproteins through autophagy to liberate amino acids and fatty acids [11]. These tumor-associated metabolic alterations are maintained by altered metabolism-related gene expression, such as lactate dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This reprogramming of the metabolic circuits has significant consequences for neighboring cells within the tumor microenvironment, including tumor-associated fibroblasts, endothelial cells, and immune cells [6, 12].

7.2 The Importance of Lactate Metabolism

The generation of lactate is a cellular process necessary for maintaining glycolytic flux and facilitating the removal of pyruvate from the cell. The interconversion of pyruvate to lactate is mediated by LDH and results in the oxidation of NADH to NAD^+. The lactate generated within a cell is then either exported from the cell via monocarboxylate transporters (MCTs) or converted back into pyruvate to fuel oxidative phosphorylation within the mitochondria (Fig. 7.1).

Lactate levels are consistently upregulated in a wide range of solid tumors [13]. Elevated lactate levels, upregulation of LDH enzymes, and the expression of MCTs are prognostic of tumor progression and metastases [14–17]. High levels of lactate in primary tumors are predictive of metastasis risk in head and neck cancer [18] and cervical cancers [19]. Serum levels of lactate dehydrogenase in patients with solid tumors are predictive of overall survival, disease progres-

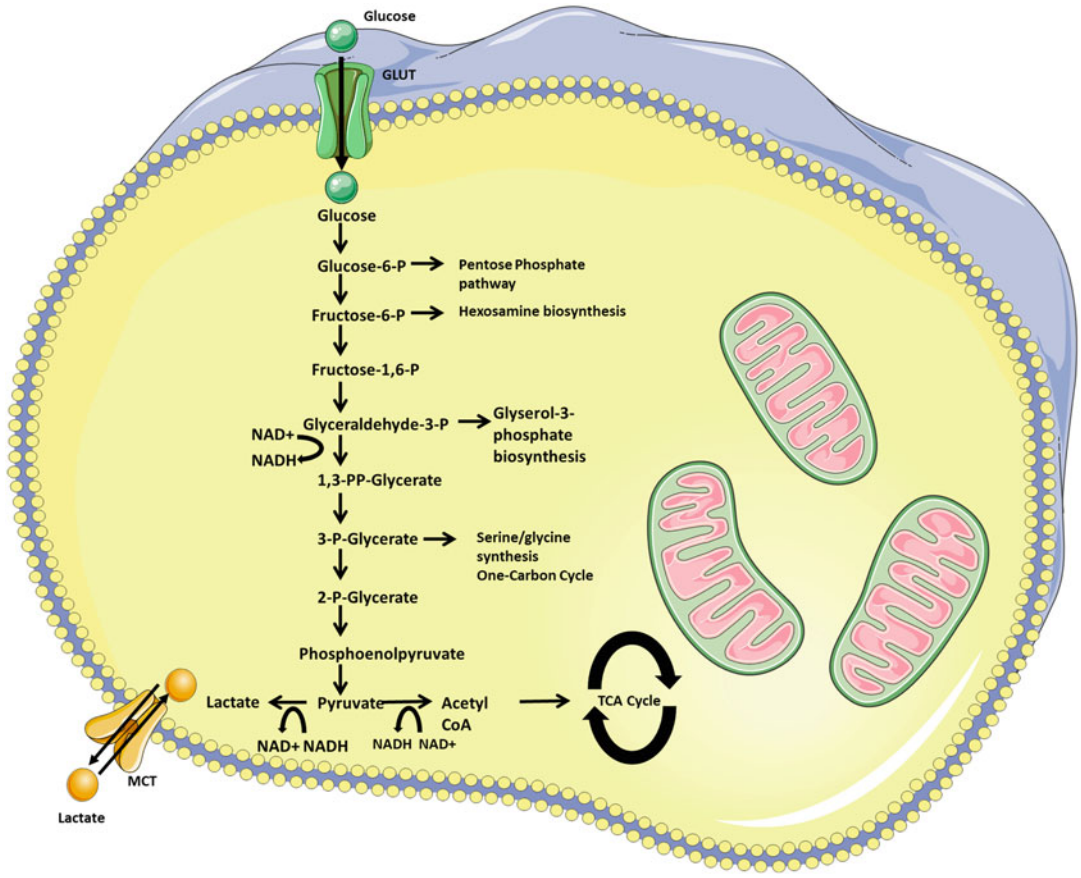

Fig. 7.1 Glycolytic intermediates fuel biosynthesis of essential molecules for tumor cell proliferation. Tumor cells favor glycolysis due to the range of intermediates produced and the ability to produce the reducing molecule NAD^+ by converting pyruvate to lactate. Detailed are the biochemical intermediates produced by glycolysis which are used for biosynthesis of essential molecules for cell proliferation. NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; MCT, monocarboxylate transporter; TCA, tricarboxylic acid cycle; GLUT, glucose transporter

sion, and recurrence-free survival [17, 20, 21]. Furthermore, suppression of lactate production within tumor cells in murine models reduces the metastatic ability of tumor cell lines [22–24].

7.3 Lactate Transport and Signaling

Lactate is transported across cell membranes via MCTs. These are a family of membrane transporters (also known as solute carrier 16 proteins), of which four members are proton-linked symporters (MCT1-MCT4) with varying tissue expression [25]. Tumors and immune cells predominantly express MCT1 and MCT4, and this expression profile appears to be characteristic of highly glycolytic cells [26]. MCTs passively transport lactate and a co-transported proton across the cell membrane. In situations where extracellular concentrations of either lactate or protons are elevated, these MCTs also facilitate the transport of lactate back into the cellular cytoplasm. This facilitates cell-cell lactate shuttles, whereby a glycolytic cell produces lactate, which in turn is taken up and utilized as an energy source by a neighboring oxidative cell [27, 28].

Extracellular lactate produced by glycolytic cells can also enter the circulation through capillaries or draining lymph. This lactate is subsequently removed from the circulation in the liver and kidney via gluconeogenesis (also referred to as the Cori cycle). Circulating lactate is transported into hepatocytes and renal cortex cells via MCTs and is converted via pyruvate back into glucose [29, 30]. Gluconeogenesis results in the consumption of ATP molecules generated from oxidative phosphorylation, and the glucose produced is either stored as glycogen in hepatocytes or exported back into the circulation where it can once again be utilized as a fuel source by glycolytic cells.

In addition to its role in glycolysis, lactate also possesses signaling and suppressor functions. Lactate is able to bind to the G-protein-coupled receptor GPR81 [31], which reduces cAMP and protein kinase A signaling, reducing proinflammatory cytokine production and inducing expression of regulatory factors such as IL-10, retinoic acid, and indoleamine 2,3-dioxygenase (IDO) [32, 33]. Lactate can also directly bind to the transmembrane domain of the mitochondrial antiviral-signaling protein (MAVS). MAVS is an innate intracellular sensor of double-stranded RNA [34]. Binding of lactate to MAVS prevents type I IFN production [35]. Lactate binding to MAVS prevents protein aggregation and provides a mechanistic link between metabolism and type I interferon responses, limiting interferon production in cells undergoing anaerobic glycolysis.

7.4 Lactate Dynamics in the Tumor Microenvironment

While elevated glycolytic flux is a well-documented characteristic of tumor cells, certain tumor cell subpopulations can utilize this lactate to fuel oxidative phosphorylation [36]. Highly glycolytic tumors have been shown to share space with low glycolytic neighboring tumors, which use lactate as a fuel source for mitochondrial respiration obtained via lactate shuttling from their glycolytic neighbors [27]. In breast cancer, signals from tumor cells can also lead to increased lactate production by stromal cells [37]. This lactate is then taken up by tumor cells, converted to pyruvate, and shuttled into the TCA cycle to fuel oxidative phosphorylation. The use of lactate as a fuel source requires an intact TCA cycle and functional mitochondria to metabolize the pyruvate generated.

While these studies highlight the importance of increased glycolytic flux in tumor cell survival and cancer progression, the exact location of this lactate remains somewhat uncertain and further research is required to directly quantify lactate levels and pH within the tumor microenvironment [38]. Direct measurements of the interstitial fluid of tumors via both in vivo and ex vivo methods indicate only a modest increase of lactate, in contrast to the dramatically elevated levels of lactate observed in whole tumor tissues [38]. These conflicting data can be reconciled if lactate preferentially accumulates within tumor cells. The proton gradient generated by the low pH of the tumor microenvironment, and relative alkaline intracellular pH of tumor cells, may favor the transport of lactate into tumor cells, thereby limiting lactate accumulation within the extracellular microenvironment [38]. Understanding the composition of the tumor microenvironment is central to untangling the individual (and potentially synergistic) effects of lactate and pH on tumor characteristics and immune cell function.

7.5 Impact of Lactate and pH on the Tumor Microenvironment

Lactate and pH have additional impacts on the tumor microenvironment beyond providing alternative energy sources for oxidative tumor cell subpopulations. Lactate has been shown to play several roles in reorganizing the physical tumor architecture and the immune landscape of many tumor types [39]. Lactate and reduced pH can promote tumor cell survival under conditions of nutrient deprivation. Glucose deprivation of the breast tumor cell line 4T1, in the absence of lactate, results in rapid apoptosis. In contrast, high

concentrations of lactate induce cell cycle arrest and autophagy, enabling 4T1 cells to survive for extended periods when deprived of glucose [40]. Lactate can act on vascular endothelial cells, activating the hypoxia-inducible factor-1α (HIF-1α) pathway to induce vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) expression, as well as stimulating autocrine NF-κB/IL-8 (CXCL8) signaling to drive angiogenesis [41, 42].

Lactate also acts on tumor-associated fibroblasts to induce the production of hyaluronic acid, which promotes the migration and extravasation of tumor cells [43]. Perhaps surprisingly, tumors can also influence sites distant from the primary tumor via metabolites. Lactate is enriched in tumor-draining lymph nodes and drives a pro-tumorigenic fibroblast phenotype in fibroblastic reticular cells by inducing activation and mitochondrial dysfunction in a pH-dependent manner [44].

7.6 The Emerging Links Between Metabolism and Effector Immune Responses

The importance of energy production and biosynthesis for the metabolic demands of activated proliferating immune cells was first documented in early studies on macrophages and neutrophils [45, 46]. However, the full extent of the links between metabolism and immune responses are only now emerging. Beyond simply meeting the energy and biosynthesis demands of activated immune cells, it is now clear that metabolic pathways directly regulate immune cell effector function, and the metabolic intermediates generated play an essential role in coordinating overall immune responses. While elevated glycolytic flux is a characteristic metabolic adaption of tumor cells, glycolysis is also a key metabolic program utilized by a variety of inflammatory immune cells, including cytotoxic lymphocytes, which migrate into the tumor microenvironment.

The upregulation of glycolytic machinery is a common feature amongst rapidly proliferating inflammatory immune cells [47–49]. Activated immune cells bear a striking resemblance to proliferating tumor cells. Immune cells require rapid production of carbon intermediates to fuel proliferation, production of effector molecules, and energy-intensive cell processes, such as migration and phagocytosis. While glycolysis is relatively inefficient in the generation of ATP, it enables the reduction of NAD^+ to NADH as well as the generation of intermediates essential for sustaining immune cell biosynthesis [50]. Proinflammatory and effector immune cells display a dramatic upregulation of glycolysis, together with an increased use of the pentose phosphate pathway, fatty acid synthesis, and amino acid metabolic pathways [50]. This distinct metabolic program supports inflammatory cytokine production, proliferation, reactive oxygen species (ROS) production, nitric oxide production, and effector cell differentiation.

Upregulation of the TCA cycle together with increased fatty acid oxidation, which reduces intracellular lipid accumulation, is associated with suppressive immune responses, the generation of immune tolerance, and the promotion of memory cell generation and survival [51–53]. These metabolic pathways are upregulated in macrophages with an M2 polarization [54], regulatory T helper cells [53], and quiescent memory T cells [55].

Intriguingly several metabolic intermediates and metabolic enzymes have been shown to have secondary signaling functions in immune cells [56–59]. This additional level of complexity facilitates the direct regulation of immune responses by metabolic processes. Hexokinase 1 has been shown to directly interact with and activate the NLRP3 inflammasome, leading to caspase activation and the processing of pro-IL-1β [60]. GAPDH binds to mRNA encoding interferon γ (IFNγ) and represses its translation; the switch to glycolysis that occurs in response to T cell activation leads to the dissociation of GAPDH allowing for translation of IFNγ [61]. Metabolic intermediates are also capable of regulating immune responses. Succinate, a metabolic intermediate of the TCA cycle, is dramatically increased upon activation of pro-inflammatory macrophages. Increased succinate

levels stabilize HIF-1α, which is required for maximal IL-1β production by macrophages [58]. Conversely, the metabolite itaconate is increased as part of an anti-inflammatory response upon diversion of aconitate away from the TCA cycle during pro-inflammatory macrophage activation. Itaconate alkylates KEAP1 leading to activation of the anti-inflammatory transcription factor Nrf2, which regulates inflammation and type I interferon responses [56]. In the context of these intimate links between metabolism and immune cell responses, the impact of lactate on immune cell function is of particular relevance for effective tumor immunity [62].

7.7 The Impact of Lactate on Immune Cell Function

Elevated lactate and decreased pH affect the phenotype and function of immune cells, polarizing the innate immune system toward tolerance and immunosuppression. It is important to note that lactate and pH can act both independently and synergistically to alter immune cell function.

Macrophages can be broadly divided into M1-like inflammatory macrophages and M2-like regulatory macrophages [63]. Lactate acts upon macrophages, independently of pH, upregulating markers associated with an M2-like phenotype and downregulating markers associated with M1-like macrophages (Fig. 7.2). Lactate induces HIF-1α signaling and drives arginase-1 and VEGF expression [64], and synergizes with hypoxia to drive activation of MAPK signaling and arginase-1 expression in tumor-associated macrophages [15]. Lactate also signals via the GPR81 receptor on macrophages to reduce NFκB and inflammasome activation, resulting in reduced production of proinflammatory cytokines including IL-6, IL-1β, and TNFα (Fig. 7.3) [32, 33, 65]. At the same time, GPR81 signaling in macrophages drives the expression of immune suppressive factors associated with

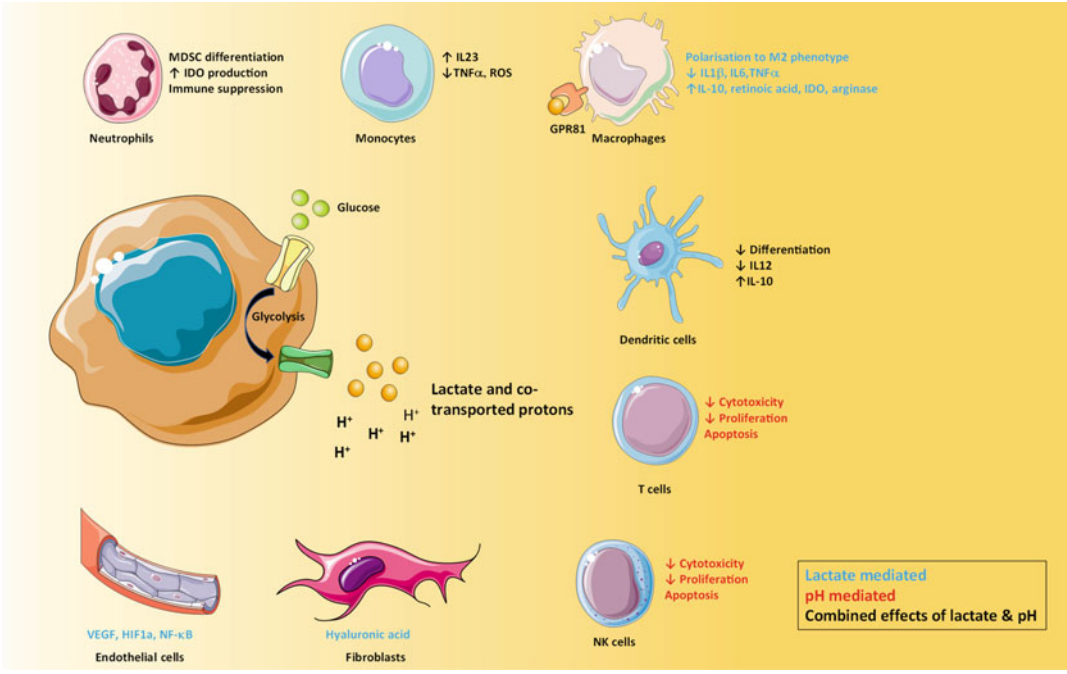

Fig. 7.2 Immunological consequences of elevated lactate and decreased pH in the tumor microenvironment. Lactate and reduced pH have differential and synergistic effects on immune cells in the tumor microenvironment. Effects mediated by lactate alone are written in blue, by pH alone in red, and combined effects in black. HIF1a, hypoxia-inducible factor 1a; IDO, indoleamine-2,3-deoxygenase; NK, natural killer; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor

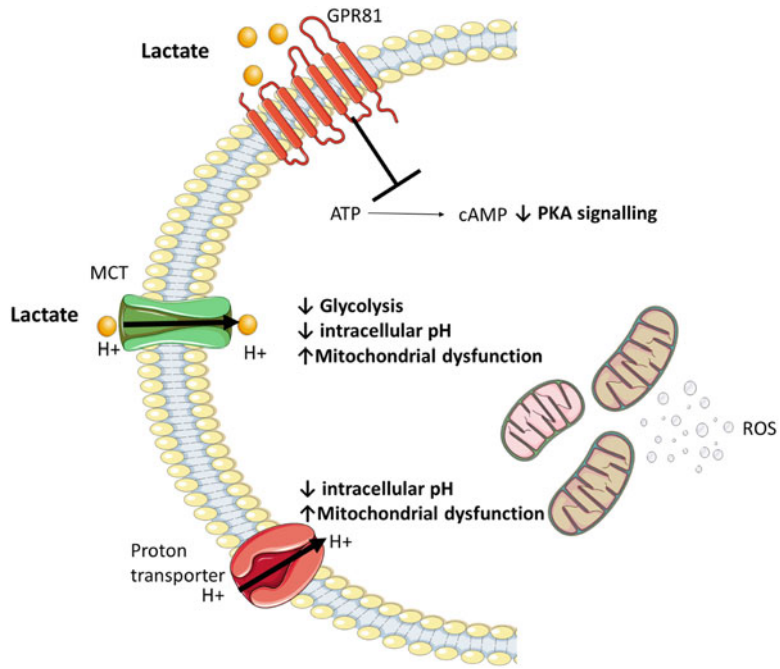

Fig. 7.3 Intracellular effects of uptake of lactate and decreased pH in the tumor microenvironment. Lactate can signal through GPR81, resulting in decreased cAMP and loss of PKA signaling. Alternatively, lactate can be absorbed into the cell, with protons, via MCTs, causing decreased intracellular pH, mitochondrial dysfunction, and reduced metabolic output. Finally, protons can be directly internalized by proton transporters, resulting in reduced pH and mitochondrial dysfunction. cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; GPR81, G protein-coupled receptor 81; MCT, monocarboxylate transporter; ROS, reactive oxygen species

M2-like phenotypes including IL-10, retinoic acid, and IDO [32, 33].

Macrophages are capable of shuttling lactate from the extracellular microenvironment via MCTs. The accumulation of intracellular lactate reduces RIG-I-like receptor signaling independently of pH by directly binding to the adaptor protein MAVS [35]. This blocks localization of MAVS to mitochondrial membranes and thereby inhibits RIG-I activation [35]. At a transcriptional level, changes in macrophage gene expression induced by lactate vary depending on the presence of lactate and/or reduced pH [66]. Lactate synergizes with low pH to induce *IL23A* transcription in monocytes, promoting the IL-23/IL-17 proinflammatory pathway [67], and likewise TNF and ROS suppression upon exposure to high levels of lactate requires the synergistic effects of both lactate and decreased pH [68] (Fig. 7.2).

A synergistic effect of lactate and decreased pH is also observed on dendritic cells [69, 70] and T cells [71]. Lactate together with a decreased pH inhibits dendritic cell differentiation as measured by CD1a, HLA-DR, and CD86 expression [69, 70]. This effect was not recapitulated by acidic pH alone (via HCl) or by the presence of lactate at pH 7.4 [69], with lactate and decreased pH acting synergistically to induce IL-10 production and suppress IL-12 production from dendritic cells [70]. In cytotoxic T lymphocytes, lactate and decreased pH induces apoptosis after 24 hours and decreases IFNγ and IL-2 production, effects not observed upon HCl treatment alone [71]. In this study, cytotoxic T lymphocyte proliferation and cytotoxic function appeared to be driven mainly by the decrease in pH associated with lactic acid treatment [71], and several other studies have highlighted the important effects of acidic microenvironments on immune cell function.

In vitro studies have highlighted the important effect of pH changes associated with lactate export on T cell and NK cell function [16, 71–74] (Fig. 7.2). T cells treated with low pH display reduced activation and cytokine production [16], while NK cells exposed to acidic microenvironment display reduced granzyme B and reduced cytotoxic effector functions [73]. Acidification of the tissue microenvironment causes a drop in intracellular pH and induces the selective cell death of T cells and NK cells, by driving increased mitochondrial dysfunction and mitochondrial ROS production (Fig. 7.3) [16, 74]. Reversing tumor acidosis has been shown to restore NK cell function and improve anti-tumor activity in vivo [72], and targeted inhibition of mitochondrial ROS production can promote NK cell survival [74] highlighting the potential for therapeutic interventions targeting metabolic pathways to improve immune cell function.

7.8 Opportunities to Target Lactate Metabolism in Cancer

The availability of immunotherapies for cancer treatment is exploding, yet many cancers and/or patients are still unresponsive. Complementary immune-activating therapies are required to increase response rates. Targeting metabolic pathways in tumors has multiple potential beneficial effects. Depriving tumor cells of essential nutrients limits their biosynthetic and proliferative capacity, reducing tumor growth dramatically. This is not a new concept in oncology where therapeutics targeting metabolism, such as methotrexate, have been used in the clinic for decades [75]. Due to the importance of tumor-derived metabolites as a component of the tumor microenvironment, targeting metabolism can create a more hospitable microenvironment for the immune system to work within and induce stress response pathways in tumor cells [76].

The broad spectrum of receptors, transporters, and catalyzing enzymes involved in tumor metabolism has led to the development of an array of metabolic therapies, which are now beginning to enter the clinic, with varying degrees of success [77, 78]. While an attractive target, metabolic therapies can also have side effects, specifically on the immune system. Metabolic changes underpin many of the immune functions we associate with tumor immunity [50], in particular T cell and NK cell activation and effector function [47, 48]. Indeed, treatments targeting metabolism, such as methotrexate, are also detrimental to the immune response. One of the other major clinical indications for the use of methotrexate is in autoimmunity where it functions as an immunosuppressant [79]. Any metabolic therapeutic approach should therefore aim to target pathways differentially used by tumor and non-tumor cells.

The glycolysis pathway provides the biochemical intermediates for several essential processes required for tumor cell growth and division [7], and the glycolytic pathway has been highlighted as a potential therapeutic target in cancer [80, 81]. However, our immune response is also dependent on glycolysis for the acquisition of effector functions, especially T and NK cells, which are the main mediators of tumor immunity [47–49]. Clinical trials of 2-deoxyglucose (2-DG), a glucose analog that reduces the rate of glycolysis in both tumor cells and immune cells, showed limited effects on tumor progression, despite promising preclinical data [82]. More recently, preclinical studies using koningic acid to partially inhibit GAPDH induced a cytotoxic response in cancer cell lines without impacting on tumor immunity [83]. This study highlights the precision and specificity required to target this pathway without impacting on immune cell function.

The production and secretion of lactate can also be targeted via several alternative therapeutic strategies that avoid the need to completely inhibit glycolysis. These alternative strategies may hold promise in avoiding the detrimental effects of complete inhibition of glycolysis on immune cells. Targeting either lactate transport via MCTs [27, 41, 42, 84] or lactate dehydrogenase enzymes [85, 86] prevents the release of lactate from tumor cells and induces cytotoxic responses. A study using an early non-selective MCT inhibitor suggests inhibition of T cell function may still be

an issue [71], and further studies are required to assess the effects of novel selective MCT and lactate dehydrogenase inhibitors on immune cell function. A specific MCT1 inhibitor, AZ3965, has shown promise in preclinical studies and is currently being trialed in solid tumors including gastric cancer and lymphoma (NCT 01791595). Furthermore, an MCT4 inhibitor is in preclinical development (AZD0095), which does not affect T cell function and when combined with checkpoint therapy improves tumor rejection in an MC-38 murine colon cancer model [87].

The hydrogen ions co-transported with lactate, which act to decrease the pH of the tumor microenvironment and suppress immune cell function, can also be therapeutically targeted. Significant clinical improvement has been reported with the use of systemic bicarbonate buffering, which neutralizes tumor acidity, reduces tumor invasiveness, and improves the immune response [72, 88, 89]. Despite these positive results from preclinical studies, translation of these strategies into clinical trials is limited by the potential for adverse events, including electrolyte imbalance, respiratory depression, and progressive vascular calcification [90]. The targeted use of bicarbonate buffering has been trialed in patients receiving trans-arterial chemoembolization for hepatocellular carcinoma, which improved tumor response rates, although had minimal effect on overall survival [91].

Decreasing intracellular pH is a consequence of the acidic microenvironment tumor-infiltrating immune cells migrate into. This decrease in pH is associated with increased mitochondrial ROS production and immune cell apoptosis [16, 74]. Reducing the accumulation of mitochondrial ROS using ROS scavengers can protect immune cells from pH-induced apoptosis ex vivo [74]. The use of mitochondria-targeted scavengers has shown some efficacy in murine models of cancer, although in these studies the effect was attributed to a direct effect on tumor cell survival [92, 93]. It remains to be seen if some of these anti–tumor effects of mitochondria–targeted scavengers in vivo are also mediated by improvements in immune cell function.

The availability of immunotherapies for cancer treatment has revolutionized the field of oncology. However, many cancers and/or patients fail to respond to these immune-activating therapies. This could be due to the inhospitable environment created by tumor metabolism, creating a toxic microenvironment for even engineered immune cells. Immunotherapies, either checkpoint inhibitors or cellular therapies, rely on the ability of immune cells to alter and maintain their metabolism to carry out effector functions. As discussed in this chapter, tumors have adapted to avoid just this. Therefore, complementary metabolic therapies are required to enhance immune-based treatments and improve patient response in solid tumors. Therapeutic approaches taking into consideration the metabolic heterogeneity of the tumor microenvironment and the metabolic demands of tumor-infiltrating immune cells in personalized models hold much promise. By harnessing the synergistic anti-tumor effects of limiting tumor growth as well as augmenting local immune cells, these metabolic approaches can complement immunotherapy and enhance tumor eradication and patient survival.

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Fatty Acid Mediators in the Tumor Microenvironment

8

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Abstract

Patients with cancer frequently overexpress inflammatory cytokines with an associated neutrophilia both of which may be downregulated by diets with high omega-3 polyunsaturated fatty acids (ω-3 PUFA). The anti-inflammatory activity of dietary ω-3 PUFA has been suggested to have anticancer properties and to improve survival of cancer patients. Currently, the majority of dietary research efforts do not differentiate between obesity and dietary fatty acid consumption as mediators of inflammatory cell expansion and tumor microenvironmental infiltration, initiation, and progression. In this chapter, we discuss the relationships between dietary lipids, inflammation, neoplasia and strategies to regulate these relationships. We posit that dietary composition, notably the ratio of ω-3 vs. ω-6 PUFA, regulates tumor initiation and progression and the frequency and sites of metastasis that, together, impact overall survival (OS). We focus on three broad topics: *first*, the role of dietary lipids in chronic inflammation and tumor initiation, progression, and regression; *second*, lipid mediators linking inflammation and cancer; and *third*, dietary lipid regulation of murine and human tumor initiation, progression, and metastasis.

Keywords

Omega 3 polyunsaturated fatty acids · Inflammation · Cancer · Tumor progression · Metastasis · Diet · Prostaglandins · Lipoxygenases · Myeloid-derived suppressor cells · Neutrophils · Myeloplasia

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_8

8.1 Introduction

Bioactive lipids include a wide variety of metabolites that regulate essential cellular functions including membrane fluidity, cellular energy storage, lipid signaling, inflammation, and immunity. These mediators have pleiotropic and opposing activities secondary to their metabolism. The biosynthesis and metabolism of fatty acids are dependent on dietary intake and composition of polyunsaturated fatty acids (PUFA). Recent studies into bioactive fatty acids and their precursors in the tumor microenvironment have emphasized the importance of improving our understanding of the cellular lipidome as a regulator of tumor initiation, progression and metastasis, inflammatory and immune responses, and their tumor therapeutic potential as targets.

Chronic inflammation has multiple roles in carcinogenesis, tumor progression, and metastasis. Evidence from preclinical and clinical studies supports a role for chronic inflammation in the initiation and progression of cancer. Further, multiple mechanisms contribute to tumor initiation including the induction of genomic instability, alterations in epigenetic events and subsequent inappropriate expression of genes, enhancing the proliferation of tumor initiated cells, resistance to apoptosis, tumor neovascularization, and tumor invasion and metastasis. Inflammation-associated reactive oxygen and nitrogen species can also result in damage to cellular components including DNA, proteins, and lipids, which may directly or indirectly contribute to malignant transformation. Overexpression, elevated secretion, or abnormal activation of proinflammatory mediators, including cytokines, growth factors (GFs), chemokines, cyclooxygenase-2 (COX-2), prostaglandins (PGs), arginase, lipoxygenases (LOX), pattern recognition receptors (toll and notch), inducible nitric oxide synthase (iNOS) and nitric oxide (NO), and a distinct network of intracellular signaling molecules including upstream kinases and transcription factors, can all contribute to tumor progression. While inflammation supports tumor development, the tumor microenvironment, including tumor and stromal cells, as well as, inflammatory/immune cells, both activate in situ, or mobilized into the tumor microenvironment, can result in an inflammatory state by aberrant expression of proinflammatory mediators. Many of the proinflammatory mediators, especially cytokines, GFs, chemokines, PGs, and leukotrienes (LTs), upregulate angiogenic switches inducing inflammatory angiogenesis and tumor-stroma-cell communication, resulting in tumor angiogenesis, local and systemic immune suppression, and tumor invasion and metastasis.

Rodent and clinical studies have shown that myeloid cell infiltration of the tumor microenvironment is associated with poor clinical outcomes, as well as, neutrophilia and lymphocytopenia. In contrast, an increased lymphocytic infiltration of tumors is associated with improved clinical outcomes. Lifestyle parameters, including obesity and diets with high amounts of saturated fat and/or omega (ω)-6 PUFAs, influence tumor leukocytic infiltration, as well as an increase in extramedullary myelopoiesis (EMM). Tumor secretion of GFs and chemokines can regulate tumor-immune-cell crosstalk; dietary lifestyle choices can also contribute to inflammation, tumor induction and progression, and tumor leukocyte infiltration. A relationship between obesity and high-fat diets (notably saturated fats in Western diets) and the regulation of inflammation, tumor induction, metastasis, and poor clinical outcomes is accepted. Further, mechanisms of dietary promotion of an inflammatory microenvironment are little studied, and few targeted drugs to inhibit the clinical sequelae have neither been identified nor studied. Similarly, adipose tissue, within the tumor microenvironment and its regulation by diet, needs additional study. Thus, modifications of obesity and dietary lipids may inform preventative or therapeutic approaches in the control of tumor-associated inflammation and neoplastic progression.

Tumor-associated adipocyte-derived elements can promote tumor growth, as well as, dedifferentiation into fibroblast-like cells. Free-fatty acids (FFAs) released by adipocytes are used by

cancer cells to support proliferation. In addition, these FFAs activate and modulate monocytes, macrophages, and vascular endothelial cells so as to favor a protumorigenic microenvironment [1, 2]. Obesity- and tumor-associated adipose tissues regulate tumor development in multiple ways, by providing energy via FFAs or through adipokines, cytokines, and miRNAs. Tumor-associated adipocytes can recruit macrophages to the tumor microenvironment and stimulate their polarization to the alternative M2 functionality via CCL2, interleukin (IL)-1β, and CXCL12 [3]. In addition, adipocytes produce inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, IL-1β, and CCL2 [3, 4], which results in inflammatory cell recruitment, infiltration, and accumulation, resulting in foci of low-grade chronic inflammation [5, 6]. Visceral adipose tissues from obese individuals are also frequently associated with increased systemic levels of CCL2, TNF-α, IL-1, IL-6, and iNOS [7]. Moreover, the level of CCL2 produced is increased due to recognition of cell-free DNA (cfDNA) from degraded adipocytes. Obese mice also have increased cfDNA release resulting in a higher accumulation of macrophages, which aggravates inflammation [8], increases angiogenesis, and supports tumor growth and progression [3].

8.2 Role of Dietary Lipids in Chronic Inflammation and Tumor Initiation, Progression, and Regression

Dietary fats can increase inflammatory responses, particularly Western diets, predominantly due to the inclusion of fatty acids (FAs) from animal sources, which are mainly saturated fatty acids (SFAs), and FAs from plants that are predominantly ω-6 PUFAs. All are proinflammatory. In contrast, FAs derived from some plant-based oils, and fatty fish, which consist mainly of ω-3 PUFA, inhibit inflammation. Rodent and clinical studies have shown that subjects given diets rich in ω-6 PUFAs have an increased risk of inflammatory mediators and diseases, including asthma, rheumatoid arthritis, and inflammatory bowel disease [9]. In contrast, diets with high levels of long chain (LC)-ω-3 PUFAs are anti-inflammatory, such that consuming individuals have a decreased risk of inflammatory diseases [9]. These clinically relevant activities are associated with the oxidization of PUFAs to either proinflammatory or pro-resolving lipid mediators (Fig. 8.1), both of which can regulate inflammation and immunity [10]. Proinflammatory mediators, notably PGs and LTs, are secreted in response to "foreign" substances and cleared by pro-resolving lipid mediators, restoring cellular and tissue homeostasis [11]. Diets with high levels of the ω-3 PUFAs, shorter chain α-linolenic acid (ALA), and more critically, LC eicosapentaenoic (EPA) and docosahexaenoic (DHA) are associated with a decrease in inflammation [12]. The beneficial effects of dietary LC-ω-3 PUFAs include their metabolism into anti-inflammatory metabolites including LTs, thromboxanes (TX), resolvins and a decrease in inflammatory cytokines. The ω-3 PUFAs differ from the ω-6 PUFAs based on the position of their double bonds in the acyl chain, such as linoleic acid (LA) as compared to the ω-6 PUFA arachidonic acid (AA) (Fig. 8.1). However, the inflammatory activities of PUFAs are not clearly separated, based on the number and placement of double bonds, counting from the methyl end of the FA (i.e., ω-3 vs. ω-6). The dietary addition of the short-chain ω-3 PUFA, ALA, an essential FA, and the main precursor of LC-ω-3 PUFAs, is proinflammatory and can result in enhanced secretion of superoxides from macrophages and neutrophils [13], and adhesion of inflammatory cells to endothelial cells [14]. Further, ALA can limit the proliferation of rodent and human lymphocytes [15–17], supporting immunosuppressive functions. Consistent with these in vitro activities, rodents provided a high-fat diet, rich in ALA, have a decreased mitogen-stimulated lymphocyte proliferation and natural killer (NK) cell activity [18].

In vitro studies using the ω-6 PUFA, AA, have revealed an increase in inflammation, including enhanced superoxide release [13], neutrophil attachment to endothelial cells [14], and increased macrophage secretion of IL-1β

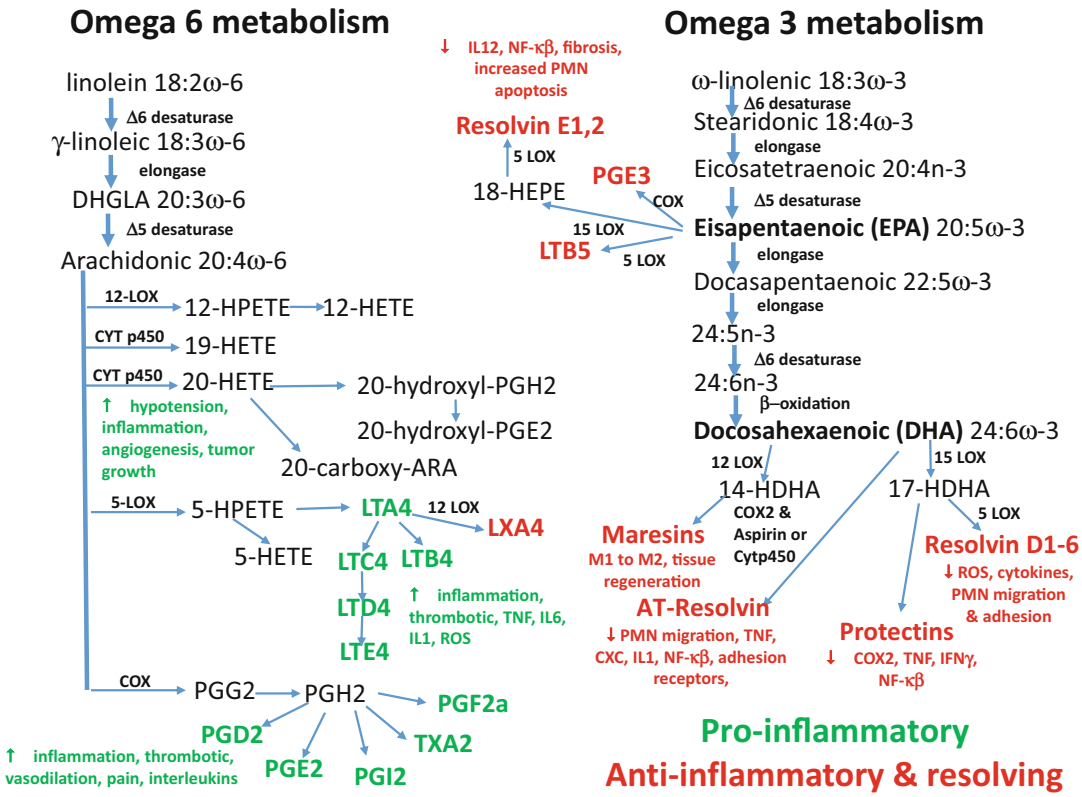

Fig. 8.1 This is an outline of eicosanoid synthesis pathways from arachidonic acid (AA) and resolving-related mediators from α-linolenic acid (ALA) and their inflammatory and anti-inflammatory functions. The abbreviations include COX, cyclooxygenase; CYT p450 cytochrome, p450; CXC chemokine subtype, HETE, hydroxyeicosatetraenoic acid; HDHA, hydroxyldocosahexaenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPDHA, hydroperoxydocosahexaenoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; IL, interleukin, IFN, interferon; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; PG, prostaglandin; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen synthetase; TNF, tumor necrosis factor, TX, thromboxane

[19]. Mice given diets with high ω-6 PUFA levels, in a dose-dependent manner, have an increased level of urinary leukotriene E4 (LTE-4) and prostaglandin E2 (PGE-2) following leukocyte stimulation in vivo [20]. Further, it has been reported that diets high in AA result in increased levels of angiotensinogen, IL-6, and monocyte chemoattractant protein (MCP)-1 and increased expression of the proinflammatory transcription factor, nuclear factor κβ (NFκβ) [21]. In studies with rats fed high AA diets for 8 weeks, a decrease in superoxide production by peritoneal macrophages was observed in response to phorbol esters [22] as well as an increase in TNF secretion by resident macrophages, although no effect on TNF production by inflammatory macrophages was observed [23]. Thus, the effects of the shorter ω-3 PUFA, ALA, on lymphocyte functions appear to be dependent on ALA levels and total PUFA diet content [24]. Studies with ALA and ω-6 PUFA contrast with the bioactivity of LC-ω-3 PUFA with 20 or more carbon atoms such as EPA and DHA, which are anti-inflammatory and immune augmenting [25]. The anti-inflammatory activity of diets incorporating LC-ω-3 PUFAs are due to a lower metabolism of ω-6 PUFA into inflammatory eicosanoids, cytokines, and the stimulation of reactive oxygen species (ROS) and nitric oxide synthase (NOS) mediators [26]. Clinically, dietary supplementation with EPA and DHA can decrease intestinal damage and improve

gut histology in patients with inflammatory bowel disease [27], as well as decrease arthritic lesions including joint pain, number of tender and swollen joints, and duration of morning stiffness [28].

Tumor blood vessels are structurally and functionally abnormal, lacking a normal hierarchical structure composed of arterioles, capillaries, and venules [29]. Tumor endothelial cells are generally loosely connected and covered by fewer and more abnormal mural pericytes [29–31]. Clinically, a poor coverage of tumor blood vessels by pericytes is related to poor patient prognosis [32–34], and pericyte dysfunction has been associated with increased numbers of metastases [35]. Prostaglandin I2 (PGI2) is an important vascular prostanoid that provides an important balance in tumor angiogenesis [36, 37]. PGI2 and agonists of PGI2 have been suggested to reduce tumor metastasis by modifying tumor angiogenesis [38]. Thus, administration of PGI 2 analogs that affect endothelium-pericyte interaction has been shown to target angiogenesis tumor microenvironment and control neoplasia progression and growth [39]. The potential interaction between vascular prostacyclin and diet is supported by the finding of increased prostacyclin production by vessel walls in patients given diets with moderate levels of LC-ω-3 PUFAs [40]. Thus, LC-ω-3 PUFAs regulation of prostanoid synthesis by vascular endothelial cells within the tumor vascular provides another potential mechanism for LC-ω-3 PUFAs control of tumor growth.

Chronic inflammation contributes to the initiation and progression of malignancy [41]. The role of inflammation in carcinogenesis was first proposed in 1863 by Rudolf Virchow, when he noticed the presence of leukocytes in neoplastic tissues [42]. Since Virchow's early observation linking inflammation and cancer, data supporting tumor initiation at sites of infection and chronic inflammation have been reported [43]. Indeed, approximately 25% of all cancers are associated with chronic infections and inflammation [44]. Although inflammation is an adaptive host defense against infection and is primarily a self-limiting process, inadequate resolution contributes to chronic pathologies including cancer [45, 46]. Evidence from laboratory- and population-based studies has suggested that organ-specific carcinogenesis is at least partly associated with inflammation [47–50]. Thus, the development of gastric, hepatic, gallbladder, prostate, and pancreatic tumors has been attributed to *Helicobacter pylori*-induced gastric inflammation, chronic hepatitis, cholecystitis, inflammatory atrophy of the prostate and chronic pancreatitis, respectively [46, 51, 52]. Patients suffering from inflammatory bowel disorders also have an increased risk of colorectal cancer [47, 53, 54], while management with anti-inflammatory drugs (COX-2 inhibitors) reduces this risk [55].

Sustained cellular injury can also induce inflammation and stimulate carcinogenesis. Inflammatory and innate immune cells are recruited to sites of infection or inflammation, such that activated myeloid cells generate ROS and reactive nitrogen species (RNS), which facilitate tumor initiation. Thus, one mechanism by which chronic inflammation supports carcinogenesis is the generation of ROS and/or RNS in inflammatory tissue and subsequent DNA damage leading to oncogene activation and/or inactivation of tumor suppressor genes. Chronic exposure to ultraviolet (UV) B radiation can induce inflammatory tissue damage [56], tumor suppressor T-cells [57], and skin cancer [56]. Mutational changes in ras and p53 have also been observed with many lipid mediators resulting in the regulation of inflammation and cancer initiation and progression [58, 59]. The activation of ras oncogene and loss-of-p53 tumor suppressor gene function have been shown to support UVB-induced mouse skin carcinogenesis [60]. ROS-induced DNA damages, including DNA strand breaks, DNA-based modifications, and DNA cross-links, result in replication errors and genomic instability contributing to tumor initiation and progression [61, 62]. NO, another reactive species, has a role in inflammation-associated carcinogenesis by direct modification of DNA and inactivation of DNA repair enzymes [63]. 8-Oxo-7,8-dihydro-20-deoxyguanosine (8-oxo-dG), which is associated with oxidative and mutagenic DNA damage [64], is produced in

association with *H. pylori*-induced gastric [65] and TNF-α-induced pulmonary carcinogenesis [66]. Peroxynitrite, formed by a reaction between NO radical and superoxide anion, causes DNA damage by forming 8-nitroguanine (8-NG) [67, 68], another biomarker of inflammation-associated cancers [69]. Thus, oxidative and nitrosative DNA damage products are associated with inflammation-driven carcinogenesis [70]. ROS and RNS can also induce lipid peroxidation resulting in other reactive species, such as manoldialdehyde and 4-hydroxynonenal (4-HNE), which are capable of forming DNA adducts [71]. Elevated intracellular ROS (e.g., superoxide anion, H_2O_2, and hydroxyl radicals) and RNS (e.g., peroxynitrite, NO, and S-nitrosothiols) also induce alterations in protein functions, including a perturbation of DNA-protein cross-links and posttranslational modification critical to maintaining cellular homeostasis. For example, NO has been shown to hyperphosphorylate and inactivate retinoblastoma protein, thereby increasing human colon cancer cell proliferation [72]. Moreover, in a mouse model of colitis, the hyperphosphorylation of retinoblastoma protein (Rb) was found to be reduced in the colons of iNOS-null mice as compared to wild-type littermates, suggesting that NO is involved in Rb hyperphosphorylation [72]. In the colon tissues of patients with ulcerative colitis, a positive correlation between iNOS levels and the phosphorylation of p53 as well as the activation of p53 transcriptional activity has been identified [73]. Thus, nitrosative stress also has a role in inflammation-associated carcinogenesis by activating activator protein-1(AP-1), a representative redox-sensitive transcription factor [74], which is involved in cell transformation and proliferation [75, 76].

Further, metabolic reprogramming from glycolysis to lipid metabolism regulates myeloid cell differentiation. For instance, IL-4-induced M2 macrophages rely on fatty acid oxidation (FAO) to proliferate, which is mediated through signal transducer and activator of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma (PPARγ)- co-activator 1β (PGC1β) [77, 78]. Indeed, the uptake of triacylglycerols followed by lipolysis is critical for M2 activation [79] and tumor-infiltrating dendritic cells (DCs) develop high levels of intracellular triglycerides [80]. This increased lipid accumulation in DCs impairs their ability to process and present antigens and results in an inhibition of the induction of antigen-specific T-cells [81, 82]. Decreasing the lipid content by inhibiting fatty acid synthesis restores DC functions and may improve the efficacy of cancer vaccines [81]. Overall, it appears that myeloid cells can use the metabolic programs that support their survival and functional demands within their microenvironment. As such, an improved understanding of these metabolic pathways may support the development of novel therapeutic targets in cancer and other chronic inflammatory diseases [83, 84].

Tumor-infiltrating myeloid derived suppressor cells (MDSCs) use FAO as their primary source of adenosine triphosphate (ATP) [85, 86], such that pharmacologic inhibition of FAO blocks the immunosuppressive functions of MDSCs, delaying tumor growth in a T-cell-dependent fashion and enhancing therapeutic responses to low-dose chemotherapy and adoptive T-cell therapy [86]. The mechanisms by which the tumor microenvironment regulates the uptake of exogenous lipids and enhances the metabolic and functional reprogramming of tumor-associated MDSCs is associated with tumor-derived GFs (granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage-colony stimulating factor (GM-CSF)) that can upregulate lipid transport receptors in tumor-infiltrating myeloid cells and increase lipid uptake in the tumor microenvironment [87]. This is associated with an enhanced oxidative metabolism and upregulated immunosuppression. Interestingly, human tumor-infiltrating and peripheral blood (PB) MDSCs also have increased levels of lipid transport proteins, supporting the development of more immunosuppressive MDSCs in vitro. Thus, tumor-derived cytokines facilitate myeloid cell lipid uptake, accumulation, and metabolism resulting in the induction of MDSC immunosuppressive functions. As such, lipids contribute to immuno-

suppressive myeloid cells (M2 macrophages, dendritic cells, and polymorphonuclear leukocyte (PMN) and monocytic (M)-MDSC) in cancer and chronic inflammatory pathologies [78–81, 83–86, 88]. Following infiltration of the tumor microenvironment, myeloid cells undergo metabolic reprogramming from glycolysis to FAO that is paralleled by the upregulation of the T-cell immunosuppressive mediators arginase I and iNOS [85, 86]. As such, metabolites in the tumor microenvironment regulate the immunometabolic induction of MDSCs. Tumor-derived inflammatory GFs including G-CSF and GM-CSF upregulate the expression of lipid transport receptors that facilitate the uptake of lipids in the tumor microenvironment, including FFAs and the triacylglycerol-carrying lipoproteins, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) [89]. The uptake, accumulation, and oxidation [86] of these lipids activate and prolong the survival of immunosuppressive MDSCs [90]. Importantly, cancer-associated MDSCs also express lipid transporters, and therefore, human peripheral blood stem cells (PBSC), cultured in lipid-rich media, develop into highly inhibitory MDSCs [91]. Consistent with other myeloid cells, substantial lipid accumulation occurs with tumor-derived MDSCs [92, 93]. MDSCs with lipid overload have greater immunosuppressive effects on $CD8^+$ T-cells, compared to MDSCs with normal lipid content. Lipid accumulation in tumor-derived MDSCs is linked to an increase in fatty acid uptake. This observation is supported by the study of Cao et al., which demonstrated an increased expression of fatty acid transport protein 4 (FATP4) in murine tumor-derived MDSCs [93]. The lipids in the MDSCs of tumor-bearing mice and cancer patients are oxidized, potentially by the oxidative activities of ROS and myeloperoxidase (MPO) [92, 94]. Inhibition of ROS and MPO can reduce the oxidation of lipids resulting in MDSCs with decreased immunosuppressive activity [92]. Studies such as these document an increase in lipids in the tumor microenvironment that are assimilated by MDSCs supporting previous reports of increased levels of serum triglycerides, LDL-cholesterol, and VLDL-cholesterol in cancer patients [95–97] and patients with chronic inflammatory diseases [95–103]. However, the origin of lipids in the tumor microenvironment is unclear, although it appears that lipids released from adipose tissue provide energy that supports tumor growth and invasion [104–106]. However, the mechanisms by which MDSCs mobilize lipids to support increasing FAO remain obscure. Adipocytes liberate fatty acids for FAO from lipids stored in lipid droplets by lipolysis, which is regulated by adipose triglyceride lipase, hormone-sensitive lipase, and lysosomal acid lipase (LAL) [107]. Recent studies showed that LAL-mediated lipolysis releases fatty acids supporting FAO in IL-4-induced M2 macrophages [79] and IL-15-driven memory T-cells [108]. Furthermore, lipids act as ligands for PPARs [109] that have a key role in the regulation of FAO [110]. In line with this, PPARγ and PPARδ, which are induced by STAT6, can regulate the alternative activation of macrophages [110].

8.3 Lipid Mediators Linking Inflammation and Cancer

8.3.1 Dietary PUFA Regulation of Myeloid Cell Functions

Diets rich in ω-6 PUFAs are proinflammatory, enhancing the expansion of myeloid cells [111] and MDSCs [112]. The increase in MDSCs is observed with both in vitro cultured murine bone marrow cells and in vivo in mice fed diets enriched in ω-6 PUFAs. In the latter studies, mice were given a linseed oil-based diet containing 45% of the shorter ω-3 PUFA, ALA, or a sunflower oil diet containing 45% of the ω-6 PUFA, LA. These studies suggested that the bioactivity of PUFAs occurred through Janus kinase-signal transducer and activator of transcription (JAK-STAT3) signaling, such that a JAK inhibitor reduced the bioactivity of PUFAs on MDSCs. Based on these and other studies, it was concluded that the inflammatory activity of PUFAs may be mediated, in part, by diet [113]. Thus, dietary fat contributes to tumor-associated

inflammation that occurs, in part, through AA metabolism [114]. A Western-style diet increases the risk of tumorigenesis via myeloid recruitment, infiltration of tumors, and subsequent activation of TNF-a, PGE2, NF-kβ, and Wnt inflammatory pathways [115]. AA can make up to 40% of the fatty acid composition of cancer cell membranes [116]. The anti-tumorigenic effects of ω-3 PUFAs may be mediated in part by their anti-inflammatory effects [117].

Recently, lipid accumulation in the adipose tissues of obese hosts have been shown to promote infiltrating macrophages with an M2 polarization shift, while M1 phenotype macrophages are observed in lean adipose tissue [118, 119]. Because dietary fish oil with LC-ω-3 PUFAs decreases PGE-2 production, LC-ω-3 PUFAs are considered anti-inflammatory. Such a diet also results in enhanced secretion of Th1-type cytokines and decreased major histocompatibility complex (MHC) II expression, lymphocyte proliferation, and NK cell activity. Consistent with these observations, the culture of human neutrophils with LC-ω-3 PUFAs has been reported to suppress superoxide production and phagocytosis [120]. Similarly, the incubation of murine peritoneal macrophages with EPA or DHA can inhibit MHC II expression [121]. In one study, human monocytes were cultured with EPA or DHA, resulting in a decreased frequency of human leukocyte antigens-DR or DP (HLA-DR or –DP) positive monocytes following addition of interferon gamma (IFN-γ) [122] and depressed antigen (Ag) presentation [123]. Similarly, adding fish oil to rodent diets can decrease superoxide and H_2O_2 secretion by macrophages [124]. Experiments comparing diets with safflower oil versus fish oil have been found to decrease peak plasma levels of the inflammatory cytokines TNF-α, IL-1β, and IL-6 following lipopolysaccharide (LPS) injection [125]. However, super-pharmacologic doses were used in this study, contrasting with the majority of rodent studies which use dietary fish oil in which EPA plus DHA comprise up to 30% of the lipid fatty acids and up to 12% of dietary energy. The conclusions from these studies have been extended using lower levels of EPA or DHA (4.4% of total FAs or 1.7% of dietary energy), documenting that these levels have anti-inflammatory activities [126].

8.3.2 T-cell Immunoregulation and PUFA

Clearly, ω-6 PUFAs are proinflammatory [127] as they are metabolized to AA and subsequently by COX-/LOX- to inflammatory lipid mediators that include PGs and LTs [128]. These *AA* metabolites have tumor-promoting actions such that the COX downstream metabolite PGE-2 can enhance tumor growth by inducing tolerogenic DCs and Tregs. 5-LOX metabolites include the four series *LTs* that can also stimulate tumor growth and progression [129]. This contrasts with LC-ω-3 fatty acids that have alternative COX/LOX activities forming metabolites with alternative bioactivities, including the three series PGEs and five series LTs. AA metabolism results in the LOX products that can stimulate the expansion and differentiation of myeloid progenitor cells [111] including MDSCs. Similarly, tolerogenic DCs contribute to T-cell regulatory functions by inhibiting their activation. In steady-state conditions, tissue-resident, immature DCs internalize, process, and present tumor Ags. These DCs, identified as DC2s, are poorly immunogenic and do not secrete proinflammatory cytokines due to the expression of low levels of costimulatory molecules. Further, DC2s secrete immunosuppressive cytokines, including IL-10 and transforming growth factor – beta (TGF-β), which are critical to the induction of T-reg cell differentiation. The secretion of indoleamine 2,3-dioxygenase (IDO) secretion by DC2s can also contribute to immune tolerance [130]. Another myeloid cell population with a similar functional profile are alternatively activated macrophages (M2s) that differentiate from monocytes by IL-4 stimulation. M2s facilitate tumor angiogenesis, support tumor progression, invasion, and metastasis, and contribute to T-cell immunosuppression by secreting IL-10, facilitating Th2 cell differentiation. This provides

a positive feedback cycle for differentiation of additional M2 macrophages, all of which can express programmed death-ligand 1 (PD-L1), further contributing to activated T-cell apoptosis [131].

Similar to M2 macrophages, MDSCs can infiltrate tumors, as well as circulate in the PB of tumor patients. MDSCs can be either of monocytic, PMN or immature in origin [132, 133]. In the blood of cancer patients, MDSCs lack lineage (LIN) markers for T and B lymphocytes (CD3 and CD19) and NK cells (CD56) and thus express an $LIN^{-}HLADR^{-}CD11b^{+}$ phenotype [134, 135] that can be further subset based on CD14 expression (monocytic), CD15 expression (PMN), or expression of $CD33^{+}CD14^{-}CD15^{-}$ (immature) cells [132, 136]. A positive correlation between the frequency of MDSCs and tumor stage has been reported for numerous tumor pathologies [133]. The function of MDSC inhibition of T-cell activation occurs via arginase, iNOS, ROS, or RNS, as well as secretion of immunosuppressive cytokines [137]. MDSCs also deplete nutrients necessary for lymphocyte function (arginine), disrupt IL-2 receptor signaling, interfere with lymphocyte trafficking, promote activation of T-regs by CD40-CD40L ligation, and suppress CD3-zeta (ζ) expression and secrete IL-10 or TGF-β [138, 139]. While few studies have examined the response of MDSCs to dietary PUFA [140], myeloid cell and myeloid progenitor cell response to dietary ω-6 PUFA as extramedullary hematopoiesis and myeloplasia has been studied [140]. Chronic inflammation has a key role in the expansion and activation of MDSCs in both tumors and various inflammatory disorders. As discussed in this chapter, chronic inflammation is a hallmark of MDSC expansion and immunosuppression. Thus, ω-6 dietary associated inflammation [140] suggests a potential role for dietary PUFA in the regulation of MDSCs. A few studies have shown that dietary PUFA can promote the expansion of MDSCs via JAK-STAT3 signaling in mice [112]. Waight et al. [141] demonstrated that G-CSF and GM-CSF increased the generation of MDSCs by downregulating the expression of interferon regulatory factor 8 (IRF-8) in myeloid progenitors via signaling via the STAT3 and STAT5 pathways. Interestingly, Yan et al. [112] demonstrated that both the ω-3 and ω-6 PUFA treatments significantly enhanced the expansion of cultured bone marrow MDSCs, PMN-MDSCs. The proliferation of T-cells decreased in a dose-dependent manner in mice given a PUFA-containing diet. Murine diets containing PUFAs increased the percentage of PMN-MDSCs in both the bone marrow and the spleen. The administration of PUFAs also stimulated the immunosuppressive properties of MDSCs isolated from mouse spleens. PUFA diets also induced the activation of JAK-STAT3 signaling and the immunosuppression of T-cells was mediated by ROS. Finally, Yan et al. [112] revealed that a PUFA-enriched diet augmented the growth of CT26 and Lewis lung carcinoma in mice. In agreement with these results, given that PUFAs activate PGE-2 production through COX-2, it is interesting to note that PGE-2 can stimulate STAT3 signaling [142]. PGE-2 is a potent inducer of MDSC functions [143] and thus might stimulate their expansion. Indeed, inhibition of COX-2 with Celebrex has been shown to slow tumor growth and inhibit MDSC expansion and numbers [144].

T-regs, a T-cell-based immunosuppressive cell, are divided into two major populations. This includes a thymic origin cell [145] and one that is induced in the PB [146] by TGF-β. Under homeostatic conditions, T-regs can limit the induction of autoimmunity, inhibit tissue destruction, and ensure the maintenance of tolerance to self-antigens [147]. In the PB of cancer patients, the frequency of T-reg cells is increased compared to normal individuals [148]. In addition, tumor-infiltrating T-regs are increased in tumors of cancer patients [149]. T-regs are phenotypically described as $CD4^{+}$ T-cells that co-express forkhead box P3 (Foxp3) and CD25 [148]. T-regs can also express checkpoint inhibitory molecules, including, but not limited to PD-1, lymphocyte activating gene-3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM3), glucocorticoid-induced tumor necrosis factor receptor (GITR), and

cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), which can all suppress T-cell responses [148, 150]. T-regs also indirectly suppress effector T-cells by depleting local IL-2 levels, which are needed for the proliferation and survival of effector T-cells [151]. Indirect suppression is also associated with the secretion of immunosuppressive cytokines, such as IL-10 and TGF-β [152].

Lipid mediators derived mainly from dietary PUFAs can also contribute to the control of inflammation. These mediators, which are collectively called specialized pro-resolving lipid mediators (SPMs), include the families of compounds termed "resolvins," "lipoxins," "maresins," and "protectins" [11]. The resolution of an inflammatory response by SPMs is characterized by lipid mediator "class switching," in which cells downregulate enzymes responsible for the production of proinflammatory lipids, including prostaglandins and leukotrienes, while upregulating enzymes responsible for the production of SPMs [153]. SPMs exert pro-resolving and anti-inflammatory activities without suppressing immunity [153, 154]. This contrasts with corticosteroid and nonsteroidal anti-inflammatory drug (NSAID) anti-inflammatory therapies. SPM's therapeutic potential is due to their activity in the nanomolar to picomolar range and secretion by many cells with minimal toxicities. LC-ω-3 PUFAs, which are precursors for SPMs, can inhibit T-cell proliferation and cytokine production [155]. Murine diets supplemented with fish oil or DHA have been reported to increase $CD4^+$ T-cell proliferation in response to a Th2 stimulus and decrease Th2 cytokine production by $CD8^+$ T-cells [156]. T-cells also express known SPM receptors, including ALX/FPR2 [157, 158], GPR32 [158], and BLT1 [159], which support their response to SPMs. Overall, SPMs show promise in regulating adaptive immune responses. Receptors for SPMs are found on DCs, T-cells, and B cells. Furthermore, SPM therapy has shown significant activity, including augmentation of pathogen clearance, resolution of inflammation, and the development of immune memory.

8.3.3 Dynamic Anti-inflammatory Activities of ω-3 PUFAs

One of the challenges with dietary studies using PUFA regulation of inflammation is that obesity is also associated with chronic low-grade inflammation and increased levels of FFA, proinflammatory cytokines, hormones, and circulating myeloid cells [160, 161]. Adipocytes can secrete metabolites that either promote or resolve an inflammatory response [162]. Thus, adipose cell hypertrophy and hyperplasia increases oxygen consumption, which can result in hypoxia [163], activation of cellular stress and inflammation in association with proinflammatory cytokine secretion [163]. Adipocyte hyperplasia also results in myeloid infiltration of adipose tissue, notably surrounding both dead and dying adipocytes, and a phenotypic shift of adipose tissue macrophages, that can release proinflammatory cytokines that induce ROS and activate inflammatory signaling pathways [164]. Obesity can also contribute to changes in the tumor microenvironment by increasing inflammatory cell infiltration and the presence of FFAs [165]. High levels of proinflammatory adipokines have also been shown to contribute to tumor infiltration of inflammatory cells within the microenvironment [166, 167] through autocrine and paracrine activation of signaling pathways including NF-κβ [168], STAT3, and extracellular regulated kinase (ERK)1/2, all of which stimulate tumor cell proliferation and inhibit apoptosis [169].

Further, LC-ω-3 PUFAs are metabolized into anti-inflammatory, bioactive SPMs, which can reduce inflammation [170]. These observations suggest that the resolving phase of inflammation is not passive but is actively downregulated by endogenous anti-inflammatory mediators [171]. This contrasts with ω-6 PUFA metabolites, including PGD-2, LTD-4, LTC-4, and LTE-4, which are inflammatory. Although AA is a precursor to LTs, its metabolite PGE-2 can also regulate macrophage and lymphocyte functions. Thus, dietary consumption of the ω-6 PUFA LA, as the precursor of AA, is causally linked to allergic diseases and provides a potential treatment strategy using LC-ω-3 PUFAs [172].

8.4 Dietary Lipid Regulation

of Murine and Human Tumor Initiation, Progression, and Metastasis

8.4.1 Dietary LC-ω-3 PUFA Controls of Murine Tumor Growth

Clinically, associations have been reported between PUFA consumption/composition and inflammation, although co-variable includes genetic susceptibility, tissue microenvironments, stress, obesity, age, caloric intake, and dietary duration. Murine models have also suggested mechanisms whereby PUFA composition can regulate tumor initiation and progression. These studies provide insight into various pathologic conditions including infections, autoimmune, inflammatory, neoplastic, and obesity conditions and a relationship with neutrophilia, splenomegaly, and multifocal, hepatic extramedullary myelopoiesis (i.e., the formation of myeloid tissue outside of the bone marrow) [173, 174]. Such inflammatory pathologies, which are associated with tumor initiation, are controlled by multiple risk factors, including hormones, obesity, diet, and age. However, following tumor initiation, inflammation is also regulated by tumor GF and chemokine secretion, as well as, additional risk factors. Thus, within the tumor microenvironment, crosstalk occurs between the immune response and inflammation, including EMM and tumor-secreted GFs.

Dietary PUFA regulation of tumor progression and metastasis has been studied in transplanted syngeneic and xenograft tumor models. In one xenograft model using MDA-MB-435 tumor cells, athymic nude mice were inoculated with tumor cells following the placing of recipients on either LA, EPA, or DHA diets. These studies revealed a significant delay in tumor growth and reduced metastases in mice fed an EPA or DHA diet, including reduced AA levels in tumor membrane phospholipids [175]. The results from one of our studies in a syngeneic tumor model, with two groups of mice receiving pair-fed isocaloric and isolipidic liquid diets documented the impact of PUFA composition on tumor growth [176]. Ten weeks following initiation of the diets, groups of mice received orthotopic injections of 4T1 mammary tumor cells. The results showed that mice consuming a LC-ω-3 PUFA diet had a significant delay in tumor initiation, slower growing tumors, and prolonged survival compared to mice given an ω-6 PUFA diet [176]. Interestingly, when mice were autopsied 35 days post-orthotopic injection, the hosts consuming the ω-6 based diets had a significantly greater number and frequency of pulmonary, hepatic, renal, cardiac, and bone marrow metastases. These results suggested that dietary PUFA composition is not only critical to tumor initiation but also modulates tumor growth and the extent of metastasis and distribution of localization metastatic sites.

As part of this study, tumors were collected 35 days after tumor injection, frozen in OCT and immunohistochemistry (IHC) performed to evaluate the frequency of proliferating cells using antibodies to KI67 (Fig. 8.2a) infiltration by macrophages, by staining with F4/80 (Fig. 8.2b) and infiltration by granulocytes and MDSCs, by staining with antibodies to neutrophil elastase (Fig. 8.2c). In every instance, the tumors in mice receiving an ω-6 based diet had a significant increase in proliferating tumor cells and infiltrating macrophages and granulocytes, as opposed to tumors from mice on an LC-ω-3 PUFA diet as described above. It is noted that there was a slight but significant difference in tumor sizes at this time, with a median tumor volume of 888.5 ± 115.2 mm^3 for the tumors from the ω-6 tumor-bearing mice and 446.3 ± 52.3 mm^3 in the ω-3 diets. This documents the effect of an ω-6 PUFA diet on innate inflammatory cell infiltration of 4T1 tumors and on tumor cell proliferation. Further, in murine studies of neoadjuvant therapy where treatment with EPA and DHA preceded surgery, the number of pulmonary metastases was significantly decreased compared to mice on an LA diet [177]. Similar results focused on immune-augmentation and therapeutic activities have been documented in R3230RC and MCF-7 breast adenocarcinoma tumor studies [178, 179], which included a reduced number of MDSCs [180]. In addition to a neoadjuvant

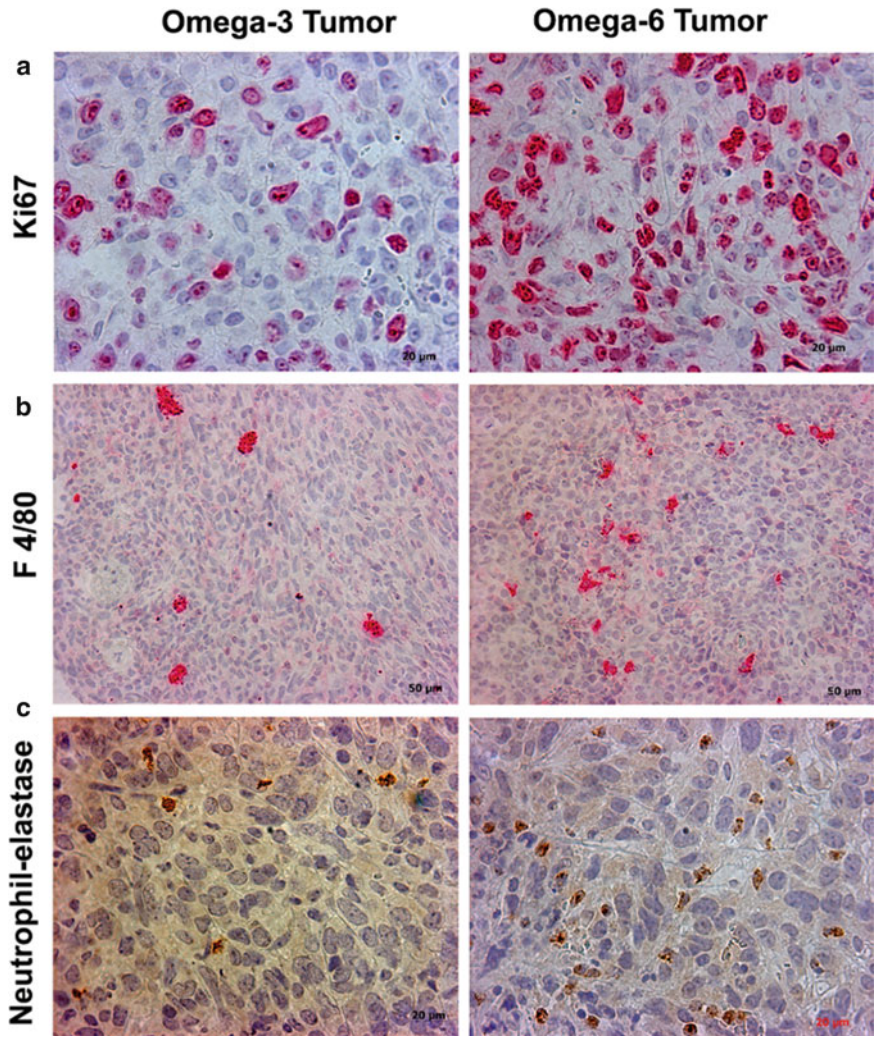

Fig. 8.2 Mice were fed omega-3 and omega-6 diets for 10 weeks prior to injection of 5000 4T1 cells in an abdominal mammary gland. Tumors were collected after 35 days from tumor injection, and IHC was performed for the evaluation of proliferating cells by Ki67 (**a**), intra-tumor macrophages by (**b**) F4/80, and neutrophil infiltration by staining with neutrophil elastase (**c**). (Images were taken at 400× magnification)

activity, LC-ω-3 PUFA diets have therapeutic potential. In a tumor survival study, mice were switched from an 8% corn oil (1% ALA) diet to an 8% canola oil (10% ALA) diet, when the average primary tumor volume became 60 mm^3. This ω-3 PUFA canola oil diet therapy significantly reduced tumor growth as compared to an ω-6 PUFA corn oil diet [181]. Based on these and other rodent studies, it appears that LC-ω-3 PUFA dietary intervention may be used with therapeutic intent [182].

Murine studies using LC-ω-3 PUFA and autochthonous chemically induced mammary tumors have confirmed and extended these observations using transplanted tumors. In an autochthonous 7, 12-dimethylbenz (α) anthracene (DMBA)-induced mammary tumor model, mice on a fish oil diet had a significantly reduced tumor incidence, slower tumor growth, and a reduced numbers of metastasis [183, 184]. The LC-ω-3 PUFA diet affected tumor induction and growth, which correlated with

reduced AA serum levels, suppressed tumor cell proliferation, protection against DNA single-strand breaks, and an increase in tumor cell apoptosis [184–186]. Similarly, a comparison of tumorigenic using a tumor model with N-methyl-N-nitrosourea (MNU)-induced rat mammary tumors and diets with differing fat composition were compared, including an SFA diet, a monounsaturated fat (MUFA) diet, an ω-6 PUFA diet, or diets with different ratios of ω-6: ω-3 PUFA. It was found that the diet incorporating a 1:1 ratio of ω-6:ω-3 PUFA could prevent mammary tumor development. Studies into causal relationships revealed that this diet group had decreased levels of COX-2 and 5-LOX transcription levels in mammary tissues and PPAR-γ levels [187]. Together, these and other studies support a role for LC-ω-3 PUFAs in regulating the metabolic inflammatory tumor microenvironment by upregulating PPAR-γ [186, 187]. Consistent with these studies, when dietary LC-ω-3 PUFA content is increased to an ω-6:ω-3 ratio of 1:14.6, as compared to a control diet of 1:0.7, a 60% decrease in tumor growth was observed [188]. Similar studies, using a therapy model with transplanted, orthotopic 4T1 mammary tumors, in which a 5% fish oil diet was initiated when the hosts had developed primary tumors that were 8–10 mm^3 in diameter, resulted in significantly reduced growth and metastases that had a correlation with decreased tumor cell proliferation [189]. A similar therapy study using C3(1) Tag mice revealed that a switch to a fish oil concentrate from a corn oil diet slowed prostate tumorigenesis and progression in association with lower estradiol, testosterone, and androgen receptor levels [190]. The ability of LC-ω-3 PUFAs to downregulate inflammatory mediators and increase tumor cell apoptosis supports the importance of its regulation in the tumor microenvironment. In vivo studies have studied the effect of dietary LC-ω-3 PUFA on inflammatory cells in animal models of both LPS inflammatory disease and tumor-induced inflammation. However, the majority of murine models have used diets that were neither isocaloric nor pair-fed, raising the question of obesity versus a role for dietary composition? Since obesity itself is inflammatory, clarifying the effects of obesity-associated inflammation, as opposed to dietary regulation of inflammation, is critical to determining the regulatory activity of dietary components. Thus, using animal models, with an isocaloric, isolipidic liquid diet that allows pair feeding and controlled dietary caloric intake, is needed to assess the impact on host weight and adipose changes, as well as dissociate effects between obesity and dietary composition.

Epidemiologic studies support the role for NSAIDs, including aspirin in reducing the incidence of cancer and can prolong survival if administered postdiagnosis [114]. Initial studies focused on colorectal cancers; however, low-dose aspirin has also demonstrated antitumor activity for other tumors, including lung, breast, prostate, and metastatic cancers [191, 192]. In addition, low-dose aspirin has been found to improve survival and provide chemopreventive benefits in combination with cytotoxic therapy and/or surgical resection [193, 194]. Many of these studies are derived from patients receiving low-dose aspirin for cardio-prevention, in which 20–30% have been suggested to obtain benefit with a decrease in cancer incidence [195]. However, studies also show that neither non-aspirin NSAIDs nor acetaminophen is associated with a reduced risk of cancer or chemoprevention [196, 197]. While the known anti-inflammatory activity of aspirin offers a rational mechanism of action, the unique antitumor mechanisms of aspirin compared with other NSAIDs is confounding. It is noted that the use of low-dose aspirin in cancer patients is limited by gastrointestinal bleeding and hemorrhagic stroke [198].

Investigations into anti-inflammatory mechanisms in cancer patients have focused on the downregulation of proinflammatory mediators, including cytokines, eicosanoids, and enzymes [114]. COX-1 and COX-2 are key targets of aspirin and are involved in the biosynthesis of proinflammatory lipid autacoids, including prostaglandins. Aspirin's anticancer activity has been suggested to be associated with the irreversible acetylation of cyclooxygenases that are overexpressed in cancer patients [199, 200].

In contrast to other NSAIDs that reversibly block COX enzymes, aspirin qualitatively alters enzymatic substrate specificity and activity of COX. A unique function of aspirin-acetylated COX is the metabolism of aspirin-triggered (AT) SPMs, including AT-lipoxin A4 (AT-LXA4) and AT-resolvins D1 (AT-RvD1) and D3 (AT-RvD3) [201–203]. Other NSAIDs do not trigger endogenous SPM production [204]. Thus, SPMs, such as resolvins and lipoxins, promote the resolution of inflammation by stimulating phagocytosis of cellular debris and counter-regulating proinflammatory cytokines without being immunosuppressive [204, 205].

The aspirin-acetylated COX facilitates the biosynthesis of aspirin-triggered SPMs from ω-3 PUFA substrates, including EPA and DHA [206]. While AT-resolvins exhibit potent anti-inflammatory activity characteristic of native resolvins, the AT forms resist rapid inactivation and have longer half-lives [204]. AT-SPMs are increased in patients who respond to the anti-inflammatory activity of aspirin compared with those that do not respond to aspirin [207]. AT-lipoxins also modulate tumor-associated macrophages and reduce bone cancer pain [208, 209]. Further, SPMs, such as resolvins, enhance cytotoxic cancer therapy by promoting the clearance of therapy-generated apoptotic tumor cells by macrophages [210]. AT-resolvins and AT-lipoxins have been shown to be critical for the anticancer activity of low-dose aspirin by resolving tumor-promoting inflammation in mice [211].

8.4.2 Dietary LC-ω-3 PUFAs and Improved Cancer Patient Outcomes

The tumor microenvironment includes, in addition to tumor cells, extracellular matrix, endothelial cells, stromal cells, fibroblasts, adipocytes, and critically infiltrating inflammatory cells (M2 macrophages and MDSCs) as well as suppressive and effector lymphocytes, all of which have a role in regulating tumor progression and metastasis. The infiltrating lymphocytes, particularly mature CD8 T-cells, serve as mediators of antitumor activities [212, 213]. In cancer patients, the infiltration of lymphocytes provides an independent, positive prognostic factor as assessed by IHC staining [214]. Studies into the type of infiltrating immune cells (e.g., $CD3^+$, $CD8^+$, and $FOXP3^+$ T-lymphocytes) and the density or location of infiltrating T-cells also provide prognostic correlations with positive (or negative) outcomes in patients with colorectal cancer (CRC) [215–222], ovarian cancer, and breast cancer [223–226]. A meta-analysis of these clinical studies assessed the impact of tumor-infiltrating leukocytes on outcomes, including one incorporating 30 studies with 2988 patients [227]. This analysis examined associations between survival and inflammatory cell ($N = 12$) and T-lymphocyte subset infiltration ($N = 18$) studies. Pooled analyses documented that a generalized tumor inflammatory infiltrate was associated with significantly improved cancer-specific survival (CS), overall survival (OS), and disease-free survival (DFS). Stratification by cellular location and T-lymphocyte subset suggested that in the tumor microenvironment, $CD3^+$, $CD8^+$, and $FoxP3^+$ cellular infiltrates were not significant prognostic markers for OS or CS. In contrast, a high frequency of infiltrating $CD8^+$ but not $CD3^+$ or $FoxP3^+$ T-cells was predictive of an increased OS. Furthermore, a high frequency of tumor-infiltrating $CD3^+$ T-cells, at the invasive tumor border, was associated with improved OS and DFS [227].

Consistent with the effects of LC-ω-3 PUFAs on tumor-infiltrating leukocytes is an inverse relationship between dietary consumption of LC-ω-3 PUFAs and the probability of developing CRC, as found in case-control studies by Murff et al. [228] and Habermann et al. [229]. However, the benefits were limited such that, in one study [228], an increased LC-ω-3 PUFA intake was associated with a reduced risk of CRCs in women. In a second trial [230], an inverse relationship was observed between low DHA intake and an increased risk of CRC in patients with genetic variants that resulted in higher proinflammatory mediators. Recently, a relationship between LC-ω-3 PUFA consumption and survival was observed

in a retrospective analysis of the CALGB 89803 randomized trial of adjuvant chemotherapy for completely resected stage III CRC ($n = 1264$) [230]. Patients in the highest quartile of LC-ω-3 PUFA dietary intake had a significantly increased DFS compared with the patients in the lowest quartile. Notably, this relationship appeared to be highest for patients with high CRC COX-2 expression [230]. Further, clinical studies have also examined adjuvant therapy with LC-ω-3 PUFA [231] such that higher consumption of LC-ω-3FA was associated with improved OS in patients with established CRC using two independent cohort studies [230, 232].

The EMT study is the only reported randomized trial of purified LC-ω-3 PUFA treatment in patients with metastatic CRC [233]. This is a phase II double-blind, randomized, placebo-controlled trial of EPA, in the FFA form, 2 g daily before surgery in patients ($n = 88$) undergoing liver resection for CRC liver metastases. In the first 18 months after resection, EPA-treated individuals obtained an OS and DFS benefit compared to the placebo cohort [233]. This preliminary observation from a "window" trial of limited LC-ω-3 PUFA use, prior to metastasis surgery, resulted in a phase III randomized trial of EPA (4 g daily in the ethyl ester form) in patients undergoing liver resection for CRC liver metastasis (the EMT2 trial). In this trial, subjects are randomized to EPA or placebo at least 2 weeks before surgery and continue the drug long term, with progression-free survival (PFS) as the primary endpoint and OS as the key secondary endpoint (ClinicalTrials.gov; NCT03428477).

The possible beneficial effects of ω-3 PUFAs in CRC incidence was first suggested in 1997 in West Coast fishermen [234]. Two years later, it was pointed out that several of the known risk factors for some cancers, including colon cancer, may be reduced by dietary ω-3 PUFAs supplementation, and implementation of clinical chemoprevention trials was encouraged [235]. Based on these observations and other studies, two randomized trials of LC-ω-3 PUFA supplementation are underway that have secondary CRC endpoints. These include the ASCEND (A Study of Cardiovascular Events in Diabetes) trial (NCT00135226), which is a 2 × 2 factorial study of long-term (median 7.5 years) LC-ω-3 PUFA (840 mg EPA/DHA ethyl ester daily) and aspirin (100 mg daily) treatment for the prevention of cardiovascular and cerebrovascular events in patients with diabetes ($n = 15{,}480$). In this study, cancer outcomes were a secondary endpoint, with the ability to continue with posttrial follow-up. In the ASCEND trial which tested ω-3 supplementation (at a dose of 1 g per day) in adults with diabetes in the United Kingdom provided generally null results. In contrast, the VITamin D and OmegA-3 Trial (VITAL) study (NCT01169259), which was a 2 × 2 factorial study of the same dose and formulation of LC-ω-3 PUFA (also 840 mg EPA/DHA ethyl ester) and vitamin D3 in 25,871 US male and female participants had different outcomes. During the overall treatment period of 5.3 years, there was a statistically nonsignificant 17% reduction in cancer death, with a hazard ratio of 0.83 [236, 237]. However, the protocol planned to account for a latency period with some analyses that excluded early follow-up. In an analysis that excluded the first 2 years of follow-up, there was a reduction in cancer deaths that was statistically significant, a 25% reduction, as well as a nonsignificant 6% reduction in cancer incidence with vitamin D. A beneficial association between higher consumption of LC-ω-3FA and a lower incidence of CRC was reported to be restricted to a subset of tumors with microsatellite instability (MSI) [238]. MSI occurs in 15% of CRC patients and is caused by a loss of DNA mismatch repair (MMR) activity [239]. Consistent with the anti-inflammatory activities of LC-ω-3FA, data support the critical role of inflammation and dysregulated anti-tumor immune response in the development of MSI tumors [240]. It should be noted that immune checkpoint inhibitor therapy has been shown to be more effective for treating cancers with MSI [241, 242]. This suggests that increased dietary consumption of LC-ω-3FA after diagnosis may benefit patients with MSI tumors [243].

As supported by our transplantable tumor studies as discussed above and shown in Fig. 8.2, IHC analyses of infiltrating lymphocytes,

particularly $CD3^+$ T-lymphocytes in primary tumors, provides a biomarker that predicts improved clinical outcomes [244–246]. Furthermore, basic histological quantification of T-lymphocyte density, cytotoxicity, and a memory phenotype, by $CD3^+$, $CD8^+$, and $CD45RO^+$ markers, respectively, demonstrated that an increase in T-lymphocyte infiltration is associated with significant improvements in DFS and OS [217, 245, 247]. In CRC, identifying the location of infiltrating cytotoxic T lymphocytes (CTLs), assessed as $CD3^+CD8^+$ T-cells within the center (CT) and invading margin (IM) of the primary tumor, predicts clinical outcomes [217]. The quantification of the density, phenotype, and location (CT or IM) of infiltrating CTL provides an immunoscore [248–250]. Indeed, the significance of the $CD3^+$ cell infiltration analysis surpasses a diagnosis of tumor stage, lymph node, and metastatic invasion, sub-setting patients into five categories based on the location in the tumor (CT and IM) of $CD3^+$ and $CD8^+$ T-cells [251, 252].

In association with immunoregulatory properties, a patient's lifestyle, preceding and following diagnosis and therapeutic interventions, is associated with controlling cancer initiation, progression [253], and responses to therapeutic interventions [254]. Specifically, patients who consume a high-fat diet (saturated fat, or ω-6 PUFAs) frequently exhibit neutrophilia that can facilitate tumor initiation, progression, and result in poor outcomes [255, 256]. Conversely, diets that contain a high LC-ω-3 PUFA content have been associated with decreased inflammation, lower EMM, and improved outcomes [173]. The improved clinical outcomes were initially suggested by epidemiological studies into the incidence and progression of breast cancer in American women of Japanese descent, as compared to Japanese women living in Japan. The results from one study indicated a significantly higher breast cancer incidence in American women of Japanese descent compared to Japanese women in Japan [257]. This observation is supported and extended by studies with female children from Japanese immigrants to America, but not the immigrants themselves, who had breast cancer rates similar to the general American population [258]. In the 1990s, dietary components were found to be implicated in these different incidences [259]. These correlative epidemiologic studies are supported by rodent studies, which demonstrated that LC-ω-3 PUFAs can reduce proinflammatory cytokines, inflammation, and cancer development [260].

Case-control studies have also shown an inverse relationship between dietary ω-6 and LC-ω-3 PUFAs ratio and the incidence of breast cancer, supporting their dietary importance [261]. An epidemiological study of 56,007 French women over 8 years revealed that the risk of breast cancer was unrelated to dietary PUFA consumption. Rather, a significant risk was associated with the ratio of dietary ω-6 versus LC-ω-3 PUFAs, which was inversely related to LC-ω-3 PUFA levels in women with the highest intake of ω-6 PUFAs, indicating interactions with PUFA consumption [262]. Subsequent studies revealed a decreased risk of developing breast cancer with dietary LC-ω-3 PUFA in a case-controlled, population-based study [263] that showed a reduction in all-cause mortality that was reduced 16–34% in women consuming high levels of LC-ω-3 PUFAs [264]. Indeed, during the last 20 years, data has accumulated supporting the observation that high ω-6 PUFA dietary consumption is proinflammatory, likely involving COX-2 secretion and NFκβ activation, resulting in an increased incidence of cancer and all-cause mortality. In contrast, consumption of high levels of LC-ω-3 PUFA were found to be protective against neoplasia, including a decreased incidence of cancer associated, all-cause mortality [265]. Indeed, in a meta-analysis of 11 independent prospective studies, it was observed that a decrease in the dietary ω-6: LC-ω-3 PUFA ratio significantly lowered the risk of breast cancer [266]. However, some studies have shown no association between heightened ω-6: LC-ω-3 PUFA ratios in the diet and breast cancer development.

Recent studies have investigated the underlying mechanisms of this observation and its relationship to innate and acquired immune cell infiltration of the tumor microenvironment. The regulatory activity of LC-ω-3 PUFA on

macrophage functions has also been documented with the use of antagonists to G protein-coupled receptor (GPR120), which is expressed by some myeloid cell populations and acts as a PUFA receptor [267]. This is supportive of a role for LC-ω-3 PUFA mediation and anti-inflammatory effects via this receptor. However, PPAR-γ also acts as a receptor for PUFAs and the regulatory mechanisms of LC-ω-3 and ω-6 PUFA on obesity [268], postmenopausal breast cancer [269], and microenvironmental inflammation [270], suggesting a need for additional studies. Further, PUFAs contribute to the regulation of bone marrow (BM) and EMH at sites such as the spleen [271, 272] and may also expand the frequency of MDSCs [112].

Unfortunately, LC-ω-3 PUFA dietary supplements can lead to various toxicities. Despite the therapeutic benefits discussed herein, there are potential risks associated with high doses. The primary adverse effects are altered platelet function. The presence of EPA and DHA leads to the production of TX A3, which is a less potent platelet activator than TX A2. Supplementation of EPA and DHA, therefore, can affect platelet activation because of the different eicosanoids produced, resulting in an antithrombotic effect that can impact blood coagulation and wound healing [273]. The impact depends on the amount and the duration of LC-ω-3 PUFA supplementation. When given in combination with other medications, such as aspirin or warfarin LC-ω-3 PUFA interactions may exacerbate adverse effects that can occur with LC-ω-3 PUFA supplementation alone [274–278]. LC-ω-3 PUFAs supplementation is contraindicated during antiplatelet and anticoagulant treatment because of the additive effect on bleeding times when administered together [279].

8.5 Future Trends or Directions

Dietary PUFA consumption may not only affect inflammation and the incidence and progression of neoplasia, but may also support responses to therapeutic interventions in cancer patients via the regulation of inflammation. In general, increased dietary ω-6 PUFA consumption is associated with a heightened risk of cancer that is suggested to be due to a proinflammatory tumor microenvironment. In contrast, an LC-ω-3 PUFA diet has potential protective effect to suppress ω-6 PUFA-associated inflammation. Nutritional recommendations are that individuals should decrease dietary ω-6 PUFA intake and increase LC-ω-3 PUFA consumption with an intake of at least 500 mg/day of LC-ω-3 PUFA [280]. PPAR-γ and GPR120 agonists also have potential for use as chemopreventive drugs, although their use may, perhaps, be better targeted toward either high-risk individuals or as part of therapeutic interventions. Both of these are receptors for LC-ω-3 PUFA [281, 282]. Regardless, we need to further study both pharmacophores and dietary regulation of PUFAs as protective and therapeutic strategies for cancer and their association with leukocyte infiltration of tumors.

Research on the role of LC-ω-3 PUFAs and SPMs on inflammation and cancer is increasing and suggests a positive role for use as an adjuvant in cancer therapy. Increased efforts are needed using high-quality randomized control trials to establish their mechanisms of action, the optimal timing for supplementation, dosage, product source, method of extraction, preparation, and quantification to obtain efficacy, which will optimize their clinical use for cancer prevention and therapy. These future trials should address these questions as well as the impact on the tumor microenvironment, specifically infiltrating cellular subtypes. We also stress the need for translational/preclinical studies that utilize isocaloric and isolipidic pair-fed diets to segregate the regulation of immunity and inflammation by obesity versus dietary PUFA. Further, care must be taken to differentiate between activity on tumor growth as opposed to metastasis, as these biologic parameters are interrelated such that, larger tumors typically have more metastases. In our experience, ω-6 PUFA diets impact not only primary tumor growth but also the extent and critically sites of metastasis, all of which are typically unstudied but highly relevant since metastasis is frequently the ultimate cause of patient mortality.

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9 Extracellular Vesicles in the Tumor Microenvironment: Various Implications in Tumor Progression

Alex C. Boomgarden, Colin Sheehan, and Crislyn D'Souza-Schorey

Abstract

Extracellular vesicle (EV) shedding is a biologically conserved cellular process across virtually every cell type. In cancer, EVs shed from tumor and stromal cells to the tumor microenvironment play a major role in determining tumor fate, which to a large extent is dictated by the biologically active cargo contained in EVs. Current understanding of various cancer-associated EVs has enabled the outlining of mechanistic connections between cargo and tumor-promoting functions. In this chapter, we describe examples of EV-mediated communication between tumor cells and stromal cells, highlighting the molecular constituents responsible for pro-tumorigenic effects. Furthermore, we discuss the roles of matrix-degrading EVs in cell invasion. Finally, we summarize research on the potential use of EVs as a novel approach to cancer therapeutics.

Keywords

Extracellular vesicles · Exosomes · Microvesicles · Tumor · Microenvironment · Cell signaling · Stromal cells · Endothelial cells · Macrophages · Cancer-associated fibroblasts · Lymphocytes · Cell proliferation · Apoptosis · Angiogenesis · Cell invasion · Cancer therapeutics

9.1 Introduction

It has long been suspected that the initiation, progression, and metastasis of a tumor are intimately linked to the nature of the surrounding tissue environment. Since Stephen Paget's early "seed and soil" theory in 1889, numerous studies have confirmed that a tumor (the "seed") is nurtured by the proximal stromal cells, which along with the extracellular matrix (ECM) comprise what is known as the tumor microenvironment (TME; the soil) [37]. Stromal cells within the TME include endothelial cells (ECs), cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), neuroendocrine (NE) cells, and various other immune cells (T-lymphocytes and natural killer (NK) cells) (Fig. 9.1a) [22]. After researchers determined that physical contact between a tumor and its associated TME promotes nearly all characteristics of cancer, numerous labs became focused on understanding the intercellular

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1_9

communication between these two entities and their ability to phenotypically influence one another [23, 85]. While extracellular vesicles (EVs) were initially believed to function solely as vehicles to shuttle unwanted components and waste out of the cell, it is now understood that through EV-cell interactions, a bidirectional cell-cell communication is achieved [30, 46].

EV shedding is an evolutionarily conversed process across nearly all cell types. By virtue of this, EVs exhibit rich heterogeneity in their size, structure, and composition [85]. While ongoing research in the field has caused for modification in nomenclature, EVs can be broadly categorized into three subgroups: (1) exosomes (50–150 nm), (2) microvesicles (MVs; 100–1000 nm), (3) and apoptotic bodies (ABs; 1000–5000 nm). For the most part, this review will discuss the roles of exosomes and microvesicles in the tumor microenvironment. Once shed into the TME, EVs can transduce signals to the parent cell (autocrine signaling), neighboring cells (paracrine signaling), or distant tissue (endocrine signaling) through various interactions (Fig. 9.1b), which include (I) ligand receptor binding, (II) transferring of biologically active cargo through endocytic uptake, (III) plasma membrane (PM) bursting and free-floating constituent-receptor interaction, and (IV) direct fusion between EV and cell PMs [13]. As a result, the characteristics and constituents of a given EV will fundamentally dictate the phenotypic change it can induce onto the recipient cell. EVs contain a highly heterogeneous mixture of nucleic acids (DNA, mRNA, pre-microRNA, microRNA), proteins, and lipids, and the exact nature of EV cargo is dependent on the parent cell it was shed from. As cell transformation or reprogramming can modulate or alter these constituents, tumor and stromal cell-derived EVs exhibit a wide range of unique functional characteristics within the TME, thus allowing a bidirectional influence between the two cell entities that is frequently seen to benefit the progression of a given cancer [85].

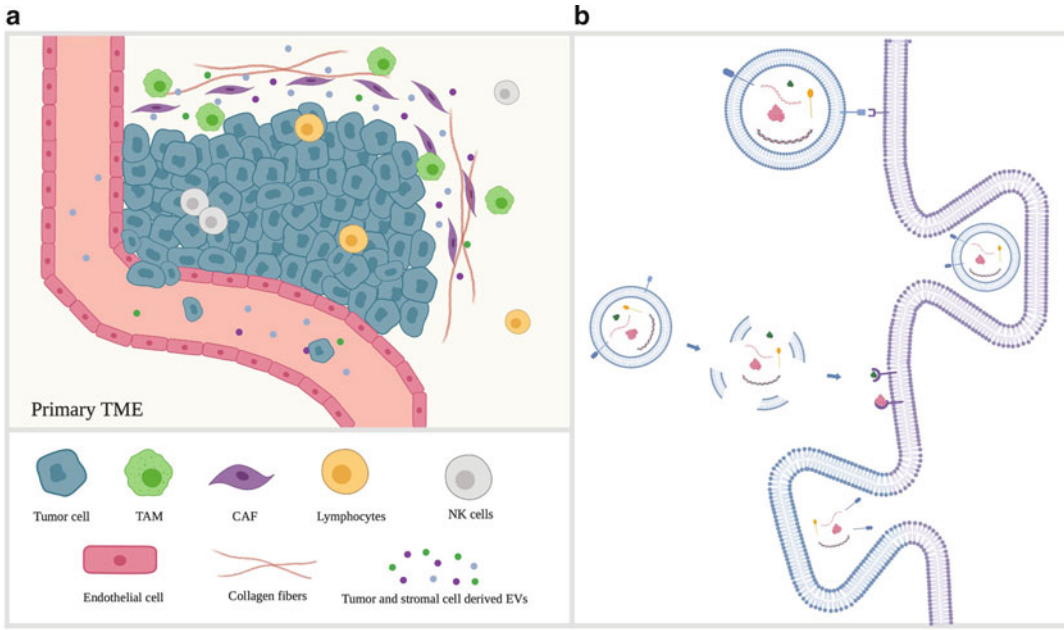

Fig. 9.1 EV shedding and communication in the tumor microenvironment (TME). (**a**) Schematic of a primary tumor and its associated TME. EVs are secreted by various cell types in the TME and include endothelial cells (ECs), cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), lymphocytes, NK cells, and collagen fibers of the ECM. (**b**) EVs interact with recipient cells. This can occur by (I) ligand receptor binding, (II) EV endocytosis, (III) EV bursting and free-floating constituent-receptor interaction, and (IV) direct fusion between EV and cell PMs

In this chapter, we begin by a brief historical perspective of EV biological research, noting hallmark findings that have led to the current understanding of EV-mediated communication within the TME. We discuss specific tumor and stromal cell-derived EVs that have demonstrated pro-tumorigenic capabilities, highlighting specific molecular traits which link to a given tumorigenic effect. We then discuss the mechanisms by which tumor cells can migrate and break through a surrounding ECM, including the secretion of certain proinvasive EVs. Finally, we outline studies that have utilized knowledge of pro-tumorigenic EVs to successfully interfere with or impede the progression of a given cancer, showcasing their potential in future cancer-related therapeutics.

9.2 Understanding EV-Mediated Communication

EV biogenesis and shedding are cellular functions evolutionarily conserved across all (human) cell types, leading to their presence in various mammalian bodily fluids [67, 87]. Among these include blood plasma, which was being studied by many scientists in the mid-1900s as a means to investigate the signaling vector(s) responsible for triggering blood clotting [25]. Contradicting the central dogma that this was a platelet-dependent process, Erwin Chargaff and Randolph West reported that a platelet-free plasma maintains the ability to induce coagulation [9]. Inspired by similar findings from the 1950s, Peter Wolf further investigated this topic by conducting a series of ultracentrifugation steps on plasma taken from various blood samples. This allowed for the isolation and visualization of vesicular structures Wolf referred to as "platelet dust," which were in fact platelet-derived EVs that can exhibit procoagulation activity [82]. This visualization was possible through newly available electron microscopy (EM), which enabled Wolf and several other labs to detect EVs across various other mammalian biological samples, including cartilage and serum [3, 5].

While these initial findings eliciting their role in normal homeostatic mechanisms gained little attention in the science community, identification of tumor cell-derived EVs (T-EVs) in 1980 sparked the interest of various labs to further investigate and characterize these membrane-bound entities as a means to understanding cancer and other pathologies [73]. Interestingly enough, their ability to impact tumor fate was implicated in some of the earliest reports involving EVs. This includes the classic study by Poste and Nicolson, which showed that "tumor membrane fragments" could transfer the highly metastatic phenotype to a recipient cell through PM fusion [53]. After nearly four decades of research, this study is considered to be among the first to report on the pro-tumorigenic capabilities of EVs in the TME. An abundance of research since has provided a more in-depth understanding of the molecular composition of certain tumor or stromal cell EVs, allowing one to draw connections between certain traits to their pro-tumorigenic effects [24, 85].

Today it is well appreciated that cancer cells in isolation are insufficient to ultimately bring death of the host organism; instead, cancer cells collude with a variety of other cell types within the tissue environment to serve their purpose. These corroborating cells support cancer cells by many processes such as increasing oxygenation and waste removal in the expanding tumor, facilitating cell invasion by ECM degradation, providing nutrients, and inhibiting antitumor immune responses. To do so, cancer cells engage in intercellular communication with other cell types through the secretion of a variety of soluble stimulatory factors and also through the release of extracellular vesicles. It is now clear that EVs such as exosomes and microvesicles take part in a dense network of interactions between cell types within the tumor microenvironment (Fig. 9.1) [8, 24]. Future functional studies conducted in vivo will likely identify new EV-mediated cross talk, as well as clarify those interactions with the most significance to cancer progression.

9.3 Pro-tumorigenic Cross Talk Between Tumor and Stromal Cells

While the ability of EVs to mediate cell-cell signaling is maintained, the process of oncogenic transformation of a given cell can affect both the production and overall constituents of its' shed vesicles, thus altering the effect the vesicles can have on neighboring tumor and stromal cells. By virtue of this, tumor-derived EVs have been shown to robustly affect the fate of a given cancer by modulating the cellular and architectural characteristics of the TME. Moreover, EVs released from non-transformed stromal cells within the TME can also modulate and condition the tumor niche, thus achieving a bidirectional influence between these two entities that can impact nearly all characteristics of cancer. These ten major characteristics include the following: (1) resisting apoptosis, (2) stimulating angiogenesis, (3) unlimited replicative potential, (4) deregulation in metabolism, (5) immune system evasion, (6) genome instability, (7) sustained proliferative signaling, (8) desensitization to growth suppressor, (9) inflammation, and (10) promoting invasion and metastasis [80]. Here, we will discuss pro-tumorigenic EVs shed from tumors and various stromal cells while noting the specific molecular constituents linked to such an effect (Table 9.1). The key stromal cell types include endothelial cells (ECs) and platelets, cancer-associated fibroblasts (CAFs), tumor-associated fibroblasts (TAMs), T-lymphocytes, and natural killer (NK) cells. Further below we summarize current literature on the influence of each of the aforementioned cell types (Table 9.2).

Endothelial Cells (ECs) and Platelets The "angiogenic switch" is a crucial transition step in tumor progression by which neovasculature is continually generated to oxygenate and remove waste in support of tumor growth. This process involves the invasion and organization of activated endothelial cells and pericytes to form branching tubules within the tumor. Angiogenesis is a latent developmental program that is exclusively activated in adult tissues during wound repair and the female menstrual cycle processes. However, cancer cells can regain access to this program either internally through deregulated signaling pathways or extrinsically by co-opting other cell types within the tumor microenvironment [6, 49].

Microvesicles (MVs) and other EVs contribute to angiogenesis by mediating intercellular communication that promotes the secretion of other pro-angiogenic factors or directly modulating endothelial cell behavior. Al-Nedawi et al. demonstrated that cancer cells expressing oncogenic EGFR transfer its signaling potential to human umbilical vein endothelial cells (HUVECs) via microvesicle delivery. Recipient cells demonstrate increased activation of downstream EGFR targets such as Akt and MAPK; further, this stimulates HUVEC expression of VEGF that appears to act in an autocrine feedback loop to sustain endothelial activation, as the effects of tumor-derived microvesicles (TMVs) could be abrogated with treatment of the VEGFR-2 inhibitor (SU5416) [1, 2]. In addition, another study found that EVs shed by breast cancer cells can harbor a unique VEGF molecule, $VEGF_{90K}$, which is formed through cross-linking smaller isoforms by acyl transferase tissue transglutaminase (tTG) enriched in MVs. These microvesicles directly promote endothelial tubulogenesis, which RNAi knockdown demonstrates VEGF is necessary for effect. In addition, Feng et al. suggest important clinical implications with their findings, as the $VEGF_{90K}$ isoform while associated with microvesicles remains insensitive to inhibition by the common VEGF drug, bevacizumab (Avastin) [19].

Other vascular cell types within the tumor microenvironment participate in angiogenic stimulating pathways. Activated platelets shed microvesicles that can be taken up by lung carcinoma cells and stimulate a variety of pro-tumorigenic effects, including upregulated expression of the pro-angiogenic factors, VEGF, IL-8, and HGF [28]. Interestingly, endothelial cells themselves appear to utilize microvesicles in order to coordinate with neighboring endothelial cells in a paracrine fashion and orchestrate complex behaviors such as tubulogenesis.

Table 9.1 Tumor cell-derived EVs

Tumor cell origin	Tumorigenic constituent	Target cell	Tumorigenic effect	References
Various EGFR-containing human cancer cells (A431, A549, DLD-1)	EGFR	Endothelial cells (ECs)	Angiogenesis stimulator	Al-Nedawi et al. [2]
Various breast cancer cells	$VEGF_{90K}$	Endothelial cells (ECs)	Promotes endothelial tubulogenesis	Feng et al. [19]
Liver carcinoma (HepG2)	miR-23a	Endothelial cells (ECs)	Pro-angiogenic stimulator	Sruthi et al. [68]
Gastric carcinoma (SGC7901)	miR-130a	Human umbilical vein endothelial cells (HUVECs)	Angiogenic stimulator	Yang et al. [86]
Mouse (4 T1, 4 T07) and human breast cancer cells (MCF10A1a)	Various miRNAs	Fibroblasts/CAFs	Fibroblast activation	Vu et al. [77]
Gastric cancer cells (HSC-44PE, 44As3)	miR-125b	Fibroblasts/CAFs	Increased fibroblast activation and chemokine expression	Naito et al. [47]
Colorectal cancer	miR-145	Macrophage-like cells	Polarized cells to an M2-like TAM phenotype	Shinohara et al. [66]
Triple-negative breast cancer (TNBC)	CCL5	Tumor cells (autocrine signaling) and macrophage-like cells	Programing of macrophages to an M2-like TAM phenotype	Rabe et al. [55]
Human melanoma	FasL	T-lymphocytes	Apoptotic signaling to T-lymphocytes	Andreola et al. [4]
Oral cancer	TGF-β	T-lymphocytes	Apoptotic signaling to T-lymphocytes	Kim et al. [32]
Brain and breast cancer cells (BT-474, MDA-MB-231)	TGF-β	Splenocyte	Apoptotic signaling to T-lymphocytes	Rong et al. [60]
Metastatic melanoma	PD-L1	CD8 T-cells	Suppresses function	Chen et al. [10]
Murine breast cancer	TGF-β	NK cells	Inhibit NK cell function	Liu et al. [40]
Acute myeloid leukemia	TGF-β	NK cells	Suppress NK cell activity and block entrance to the TME	Szczepanski et al. [71]

Table 9.2 Stromal cell-derived EVs

Stromal cell origin	Tumorigenic constituent	Target cell	Tumorigenic effect	References
Platelets	VEGF, IL-8, and HGF	Lung carcinoma	Metastasis and angiogenesis	Janowska-Wieczorek et al. [28]
Immune cells	IL-8 (immune cells)miRNA and phosphor-STAT5 (endothelial cells)	Endothelial cells (paracrine and autocrine)	Activates endothelial cells and promotes 3D tube formation	Lombardo et al. [41]
CAF	CD81	Breast cancer	Increased migration	Luga et al. [42]
CAF	ANXA6/LRP1/TSP1 complex	Pancreatic ductal adenocarcinoma (PDA)	Increased aggressiveness	Leca et al. [38]
CAF	lncRNA H19	Colorectal cancer (CRC)	Promotes stemness and chemoresistance	Ren et al. [57]
CAF	Various other suspected transcription factors	Oral squamous cell carcinoma (OSCC)	Induce migration and invasion	Dourado et al. [17]
TAM	Apolipoprotein E (ApoE)	Gastric cancer (GC) cell	Migration potential enhanced	Zheng et al. [89]
TAM	miR-29a-3p, miR-21-5p	T-cells	Immune-suppressive TME, promoting epithelial ovarian cancer (EOC) progression	Zhou et al. [90]
TAM	HIF-1α-stabilizing long noncoding RNA (HISLA)	Breast cancer cells	Promotes aerobic glycolysis and apoptotic resistance	Chen et al. [11]

Lombardo et al. demonstrate that IL-3, an inflammatory cytokine produced by immune cells and secreted in tumor microenvironments, stimulates microvesicle shedding by endothelial cells; these microvesicles transfer miRNA and phospho-STAT5 cargo, which activates recipient endothelial cells and promotes 3D tube formation [41].

Cancer-Associated Fibroblasts (CAFs) As cancers are sometimes referred to as a wound that doesn't heal, it is no surprise that stromal fibroblasts, which function in tissue repair and regulation of inflammation, play an important role in cancer progression [31]. As depicted in Fig. 9.1, circulating tumor EVs in the TME are involved in various cell signaling pathways, which cause an increase in the concentration of specific TME-associated cell types, or "cell states." Among these include fibroblasts that are triggered to become activated and remain as such and in the context of cancer are commonly referred to as cancer/carcinoma-associated fibroblasts or CAFs. Frequently seen as the most abundant cell in the TME, CAFs are well known for their protumorigenic functions, which include angiogenesis stimulation and immune system evasion [65]. The induction of a CAF-like phenotype, which involves the transition from a dormant, normal fibroblast to an alpha-smooth muscle actin- (α-SMA) or chemokine-expressing active form, is understood to be heavily dependent on interaction with tumor cell-derived EVs [47].

Similar to TME ECs, specific miRNA-containing EVs appear to be responsible for this CAF phenotypic transition. Among these include miR-125-containing EVs, which following their release from mouse and human breast cancer cell lines were found to elevate fibroblasts activation [77]. Furthermore, Naito et al. found that while EVs shed from high-metastatic diffuse-type gastric cancer (DGC), cancer cell can strongly induce fibroblast activation; the same effect is not seen in those shed from a low-metastatic DGC [47]. As stated in the previous section, stromal cell-derived EVs in the TME have also been found to promote the growth and progression of a given tumor, which includes those derived from CAFs. In a study involving human primary CAFs, shed vesicles profoundly increased the proliferation, migration, and invasion of oral squamous cell carcinoma [17]. Ren et al. also demonstrated that through exosomal transfer of noncoding RNA (lncRNA) H19, CAFs promoted stemness and chemoresistance of colorectal cancer (CRC) [57]. Various other studies have reported similar findings in which CAF-derived EVs effectively enhance the aggressiveness of differing cancer cell types in a cargo-dependent manner [38, 42].

Tumor-Associated Macrophages (TAMs) While their phagocytic, proinflammatory, and tissue repair functions of macrophages make them vital to our immune system, prolonged exposure to cancer cells and tumor EVs can cause macrophages (or TAMs) to take on a polarized, alternatively activated M2-like phenotype. These TAMs can then exhibit a number of protumorigenic effects, including the promotion of tumor growth and progression, angiogenesis, and immunosuppression [51, 84]. Similar to CAFs, T-EVs harboring specific miRNAs have been shown to play a role in inducing the cellular reprogramming enabling the acquisition of an M2-like TAM phenotype. Shinohara et al. demonstrated that colorectal cancer-derived EVs containing miR-145 could downregulate histone deacetylase 11 in recipient macrophages, thereby polarizing them to a M2-like state [66]. This is seen as well in a manuscript in revision by Rabe et al., in which triple-negative breast cancer (TNBC)-derived EVs were found to induce a Toll-like receptor (TLR2 and 3)-mediated macrophage reprogramming to an M2-like, TAM phenotype [55].

The ability of certain TAMs to exhibit protumorigenic effects is in part achieved through EVs shedding. In a recent study, Zheng et al. demonstrated that apolipoprotein E (ApoE)-rich M2-like TAM exosomes, which are enriched in human gastric cancer (GC) patients, elevated migration of GC cells in vitro and in vivo [89]. Zhou et al. separately utilized microarray analysis to identify specific miRNAs (miR-29a-3p and miR-21-5p) within TAM exosomes, which

directly reduced STAT3 expression, disrupted the ratio of regulatory T-cells (Treg) and T helper 17 (TH17) cells, and promoted EOC progression [90]. Chen et al. demonstrated that TAM EV-mediated transmission of HIF-1α-stabilizing long noncoding RNA (HISLA) to breast cancer cells consequentially enhanced aerobic glycolysis and apoptotic resistance [11].

Lymphocytes and NK Cells T-lymphocytes (T-cells) are immune cells universally known for their ability to target and destroy various pathological entities, including cancer cells. However, certain transformed cells can adopt the ability to evade or suppress an immune system-mediated attack, thus allowing for the development of a tumor and later metastasize to neighboring tissues [75]. Researchers have found that this immunosuppression can partially be achieved through EV-mediated signaling to certain cancer-fighting immune cells. In doing so, EVs have been found to induce apoptosis, suppress function, or inhibit growth or proliferation of certain immune cells [16, 43].

EV constituents linked to apoptotic signaling include Fas ligands (FasL) [4, 32] and tumor necrosis factor-α (TNF-α) [78]. Both of which are so-called death ligands that are found on EVs and can bind to specific immune cell death receptors, thereby inducing necrosis- or caspase-dependent cell death [79]. EVs can also provide immune system evasion by suppressing immune cell growth or function [16]. A frequent culprit in EVs that is capable of doing so is the transforming growth factor-β (TGF-β), which has been shown to target T-lymphocytes (such as CD8+) in glioblastoma [79]. Rong et al. showed that under hypoxic conditions, breast cancer cells will enhance the release of TGF-β-containing exosomes, thereby suppressing T-cell proliferation [60]. Another example of this is exosomes that contain programmed death-ligand 1 (PD-L1), which have been found to suppress CD8 T-cell function and consequentially immune evasion [10].

As T-cells are not the only immune cell capable of targeting and destroying cancer cells, EV-mediated immunosuppression can involve other cell types, including the natural killer (NK) cell. Just as in the case of T-cells, Szczepanksi et al. found that TMVs released from patients with acute myeloid leukemia contained elevated levels of TGF-β. As a result, these TMVs suppress cytotoxic activity and entrance of natural killer (NK) cells to the TME [71]. While not directly linked to TGF-β, Liu et al. reported similar findings in which murine mammary carcinoma-derived exosomes exhibited NK cell suppression, thereby promoting tumor cell growth [40].

9.4 Invasive Tumor Cell Behaviors

The transition of cancer from a local to metastatic disease requires cancer cells to break through the basement membrane and initiate invasion through the stromal layer. This event is heavily reliant upon the action of matrix-degrading proteases such as cysteine cathepsins and metalloproteases to remodel the ECM and carve a path for migratory cancer cells [7, 76]. ECM degradation can take place by the actions of specialized membrane foci, such as invadopodia, or through the assistance of protease-loaded EVs [45, 48, 58, 64]. In this section, we will discuss potential mechanisms by which tumor cells can become invasive and EVs might facilitate such a process.

During tissue invasion, cancer cells migrate by a range of different behaviors including single cell migration, collective migration, mesenchymal cell migration, and amoeboid migration. During mesenchymal migration, carcinoma cells undergo an epithelial-mesenchymal transition (EMT), gaining transcriptional access to express membrane-bound matrix metalloproteases (MT1-MMP) that is normally restricted to mesenchymal cell types [20]. At the plasma membrane, MT1-MMP can activate dormant, stromal pro-MMP enzymes and efficiently cut through the dense network of ECM obstructing a cancer cell's path. Mesenchymal cells traffic MT1-MMP to the site of specialized cell membrane structures, called invadopodia, through the intracellular guidance of the v-SNARE, VAMP7, and are dependent on the activity of a network of small GTPases regulating endosomal trafficking and

dynamics [52, 63, 69]. There are studies to show that invadopodia are sites for multivesicular endosome (MVE) docking and release of exosomes that contain proteases and other molecular cargo to promote cell invasion [26]. Further, it was also shown that exosome secretion facilitates directional sensing of cells during migration and in response to chemotactic gradients [70].

Amoeboid cells are round in shape and move via the protrusion and retraction of membrane blebs in a behavior reminiscent of the amoeba, *Dictyostelium discoideum*. Amoeboid cell movement is dependent on the activity of the small GTPase, RhoA, as well as its downstream effector kinase, Rho-associated protein kinase (ROCK) [18, 62]. Related to this, microvesicle shedding also appears to increase dramatically as invasive tumor cells adopt amoeboid phenotypes [64]. Previous thinking was that amoeboid cell movement occurred in the absence of matrix metalloproteases, as studies on cell invasion in the presence of protease inhibitors found that some tumor cells remained capable of invading compliant matrices but had preferentially turned toward an amoeboid phenotype [15, 62]. However, it is now known that tumor cells displaying amoeboid behavior can continue to utilize matrix metalloproteases to facilitate invasion while its activity remains available [34]. Thus while exosomes and invadopodia facilitate mesenchymal cell migration, it appears that microvesicles may act as the invasive structure for amoeboid cells. When isolated from the conditioned media of invasive cell lines and applied to protein matrices, microvesicles produce visible degradation of the underlying matrix and also are found to lack the common biomarkers of invadopodia, such as cortactin [13, 64]. In the case of microvesicles, MT1-MMP is trafficked to the cell surface through its association with a tetraspanin, CD9, and by the guidance of a different v-SNARE, VAMP3. Further, inhibiting any components involved with the formation of invasive microvesicles, such as through knockdown of VAMP3, MT1-MMP, or fascin, has been shown to significantly hinder tumor cell migration through in vitro 3D gelatin matrices [13, 14]. Hence, amoeboid cells can utilize metalloprotease activity to facilitate cell movement and do so through invasive microvesicle shedding.

Interestingly, a study with the invasive melanoma line, LOX, demonstrates how tumor cells can toggle between mesenchymal and amoeboid cell movement based on their surrounding environment, presumably through the use of mechanosensory machinery. When applied to thin, stiff matrices (5% gelatin), the LOX cells demonstrate a mesenchymal phenotype and create punctate degradative patterns in the underlying matrix through invadopodia structures. However, when applied to thick, soft matrices (1% gelatin), LOX cells revert to an amoeboid phenotype and cut large paths through the matrix by shedding protease-loaded TMVs. In this system, Rac1 and ARF6 act upstream of RhoA to modulate its activity. When applied to stiff matrices, enhanced Rac1 activity inhibits RhoA and consequently TMV shedding; however, when applied to soft matrices, Rac1 is inhibited to release RhoA activity and promote an amoeboid phenotype [64].

9.5 Targeting or Utilization of Certain EVs as Novel Approach to Cancer Therapeutics

As it has become more apparent that tumor and stromal cell-derived EVs can be directly involved in cancer progression and establishment of a tumor niche, several labs have become interested in specific pro-tumorigenic EVs as an avenue to develop novel approaches to cancer treatment and therapeutics [12]. As a result, researchers have found that by suppressing the release or function of specific EVs, one can impede or negatively affect the progression of a given cancer. Furthermore, various labs have demonstrated that the utilization or modification of specific pro-tumorigenic EVs can be an effective approach to induce an anti-tumorigenic effect on a given cancer or its surrounding TME.

Targeting the Function or Release of Pro-tumorigenic EVs After it became apparent that

EVs can harbor and deliver highly metastatic molecular cargos, several studies began to show that by suppressing their function or inhibiting their release, such cancer-related traits can be reduced or impeded. TME EVs that have received much attention in this regard are those containing specific miRNAs, which as we have described are capable of modulating gene expression to promote tumorigenicity. By suppressing the function of miRNA-packed EVs, researchers have found that tumor malignancy can be reduced, while sensitivity to anticancer drugs is enhanced. This is seen in Guan et al., in which the ability of proton-pump inhibitors (PPIs) to inhibit GC progression (in vitro and in vivo) appeared to be largely due to the inhibition of miRNA-containing exosome and release and prevention of CAF reprogramming [21]. Similar results were shown in Qin et al., in which the absence of lung cancer cell-derived exosomes containing miR-100-5p was shown to enhance sensitivity of cisplatin [54]. This has prompted other labs to identify drug inhibitors capable of preventing the release of cancer-related EVs, among which include sulfisoxazole (SFX) [27]. After seeing that this oral antibiotic exhibited significant anti-tumorigenic effects in mouse breast cancer models, Im et al. determined that this was in part due to sulfisoxazole-mediated inhibition of T-EV release through endothelin receptor A (ETA) interference. Interestingly, cannabidiol (CBD) has also demonstrated the ability to inhibit T-EV release from the following cancer cell lines: prostate cancer (PC3), hepatocellular carcinoma (HEPG2), and breast adenocarcinoma (MDA-MB-231) [33]. This potentially has major implications, providing a partial explanation for certain anticancer effects following CBD treatment [44].

Other approaches taken in targeting the release of pro-tumorigenic EVs involve the modulation of TME pH. As the TME is known to be an acidic environment, Parolini et al. chose to investigate how this low-pH environment affects cancer cell PM and their ability to release and uptake exosomes. By modulating the pH, metastatic melanoma exhibited changes in PM structure and composition, which consequentially reduced exosome release and uptake [50]. Despite more work being required to draw further connections to EVs, TME pH modulation has been shown to bring on various other anti-tumorigenic effects, including an enhanced immune response and elevated sensitivity to certain drug treatments [35, 88].

Utilizing/Modification of Pro-tumorigenic EVs

As discussed above, EVs within the TME can induce a pro-tumorigenic signal to a recipient cell through their ability to interact and deposit their biologically active cargo (Fig. 9.1b). Researchers have recognized this as an opportunity to utilize such EVs as a vector for delivering cytotoxic drugs, along with specific molecular constituents capable of increasing expression of apoptosis inducers and tumor suppressors.

Through their cancer or stromal cell targets and innate biocompatibility, many have utilized TME-associated EVs as vectors for anticancer drug delivery. This has been shown to enhance the overall effectiveness of such drugs while reducing their off-target side effects [12, 29]. In a study involving mice, Tang et al. showed that the chemotherapeutic effectiveness of methotrexate and cisplatin significantly increased. While doing so, he also found that mice no longer experienced off-target effects, such as hair loss, weight changes, and liver and kidney function aberrations [72]. The ability to reduce toxicity to normal healthy tissue was also shown in Toffoli et al., in which exosomes containing doxorubicin were reduced by ~40% in cardiac tissue, thus preventing toxicity and maintaining normal cardiac tissue [74]. The ability to enhance cytotoxicity and overall impede cancer cell growth has been seen in studies involving the delivery of paclitaxel. Paclitaxel has been considered to be an important anticancer drug used in chemotherapeutics through its ability to arrest the cell cycle and interfere with cell division [81]. Interestingly, Saari et al. demonstrated that by packaging paclitaxel in EVs derived from prostate cancer cells, paclitaxel can be directly delivered to a target cell through endocytosis, thereby increasing its overall cytotoxicity

[61]. EV modification for targeted drug delivery also includes those derived from TME stromal cells, such as macrophages [56].

After discovering that specific miRNA constituents are responsible for certain EV-mediated pro-tumorigenic effects, several labs began testing whether miRNAs capable of inducing anti-tumorigenic gene expression could produce the opposite effect. As mentioned in an earlier section, EVs expressing certain "death ligands" can provide some level of immunosuppression by inducing apoptosis to proximal immune cell [16, 43]. However, scientist realized that such ligands can be expressed by TME-associated EVs to induce cell death to tumor cells. This includes work by Rivoltini et al., in which exosomes modified to contain TRIAL, a TNF-related apoptosis-inducing ligand, presented its signal to tumor sites to induce cancer cell death [59]. In addition, Li et al. showed that modified exosomes that contain tumor suppressor LRRC4 can be used to re-establish its expression in glioblastoma multiforme (GBM) cells, thereby robustly enhancing antitumor immunity [39].

9.6 Concluding Remarks

In summary, the understanding of molecular constituents has enabled the outlining of mechanistic connections between certain EVs subtypes and their pro-tumorigenic function. Overall, this has shed light on the three areas of research: (1) EV cross talk between tumor and stromal cells, (2) EV-mediated ECM invasion and further metastasis, and (3) the ability to utilize or modify specific EVs as an anticancer therapeutic. While much progress has been made in these aforementioned areas, additional research is necessary to unveil and exploit the rich diversity in composition, function, and potential for utilization that surrounds EVs in the TME. In considering specific EVs in the TME, it has been largely in the context of specific cell targets and a given tumorigenic effect; however, it is important to note that the signaling capabilities are far more complex. EVs constituents are highly heterogeneous, thereby enabling them to contain multiple signaling molecules corresponding to various different cell types. Therefore, a single EVs can induce a wide variety of pro-tumorigenic (and even anti-tumorigenic) effects depending on the cell type in which it can interact with [24]. Further characterization and assessment of EVs has enabled elucidation of their diagnostic potential [36]. Additional research is required for further understanding of specific EV biomarkers associated with a given cancer subtype. An area of EV research that also deserves attention involves investigating how metabolic changes are associated with EV release. A recent study has confirmed that the concentration of EVs released by microglial cells is dependent on the cellular availability of glutamate and its downstream product, α-ketoglutarate. Furthermore, overexpression of glutaminase-1 (GLS-1) increases EV release in an in vivo transgenic mouse model [83]. Modulation of such metabolites may prove as an effective approach to targeting the release of pro-tumorigenic EVs into the TME. Further, while modified pro-tumorigenic EVs have been successfully used to alter the growth and progression of tumors, the ability to implement them as cancer therapeutics is far more complex. Nonetheless, the release of EVs into the TME represents a potentially impactful avenue that can be harnessed for practical application in the clinic.

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Index

A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1259, https://doi.org/10.1007/978-3-030-43093-1