Mechanisms of Anti-angiogenic Therapy

Roser Pons-Cursach and Oriol Casanovas

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Abstract

Angiogenesis inhibition is a promising approach to fight cancer. This strategy offers some advantages in comparison with conventional drugs, such as the inhibition of single vessels that can induce the death of many tumor cells. Moreover, this therapy can be used in the treatment of a wide range of solid

R. Pons-Cursach · O. Casanovas (✉)
Tumor Angiogenesis Group, ProCURE, Catalan Institute of Oncology-IDIBELL, Barcelona, Spain
e-mail: rpons@iconcologia.net; ocasanovas@iconcologia.net

D. Marmé (ed.), *Tumor Angiogenesis*,
https://doi.org/10.1007/978-3-319-33673-2_2

tumors and may produce less resistance. Since angiogenesis is a complex process, it can be inhibited at different levels. The most established therapy is the inhibition of angiogenic signaling. Vascular endothelial growth factor (VEGF) pathway is the most important signaling pathway in the angiogenesis process, and for this reason, many inhibitors have been developed to block the action of VEGF or its receptors, VEGFRs. Another approach is the inhibition of endothelial progenitor cells (EPCs), mobilized from the bone marrow to the tumor site in hypoxia conditions, which contribute to the formation of new blood vessels and the pre-metastatic niche. The interaction between extracellular matrix and endothelial cells is very important during angiogenesis, so the inhibition of this interaction produces anti-angiogenic effects. An alternative strategy is based in the regression of preexisting tumor vasculature, which presents abnormalities in the structure and function in comparison with normal vessels. In this case, vascular-disrupting agents (VDAs) can cease the blood flow within minutes and lead to the formation of central necrosis. Finally, tumor vessel normalization produced after anti-angiogenic therapies may reduce the metastatic dissemination and improve delivery of drugs to the tumor.

Keywords

Angiogenesis · Anti-angiogenic drugs · VEGF · Endothelial progenitor cells (EPCs) · Integrins · Extracellular matrix · Basement membrane · Endothelial cells · Pericytes · Vascular-disrupting agents (VDAs) · Vascular normalization

Introduction

The treatment of solid tumors with chemotherapeutic drugs is based on the expectation that drugs at low doses will preferentially kill rapidly dividing tumor cells, rather than normal cells. However, chemotherapeutic drugs are not selective for tumor cells and produce toxicities in normal tissues with high proliferation, such as the bone marrow, gastrointestinal tract, and hair follicles. Moreover, the efficiency of these drugs is reduced due to the lack of accumulation of drug at the tumor site produced by the high pressure and the irregular vasculature of the tumor (Bosslet et al. 1998).

It has been recognized for decades that most tumors are highly vascular. This concept was introduced by *Ide and Algire* (Algire and Chalkley 1945; Ide et al. 1939) and confirmed by *Folkman's group* in the 1960s (Folkman et al. 1963). These studies proposed that when tumors acquire a size of 1–2 mm, the inadequacy of nutrient supply and metabolic waste clearance by vessels produce hypoxia and acidosis. At this moment, tumors initiate molecular signals in order to induce angiogenesis and continue growing. Therefore, angiogenesis is an essential process for tumor development. Thus, vascular targeting was proposed as a new approach to fight the limitations of conventional drugs. This promising strategy leads the tumor cell death by the lack of nutrients and oxygen. Importantly, anti-vascular therapies have some advantages in comparison with conventional drugs. Firstly, removing one blood vessel triggers the cell death of all tumor cells supplied by this vessel. Secondly, anti-vascular targeting can be used in the treatment of a wide range of solid tumors. Finally, anti-vascular treatments may produce less therapy resistance because endothelial cells and pericytes are genetically more stable than tumor cells.

Given many mechanisms are implicated in the formation of new blood vessels, tumor vasculature can be inhibited at different levels (Fig. 1). The inhibition of endothelial cells, pericytes, or extracellular matrix (ECM) remodeling produces tumor blood vessel reduction. Different classes of compounds are currently used for targeting the different anti-angiogenic mechanisms mainly small molecules and antibodies. In pharmaceutical biotechnology, antibodies are the binding molecule class, most of them used for tumor diagnosis and therapy. Nevertheless, antibodies present some disadvantages, such as the requirement for an expensive mammalian cell production system,

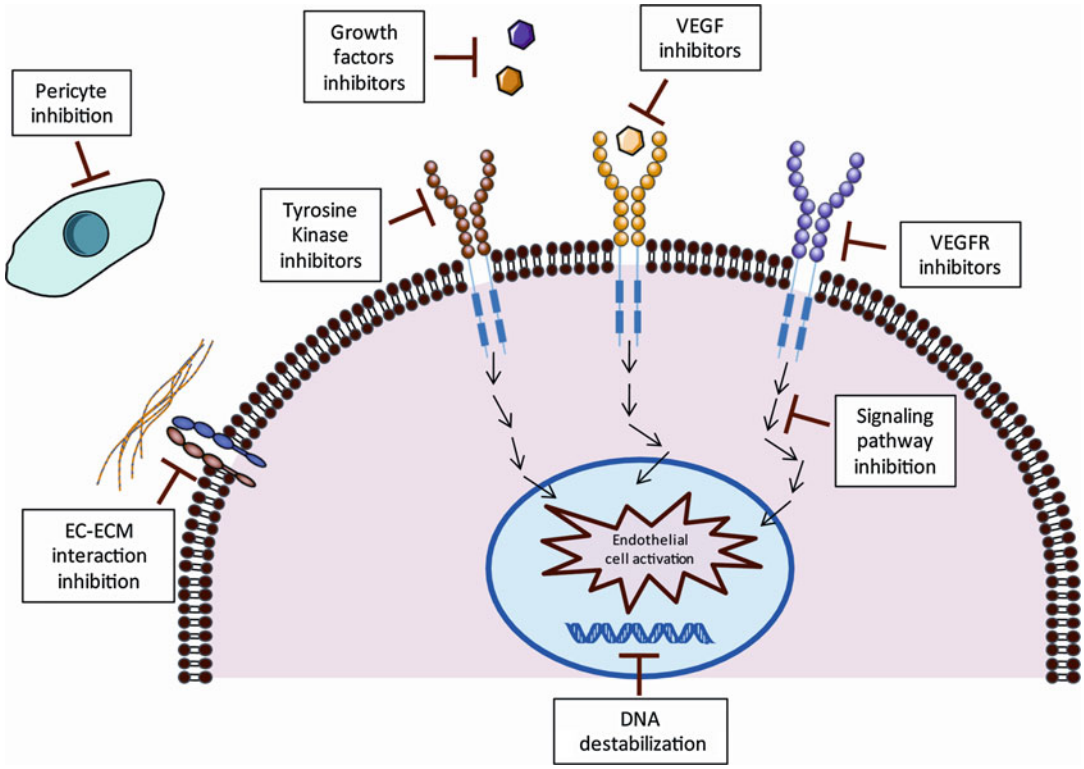

Fig. 1 *Strategies of angiogenesis inhibition*. Angiogenesis process is a complex mechanism and many molecules are implicated, so it can be inhibited at different levels. Angiogenic signaling can be blocked by VEGF and other growth factor inhibitors and by VEGFR or other tyrosine kinase receptor inhibitors. The interactions between endothelial cells and extracellular matrix can also be interfered by integrin inhibitors. Finally, the inhibition of pericytes produces anti-angiogenic effects

low expression yields, dependence on disulfide bonds for stability, and the tendency to aggregate. For this reason, other classes of molecules are being investigated for tumor-targeting applications, such as small globular proteins, peptides, and aptamers (Hey et al. 2005).

In the recent years, many progresses have been made to understand the mechanism of action of anti-angiogenic drugs. Many of those approaches have been obtained evaluating the effects of the anti-angiogenic inhibitors on tumor blood vessels in preclinical and clinical studies. Importantly, not all the angiogenesis inhibitors have the same cellular actions. Angiogenesis inhibitors have multiple effects and not all have the same therapeutic relevance. For example, vascular endothelial growth factor (VEGF) inhibitors influence endothelial cell survival, migration, growth, plasma leakage, blood flow, and recruitment of leukocytes and stem cells (Kamba and McDonald 2007).

The effects of angiogenesis inhibitors on tumor blood vessels can be classified into three categories: (1) inhibition of tumor blood vessels, (2) regression of tumor blood vessels, and (3) normalization of tumor blood vessels.

Inhibition of Angiogenic Signaling

Commonly, endothelial cells are activated by tyrosine kinase (TK) receptors and consequently their corresponding signaling pathway. Growth factors stimulate the endothelial cells to form new blood vessels. Therefore, growth factors, their receptors, and subsequent signaling

cascades are promising targets in angiogenesis inhibition.

The most important growth factor implicated in angiogenesis is VEGF, and many inhibitors have been developed to block the action of this molecule (Ferrara 1999). Interestingly, different approaches have been developed to inhibit VEGF or its signaling pathway, such as the neutralization of the ligand by anti-VEGF antibodies, soluble receptors, or oligonucleotide aptamers, the inhibition of VEGF receptors by antibodies or small-molecule inhibitors of TK phosphorylation, and the inhibition of the intracellular signaling pathway directly. Finally, the inhibition of pro-angiogenic signaling tilts the balance in favor of endothelial cell apoptosis and regression.

Importantly, many drugs have been developed to block growth factors and tyrosine kinase receptors, and some of them have been approved by the Food and Drug Administration (FDA) for the treatment of some tumor types or are currently in clinical development (Krause and Van Etten 2005). Clinical trials have been focused in the study of the two main angiogenesis pathways: VEGF pathway and platelet-derived growth factor (PDGF) pathway.

Vascular Endothelial Growth Factor Inhibitors

VEGF pathway is the most important process in angiogenesis regulation. The VEGF produced by tumor and stroma cells interacts with their TK receptors expressed by endothelial cells promoting proliferation, migration, and invasion, leading to angiogenesis. VEGF is a homodimeric protein and five different isoforms have been described. Equally, VEGF receptor (VEGFR) is divided in three different isoforms with different roles in angiogenesis (Hicklin and Ellis 2005).

VEGF pathway can be blocked inhibiting the VEGF ligand or inhibiting the VEGFR. The most important anti-VEGF drug used in clinics is the monoclonal antibody bevacizumab. Bevacizumab is a recombinant humanized monoclonal antibody against VEGF-A. The first evidence of targeting VEGF-A inhibit tumor growth was observed in a mouse model in 1993 using a monoclonal antibody anti-VEGF-A (Kim et al. 1993). Moreover, bevacizumab was the first clinically available angiogenesis inhibitor in the United States. This drug was approved for the treatment of certain lung cancers, renal cancers, ovarian cancers, and glioblastoma multiforme of the brain (Shih and Lindley 2006).

Tyrosine Kinase Inhibitors

TK inhibitors are useful in the treatment of cancer because they present dual effect; they block tumor cell proliferation and the pro-angiogenic signaling pathway. Given different TK receptors are expressed in both tumor and endothelial cells and TK inhibitors often target more than one type of receptors, the inhibition of TK can affect both types of cells (Krause and Van Etten 2005). A wide range of TK inhibitors have been developed and approved for the treatment of several cancers. The efficacy of TK inhibitors can vary depending on the expression levels of the different types of growth factors and TK receptors; therefore, different types of tumors may respond differently to these drugs. Several approaches have been proposed to target growth factors and their receptors, such as compounds that bind to ATP-binding site of the TK receptors and block receptor activation, or antibodies that bind to the growth factors or their receptors, preventing binding and subsequent receptor activation (Hartmann et al. 2009). The most important anti-VEGFR and PDGF TK inhibitors are sorafenib, sunitinib, and pazopanib.

Sorafenib is a synthetic compound which inhibits the angiogenesis process and also the growth signaling. Sorafenib has a dual inhibition: it inhibits rapid accelerated fibrosarcoma kinase (RAF kinase), a critical component of the RAF/MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway that control cell division and proliferation, as well as VEGFR-2 and PDGF receptor-beta (PDGFRB) signaling pathway that blocks angiogenesis process (Kelly et al. 2010). Sorafenib was approved

in the treatment of advanced renal cell carcinoma, hepatocellular carcinoma, and radiation-resistant advanced thyroid carcinoma.

Sunitinib is another TK inhibitor which blocks angiogenesis and cell proliferation. The therapeutic effect is produced by the inhibition of VEGFR-2, PDGFRB, and c-kit. Sunitinib was approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST) (Gan et al. 2009).

In the same way, pazopanib inhibits tumor angiogenesis, blocking VEGFR-1, VEGFR-2, and VEGFR-3, c-kit, and PDGFR. Pazopanib has been approved for the treatment of renal cell carcinoma and soft tissue sarcoma (Verweij and Sleijfer 2013).

Inhibition of Vascular Progenitor Cells

Two processes contribute to the formation of new blood vessels: vasculogenesis and angiogenesis (Risau 1997). In vasculogenesis, new vessels are originated by the differentiation of mesenchymal cells (angioblasts) into endothelial cells, while, in angiogenesis, endothelial cells from the blood vessels proliferate and sprout to constitute the new vascular structures. However, the discoveries about the idea that circulating vascular progenitors are involved in angiogenesis have changed this dogma (Moore 2002). Moreover, hematopoietic cells can contribute to the maintenance and initiation of these processes, being essential for neoangiogenesis.

Endothelial progenitor cells (EPCs) are derived from the bone marrow and contribute to many processes, such as wound healing, myocardial ischemia, neovascularization, and tumor growth. EPCs are defined by the co-expression of the markers CD34, CD309, and CD133. In physiological conditions, EPCs are quiescent, but in response to a vascular injury, they acquire the ability to circulate in the peripheral blood, proliferate, and differentiate into mature endothelial cells. In this process, the damaged site releases growth factors and cytokines that promote the migration of EPCs to the local endothelium, contributing to neovasculature (Peichev et al. 2000).

Therefore, acknowledging that EPCs contribute to tumor angiogenesis provides the basis for new therapies and monitoring strategies for several types of malignancies.

EPCs in Tumor Angiogenesis

In normal conditions, vascular injury or hypoxia mobilizes EPCs from the bone marrow by the secretion of paracrine factors, such as VEGF and stromal cell-derived factor-1 (SDF-1). In pathological conditions, such as tumors, the chronic state of hypoxia or inflammation produces constitutively activation of EPCs (Fig. 2a). EPCs can contribute to the formation of new tumor blood vessels by secreting pro-angiogenic growth factors and also due to their ability to form new vessels. Moreover, EPCs contribute maintaining the anti-inflammatory state. EPCs are mobilized from the bone marrow in different types of malignancies, such as hepatocellular carcinoma and lung, pancreatic, and breast cancer (Ono et al. 2014). Several studies have demonstrated that EPC levels are higher in tumor tissue and peripheral blood of cancer patients than in healthy donors. Thus, circulating EPCs may be used as predictors of the malignancy grade of some tumors. In fact, studies have observed that EPC levels decrease in patients who respond to cancer treatments. Interestingly, circulating EPCs have been proposed as predictive biomarkers for gastric patients treated with chemotherapy (Ahn et al. 2010).

EPCs and Tumor Microenvironment

Tumor microenvironment interacts direct or indirectly with different cell types, such as cancer cells, EPCs, inflammatory immune cells, and endothelial cells. Hypoxia, characteristic of tumors, induces the formation of new blood vessels supplying oxygen to tumor mass. In hypoxia, the transcriptional activation of hypoxia-inducible factor (HIF) activates the transcription of genes required for tumor progression, such as VEGF, PDGF, SDF-1, and C-X-C chemokine receptor

type 4 (CXCR4) (Tang et al. 2016). Studies have demonstrated that the loss of function of HIF inhibits EPC proliferation and differentiation, reducing their ability to form new vessels. The avoidance of the immune response and the maintenance of the chronic state of inflammation are some of the hallmarks of cancer described by *Hanahan and Weinberg* (Hanahan and Weinberg 2011). During the inflammation process, damaged or hypoxic tissue releases cytokines, producing a molecular gradient which guides EPCs to the inflamed tissue (Fig. 2b). Moreover, high plasma levels of VEGF, secreted by both tumor and stroma cells, promote the mobilization of EPCs from the bone marrow and their proliferation (Lyden et al. 2001). Interestingly, the remodeling of the basement membrane (BM) in the first stages of neoplastic transformation can produce the mobilization of EPCs by VEGF and angiopoietin-1 (Ang-1). The depletion of VEGF and Ang-2 activity inhibits tumor growth and EPC recruitment.

Moreover, possible roles of EPCs in the induction of tumor invasion have been proposed. Endothelial cells and immune cells secrete paracrine factors that promote mobilization of EPCs

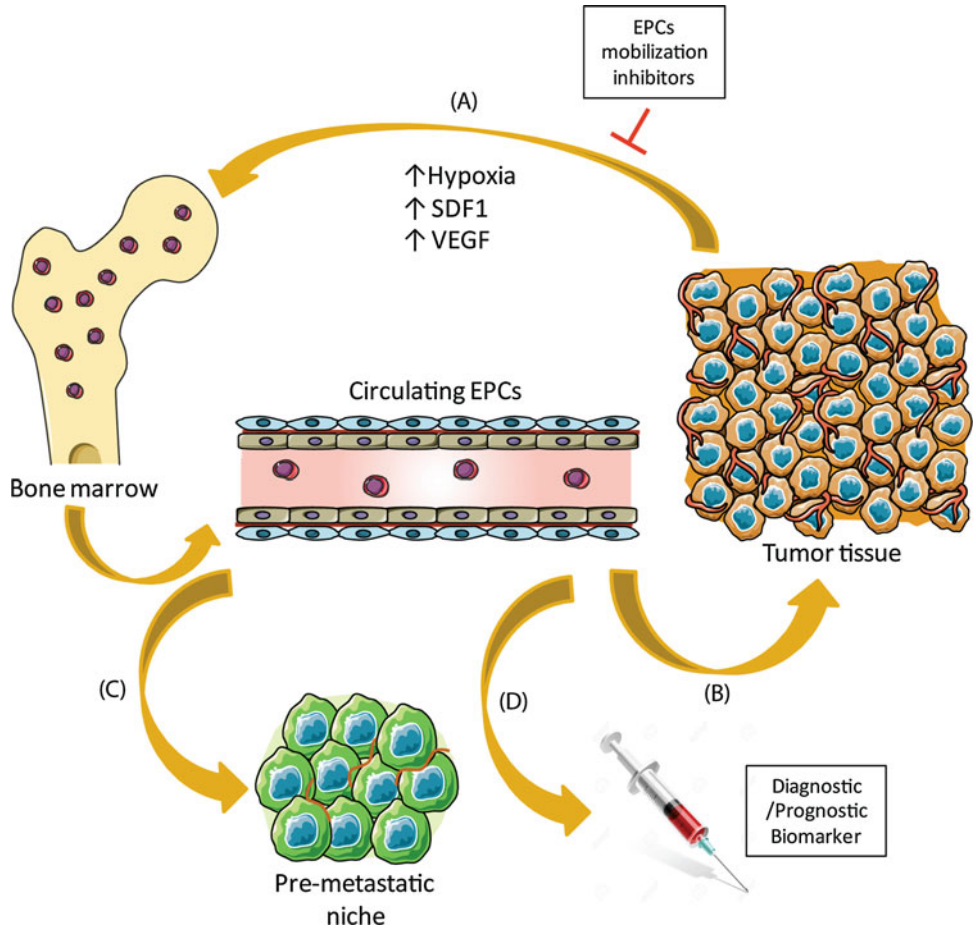

Fig. 2 *Inhibition of endothelial progenitor cell recruitment in tumor angiogenesis*. (**a**) Chronic hypoxia present in tumors mobilizes EPCs from the bone marrow to the circulation by the secretion of paracrine factors, such as VEGF and SDF-1. Therefore, the inhibition of VEGF or SDF-1 prevents the mobilization of EPCs to the tumor site. (**b**) Circulating EPCs home to hypoxic sites and contribute to the generation of new blood vessels. (**c**) However, circulating EPCs can also contribute to pre-metastatic niche formation before the arrival of malignant metastatic cells. (**d**) Levels of circulating EPCs have been proposed as diagnostic and prognostic biomarker

to form new blood vessels. HIF-1, upregulated in tumor hypoxia, induces the release of SDF-1 from tumor cells, endothelial cells, and stroma fibroblasts (Mohle et al. 1998). Additionally, EPCs highly express CXCR4, SDF-1 receptor. And thus, the gradient of SDF-1, produced in hypoxia, attracts the EPCs to the tumor tissue contributing to the generation of new blood vessels. Moreover, EPCs can also secrete SDF-1 and VEGF-1 during tumor progression. The interaction between SDF-1, secreted by EPCs, and CXCR4 expressed in tumor cells may contribute to the extravasation and development of a pre-metastatic niche before the arrival of malignant metastatic cells (Jin et al. 2012) (Fig. 2c). SDF-1 produced by the immune system may attract EPCs to distant sites and induce SDF-1 production spontaneously, promoting the spread of tumor cells to other sites. Moreover, the ability of EPCs to activate metalloproteinase-9 (MMP-9) could induce tumor cell migration and invasion. Thus, EPCs could facilitate the pre-metastatic niche formation by the secretion of SDF-1.

Therapeutic Strategies

EPCs in Cancer Diagnosis and Prognosis

Given that EPCs are mobilized from the bone marrow to tumor tissues and circulation, different studies have been proposed that they may be useful as diagnostic and prognostic factor (Fig. 2d). Numerous laboratories have studied whether variation in the number of EPCs in peripheral blood could represent a tool to predict a pathological state. Importantly, the number of circulating EPCs can vary between patients and healthy people and between patients affected by the same pathology. Moreover, the number of circulating EPCs could be correlated with clinical outcomes in some cases.

In cancer patients, EPC levels are higher than in healthy people, due to their mobilization from the bone marrow to contribute in new vessel formation. Therefore, circulating EPC levels could be useful as a diagnostic and prognostic tool to monitor the clinical state of patients. For example, EPC levels correlate with poor overall survival in non-small cell lung cancer (NSCLC). Moreover, NSCLC patients with partial or complete remission after anticancer therapy present lower levels of circulating EPCs than patients with stable or progressive disease, suggesting that EPC levels correlate with the efficacy of treatment (Dome et al. 2006). In addition, EPCs have been proposed as biomarkers to monitor the progression of the disease and discriminate between good and bad response to therapies. Several studies have demonstrated that EPCs may be used as a good marker to evaluate the response of colorectal cancer patients to anti-angiogenic drugs (Matsusaka et al. 2011). Additionally, chemotherapy reduces the number of circulating EPCs in breast cancer patients with high levels of EPCs, although it induces the mobilization of EPCs from the bone marrow at the same time. Therefore, the combination of anti-angiogenic therapies and chemotherapy could be useful to avoid the possible pro-angiogenic effects of EPC mobilization after chemotherapy. Another approach is the possible use of EPCs in the evaluation of tumor stage. In late-stage gastric cancer patients, the number of EPCs in cancer tissue and adjacent tissue was lower than in early-stage patients. Furthermore, levels of other molecules, such as VEGF and hematopoietic progenitor cells, are higher in cancer patients, and the combination of all of them may be considered to monitor the progression of the disease (Nowak et al. 2010). Nevertheless, further studies are needed to confirm the use of EPCs as a diagnostic and prognostic factor in cancer.

EPCs in Anti-Angiogenic Treatments

Given that EPC mobilization contributes to tumor angiogenesis and metastasis, blocking this process would inhibit the formation of new blood vessels and the metastatic niche (Moccia et al. 2015). One strategy could consist in blocking the molecules involved in the homing of EPCs to tumor vasculature or the factors responsible for their recruitment from the bone marrow (Fig. 2a). Given that SDF-1 is the most important regulator of EPC mobilization and CXCR4 disruption is essential for the mobilization of EPCs to the

circulation, different agonists and antagonists of CXCR4 have been proposed, such as small peptide antagonists and agonists, non-peptide CXCR4 antagonists, antibodies to CXCR4, and modified antagonists for SDF-1 (Burger and Peled 2009). All of these compounds act preventing the gradient of chemokines that allows the homing of EPCs to the tumor site.

Another important factor involved in the EPC mobilization is VEGF. Most anti-angiogenic therapies are designed to inhibit the interaction between VEGF and VEGFR using neutralizing antibodies, soluble receptors, and small-molecule inhibitors. Preclinical studies demonstrated that VEGF inhibition negatively modulates EPC-mediated vasculogenesis (Kerbel and Folkman 2002). Nevertheless, some clinical studies have suggested the possible role of EPCs in the acquisition of tumor resistance to vascular-disrupting agents (VDAs) (Taylor et al. 2012). For this reason, combinational treatments of anti-VEGF or VDAs with EPC-targeting drugs should be evaluated.

Inhibition of Extracellular Matrix Remodeling

The communication between cells and their microenvironment is very important in the control of development and homeostasis. Cell-ECM interactions regulate many processes, such as morphogenesis, differentiation, and organ architecture. Bidirectional communication between components of the ECM and cells led the cell to sense their microenvironment, transfer this molecular information from outside the cells to inside, and finally initiate cellular response. Additionally, the transfer of biochemical information from cells to the ECM is also important, because cells can response altering their local microenvironment by inside-out communication. Integrins are the most important cell adhesion receptors that mediate this bidirectional communication system (Stupack and Cheresh 2004).

Given the importance of cell-ECM interactions in the control of cell behavior, it is not surprising that cell-ECM interactions play a critical role in regulating angiogenesis, tumor growth, and metastasis. Consequently, affecting cell-ECM interactions may produce anti-angiogenic effects and could be used as anticancer drugs (Yang et al. 2003).

Importantly, targeting the link between endothelial cells and ECM-inhibiting integrins may be more effective (Serini et al. 2006). Finally, another approach is the inhibition of the interaction between endothelial cells and pericytes or adjacent tumor cells by the inhibition of N-cadherin-mediated junctions.

Extracellular Matrix in Angiogenesis

Composition and Structural Organization of Vascular Extracellular Matrix

ECM composition and organization is essential in the regulation of angiogenesis. This was evidenced in mice with alterations in the expression and functions of ECM molecules such as collagen, fibronectin, and laminin. It has been observed that mice with mutations in these proteins exhibit vascular abnormalities (Hirsch et al. 2000).

The ECM of vessels is constituted by BM. BMs are mainly composed by two multidomain glycoproteins, collagen type IV and laminin, which are interconnected with proteoglycans such as nidogen and perlecan (Kalluri 2003). Other minor components such as collagen type VIII, XV, and XVIII also constitute the BM. The composition and structural integrity of the BM can vary between tissue compartment and during developmental and pathological processes. Additionally, BMs provide critical binding sites for other ECM proteins, integrin receptors, growth factors, and cell surface proteoglycans. The interactions between cells and these BM components regulate many signaling pathways including phosphoinositide 3-kinase (PI3K)-AKT8 virus oncogene cellular homolog (AKT), mitogen-activated protein kinase (MAPK)-ERK, and Jun amino-terminal kinase (JNK) cascades, which are implicated in angiogenesis processes, such as cell adhesion, migration, invasion, proliferation, survival, and differentiation of endothelial cells (Chen et al. 2004). BMs also include the surrounding interstitial matrix composed by distinct

collagens such as type I, II, and III as well as fibronectin, fibrinogen, thrombospondin, vitronectin, and elastin. This interstitial matrix presents multiple protein-binding sites creating an elaborate network. Moreover, interstitial matrix can function as a reservoir of regulatory molecules including angiogenic growth factors, cytokines, motility factors, proteolytic enzymes, and protease inhibitors (Mott and Werb 2004). For example, two binding domains to fibronectin were identified in VEGF molecule, whose function is to enhance the activity of VEGF. Therefore, the interaction between ECM components, integrins, and growth factors controls the development of new blood vessels and may constitute a good target for anti-angiogenic therapies.

Cell Adhesion Receptors

In general, angiogenesis is organized in different steps and in all of them, cell adhesion has an important role (D'Amore and Thompson 1987). Firstly, in the initiation phase, growth factors and cytokines are released, initiating signaling transduction pathways that lead in endothelial cell activation. At this point, endothelial cells acquire an invasive phenotype, which produce cell-cell dissociation, extracellular matrix remodeling by the protease secretion, and alterations in the expression of cell surface adhesion receptors. The activated endothelial cells interact with the remodeled BM components and start to invade the local interstitium. After that, endothelial cells remodel their microenvironment again and interact with the modified interstitial ECM components leading to morphogenesis and cellular reorganization into tubelike structures. Finally, in the maturation phase, endothelial cells start to express new matrix components, reorganizing cell-cell interactions with pericytes and differentiating into functional blood vessels.

Given the changing interactions of endothelial cells with the ECM components in all the angiogenesis process, cell adhesion molecules may have a crucial role in this process. The main families of cell adhesion molecules include cadherins, selectins, immunoglobulin supergene family members, and integrins (Brooks 1996). Interestingly, recent studies have demonstrated the possible contribution of cell surface receptor tyrosine kinase and various proteoglycans in the regulation of cell adhesion and the possible implications in the angiogenesis process (Beauvais et al. 2009).

Integrins

Given the importance of bidirectional communication between endothelial cells and their microenvironment in the regulation of angiogenesis, the integrin family could play crucial roles in this process. Integrins are heterodimeric receptors whose function is to mediate cell-ECM interactions. The first studies about the possible importance of cell-ECM interactions in regulating cellular processes were done in the 1980s by several investigators, notably Drs. *Hynes*, *Ruoslathi*, *and Springer* (Xiong et al. 2003; Hynes 2004; Pytela et al. 1985). In these studies, some of the first described integrins were isolated and cloned, such as fibronectin receptor α5β1 and the platelet fibrinogen receptor αIIbβ3. The observation of the integrins connecting ECM molecules to the cytoskeletal provided important evidence for the concept that cells communicate bidirectionally with the local microenvironment. To date, 24 integrin heterodimers have been identified resulting between the interaction of 18α and 8β subunits, each with specific distribution and functions. In general, integrins are organized into three domains, including extracellular cation-dependent ligand-binding domain, transmembrane regions, and an intracellular tail known to interact with cytoskeletal components. Moreover, integrins contain metal ion-dependent adhesion sites (MIDAS), which are needed for their binding to integrin ligands (Mould and Humphries 2004). Importantly, many function-blocking integrin antagonists are directed to the MIDAS region blocking the interaction between the integrins with their ligands.

Integrins are not only attaching the cells to the microenvironment, but they can sense and respond to their microenvironment inducing signaling cascades. Integrin response relies on the recruitment of adaptor proteins that behave as molecular hubs for intracellular signaling and organize complex signaling networks

(DeMali et al. 2003). Importantly, the capacity of integrins to recognize and bind to their ligands depends on their activation state. Conformational changes within the cytoplasmic tails of integrins induced by intracellular signaling events cause integrin activation and enhanced ligand binding through a processed termed inside-out signaling. In contrast, molecular information of outside the cell can be transmitted to the cell interior in a process called outside-in signaling.

Different studies have demonstrated and confirmed the importance of integrins in angiogenesis and blood vessel formation. For endothelial cells, integrins are the most important partners for growth factor receptors. For example, it is well established that some growth factors, such as VEGFs, are able to activate various integrins (Byzova et al. 2000). However, the relationship between the integrin and growth factor receptor seems to be more complex than unidirectional activation. Integrins can induce growth factor signaling and vice versa; growth factors can modulate integrin activity. The best known example is the cross talk between VEGFR2 and αvβ3 integrin, which controls the adhesion and migration of endothelial cells during vascular development and VEGF-induced angiogenesis. Moreover, treatment with VEGF or integrin ligation induces the formation of a complex between these two receptors. Also the cross talk or complex could be a good target for angiogenesis regulation. A study has shown that the bone morphogenic protein antagonist, gremlin, binds to VEGFR2 stimulating the interaction with αvβ3 integrin, and finally, it induces angiogenesis (Ravelli et al. 2013).

During angiogenesis, endothelial cells interact with various ECM proteins, and therefore they need to express more than one integrin during their migration, induced by VEGF/VEGFR2. For this reason, VEGFR2 may also interact with other integrins such as β1 integrin. This synergism is mediated by tetraspanin CD63, which interacts with β1 integrins and VEGFR2, and functions as a regulator of the complex between these two receptors (Tugues et al. 2013).

Therefore, the interaction between many integrins and growth factor tyrosine kinase receptors in a coordinated manner is necessary for the correct adhesion and migration of endothelial cells during angiogenesis. Consequently, blocking integrin interactions and complex formation may inhibit angiogenesis.

Other Receptors

Apart from integrins, there are other adhesion molecules which mediate cell-cell interactions, such as cadherins, selectins, and immunoglobulin family members (Brooks 1996). During angiogenesis, endothelial cells express a particular adhesion molecule in each angiogenesis step.

The first evidence of vascular endothelial cadherin (VE-cadherin) implication in angiogenesis came from experiments using functional blocking antibodies in in vitro angiogenesis (Matsumura et al. 1997). From that moment, many studies have demonstrated the implications of VE-cadherin in the angiogenesis process and in the tumor angiogenesis. During angiogenesis, VE-cadherin expression on the adherens junction disappears and epitopes are unmasked. Moreover, VE-cadherin is also implicated in vascular proliferation and in lumen formation (Nelson and Chen 2003).

E-selectin is an endothelial membrane glycoprotein implicated in the adhesion of leucocytes to cytokine-activated endothelial cells. The possible implications of E-selectin in angiogenesis come from the observation that antibodies against this protein inhibit capillary-like tubes in vitro (Nguyen et al. 1993). Additionally, E-selectin is expressed in proliferating endothelial cells in hemangioma tumors suggesting the possible participation in angiogenesis (Kraling et al. 1996). Therefore, there is evidence of the association of E-selectin with the angiogenesis process, but the mechanism by which E-selectin contributes is still unknown.

More studies are needed to elucidate the potential roles of E-selectin, VE-cadherin, and other unknown adhesion molecules in angiogenesis. Deeper insight into the mechanism by which they regulate endothelial cell interactions will contribute to the development of new anti-angiogenic drugs.

Targeting Angiogenic Microenvironment

Inhibition of the Extracellular Matrix

As interactions between cells and ECM play an important role in angiogenesis regulation, structural and biochemical modifications of this ECM may affect the cell behavior and angiogenesis (Seiki et al. 2003). Several studies have demonstrated that the composition, structural integrity, and biochemical characteristics of the ECM are modified during many pathological processes such as tumor growth, invasion, metastasis, and angiogenesis. New ECM proteins are secreted by inflammatory cells, stromal fibroblasts, tumor cells, and endothelial cells altering the microenvironment composition. The changes in the ECM composition can also alter the integrin ligands within the local microenvironment (McCarthy et al. 2004). Therefore, alterations in the composition or biochemical characteristics of ECM proteins consequently alter the integrin mediated cross talk between cells and the ECM (Fig. 3a).

The remodeling of the ECM is a well-studied process that affects cellular behavior. Matrix-degrading enzymes are important in regulating invasive cellular processes such as angiogenesis, tumor invasion, and metastasis. Some studies demonstrated that deficient mice in MMPs, such as MMP-2 and MMP-9, present defects in tumor growth and angiogenesis (Masson et al. 2005). Additionally, other studies showed that tissue inhibitor of MMPs (TIMPs) as well as synthetic inhibitors of serine proteases, such as

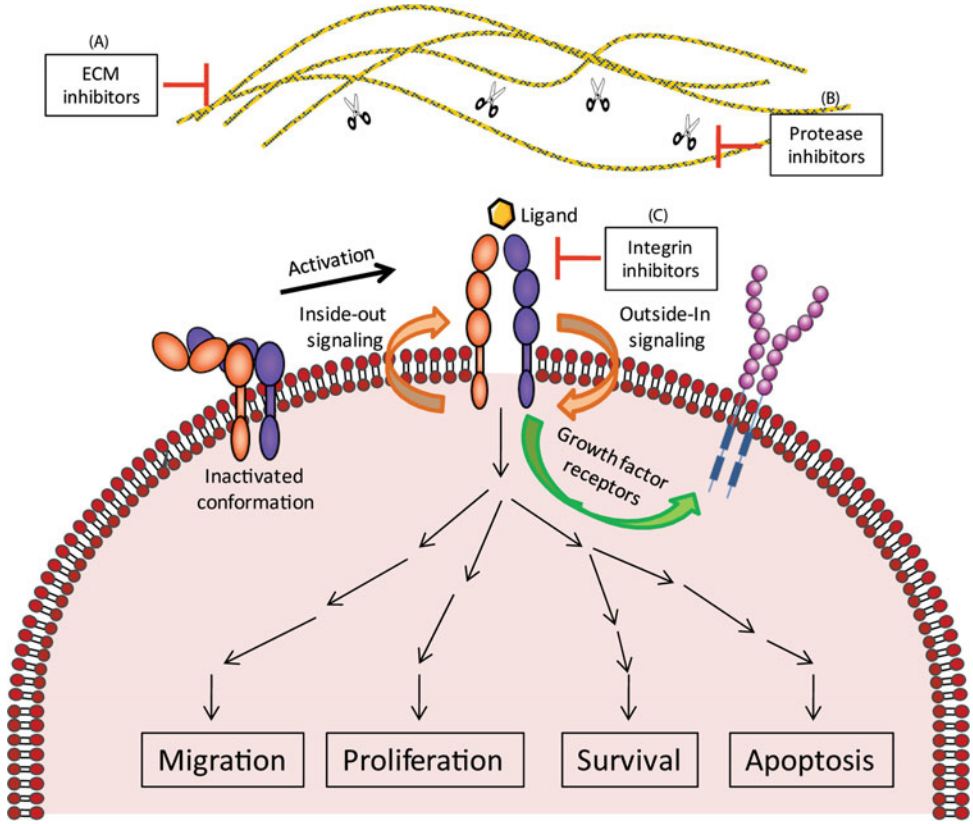

Fig. 3 *Targeting of extracellular matrix contribution in angiogenesis process*. Bidirectional communication between endothelial cells and extracellular matrix (inside-out and outside-in signaling) is important in the angiogenesis process. (**a**) Inhibition of extracellular matrix components or (**b**) protease activity produces anti-angiogenic effects. Integrins mediate the interaction between extracellular matrix and endothelial cells. (**c**) Alterations in expression, ligand binding, or activation state of integrins affect the angiogenesis process

urokinase-type plasminogen activator (uPA), can inhibit angiogenesis and tumor growth. Moreover, proteolytic enzymes can regulate invasive cellular behavior by altering ECM, creating a less restrictive microenvironment through which tumor and endothelial cells can migrate (Noel et al. 2004) (Fig. 3b). Proteolytic enzymes can also stimulate an invasive cell phenotype exposing integrin ligands of the ECM that regulate motility, proliferation, and gene expression. In addition, many other mechanisms by which proteolytic enzymes can induce invasive cellular behavior have been proposed such as a release of growth factors like VEGF by cleaving matrix-bound sites.

Many laboratories have tried to find natural angiogenesis inhibitors derived from ECM proteins. For example, arrestin, canstatin, and tumstatin inhibit angiogenesis through binding to αvβ3 integrin and β1 integrins (Petitclerc et al. 2000). Therefore, ECM fragments can inhibit the interaction between integrins and their microenvironment affecting the adhesion of endothelial cells and in consequence blocking angiogenesis.

Inhibition of Integrins

As mentioned before, integrins represent important possible targets to inhibit angiogenesis. Many integrin receptors play key roles in angiogenesis, including α1β1, α2β1, α3β1, α4β1, α5β1, α6β4, αvβ3, and αvβ5 (Hwang and Varner 2004). Granted that integrins are well studied, many strategies could be designed to alter the expression, ligand binding, or activation state by siRNA, small-molecule inhibitors, peptide memetics, and antibodies (Fig. 3c). Preclinical and clinical trials have tested many of these approaches for the control of angiogenesis and tumor progression. For example, clinical trials have studied various monoclonal antibodies directed to integrin receptors for the treatment of human cancers, such as Vitaxin (humanized monoclonal antibody directed to αvβ3 integrin) (Gutheil et al. 2000), CNTO 95 (monoclonal antibody against αv) (Trikha et al. 2004), and monoclonal antibody M200 (monoclonal antibody directed to the fibronectin receptor α5β1). Therefore, some of these anti-integrin drugs may be useful in the treatment of angiogenic neoplasm.

To optimize the effectiveness and specificity of integrin antagonists, it is very important to understand the particular functions of integrin inhibitors and identify the particular cell type in which the integrin targets are expressed. These aspects are very important given that binding specificity and functions can vary for a particular integrin, as well as a variety of cell types contribute to the regulation of angiogenesis, including endothelial progenitors, inflammatory cells, stromal fibroblasts, pericytes, and tumor cells (Jung et al. 2002). Interestingly, integrin conformation could change depending on the concentration and affinities of the integrin-binding molecule leading to activation or inhibition of integrin function.

β1 and α1 integrins play important roles in angiogenesis. In detail, mice-harboring mutation in several collagen molecules, which represent ligands for β1 integrins, presented defects in vascular development (Marneros and Olsen 2005). Collagen-binding integrins α1β1 and α2β1 contribute to the regulation of VEGF-induced angiogenesis. Therefore, the inhibition of collagen-binding integrins α1β1 and α2β1 may inhibit angiogenesis. Studies have demonstrated that mice treated with function-blocking antibodies against α1 or α2 integrin partially inhibited angiogenesis in vivo (Senger et al. 1997). This inhibition disrupted endothelial cell adhesion to collagen, suggesting the possibility that collagen-binding β1 integrin-signaling cascades play roles in VEGF-dependent angiogenesis.

Another important β1 integrin implicated in angiogenesis is the laminin-binding α3β1 integrin. α3β1 integrin is expressed in endothelial cells and angiogenic blood vessels and bound to laminin and collagen. α3β1 modulates angiogenesis by the association with thrombospondin-1 (TSP-1), an endogenous angiogenesis inhibitor, and uPA receptor (uPAR), receptor associated with proliferation and motility in angiogenesis (Short et al. 2005).

Finally, fibronectin receptors α4β1 and α5β1 are the last β1 integrin receptors implicated in new blood vessel formation. Studies in null mice for α4 and α5 suggested a role of these integrins in vascular development and blood vessel formation. Integrin α4β1 can serve as a counter-receptor for

the cell adhesion molecule vascular cell adhesion molecule-1 (VCAM-1), adhesion molecules expressed by endothelial cells during angiogenesis (Yang et al. 1995). Moreover, studies have suggested that α5β1 integrin is highly expressed in angiogenic blood vessels and can regulate cell survival and apoptosis. Antagonists of α5β1 produce an endothelial cell function inhibition in vitro and an inhibition of angiogenesis in vivo (Kim et al. 2000).

Perhaps the most studied integrin in angiogenesis is the αv integrin subfamily. Studies have shown that angiogenesis induced by fibroblast growth factor-basic (bFGF) and tumor necrosis factor-α (TNF-α) requires the integrin αvβ3 function, whereas angiogenesis induced by VEGF or transforming growth factor-α (TGF-α) requires αvβ5 integrin function (Friedlander et al. 1995). Moreover, other non-ECM proteins may also bind to αv integrins regulating angiogenesis, such as MMP-2 and uPAR. Given the evidence that αvβ3 plays significant roles in angiogenesis, many studies have been focused on the mechanisms by which αvβ3 regulates new blood vessel formation. Studies have shown that αvβ3 does not only facilitate endothelial cell adhesion and migration, it can also regulate endothelial cell survival and apoptosis. Studies in melanoma demonstrated that blocking αvβ3 induced apoptosis on human melanoma tumors suggesting that αvβ3 integrin may regulate apoptosis in both tumor cells and endothelial cells. Moreover, several studies have shown the possible association between αvβ3 integrin and VEGF or VEGFR (De et al. 2005). Importantly, αvβ3 integrin is expressed in angiogenic endothelial cells but not in quiescent endothelial cells. Therefore, monoclonal antibodies against αvβ3 integrin such as LM609 can inhibit the invasive and proliferative phenotype of endothelial cells suppressing angiogenesis (Drake et al. 1995).

Regression of Tumor Blood Vessels

The most typical approximation about inhibition of blood vessels is the inhibition of the formation of new vessels. However, preexisting tumor vasculature can be inhibited resulting in a regression of the tumor vessels. In this context, the cessation of blood flow would trigger death of endothelial cells by apoptosis or necrosis, leading to the regression of vessels, and finally tumor cell death. Several approximations aim to regress tumor blood vessels, which include vascular disruption and reduction of endothelial cells. VDAs, divided into flavonoids and tubulin-binding agents (TBAs), can cease blood flow in tumors in minutes and lead to the formation of extensive central necrosis. Moreover, directing tissue factor to antigenic epitopes expressed in tumor blood vessels would induce intravascular coagulation and cessation of blood flow (Huang et al. 1997). Growth factors, such as VEGF, not only induce the formation of new blood vessels but also regulate endothelial cell survival in existing vessels. Targeting these growth factors by anti-VEGF drugs or others, such as endogenous inhibitors TSP-1, endostatin, angiostatin, and tumstatin, decreases the permeability of tumor vessels and also increases apoptosis of endothelial cells (Inai et al. 2004). Finally, cytotoxic chemotherapeutic drugs can induce endothelial cell apoptosis besides tumor cell death. The anti-vascular effect of chemotherapeutic drugs is due to the cytotoxicity on proliferating endothelial cells contributing to their anticancer action.

Vascular-Disrupting Agents

Regression therapy has focused on the development of agents that inhibit the abnormal vasculature present in the tumor at the time of detection and treatment. This strategy uses VDAs to cause a rapid and catastrophic shutdown in the vascular function resulting in death of cells supplied by those vessels as a result of oxygen and nutrient deprivation. This approximation presents some differences in comparison with current anti-angiogenic strategies. While anti-angiogenic therapies interfere with new vessel formation preventing tumor growth and limiting metastatic potential, VDA attacks established tumor vasculature destroying tumor masses as well as preventing progression. Given these differences, the therapeutic applications of anti-angiogenics and

VDAs are complimentary and not redundant (Chaplin et al. 2006).

VDA Concept

VDAs are ligand-specific or small molecules that selectively target preexisting tumor vasculature and rapidly shut down the blood flow of tumor tissue. They produce ischemia and consequently tumor cell death. The efficacy of VDAs is variable depending on tumor type and vessel fragility, instability, and cell-to-cell endothelial junctions' defects of tumor blood vessels (Siemann et al. 2005).

Ligand-specific VDAs are antibodies, peptides, and growth factors, which selectively bind to the endothelium. The conjugation of a toxin or a procoagulant factor with them induces endothelial cell death (Thorpe et al. 2003).

The two main categories of small-molecules VDAs are flavonoids and TBAs (Lippert 2007). Flavonoids produce partial derangement of the actin cytoskeleton, DNA strand break, and apoptosis of endothelial cells, along with macrophage activation and cytokine release. TBAs induce tubulin depolymerization and disorganization of both tubulin and actin cytoskeleton, due to their binding to different sites of tubulin.

History of VDAs

Given tumoral blood vessels present functional and morphological differences in comparison to normal vessels, tumor vasculature may in principle be killed specifically, leading to tumor cell death. The first evidence was shown by *Denekamp* et al. in in vivo models. This study demonstrated that endothelial cells from tumors proliferated faster than endothelial cells from normal tissues (Denekamp and Hobson 1982). Later, this evidence was confirmed in human tumors (Eberhard et al. 2000). Based on their studies, *Denekamp* proposed vascular disruption approach to treat cancer and continued investigating the vascular collapse necessary to produce antitumoral effects (Denekamp et al. 1983). Around 1980, different studies demonstrated that some of the emergent cancer treatments presented antivascular effects. Many laboratories were interested in the identification of new molecules expressed only in the tumor vasculature to develop new drugs for cancer therapy. More recently, studies by *Burrows and Thorpe* in animal models have shown the efficacy of drugs against tumoral vascular endothelium (Burrows and Thorpe 1993).

Types of Vascular-Disrupting Agents

Flavonoids

Flavonoids are polyphenolic compounds found in a variety of vegetables, fruits, tea, and red wine. They present many pharmacological applications due to their inhibition of tumor cell proliferation and their anti-angiogenic effects (Andrea et al. 2013).

The first VDA described was flavone acetic acid (FAA). The studies in animal models were promising, but early phase clinical studies demonstrated negative results in humans (Hasani and Leighl 2011). After that, some laboratories tried to synthesize and develop many FAA analogs leading to the identification of 5,6-dimethylxanthenone-4-acetic acid ASA404 (DMXAA, Vadimezan, Novartis) and xhantenone-4-acetic acid (XAA) analogs. The development of these analogs demonstrated that vascular-disrupting activity was primarily dependent on the position of the substituents rather than on their nature. In vitro studies demonstrated that derivations of XAA analogs could stimulate human leukocytes to produce interleukin-6 (IL-6) and IL-8 and inhibit tube formation in human endothelial-like cells. However, studies using mouse models showed that the most active compound in human cells was inactive in murine models, suggesting the need for the use of appropriate in vivo animal models in selecting clinical candidates (Tijono et al. 2013).

More recently, in vivo studies have shown that ASA404 can shut down the tumor vasculature and inhibit rapidly the blood flow leading to necrosis of the tumor and hypoxia, after an hour of administration. Moreover, this flavonoid induces apoptosis of endothelial cells in the tumor vasculature and increases the vascular permeability

(Zhao et al. 2005). The mechanism of action of flavonoids as VDAs seems to be associated with the induction of local cytokine production such as TNF-α, interferons, and interleukins through the activation of the NF-xB pathway in monocytes, macrophages, vascular endothelial cells, and tumor cells (Roberts et al. 2007). Therefore, ASA404 produces the disruption of the tumor vasculature, the induction of innate immune cells, and the activation of platelets. Additionally, platelet activation induces the release of von Willebrand factor and consequently generates hypoxia. Finally, hypoxia promotes the release of VEGF that contributes with TNF-α to a positive feedback loop that increases vascular permeability, leading to tumor hemorrhagic necrosis.

Tubulin Binding Agents

TBAs were originally used as antimitotics against cancer, but anti-vascular activities were also identified. TBAs bind to tubulin and induce microtubules polymerization and stabilization or microtubule depolymerization and instability (Jordan and Wilson 2004). These VDAs modify the cytoskeleton organization of endothelial cells changing endothelial shape and leading to vessel blockage, reduction in blood flow, and disruption of the endothelial cell layer. Moreover, the exposure of the BM activates the coagulation cascade increasing the vessel permeability. Indeed, TBAs at low concentrations can affect microtubule dynamics inhibiting their contacts, such as focal adhesions and adherens junctions. Thus, TBAs inhibit endothelial cell adhesion, motility, and cell-cell interactions (Schwartz 2009).

TBAs can also interfere with the normal organization of actin stress fibers resulting in the loss of cell polarity and in the inhibition of cell contractility. Moreover, TBAs can act on focal adhesion kinase (FAK) and VE-cadherin signaling pathways disrupting adherens junction assembly. Importantly, these adherens are critical for angiogenic sprouting and for the maintenance of vascular integrity (Vincent et al. 2005).

Combrastatin-A4 (CA-4) is one of the most well-known TBAs. CA-4, isolated from the African tree *Combretum caffrum*, emerged as a promising VDA. The mechanism of action and the anti-vascular effect have been investigated in vitro and in various tumor models (Dark et al. 1997). Moreover, CA-4P (soluble prodrug of CA-4) was approved for the treatment of various thyroid cancers. However, CA-4P presented some side effects such as enhanced pain. After that, many synthetic analogs were synthesized to modify CA-4 structure in order to improve the activity and reduce adverse reactions, such as TR-644 and BNC105. In particular, TR-644 presents higher microtubule depolymerizing activity. In animal tumor models, TR-644 significantly reduced the number of vessels after 24 h from the administration of a single dose (Porcu et al. 2013).

Therapeutic Approaches of VDAs

VDAs as Monotherapy

Three different phase I clinical trials demonstrated the antitumoral activity of ASA404 at well-tolerated doses (Baguley and Siemann 2010). In phase II trials, ASA404 was administrated in combination with taxanes and carboplatin in different types of cancers. Despite the results seemed promising with improved tumor response and median survival increase, they were not demonstrated statistical significant. Finally, phase III trials failed to demonstrate survival advantages or improvement of overall survival (Lorusso et al. 2011).

Until now, phase I trials with TBAs, such as AVE8062, OXi4503, MPC-6827, ABT-751, and BNC105P, have been carried out in patients resistant to traditional therapies, with advanced solid tumors (Innocenti et al. 2013). AVE8062, CA-4 analog, causes a rapid and irreversible reduction of blood flow in different experimental tumor models. The administration of this analog as monotherapy in patients with advanced solid tumors produces antitumor effects and increases circulating endothelial cells, MMPs, and VEGF. Nevertheless, phase III trials as first lines in NSCLC and as second line in soft tissue sarcoma failed, and its development was stopped. MPC-6827 was evaluated in phase II trials, and although it was well tolerated, its activity was limited. Finally, BCN105P and CYT997 are two

other promising TBAs in clinical development (Burge et al. 2013).

Resistance to VDA Treatments

Despite, VDAs have demonstrated their efficacy in the treatment of cancer; preclinical studies and clinical trials have shown the existence of a residual viable tumor rim after treatment of solid tumors with VDAs (Wu et al. 2013). Therefore, surviving tumor cells after VDA treatments can induce resistance to this therapy. Several mechanisms to explain tumor resistance have been proposed related to hypoxia, tumor-associated macrophages, and bone marrow-derived circulating endothelial progenitor cells (Welford et al. 2011). Additionally, a variety of magnetic resonance imaging (MRI) studies have allowed to observe and to quantify the tumor resistance process. Many strategies have been suggested to improve the antitumor effects of VDAs and to prevent the acquisition of tumor resistance. The most promising strategy is the combination of VDAs with other approaches including anti-angiogenic agents, chemotherapy, and radiotherapy.

VDAs in Combination Therapy

Greater antitumor effects have been achieved when conventional chemotherapy is combined with VDAs. Cells comprising the viable rim of tumor tissue that survives after VDA treatment have a high proliferation rate and excellent nutrition. Therefore, VDA-resistant tumors show enhanced accessibility to systemically administrated agents making the surviving tumor cells susceptible to be killed by radiation and anticancer drugs (Chung et al. 2008). Therefore, the combination of VDAs with conventional therapies may improve the therapeutic potential of each strategy used as monotherapy.

Different studies have reported enhancements in antitumor activities when VDAs were combined with standard chemotherapy (Grosios et al. 2000), principally by post-chemotherapy administration.

In vivo studies in mice models demonstrated the synergism of VEGF inhibition with VDAs. Studies in patients treated with bevacizumab 4 h after CA4-P significantly reduced vascular permeability and tumor perfusion (Cesca et al. 2013). Moreover, phase II clinical trial has demonstrated that the combination of bevacizumab and CA4-P in recurrent ovarian cancer increases significantly the progression-free survival in comparison to bevacizumab alone (Mitrus et al. 2009).

Numerous studies have shown the synergistic effects of ASA404 with carboplatin and paclitaxel, but phase III trial failed (Farace et al. 2007). Another preclinical study investigated the effects of combining ASA404 and everolimus in renal cell carcinoma. The combination produced extensive necrosis and a reduction in the viable rim with respect to ASA404 alone (Wilczynski et al. 2011).

Importantly, the incorrect schedule of combined treatment can cause therapeutic failure. Therefore, more studies are needed to find the best approach and increase the therapeutic potential of the combination therapy.

Normalization of Tumor Vasculature and Microenvironment

Traditional anti-angiogenic strategies try to inhibit new vessel formation or to destroy existing vessels. Nevertheless, it is known that these therapies have insufficient efficacy and tumors can acquire resistance (Ferrara 2010). In the 1990s, preclinical studies showed the concept of vascular normalization. In these studies, VEGF signaling inhibition transiently repaired the vascular abnormalities improving tumor oxygenation and decreasing interstitial fluid pressure. Historically, vessel normalization was initially identified as vessel remodeling in human tumor xenografts. Several studies in mouse models have demonstrated the positive effects of promoting vascular normalization, such as improving tumor vessel perfusion and oxygenation (Fig. 4). Importantly, vascular normalization reduced metastasis and improved the efficacy of chemotherapies and immunotherapies (Mazzone et al. 2009).

Therefore, tumor vessel normalization may reduce the metastatic dissemination and improve

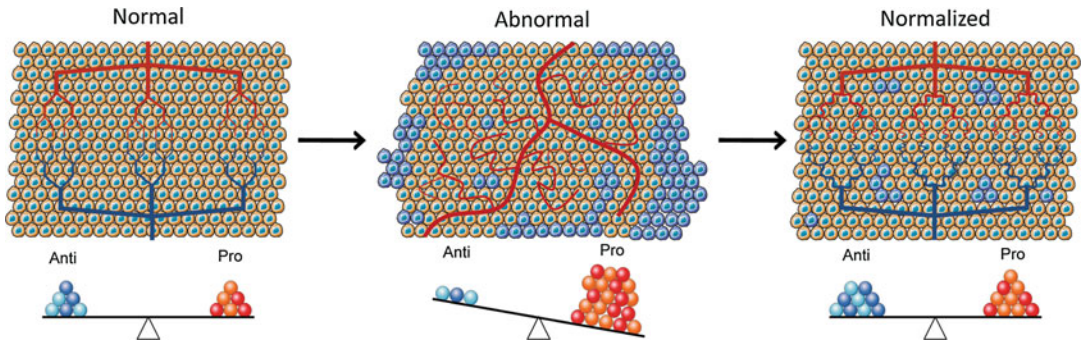

Fig. 4 *Normalization of tumor vessels in response to anti-angiogenic therapies*. (**a**) Vasculature of the tumors is structurally and functionally abnormal. Anti-angiogenic therapies improve both structure and function of the tumor vessels normalizing the tumor vasculature. (**b**) The abnormal vasculature reflects the changes in the balance of pro-angiogenic and anti-angiogenic factors in the tissue

the response to conventional therapies. The possible advantages of vessel normalization compared to traditional therapies have been debated. Nevertheless, increasing evidence indicates that vessel normalization might complement current anti-angiogenic strategies.

Abnormalities of Tumor Vessels

Overexpression of VEGF and other pro-angiogenic factors, induced in hypoxia conditions, leads to formation of a new vasculature that is structurally and functionally abnormal. Moreover, these abnormalities are exacerbated as a tumor continues to grow (Gazit et al. 1997).

Structural Abnormalities

Differentially to normal vessels, tumor vessels are tortuous; they branch irregularly in a chaotic network of tangles connecting randomly with other vessels and with stroma (Nagy et al. 2010). Moreover, tumor vessels are very heterogeneous, the vessel diameter is irregular, some vessels are oversized, and others are more immature. These anomalies have been observed in a wide range of tumor types.

The endothelial cells are also abnormal. Activated tumor endothelial cells lose their polarity, allowing endothelial cells to detach from BM and stack upon each other. Moreover, tumor endothelial cells produce extension into the lumen and form sprouts, with leading tip cells penetrating deep into the tissue. Additionally, these endothelial cells contain multiple fenestrations and other transendothelial channels, resulting in hemorrhage and increased interstitial fluid pressure (Jain 1988).

Tumor vessels are entirely affected. Therefore, all the vessel components are affected, such as pericytes or BM. Activated pericytes in cancers lose their association with endothelial cells and activate processes inside the stroma. Moreover, they change their shape and express more immature markers (Morikawa et al. 2002). In addition, vessel coverage by pericytes in tumor vessels is reduced. The pericyte-deficient condition compromises the vessel wall, favoring the intravasation of tumor cells. Finally, the tumor vessel BM often loses their interaction with endothelial cells and presents an aberrant thickness (Baluk et al. 2005).

Functional Abnormalities

The increased vascular resistance and the improper vasoregulation in tumors compromise the blood flow. Moreover, in tumors, the interstitial fluid pressure is increased and the perfusion is heterogeneous. These flow patterns create an obstacle to a uniform delivery of nutrients and drugs. In fact, properties of vascular barriers can also determine the penetration of the drugs into the tumor. Importantly, hypoxia induced by radiation and chemotherapeutics can reduce the efficacy of conventional anticancer treatments. The high metabolic demand of tumor cells produces

an excess of pro-angiogenic factors. Pro-angiogenic factors induce the formation of abnormal vessels, and these abnormal vessels are unable to fulfill the entire tumor requirements, creating a self-reinforcing vicious cycle (Rey and Semenza 2010).

The abnormal vessel structure and function increase interstitial hypertension, hypoxia, and acidosis, creating a favorable environment for tumor progression and metastasis. Tumor cells to invade must cross the tumor-blood barrier by invading the vascular BM, transmigrating across the endothelium, surviving in the blood, and finally extravasating and growing at a distant site (Kienast et al. 2010). Abnormal tumor vessels facilitate this process because the physical barrier does not exist and also because tumor endothelial cells produce proteinases, adhesion molecules, and other factors that facilitate the tumor cell migration (Sullivan and Graham 2007).

Therefore, tumor vessel abnormalities can provide a favorable environment for invasion and metastasis of tumor cells and can influence tumor responsiveness to conventional anticancer treatment.

Tumor Vessel Normalization

In tumors, the oncogenic, hypoxic, metabolic, and inflammatory pathways stimulate the production of angiogenic inductors, tipping the balance in favor of forming new vessels. Indeed, in contrast to physiological angiogenesis, the stimulation of angiogenesis persists and, consequently, tumor vessels become increasingly abnormal. Thus, targeting different components of the tumor vessel wall may restore this balance (Jain 2005).

Normalization of Endothelial Cells

VEGF is the most important and well-known pro-angiogenic factor and is implicated in many angiogenic aspects, such as endothelial cell growth, migration, and permeability. Moreover, preclinical studies have associated high levels of VEGF with vessel abnormalities. Therefore, the inhibition of the VEGF or its signaling pathway may decrease vessel abnormalities. Anti-VEGF drugs induce transient vessel normalization in preclinical models by reducing the enlarged size and tortuosity of vessels, increasing vessel maturation, enhancing pericyte coverage, and normalizing the BM (Baffert et al. 2006).

Moreover, some studies have demonstrated that blocking VEGF reduces interstitial fluid pressure, while transiently it increases perfusion, oxygenation, and drug delivery (Dickson et al. 2007). However, the prolongation of anti-VEGF treatment can finally destroy tumor vessels, and tumors can become resistant by induction of other pathways. Therefore, vessel normalization is limited to a transient window, and for this reason, some studies did not report some benefits of vessel normalization (Franco et al. 2006). In fact, new studies will help to understand whether and how the normalization window by VEGF blockade can be prolonged to enhance the benefits.

Prolyl hydroxylase domain-containing protein 2 (PHD2) is another example of angiogenic molecule that induces vessel normalization. PHD2 is an oxygen-sensing enzyme that hydroxylates HIFs when sufficient oxygen is available, and once HIFs are hydroxylated, they are targeted for proteosomal degradation. Under hypoxia conditions, PHD2 is inactive and HIFs can induce the response to increase the oxygen supply. Mouse deficiency for PHD2 in endothelial cells demonstrated that inhibition of PHD2 does not affect physiological angiogenesis but induces normalization of tumor vessels by reducing leakage, tortuosity, and remodeling and increasing endothelial cell quiescence, barrier tightening, and vessel maturation (De Bock et al. 2009). Moreover, these changes increase tumor perfusion, reduce tumor hypoxia, and shift tumor metabolism to a more aerobic glycolysis. Additionally, PHD2 deficiency decreases the invasion, intravasation, and metastasis by the induction of junctional molecules in endothelial cells, which provides a more impenetrable blood barrier.

Normalization of Vessel Microenvironment

The molecules implicated in the pericyte coverage, such as PDGFB, can also regulate the tumor vessel normalization. Sprouting endothelial cells

release PDGFB to chemo-attract pericytes that express its receptor, PDGFRB. The inhibition of PDGFB signaling causes pericyte deficiency, leading to vessel leakage, tortuosity, microaneurysm formation, and bleeding. Moreover, deficiencies in PDGFB or in pericytes form abnormal vessels (Huang et al. 2010).

Preclinical studies and studies in patients have demonstrated that deficiencies in pericyte coverage disassemble the vessel wall and promote metastasis (Yonenaga et al. 2005). In addition, overexpression of PDGFD normalizes tumor vessels and increases drug delivery. However, the inhibition of PDGFRB improves drug delivery and chemotherapy (Hellberg et al. 2010). Therefore, future studies are necessary to understand the possible role of PDGF inhibition in cancer treatment.

Regulator of G-protein signaling 5 (RGS5) is a molecule produced by activated pericytes and hypoxic endothelial cells, and its inhibition results in vessel normalization. Loss of RGS5 in pancreatic cancer model produces vessel normalization, with smaller and less leaky microvessels (Hamzah et al. 2008).

ANG-TIE (tunica interna endothelial cell kinase) receptor axis controls vessel maturation and regulates vessel normalization. The interaction between Ang-2 and its receptor, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2), on endothelial cells destabilizes vessels and promotes leakiness, whereas Ang-1, released by pericytes, induces the formation of pericyte coverage (Thurston et al. 1999). Consequently, vessel normalization is produced in glioblastomas with the inhibition of Ang-1 or the overexpression of Ang-2.

Finally, both genetic and pharmacological studies have shown that establishing perivascular gradients of nitric oxide (NO) normalizes tumor vessels. NO stimulates angiogenesis by endothelial NO synthase (eNOS), promoting the formation of stable vessels. However, tumor cells express neuronal NOS (nNOS) in human glioblastoma xenograft model, destabilizing the NO gradients. Finally, blocking nNOS in tumor cells restores the NO gradient and normalizes vessel phenotype (Kashiwagi et al. 2008).

Therapeutic Implications of Vascular Normalization

Rakesh Jain introduced the concept of vessel normalization in 2001 (Jain 2001). For this reason, the most compelling evidence about the vessel normalization stems from preclinical studies, and the translation to clinical studies has not been fully demonstrated yet. Moreover, it is difficult to obtain biopsy samples from patients during the treatment to evaluate vessel normalization. Therefore, some questions have not been answered yet, such as whether vessel normalization can be used as monotherapy or in combination with anti-angiogenic or cytotoxic therapies or whether vessel normalization can prevent metastasis.

Nevertheless, some signs of vessel normalization have been observed in cancer patients. For example, human biopsies of tumors show similar abnormalities to mouse tumors, such as high interstitial fluid pressure (Bullitt et al. 2004). Moreover, clinical studies have shown that anti-VEGF therapies induce some characteristics of vessel normalization, such as reduced numbers and size of immature tumor vessels and increased pericyte coverage, accompanied by decreased permeability, an edema, and interstitial fluid pressure. Additionally, vessel normalization was observed in patients treated with bevacizumab, but they do not demonstrate whether the beneficial effect on tumor growth inhibition was produced by normalization (Willett et al. 2009).

Vessel normalization in cancer patients has also been visualized by MRI in patients treated with anti-VEGF therapies. In patients treated with cediranib, pan-VEGFR tyrosine kinase inhibitor, MRI reveals a decrease in vessel diameter, vascular permeability, and an edema. Moreover, MRI studies have showed that survival of patients with recurrent glioblastoma, treated with cediranib, correlates with vascular normalization index. These results suggest that vessel normalization may predict efficacy of therapy (Sorensen et al. 2009).

Combination of bevacizumab with cytotoxic or cytokine therapy is approved in the treatment of some solid tumors. Some mechanisms have been proposed to explain the benefits of

combination therapy, including the improved cytotoxic drug delivery and efficacy by vessel normalization. This hypothesis is supported by preclinical findings in which VEGF blockade increases the penetration of molecules into the tumor by restoring the fluid pressure and inducing a more uniform distribution of blood flow (Wildiers et al. 2003). Moreover, the vessel normalization after VEGF blockade increases the accessibility of immune cells into the tumor (Shrimali et al. 2010). Given increased drug delivery after VEGF blockade has not been observed in all preclinical models; more investigations are required to pinpoint the effects of vessel normalization induced by bevacizumab.

Conclusion

Many mechanisms are implicated in the formation of new blood vessels. For this reason, tumor vasculature can be inhibited at different levels. Anti-angiogenics can produce the inhibition, regression, or normalization of tumor blood vessels. Inhibition of angiogenic signaling is the most utilized approach. Both the inhibition of VEGF and VEGFR by monoclonal antibodies or small molecules is effectively used in the clinic for the treatment of different solid tumors. On the other hand, EPCs contribute to the formation of new blood vessel and the metastatic niche, so the inhibition of EPC mobilization produces antitumor effects. Moreover, levels of circulating EPCs have been proposed as a diagnostic and prognostic biomarker of cancer. The bidirectional communication of endothelial cells and ECM mediated by integrins is important during the angiogenesis process. Therefore, the inhibition of integrins and ECM can block new vessel formation. Another strategy is the inhibition of the preexisting tumor vasculature by VDAs. VDAs cause a rapid and catastrophic shutdown in the vascular function resulting in death of tumor cells supplied by those vessels. Clinical trials have demonstrated the antitumoral activity of VDAs as monotherapy and in combination with other conventional therapies. Finally, normalization of the abnormal tumor vasculature is produced after anti-angiogenic therapies and improves tumor oxygenation and decreases interstitial fluid pressure, which may reduce metastatic dissemination and improve delivery of drugs to the tumor.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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