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Tumor microenvironment and angiogenesis

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1. ABSTRACT

The tumor microenvironment is a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immune cells. Tumor growth and metastasis formation are dependent on the growth of blood vessels into the tumor mass. The tumor microenvironment contributes to this pathological angiogenic process. The extracellular matrix and basement membranes are a source for endogenous angiogenesis inhibitors, such as endostatin. On the other hand, many extracellular matrix molecules can promote angiogenesis by stabilizing blood vessels and sequestering pro-angiogenic growth factors. The majority of stromal cells in carcinomas are fibroblasts. Carcinoma-associated fibroblasts show a distinct phenotype from normal fibroblasts. The mechanisms how the tumor-associated fibroblasts regulate angiogenesis are not fully known, but they are suggested to be an important source for growth factors and cytokines recruiting endothelial cells. The immune cells, particularly macrophages and neutrophils are another source for angiogenesis-regulating chemokines, growth factors and proteases. Taken together, the tumor microenvironment is a complex unorganized tissue of various cell types and extracellular matrix that can regulate the pathological angiogenic switch.

2. INTRODUCTION

Tumorigenesis is a complex multi-step process, and these steps reflect alterations that drive the progressive transformation of normal cells into highly malignant ones. However, the tumor growth is not just determined by the malignant tumor cells, but instead various cell types and the extracellular matrix (ECM) of the tumor tissue affect the outcome (1). Angiogenesis, the formation of new blood vessels from pre-existing ones, is one of the key events in tumor progression. In addition to providing structural and functional support, the extracellular matrix can modulate vascular endothelial cell behavior. The ECM is a rich source of angiogenesis inhibitors and a storage place for angiogenesis promoters. In physiological conditions the angiogenesis inhibitors counteract the activity of the promoters, thus keeping the angiogenic switch at balance. During pathological events, particularly cancer progression, the angiogenic switch is turned on (2). The evidence available indicates that different types of tumor cells use distinct molecular strategies to activate the angiogenic switch. This raises the question of whether a single anti-angiogenic therapeutic will be sufficient enough to treat all tumor types or whether a cocktail of such therapeutics needs to be developed, each responding to a distinct program of angiogenesis (1). In addition to endothelial

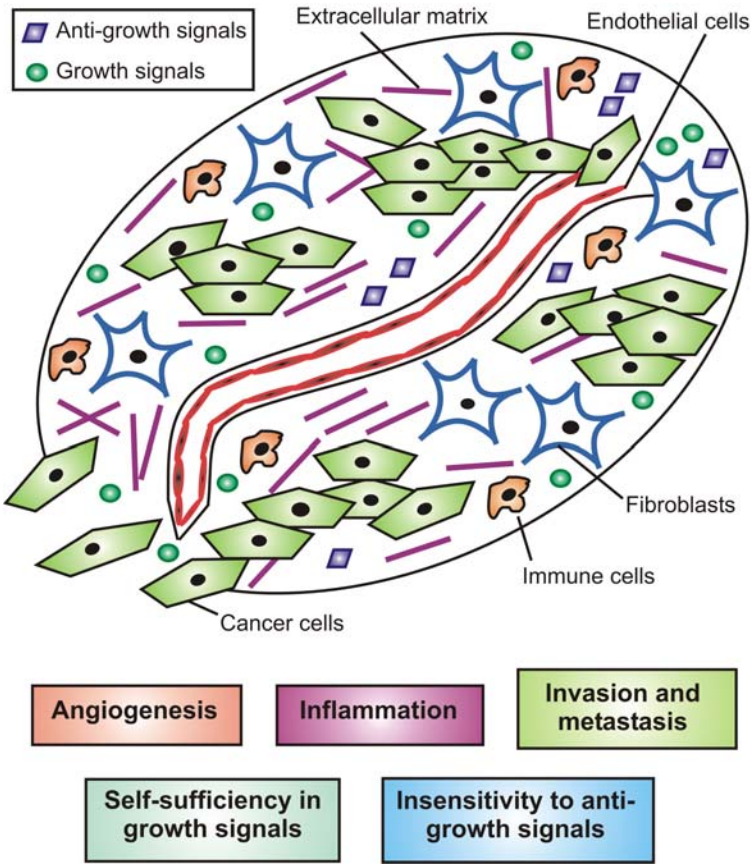

Figure 1. Characteristics of the carcinoma tissue. The tumor microenvironment is a complex scaffold of extracellular matrix and various cell types. In addition to the carcinoma cells, endothelial cells, fibroblasts and immune cells, as well as extracellular matrix molecules, contribute to the carcinoma process. Tumor tissue is characterized by self-sufficiency in growth signals and insensitivity to anti-growth cues, angiogenesis, inflammation and ability to cell invasion and metastasis. The different cell types in the tumor can secrete and remodel the anti-growth and growth signals, and respond to stimuli secreted by other cells. That creates a favorable environment for tumor growth and spread. The figure is a modification from Hanahan & Weinberg review (1).

cells, fibroblasts and inflammatory cells contribute to tumor growth. Fibroblasts affect the tumor microenvironment in several ways: they synthesize ECM and basement membrane components, regulate ECM turnover by secreting proteases, and contribute to epithelial cell behavior by secretion of growth factors and by direct interactions between mesenchymal andepithelial cells (3). The role of a special sub-population of fibroblasts, called cancer-associated fibroblasts is under active research now, but yet their exact role and mechanism is not completely understood. The concept that also inflammation is critically connected to tumor progression is widely accepted. Many cancers are suggested to arise from sites of inflammation, infection and chronic irritation. The inflammatory cells contribute into the orchestration of the tumor microenvironment, foster proliferation, survival and migration of other cell types, and in addition provide chemokines and other signaling molecules (4). *Summa summarum*, the tumor microenvironment consists of a complex network of extracellular matrix components and different cell types that regulate each other and contribute to the pathological tumor angiogenesis, and to the overall tumor progression (Figure 1). In this review, we provide an overview of the relationships between the tumor microenvironment and angiogenesis.

3. TUMOR ANGIOGENESIS

Judah Folkman launched the hypothesis that tumor growth depends on angiogenesis already 37 years ago (5). As all cells need to be located within a close proximity of blood vessels providing oxygen and nutrients, solid tumors cannot grow beyond a few millimetres in diameter without being able to recruit their own blood supply (6, 7). Angiogenesis starts with the separation of endothelial cells from pericytes (cells that are located within the basement membrane of capillary vessels stabilizing the vessel wall) and the vascular basement membrane, processing of the vascular ECM, cell invasion and migration across the basement membranes, and results in novel vascular extensions into the tumor body (6). In addition to this sprouting angiogenesis, several other mechanisms of neovascularization have been identified in tumors, including the recruitment of progenitor endothelial

cells, intussusceptive angiogenesis, vessel co-option, vasculogenic mimicry and lymphangiogenesis (8). Angiogenesis depends plausibly on a delicate balance between endogenous stimulators and inhibitors. During tumor progression this balance is disrupted favoring pro-angiogenic events, *i.e.* the angiogenic switch is turned on. In adult individuals the normal physiological status of the angiogenic switch is either off or at balance and thus angiogenesis does not occur. It is speculated that some individuals might be more susceptible to turn the angiogenic switch on in pathological conditions depending on whether their physiological angiogenic state is at balance rather than completely off (2). Therefore it is crucially important to understand how the angiogenic switch is maintained at balance or off, and what happens when it is pathologically turned on. Different types of tumor cells use distinct molecular mechanisms to activate the angiogenic switch (1). The vascular system is highly heterogeneous in different organs and tissues. The organ microenvironment can directly contribute to the maintenance and induction of the angiogenic factors (9). It has been shown that the phenotype of tumor-associated microvessels is different from both normal and non-tumor-associated angiogenic vessels. The expression of several adhesion molecules, such as E-selectin and VE-cadherin, is lost or diminished in tumor vessels (10), while others are overexpressed, such as integrin alphaVbeta3 (10) and heparin sulphate proteoglycan CD44 family members (11). Thus, it seems possible to target the anti-angiogenic therapy to the tumor vasculature without any harmful effects on the normal vasculature. The predominant mode of action of the anti-angiogenic agents clinically tested to date has been cytostatic; the inhibition of tumor vasculature causes tumor dormancy. Many of these cytostatic agents have been shown to have reversibility of their activity upon removal of the agent. Knowing the speed of vascular regrowth in tumors after cessation of treatment is therefore of high clinical relevance. A recent article determined how rapidly and to what extent tumor blood vessels regrow after removal of anti-VEGF therapy. As fast as one day after drug removal, endothelial sprouts started growing into the empty sleeves of basement membranes that were not destroyed by the anti-angiogenic treatment. Furthermore, also pericytes survived the treatment. This suggests that anti-angiogenic therapy could be more effective if pericytes and basement membrane sleeves could be targeted as well (12).

The endogenous molecules affecting the angiogenic balance are released by the tumor cells and various other cell types or the extracellular matrix in the tumor microenvironment. Stimulators of angiogenesis include hypoxic conditions that activate hypoxia inducible factor HIF-1alpha, which itself can upregulate angiogenic proteins, various growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and plateled derived growth factor (PDGF), as well as angiogenic oncogenes such as Ras. In addition to the above pro-angiogenic factors, the angiogenic phenotype is characterized by tumor expression of pro-angiogenic proteins such as interleukin-8 (IL-8), placental growth factor (PIGF), and transforming growth factor-beta (TGF-beta) (13, 14). Inhibitors of angiogenesis include various anti-angiogenic peptides, hormone metabolites and apoptosis modulators, such as p53 and PTEN (13, 15, 16). p53 also affects directly the survival of tumor cells in hypoxic conditions, as cells expressing wild type p53 will apoptose during hypoxia, whereas p53 mutant or null cells do not (17), demonstrating how hypoxia can select for the survival of p53 mutant cells. Various endogenous anti-angiogenic factors have been described, many of which are fragments of naturally occurring basement membrane and extracellular matrix components. Particularly the non-collagenous parts of many basement membrane collagens function as inhibitors of angiogenesis once they are cleaved from the parent molecule (2). In addition, there are many so called non-classic endogenous regulators of angiogenesis that will be only briefly mentioned here. Some of them have been shown to have a role in tumor angiogenesis; erythropoietin, endothelins, adrenomedullin, proadrenomedullin N-terminal 20 peptide and neuropeptide Y stimulate tumor angiogenesis, whereas somatostatin inhibits it (18).

4. EXTRACELLULAR MATRIX IN ANGIOGENESIS

The extracellular matrix is a three-dimensional structure of heterogeneous macromolecules. In addition to providing structural support to cells and tissues, it supports adhesion of cells, transmits signals through adhesion receptors, and binds, stores and presents growth factors and other biologically active molecules. Basement membranes (BM) are specialized sheet-like extracellular matrix structures that are closely attached to cells. They function as barriers, polarize cells, shape tissue structures, guide and support migrating cells (19). Blood vessel endothelial cells are supported by vascular basement membranes (20). In addition to providing structural and functional support, vascular basement membrane components can modulate endothelial cell behavior (21). The main constituent of basement membranes is type IV collagen that forms a network together with other basement membrane molecules, such as laminins, nidogens, fibulins, SPARC (secreted protein acidic and rich in cysteine), fibronectin, type XV and XVIII collagens, and heparan sulphate proteoglycans (HSPGs), such as perlecan (22). Different cells and even different types of tumor cells secrete a characteristic pattern of matrix proteins (23). The constituents of the basement membranes can vary even within the same tissue; not all vascular basement membranes are the same, for example. The BMs can also have some structural abnormalities in pathological conditions, such as in the blood vessels and endothelial sprouts in tumors (20, 24). The abnormalities of the basement membrane in tumor vessels make the vessels dynamic. Although the tumor vessels are almost completely covered by basement membranes, the membrane has conspicuous structural abnormalities, including a loose association with endothelial cells and pericytes, broad extensions away from the vessel wall, and multiple layers visible by electron microscopy (24). Interestingly, basement membranes can become structurally altered if only one of the BM components is absent. This is the case in collagen XVIII deficient mice. The basement membrane

of these mice is broadened, leading to hydrocephalus and kidney problems (25).

The extracellular matrix functions as a storage place for stimulators and inhibitors of angiogenesis. These biologically active molecules are liberated or activated by proteases. Particularly matrix metalloproteases (MMPs) are involved in several steps of angiogenesis and cancer progression. Not only do they degrade basement membranes and other physical barriers, enabling tumor endothelial cells to migrate and metastatic cells to spread, but they also affect cellular and immune processes (26). MMPs generate growth-promoting signals. They can release precursors of growth factors (27), and through their effects on the extracellular matrix composition, as well as indirectly, they regulate proliferative signals through integrins (28). VEGF, the most potent and best studied angiogenesis promoter, gets activated and liberated from the ECM by MMPs (29, 30). The bioavailability of many growth factors is regulated by the balance of their binding capacity onto HSPGs and the action of extracellular matrix degrading enzymes, which can release heparin-binding growth factors from the matrix to exert their effect (31). Many angiogenesis inhibitors are stored as cryptic fragments within larger precursor matrix molecules that are not themselves anti-angiogenic (2). The regulation of proteolytic processing of these matrix precursors plays an important role in the vascularization of tumors. The activity of MMPs on non-matrix substrates, such as chemokines, growth factors, growth factor receptors, adhesion molecules and apoptosis mediators, is essential for the rapid and critical cellular responses required for angiogenesis, tumor growth and progression (26). MMPs seem to have dual or even opposite effects on tumor angiogenesis; on one hand by facilitating extracellular matrix degradation, enabling endothelial cells to invade the stroma and facilitating neovascularization (32), but on the other hand by blocking pathological angiogenesis by releasing cryptic inhibitors of endothelial cell growth, such as endostatin derived from collagen XVIII (33, 34), angiostatin derived from plasminogen (35, 36, 37) and tumstatin derived from type IV collagen (38). A good example of the complex network regulating cancer development is the fact that the same MMP, such as MMP-9 can be an initial activator of the angiogenic switch by promoting the release of VEGF (39) and an inhibitor of the angiogenic switch by liberating endogenous angiogenesis inhibitors from their parent matrix molecules. Furthermore, increased MMP-9 expression reduces tumor growth and vasculature (40). MMP-9 deficient mice have shown accelerated growth of tumors, at least partially because the mice cannot cleave tumstatin or other cryptic angiogenesis inhibitors from the parent molecules (38). Metastasis have been shown to decrease in MMP-9 knockout mice using many experimental mouse models (41), but surprisingly MMP-9 down-regulation in fibrosarcoma cells resulted in increased extravasation and metastasis (42). To make things even more complex, the regulation between MMPs and endogenous angiogenesis inhibitors seems to be reciprocal in some cases, since some inhibitors cleaved by MMPs are able to regulate the activity of certain MMPs, possibly generating regulatory feedback loops (43, 44, 45, 46).

MMPs are not the only protease family implicated in tumor angiogenesis. The plasminogen activator-plasmin(ogen) system consists of serine protease activities. In particular, plasminogen activator inhibitor type-1 (PAI-1) and urokinase-type plasminogen activator (uPA) are important regulators of tumor angiogenesis, as well as tumor invasion and metastasis (47). Tumor vascularization and invasion are inhibited in PAI-1 deficient mice. Invasion and angiogenesis are restored by exogenous administration of PAI-1 (48). uPA/uPAR/plasmin antagonists are being developed as therapeutic strategies to inhibit tumor angiogenesis (49). On the other hand, plasminogen is the parent molecule of angiogenesis inhibitor angiostatin (50). Cathepsins include serine, cysteine, and aspartyl type proteases. Increased cathepsin activity is associated with angiogenic vasculature and invasive fronts of carcinomas, and differential expression is found in immune, endothelial, and cancer cells. A broad-spectrum cathepsin inhibitor impairs angiogenic switching in progenitor lesions, as well as inhibits tumor growth, vascularity, and invasiveness (51). Trypsins and human tissue kallikreins are serine proteases that are also involved in angiogenesis (52). Thus, these protease families may be potential therapeutic targets in human cancers as well.

4.1. Angiogenesis activators associated with the ECM

The extracellular matrix around the vasculature and its remodelling events promote angiogenesis in two main ways. First, many ECM proteins, including collagens, laminins and fibronectin, have pro-angiogenic properties; they promote endothelial cell survival, proliferation, migration and/or tube formation. Second, many of the pro-angiogenic factors, such as VEGF, bFGF and TGF-beta are sequestered in the heparin-like glycosaminoglycans of ECM and they can be mobilized during ECM degradation by proteases secreted by tumor or stromal cells (53). A surprising feature of angiogenesis has been the shared mechanisms and signalling pathways between the vasculature and nervous system (13, 54).

Vascular endothelial growth factors, particularly VEGF-A, are probably the most potent pro-angiogenic factors described to date. Thus, a lot of research has focused on the VEGF family members and their receptors, VEGFR-1, -2 and -3, in cancer progression. VEGF is secreted by the tumor cells and binds to its receptors VEGF-R2 and neuropilin on endothelial cell surface (13). By binding to VEGF-receptors on the endothelial cell surface, VEGF-A mediates vascular leakage, endothelial cell proliferation and migration (55). The growing vessel sprouts are guided by a VEGF gradient (54). In addition, autocrine VEGF-A signalling contributes to the invasiveness of carcinomas by affecting the survival and migration of the carcinoma cells themselves (56). An anti-VEGF monoclonal antibody (bevacizumab, Avastin) is approved by the U.S. Food and Drug Administration as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy (57). VEGF-C and -D have been implicated in the development and maintenance of lymphatic vasculature that has been hypothesized to be involved in tumor metastasis (58, 59). Other angiogenic

factors include fibroblast growth factors, platelet derived growth factors, transforming growth factor-beta and angiopoietins. Fibroblast growth factors are heparin binding mitogens. Basic fibroblast growth factor (bFGF) is secreted by tumor cells, and it binds to tyrosine kinase receptors (FGFR1 and FGFR2) on endothelial cells. The binding activates the MAPK signalling pathway leading to endothelial cell proliferation (60). Platelet derived growth factor (PDGF) has not attracted as much interest as VEGF or bFGF, but evidence is accumulating that it has a role in tumor angiogenesis as well. Some tumors secrete PDGF that can up-regulate its own receptor on endothelial cells (13). PDGF has been found to stimulate angiogenesis and recruitment of pericytes in tumors (61). The combination of VEGF/VEGFR inhibitors and PDGF/PDGFR antagonists is an attractive concept for the inhibition of tumor angiogenesis, as pericytes seem to be able to survive VEGF inhibition, and possibly pericytes might confer resistance to VEGF/VEGFR antagonists (12, 62). Both pro- and anti-angiogenic properties have been described for TGF-beta. It can directly affect the endothelial cells or function via other cell types. TGF-beta activates Smad signalling cascades. At low doses, TGF-beta induces the angiogenic switch by upregulating angiogenic factors and ECM degrading proteases, whereas at high doses, TGF-beta inhibits endothelial cell growth, facilitates basement membrane reformation and differentiates smooth muscle cells (14). TGF-beta signalling stimulates angiogenesis and thus promotes tumor growth and metastasis (63), and blocking TGF-beta activity by a neutralizing antibody reduces blood vessels and inhibits tumor angiogenesis (64). Angiopoietin-1 and -2 bind to Tie2, a tyrosine kinase receptor on endothelial cells. Angiopoietin-1 helps to maintain the normalized state in blood vessels and has anti-inflammatory activity, but in the tumor microenvironment the abundant angiopoietin-2 competes for Tie2 receptor binding resulting in proinflammatory response and destabilization of pre-existing vessels. Angiopoietin-2 can facilitate angiogenesis and lymphangiogenesis by increasing basement membrane degradation and endothelial cell migration (13, 54, 65).

The ECM is not just a passive storage and sequestering place for vascular growth factors; the ECM components as such play an important role in tumor angiogenesis. Many of them, including collagen I, III, XV, fibronectin, fibulin-1, perlecan, laminin-1 and -8 promote angiogenesis, and stabilize blood vessels during angiogenesis. Particularly fibronectin seems to play an important role in these processes. Fibronectin promotes endothelial cell survival and migration. In addition, it binds to VEGF and enhances VEGF-induced endothelial cell migration (53, 66). Interestingly, fibronectin might also control ECM remodelling events, as the inhibition of fibronectin matrix deposition also inhibits the deposition and retention of other ECM components that affect angiogenesis, such as thrombospondin-1 and collagen I and III (67). The backbone of basement membranes, collagen IV also regulates angiogenesis. MMP-cleavage exposes cryptic proangiogenic epitopes on collagen IV, which are needed for angiogenesis and tumor growth (68).

4.2. ECM derived angiogenesis inhibitors

Both intact ECM molecules and particularly cryptic fragments of ECM have proven to possess anti-angiogenic activity. The striking feature of the ECM associated inhibitors of angiogenesis is the large number of inhibitors that are proteolytically cleaved from a larger parent molecule to gain their anti-angiogenic properties. The parent molecules usually are not anti-angiogenic or they might even promote vascular growth, as discussed in the previous chapter (2, 53). Thrombospondin-1 was the first protein to be recognized as a naturally occurring endogenous inhibitor of angiogenesis (69). It is a large multifunctional extracellular matrix glycoprotein that regulates several biological events in addition to angiogenesis, such as cell adhesion, proliferation and survival, TGF-beta activation, and protease activation (70). It has been shown to inhibit tumor angiogenesis, growth and metastasis (71, 72). Thrombospondin-2 has anti-angiogenic activity as well (73). Implanted melanoma and testicular teratocarcinoma tumors grow faster in thrombospondin-1 null mice, showing that also endogenous levels are sufficient to inhibit angiogenesis (74). Many other endogenous inhibitors of angiogenesis are cryptic fragments from larger molecules. These inhibitors can be divided into two major classes: matrix derived and non-matrix derived inhibitors. Endostatin is a matrix derived anti-angiogenic fragment from collagen XVIII. The non-collagenous endostatin-like fragment of collagen XV also possesses similar anti-angiogenic activity. Arresten, canstatin and tumstatin are angiogenesis inhibitors cleaved from collagen IV (75). Other matrix derived endogenous inhibitors of angiogenesis include endorepellin from perlecan (76), decorin (77), fibulin fragments (78), and the fibronectin derived fragment anastellin (79, 80). Angiostatin is a non-matrix inhibitor of angiogenesis derived from plasminogen, and the first cryptic fragment of a larger parent molecule possessing novel anti-angiogenic properties that the intact molecule does not have (50). Other non-matrix derived endogenous inhibitors include cleaved antithrombin III and prothrombin kringle 2 (81, 82), chondromodulin I (83), interferons (84), interleukins (85), kringle 1-2 domains of tissue type plasminogen activator (86), pigment epithelium-derived factor PEDF (87), the non-catalytic C-terminal hemopexin-like domain of MMP-2 called PEX (88), platelet factor-4 (89), tissue inhibitors of matrix metalloproteases (TIMPs) (90), troponin I (91) and vasostatin (92). Although they are not directly cleaved from ECM molecules, many of them are stored within the ECM or otherwise regulated by it.

Endostatin is probably the most studied cryptic endogenous inhibitor of angiogenesis. The discovery of endostatin in 1997 (33) raised high hopes for cancer cure and a lot of hype in the media as well. The idea of the crucial importance of angiogenesis in cancer growth was indeed revolutionary (5). Endostatin has the broadest anti-cancer spectrum of the endogenous angiogenesis inhibitors. It affects over 12 % of the angiogenesis regulatory human genes (93), and has shown no toxicity in human patients even in long-term use. There are growing numbers of tumor types whose growth is inhibited by endostatin (94). Unfortunately endostatin has not yet become the miracle

cancer drug that scientists and clinicians hoped for when its anti-cancer properties were first discovered, although it has been approved for the clinical use in the treatment of lung cancer in China (94). In fact, it has recently been discovered in some clinical studies that overexpression of collagen XVIII, and thus elevated levels of circulating serum endostatin, is actually associated with poor outcome in non-small cell lung cancer (95). Some studies have found that circulating endostatin levels are normal in patients with head and neck squamous cell carcinoma (96), and that serum endostatin levels have no prognostic significance in patients with hepatocellular carcinoma (97). However, the majority of evidence is supporting the role of a potent anti-cancer drug for endostatin. Particularly interesting is the fact that individuals with Down syndrome, who have higher levels of circulating endostatin, seem to be the most protected of all humans against cancer. This notion is supported by mouse models with similar overexpression of endostatin that show markedly reduced tumor growth (98). All these recent data about endostatin emphasize how the actions of endostatin seem to be more complex than originally thought, and how a lot of work still needs to be done. In addition, endothelial cells are not the only targets of endostatin. A growing number of papers report that the efficacy of endostatin extends beyond its anti-angiogenic activity and includes anti-tumorigenic activity as well (43, 45, 99). Some tumor cells seem to be able to internalize endostatin (100) while in other tumor cells endostatin remains on the cell surface (101). Platelets are a novel source for endostatin and possible target for cancer treatments. They seem to store endostatin intracellularly and be able to release it into the tumor vasculature (13). Despite of all the research, very little is known about the physiological role of endostatin. Strikingly, during embryonic development endostatin enhances endothelial cell proliferation and migration (102).

It should be noted that even though all the collagen derived anti-angiogenic molecules are about the same size, come from similar sources and have amino acid sequence similarities (33, 103, 104, 105), they function via distinct mechanisms, bind different cell surface receptors, affect different parts of the angiogenic process and possibly affect other cell types in addition to the endothelial cells. Endostatin binds to integrin alpha5beta1 and inhibits endothelial cell migration by interfering with the signaling pathways via ERK1 and p38. Tumstatin on the other hand binds to integrin alphaVbeta3 and inhibits the PI3-kinase/Akt/mTOR/4EBP1 signaling pathway, resulting in decreased endothelial cell proliferation (106). Canstatin has been shown to inhibit Akt activation and to induce Fas-dependent apoptosis in endothelial cells (107). In addition, it triggers a crucial mitochondrial apoptotic mechanism in both endothelial and tumor cells, which is mediated through cross-talk between alphaVbeta3- and alphaVbeta5-integrins (108). Arresten binds to integrin alpha1beta1 (105) that is a crucial receptor for the anti-angiogenic function (109). Studies with canstatin and tumstatin show that even within the same molecule, different parts can participate in the inhibition of angiogenesis or tumor growth in distinct ways. The C-terminal part of canstatin is the domain mainly associated with the specific inhibition of proliferation of endothelial cells, whereas the N-terminal part of canstatin is associated with the potential apoptosis-inducing activity on endothelial cells (110, 111). Tumstatin has two separate binding sites to integrin alphaVbeta3, the N-terminal one being associated with anti-angiogenic properties and the C-terminal one with anti-tumor activity (110, 113, 114). In the case of endostatin, both the anti-tumor and the anti-angiogenic activities are located within a 27-amino acid peptide in the amino-terminal part of the endostatin molecule (115). Arresten possess two binding sites on endothelial cell surface, a high-affinity one and a low-affinity one. In addition to the functionally relevant and high affinity binding to integrin alpha1beta1 (109), arresten also binds to heparan sulphate proteoglycans on the endothelial cells, but it is not yet known whether this binding is of functional significance (105). Recent studies have shown that HSPGs are important in many ways in the regulation of angiogenesis. They are large multifunctional molecules that are associated either with cell membranes or with extracellular matrix. In the previous chapter we discussed how HSPGs sequester pro-angiogenic growth factors, but once the HSPGs get cleaved, the cleavage products can have anti-angiogenic properties. Particularly perlecan has been shown to have both angiogenic and anti-angiogenic effects. The anti-angiogenic cryptic fragment of perlecan has been named endorepellin (116). Similarly, intact fibronectins usually associate with promotion of angiogenesis, whereas fibronectin fragment named anastellin inhibits angiogenesis, tumor growth and metastasis (80). Decorin is another ECM protein that inhibits tumor growth and angiogenesis, possibly via suppression of VEGF (77).

4.3. ECM-endothelial cell interactions via integrins in tumor angiogenesis

Angiogenesis is also regulated by integrins, a group of heterodimeric transmembrane cell surface receptors. They can mediate cell adhesion to the components of the extracellular matrix and to other cells, as well as make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways (117, 118). The endothelial cells are among the most anchorage-dependent cells. Integrins facilitate endothelial cell binding to the ECM. Thus, the up-regulation of endothelial cell integrins by pro-angiogenic factors sustains cell viability, increases cell sensitivity to growth factors and is required for migration (13). Endothelial cells have been reported to express up to ten different integrins; alpha1beta1, alpha2beta1, alpha3beta1, alpha5beta1, alpha6beta1, alpha6beta4, alphaVbeta1, alphaVbeta3, alphaVbeta5 and alphaVbeta8 (119). During angiogenesis cells alter their cell surface integrins by overall increase in the expression level and transcriptional shifting of integrin expression from collagen and laminin binding integrins to integrins that bind fibrinogen, fibronectins, vitronectin and proteolytically cleaved forms of collagens. For instance, integrins alpha1beta1 and alpha6beta4 are usually down-regulated, and integrins alpha5beta1 and alphaVbeta3 are up-regulated or expressed *de novo* (120). Pathological angiogenesis is often associated with up-regulation of certain integrins, for instance alpha5beta1 (98, 121, 122). As discussed

previously, many endogenous inhibitors of angiogenesis function via integrin binding; arresten binds to integrin alpha1beta1, endostatin to alpha5beta1 and tumstatin to alphaVbeta3. Interestingly, the binding of integrin alpha4beta1 to thrombospondins results in a pro-angiogenic effect (123), although thrombospondins usually are considered to have anti-angiogenic activity. In addition to the biologically active non-collagenous domains, the central triple helical collagen domains also interact with cells via alpha1beta1 and alpha2beta1 integrins (124, 125). Collagen IV contains cryptic integrin binding sites in addition to the anti-angiogenic fragments that require cleavage to obtain their activity. During angiogenesis these sites are exposed and induce a switch in integrin recognition, with loss of alpha1beta1 binding sites and gain of alphaVbeta3 binding (68). AlphaVbeta3 integrin is selectively expressed on growing blood vessels. Particularly the bFGF induced angiogenesis is dependent on it (126). The integrin alphaVbeta3 deficient mice show normal developmental angiogenesis, but instead they exhibit increased pathological angiogenesis (127). Indeed, blockage of integrin alphaVbeta3 as well as alphaVbeta5 disrupts tumor angiogenesis (126, 128). Both integrins alphaVbeta3 and alpha5beta1 seem to mediate proapoptotic signals when they are unligated or occupied by a soluble ligand (38, 106, 129), and to inhibit VEGF-specific angiogenesis by decreasing VEGF-R2 expression (127, 130). Recently is has been discovered that alphaVbeta3 integrin binds to MMP-2 and thus this co-operation may regulate endothelial cell migration and other functions necessary for angiogenesis (131). The loss of integrin beta4 significantly inhibits tumor angiogenesis suggesting a role for integrin alpha6beta4, although its expression is usually down-regulated during angiogenesis (132). Integrins alpha2beta1 and alpha1beta1 are known to promote cell migration, proliferation and matrix reorganization, and thus they are important in non-quiescent cells during dynamic situations, such as angiogenesis. VEGF significantly induces their expression on the endothelial cell surface. Inhibiting the function of integrins alpha1beta1 and alpha2beta1 by antibodies leads to selective inhibition of VEGF-driven angiogenesis *in vivo* without any effects on the pre-existing vasculature. Therefore, it has been suggested that integrins alpha1beta1 and alpha2beta1 are of particular importance in pathological angiogenesis (133). When tumors were implanted into integrin alpha1 knockout mice, it was unexpectedly discovered that the tumors were growing more slowly in the null mice. It was suggested that this might be due to the upregulation of MMPs leading to an increased amount of angiostatin, an inhibitor of angiogenesis proteolytically derived from plasminogen (134). Taken together, the integrin experiments demonstrate a few principles: (i) integrins may be either positive or negative regulators of angiogenesis, (ii) integrin binding to soluble or insoluble/immobilized ligands may result in distinct outcomes in angiogenesis and (iii) the absence of an integrin is not the same as the presence of a dysregulated integrin.

5. REGULATION OF ANGIOGENESIS BY TUMOR ASSOCIATED FIBROBLASTS

Fibroblasts synthesize ECM and basement membrane components, regulate ECM degradation, epithelial differentiation and behavior, inflammation, wound healing and tissue fibrosis as well as are involved in cancer progression. Fibroblasts and myofibroblasts often represent the majority of the stromal cells within various types of carcinomas, yet the specific contributions of these cells to tumor growth are poorly understood. Stromal fibroblast fractions, named carcinoma-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs) seem to critically differ from the normal fibroblast populations in their ability to promote tumor growth (3, 135). This tumor promoting ability was first demonstrated using fibroblasts from human prostate carcinomas (136). Later it was discovered that this phenomenon happens with other tumor types as well; CAFs from invasive human breast carcinomas are more competent to promote the growth of mammary carcinoma cells and to enhance tumor angiogenesis than are comparable fibroblasts derived from outside of these tumor masses (137). CAFs are usually quite heterogenic in their phenotype. Large numbers of CAFs have myofibroblast-like characteristics and can be identified by the expression of alpha-smooth muscle actin (alpha-SMA). Fibroblast activation protein (FAP) seems to be a unique marker for CAFs, although activated fibroblasts express it also. Other fibroblast markers include vimentin, type I collagen, alpha1beta1 integrin, FSP1 and platelet derived growth factor receptor-beta, but these markers are far from being specific to fibroblasts, and moreover their expression cannot be used to distinguish between activated (CAFs) or resting (normal) fibroblasts (3, 135, 138, 139, 140).

The mechanisms how CAFs contribute to tumor angiogenesis are not well understood. One possibility is that they promote angiogenesis by their production of large amounts of ECM proteins. Some recent publications shed light onto this question. A population of CAFs secrete elevated levels of a cytokine called stromal cell-derived factor 1 (SDF-1 or CXCL12), which plays a central role in the promotion of tumor growth and angiogenesis. CAF-derived SDF-1 not only stimulates carcinoma cell growth directly via the CXCR4 receptor on tumor cells but also recruits endothelial progenitor cells into tumors, thereby promoting angiogenesis (137). SDF-1/CXCL12 produced by immune cells also induces VEGF-A expression in endothelial cells (141), so it is possible that SDF-1/CXCL12 from fibroblasts has a similar effect. Overall, VEGF production by stromal fibroblasts plays an important role in tumor angiogenesis. VEGF has an important role in the emergence of ECM alterations characteristic to tumors. In addition to promoting endothelial cell proliferation, VEGF induces vascular permeability resulting in an influx of endothelial cells, fibroblasts and inflammatory cells, all of which contribute to the altered ECM composition favoring tumor angiogenesis (142). Fibroblasts and inflammatory cells are the main sources of VEGF in tumors, although cancer cells produce it too (143). However, VEGF is also expressed by normal tissue

fibroblasts, raising the question of how the VEGF activity of fibroblasts is regulated. The latent VEGF angiogenic activity of fibroblasts is activated by cancer cells, resulting in tumor-selective utilization of fibroblast-derived VEGF. Through the production of VEGF, fibroblasts promote angiogenesis and growth of human pancreas cancer. These molecular mechanisms that trigger angiogenesis are effective at least in human primary fibroblasts and human colorectal tissue. It seems that fibroblasts are involved in the production and storage of latent VEGF in the extracellular environment for urgent use in angiogenesis. MMPs from carcinoma cells are needed to liberate the VEGF angiogenic activity (30). The tumor cells can also stimulate the fibroblasts to express and release MMPs and other proteases in a paracrine manner through secretion of interleukins, interferons and growth factors (144, 145). On the other hand, fibroblasts can be the source of endogenous angiogenesis inhibitors; for instance fibroblasts secrete and deposit thrombospondin-1 into the extracellular matrix (146).

The origin of CAFs is also unclear. There has been some evidence that they might originate from carcinoma cells via epithelial-mesenchymal transition (EMT); in many human epithelial cancers the CAFs nearby the tumor cells carry the same p53 mutation as the primary cancer cells (147). However, in many cancers CAFs do not exhibit karyotypic alterations, such as aneuploidy, and they are not tumorigenic (135). Other possible sources for CAFs are normal fibroblasts, myofibroblasts, preadipocytes, smooth muscle cells or bone-marrow derived progenitor cells (135, 148, 149). A recent paper demonstrated that endothelial cells can also be a source for CAFs via endothelial to mesenchymal transition (EndMT) (150). In the liver metastases of colorectal cancer, the CAFs originate from the resident fibroblasts. The CAFs in the metastatic sites contribute to angiogenesis by TNF-alpha mediated increase in the production of IL-8, a chemokine that is related to invasion and angiogenesis (151).

6. REGULATION OF ANGIOGENESIS BY TUMOR ASSOCIATED IMMUNE CELLS

Inflammation is a crucial function of the immune system that protects against pathogens and initiates specific immunity. Chronic inflammation is associated with most human cancers. The immune cells seem to have a dual role in cancer progression. On one hand, the immune system is capable of recognizing and attacking cancer cells. The tumor cells need to develop ways to escape the immune surveillance (41, 152). On the other hand, strong evidence suggests that cancer associated inflammation promotes tumor growth and progression. Chronic inflammation increases the risk of certain cancers; this is best characterized in cases of chronic ulcerative colitis and Crohn's disease that clearly associate with colorectal cancers. Furthermore, many cancers arise at the sites of chronic inflammation, inflammatory cells are abundantly found in cancers, cancer cells produce inflammation regulators, and the long-term use of non-steroidal anti-inflammatory drugs reduce the risk of some cancers. The deletion or inhibition of inflammatory mediators inhibits cancer development in experimental models. Genetic variations of inflammatory genes can alter susceptibility to cancer and the severity of the disease (4, 153). The inflammation associated with neoplasia is usually type 2 inflammation promoting cell proliferation by producing growth factors and products of the arginase pathway, scavenging debris by expressing scavenger receptors, and promoting angiogenesis and tissue remodelling (154, 155). The cancer associated inflammation starts with the migration and infiltration of leucocytes (neutrophilic and eosinophilic granulocytes) from the blood stream to the site of inflammation followed by the infiltration of monocytes, plasma cells and lymphocytes. Tumor cells produce various growth factors, cytokines and chemokines, *e.g.* VEGF, bFGF and PDGF that attract diverse populations of leucocytes and stimulate migration of mast cells. The immune cells are then able to produce a specific array of cytokines and chemokines, which can act as mitogens for carcinoma cells as well as endothelial cells and fibroblasts. The immune cells also produce various ECM degrading proteases that provide space for neovascular sprouts, and liberate biologically active molecules affecting angiogenesis. This stimulates angiogenesis and lymphangiogenesis, as well as enables metastatic spread via engagement with either blood or lymphatic vessels (4, 156). The tumor associated macrophages (TAMs) derived from monocytes are probably the key cells in chronic tumor associated inflammation. They particularly accumulate into the hypoxic areas of tumors (157). Once activated, the macrophages are the main source for cytokines, chemokines, growth factors and proteases that profoundly affect endothelial, epithelial and mesenchymal cells in the tumor microenvironment (4, 153, 158). For instance, during melanoma development, TAMs produce TFG-beta, TNF-alpha, IL-1alpha, arachidonate metabolites and extracellular proteases, which induce melanocytes to express IL-8 and VEGF-A thereby inducing vascular angiogenesis (159). Thus, macrophage infiltration is closely associated with the depth of melanoma cell invasion partially via macrophage-regulated tumor-associated angiogenesis (160). Another good example of the ability of TAMs to regulate tumor angiogenesis is during human cervical carcinogenesis, when TAMs express VEGF-C and VEGF-D that are participating in lymphangiogenesis and the formation of lymphatic metastases (161). It should be noted that the inflammatory cells and reactions are as diverse as the tumors. For instance, subpopulations of TAMs have been described with variable capacities to produce cytokines TNF-alpha, IL-1 and IL-6 (162). In addition to macrophages, other inflammatory cells; mast cells, neutrophils, eosinophils and activated T lymphocytes contribute to tumor angiogenesis. Mast cells are able to up-regulate angiogenesis in squamous cell epithelia (163). In a mouse model of pancreatic islet carcinogenesis, neutrophils and macrophages are the major sources of MMP-9 and thus mediate the angiogenic switch. MMP-9-expressing neutrophils are predominantly found inside angiogenic islet dysplasias and tumors, whereas MMP-9-expressing macrophages are localized along the periphery of the lesions. Furthermore, depletion of neutrophils significantly suppresses the association of VEGF to the VEGF-receptor and inhibits angiogenesis. Thus infiltrating neutrophils can play a crucial role in activating the angiogenic switch

during early carcinogenesis (164). The disruption of inflammatory cell influx by two mechanistically distinct anti-inflammatory drugs (cortisone and ibuprofen) inhibits angiogenesis indicating a direct pro-angiogenic role for neutrophil-like leukocytes (165).

Chemokines affect inflammation and cancer through leukocyte attraction and angiogenesis. They are the largest family of cytokines, and are identified by the location of cysteine residues near the amino terminus; hence the abbreviations CXC or CC are used. Some chemokines, such as CXCL8/IL-8 and CXCL6/granulocyte chemotactic protein-2, are proangiogenic, whereas other chemokines, such as CXCL10/IFN-gamma induced protein-10 and CXCL4/platelet factor-4, possess angiostatic properties (4, 166). Most chemokines activate leukocytes via binding to G protein coupled receptors designated CXCR or CCR (167). It is not always clear whether the angiogenic or angiostatic effects of chemokines are direct or indirect. For example, a chemokine called CXCL12/stromal cell derived factor-1 induces VEGF-A expression in endothelial cells, VEGF-A in turn upregulates endothelial cell expression of CXC-receptor-4, to which angiostatic chemokines usually bind to (141). Another example is the highly angiostatic chemokine, CXCL4L1/platelet factor-4 variant (PF-4var) that was isolated from platelets, and also produced by mesenchymal tumor cells and induced in monocytes. CXCL4L1/PF-4var, but not CXCL4/PF-4, was co-induced with the angiogenic chemokine CXCL6/granulocyte chemotactic protein-2 (GCP-2) by cytokines, *e.g.*, IL-1beta and IL-17, in sarcoma cells, but not in fibroblasts. Furthermore, the induction of CXCL6/GCP-2 in endothelial cells was enhanced by TNF-alpha but inhibited by IFN-gamma, which synergized with IL-1beta to produce the angiostatic CXCL10/IFN-gamma-induced protein-10 (166). Thus, the delicate equilibrium between angiostatic and angiogenic chemokines during inflammation and tumor progression is rather complex and differs depending on the chemokine, cell type, and stimulus. Selective intervention in this network may dramatically disturb the balance and turn the angiogenic switch on or off.

6. ANGIOGENESIS AND METASTASIS

At some point of the development of most human cancers, pioneer cells move out from the primary tumor mass, invade the tissue, intravasate into the blood or lymphatic circulation and travel to distant sites where they extravasate and may found new colonies, metastases. Metastases are the cause of about 90% of human cancer deaths. During invasion and metastasis, the physical coupling of cells to each other and to the microenvironment changes (168). The metastatic cells break various physical barriers consisting of basement membranes, extracellular matrix and layers of tightly associated cells (169). It has been speculated that the basement membranes of tumor blood vessels might be incomplete or absent, thus enabling an easier route for the cancer cells to start metastasizing. However, it seems that the basement membranes actually cover most tumor vessels but it is structurally abnormal, consistent with the dynamic nature of endothelial cells and pericytes in tumors. Despite the extensive vessel coverage, the basement membrane has profound structural abnormalities, including a loose association with endothelial cells and pericytes, broad extensions away from the vessel wall, and multiple layering (24). Nevertheless, the expanding endothelial cell surface gives the tumor cells more opportunities to enter the circulation and start metastasizing.

Epithelial to mesenchymal transition (EMT) is a biological process where epithelial cells loose their epithelial characteristics and undergo a transition into a mesenchymal phenotype. EMT is one possible mechanism in the acquisition of an invasive phenotype leading to metastasis. Extracellular stimuli such as growth factors contribute to the EMT. The most studied EMT potentiator is TGF-beta (170). The cleavage of cell adhesion molecules by MMPs and the liberation of TGF-beta play a role in the EMT associated with cancer (41). Angiogenesis inhibitors cleaved by MMPs can also suppress metastasis formation (50). It is not just the cleavage of adhesion molecules and extracellular matrix bound factors that induce EMT; intact matrix components such as collagen I promote EMT in lung cancer. This happens via activation of autocrine TGF-beta signalling. Interestingly, epidermal growth factor (EGF) seems to initiate EMT also via TGF-beta dependent mechanism (171). Hypoxia, angiogenesis and particularly lymphangiogenesis have been shown to be involved in the spread and metastasis of primary tumors (172, 173). It is not entirely clear whether there are differences in hypoxia and angiogenesis at different metastatic sites, and what might be the mechanisms of such differences. Comparisons of gene expression profiles of primary breast tumors and metastases from different locations reveal that the metastases are strikingly similar to the primary tumor (174, 175) suggesting that the influence of the local tumor microenvironment of the metastatic site is less important than the primary tumor cell biology. Furthermore, the endothelial cells in different organs and vascular beds possess considerable structural and functional heterogeneity; the gene expression patterns between endothelial cells from larger vessels and microvascular endothelial cells, as well as endothelial cells from different organs are pervasively different (176). The tissue specific differences in endothelial cells might be due to the differences in the tissue microenvironment, *i.e.* through distinct soluble factors, cell-cell and cell-matrix contacts (177, 178). Very recently Watnick and colleagues discovered that certain human tumors create a metastasis favoring niche by producing a novel protein that specifically represses thrombospondin-1 in the stromal tissue to which the tumor is subsequently able to metastasize. The mechanism of this formation of a metastatic niche is not yet uncovered, but it gives further insight to the connections between metastasis and angiogenesis. Such a mechanism can facilitate the initiation of angiogenesis by metastatic tumor cells (13).

7. CONCLUSIONS AND PERSPECTIVES

It has become more and more clear how important the tumor microenvironment is for the tumor

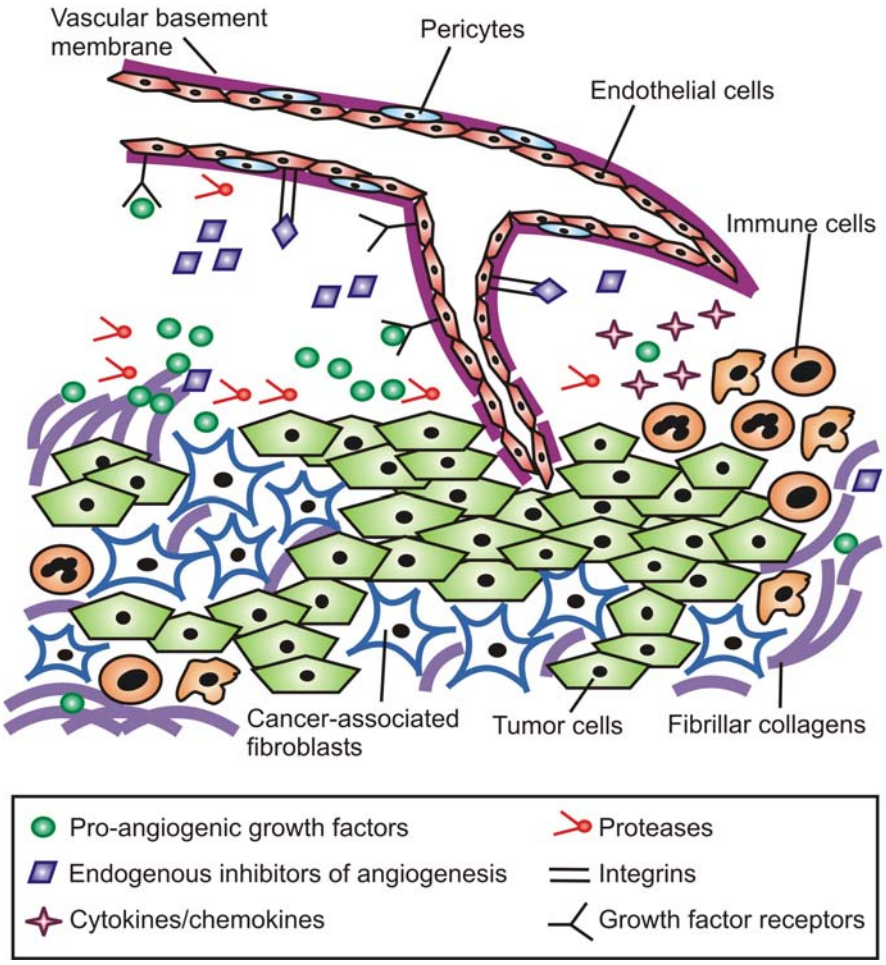

Figure 2. A summary of the angiogenesis-related processes and molecules in the tumor microenvironment. Angiogenesis is crucial for tumor development; without sufficient blood supply the tumors remain small and dormant. Cancer cells together with cancer-associated immune cells and cancer-associated fibroblasts orchestrate the production and remodelling of extracellular matrix proteins, proteases, growth factors, cytokines and chemokines that can turn the pathological angiogenic switch on and result in vascularized and malignant tumors. The endothelial cell layer of mature and sprouting vessels is stabilized by pericytes and surrounded with vascular basement membranes. The cleavage of basement membrane results in the liberation of cryptic endogenous inhibitors of angiogenesis, of which endostatin is probably the best known. On the other hand, many pro-angiogenic growth factors, such as VEGF and bFGF, and chemokines are stored and sequestered within the extracellular matrix. The physiological balance of these pro- and anti-angiogenic factors is disrupted during cancer progression. The negative and positive regulators of angiogenesis bind to different endothelial cell surface receptors, like integrins and specific growth factor receptors. Certain proteases produced by the various cells in the tumor microenvironment are crucially important in regulating the angiogenic switch, because proteolysis modulates the activity of the extracellular matrix molecules, growth factors and chemokines in addition to facilitating cell invasion by breaking physical barriers.

growth. The different cell types and ECM components contributing to the tumor microenvironment and affecting the tumor vasculature are summarized in Figure 2. The role of tumor angiogenesis and the ability of the extracellular matrix to serve as a source for pro- and anti-angiogenic cues have been known for a while now, but only quite recently scientists have really realized the fundamental importance of fibroblasts and immune cells in cancer growth and progression (3, 4, 135). Targeting the function or regulation of any of these cancer-associated cells or ECM molecules could be a therapeutic strategy. For example, zolendronic acid (a bisphosphonate compound) suppresses MMP-9 expression in tumor-associated mast cells and reduces the association of VEGF with its receptor on endothelial cells (179). Cancer-associated fibroblasts are attractive targets for cancer treatment as well in the future, once more in-depth knowledge is gathered about the function and mechanism of CAFs. In addition to secretion of growth factors, all the cell types in the tumor microenvironment secrete a characteristic pattern of ECM proteins that can affect angiogenesis either as intact molecules or as cryptic proteolytically cleaved fragments. Particularly the basement membrane derived angiogenesis inhibitors have been under enthusiastic research. It has

been speculated that certain individuals might be more susceptible to cancer growth, because they have lower levels of circulating or local endogenous inhibitors of angiogenesis, and thus their angiogenic switch might turn on more easily. This view is supported by several facts. Individuals with Down syndrome, who have higher levels of circulating endostatin, seem to be well protected against cancer. In addition, mouse models with similar overexpression of endostatin show significantly reduced tumor growth, and mice deficient in thrombospondin show much more rapid tumor growth (98). The majority of anti-angiogenic therapies in clinical use block VEGF. However, VEGF is not the only growth factor affecting tumor angiogenesis. Moreover, tumor cells might shift their growth factor production over time during VEGF blocking treatments. Therefore combination therapies are probably needed. In addition, very little is known about the role of some cell types; for instance platelets have been suggested to have a role in angiogenesis. They appear to scavenge and store both pro- and anti-angiogenic regulators, which seem to be released within the tumor vasculature, but the putative role of platelet release of angiogenesis regulatory molecules in tumors remains to be elucidated (13, 180).

All this new data emphasizes the importance of the tumor microenvironment, and the active communication of the tumor cells and the microenvironment with each other. Therefore, the conventional and still widely used tumor burden assay where tumor cells are subcutaneously injected into mice might not give relevant information on the real-life situation. Similarly, results from studies performed by two-dimensional monolayer cell culture methods might not tell us much about the carcinoma process *in vivo*, because the cells might behave completely differently in the complex three-dimensional tumor microenvironment. A good tool to solve these problems is the use of organotypic carcinoma system, which was originally developed by Fusenig and colleagues (181). The organotypic models are generated by plating carcinoma cells onto a synthetic stroma usually composed of a collagen gel embedded with fibroblasts. Thus the carcinoma cells grow in a three dimensional environment surrounded by ECM components and fibroblasts. The tumor invasion can be quantitated with various methods (182, 183), and the EMT-related changes in the cell phenotypes can be detected. The organotypic systems can also be transplanted on to the back muscle fascia of nude mice (182). Thus the organotypic method more closely mimics the *in vivo* situation and gives scientists a better tool to understand the complex tumor microenvironment and to develop effective treatment strategies for cancer.

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Abbreviations: BM: basement membrane, CAF: cancer-associated fibroblast, ECM: extracellular matrix, EMT: epithelial to mesenchymal transition, FGF: fibroblast growth factor, HIF: hypoxia inducible factor, HSPG: heparan sulphate proteoglycan, IFN: interferon, IL: interleukin, MMP: matrix metalloprotease, PAI: plasminogen activator inhibitor, PAR: plasminogen activator receptor, PDGF: platelet derived growth factor, PF: platelet factor, TAM: tumor-associated macrophage, TGF: transforming growth factor, TNF: tumor necrosis factor, uPA: urokinase-type plasminogen activator, VEGF: vascular endothelial growth factor, VEGFR: vascular endothelial growth factor receptor.

Key Words: Extracellular Matrix, Fibroblast, Immune Cell, Integrin, Microenvironment, Tumor Angiogenesis, Review

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