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Commentary

Angiogenesis: regulators and clinical applications

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Abstract

Angiogenesis is a fundamental process in reproduction and wound healing. Under these conditions, neovascularization is tightly regulated. Unregulated angiogenesis may lead to several angiogenic diseases and is thought to be indispensable for solid tumor growth and metastasis. The construction of a vascular network requires different sequential steps including the release of proteases from "activated" endothelial cells with subsequent degradation of the basement membrane surrounding the existing vessel, migration of endothelial cells into the interstitial space, endothelial cell proliferation, and differentiation into mature blood vessels. These processes are mediated by a wide range of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules. Finally, when sufficient neovascularization has occurred, angiogenic factors are down-regulated or the local concentration of inhibitors increases. As a result, the endothelial cells become quiescent, and the vessels remain or regress if no longer needed. Thus, angiogenesis requires many interactions that must be tightly regulated in a spatial and temporal manner. Each of these processes presents possible targets for therapeutic intervention. Synthetic inhibitors of cell invasion (marimastat, Neovastat, AG-3340), adhesion (Vitaxin), or proliferation (TNP-470, thalidomide, Combretastatin A-4), or compounds that interfere with angiogenic growth factors (interferon-α, suramin, and analogues) or their receptors (SU6668, SU5416), as well as endogenous inhibitors of angiogenesis (endostatin, interleukin-12) are being evaluated in clinical trials against a variety of solid tumors. As basic knowledge about the control of angiogenesis and its role in tumor growth and metastasis increases, it may be possible in the future to develop specific anti-angiogenic agents that offer a potential therapy for cancer and angiogenic diseases.

Keywords: Angiogenesis; Growth factors; Proteolytic enzymes; Endothelial cell proliferation and migration; Adhesion molecules; Tumor growth and metastasis

1. Introduction

Angiogenesis is the process of generating new capillary blood vessels. In the adult, the proliferation rate of endothelial cells is very low compared with that of many other cell types in the body. Physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing [1]. Unregulated angiogenesis may result in different pathologies [2], such as rheumatoid arthritis [3], diabetic retinopathy [4], psoriasis and juvenile hemangiomas [5]. Finally, tumor growth and metastasis are angiogenesis-dependent [6]. A growing tumor needs an extensive network of capillaries to provide nutrients and oxygen. In addition, the new intratumoral blood vessels provide a way for tumor cells to enter the circulation and to metastasize to distant organs. Thus, every organ system may involve diseases in which angiogenesis is an important component.

Angiogenesis is a complex process involving extensive interplay between cells, soluble factors, and ECM components. In this review, the regulation of key mediators of

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Abbreviations: Ab, antibody; Ang, angiopoietin; COX, cyclooxygenase; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; HGF/SF, hepatocyte growth factor/scatter factor; HIF-1α, hypoxia-inducible factor-1α; HS, heparan sulfate; HSPG, heparan sulfate containing proteoglycan; IFN, interferon; IL, interleukin; LLC, Lewis lung carcinoma; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PD-ECGF, platelet-derived endothelial cell growth factor; PDGF, platelet-derived growth factor; PEDF, pigment epithelium-derived factor; PF-4, platelet factor-4; PlGF, placenta growth factor; TGF-β, transforming growth factor β; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor α; TP, thymidine phosphorylase; tPA, tissue-type plasminogen activator; TSP-1, thrombospondin-1; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VEGI, vascular endothelial cell growth inhibitor; VCAM-1, vascular cell adhesion molecule-1; VHL, von Hippel-Lindau; *and* vWF, von Willebrand factor.

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Table 1
Overview of the different MMPs and their substrates [11,188,189]

Enzyme	MMP No.	Main substrates
Group I		
Matrilysin	MMP-7	Non-fibrillar collagen, gelatin, laminin, fibronectin, proteoglycans, proMMP-1, -9
Group II		
Interstitial collagenase	MMP-1	Fibrillar collagens (types I, II, III, VII, and X), proMMP-2, -9
Neutrophil collagenase	MMP-8	Fibrillar collagens
Collagenase-3	MMP-13	Fibrillar collagens
Stromelysin-1	MMP-3	Non-fibrillar collagen, gelatin, laminin, fibronectin, proteoglycans, proMMP-1, -9, -13
Stromelysin-2	MMP-10	Non-fibrillar collagen, gelatin, laminin, fibronectin, proteoglycans, proMMP-1
Stromelysin-3	MMP-11	Weak activity against non-fibrillar collagen, laminin, fibronectin
Metalloelastase	MMP-12	Elastin
(Unnamed)	MMP-19	Not known
Enamelysin	MMP-20	Amelogenin
Group III		
Gelatinase A	MMP-2	Gelatin, types IV, V, and I collagen, laminin, fibronectin, proMMP-9, -13
Gelatinase B	MMP-9	Gelatin, types IV and V collagen
Group IV		
MT1-MMP	MMP-14	proMMP-2, -13, gelatin, fibrillar collagens, laminin, fibronectin
MT2-MMP	MMP-15	proMMP-2, gelatin, fibrillar collagens, laminin, fibronectin
MT3-MMP	MMP-16	proMMP-2
MT4-MMP	MMP-17	Not known

angiogenesis and their effect on tumor growth and metastasis will be highlighted, and recent advances in the development of specific antagonists with promising antitumor activity will be discussed.

2. Regulation of angiogenesis

2.1. Basement membrane breakdown: proteolytic enzymes

To initiate the formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighboring tissue [7]. These processes of endothelial cell invasion and migration require the cooperative activity of the PA system and the MMPs.

The uPAs and tPAs are serine proteases that convert plasminogen into plasmin. The fibrinolytic activity in blood is mainly regulated by tPA, whereas the activation of plasminogen in tissues is regulated by uPA [7,8]. uPA is secreted as an inactive single-chain proenzyme. Secreted pro-uPA binds to the uPAR present on many different cell types. Cleavage of pro-uPA by plasmin, Factor XIIa, or cathepsin B yields the active enzyme consisting of two disulfide-linked chains [8]. The interaction of uPA with its receptor concentrates the enzyme activity to the so-called "focal attachment sites" on the cell surface and stimulates signal transduction through the uPAR, leading to induction of cell migration and invasion [9]. Plasmin has broad substrate specificity and degrades several ECM components, including fibrin, fibronectin, laminin, and the protein core of proteoglycans [7]. In addition, plasmin may activate several MMPs such as MMP-1, MMP-3, and MMP-9 [10].

The metalloproteinase family consists of at least 16 members, which are expressed as latent enzymes with a similar domain structure [11]. They all contain a pre-domain, which is a signal peptide for secretion, a pro-domain, which is removed when the enzyme is proteolytically activated, a catalytic domain containing a zinc ion, and besides matrilysin, a "hemopexin" domain, which contains a binding site for TIMPs. One specific class of MMPs, the gelatinases, also contain a "fibronectin" domain that is inserted in the catalytic domain. MMPs are soluble, secreted enzymes with the exception of the recently discovered MT-MMP group that contain a transmembrane domain at the carboxy-terminal end and are located at the cell surface [11]. MMPs have been classified according to their domain structure (Table 1) or substrate specificity.

The activity of both PAs and MMPs is controlled at three levels: (i) the expression of uPA, uPAR, and MMPs is up-regulated by angiogenic growth factors [12–16] and cytokines [17]; (ii) pro-MMPs and pro-uPA need to be activated proteolytically [10]; and (iii) the activity of MMPs, plasmin, and uPA is regulated by, respectively, TIMPs [18], α_2-antiplasmin, and PAIs [9,19]. PAs and MMPs are secreted together with their inhibitors, ensuring a stringent control of local proteolytic activity, in order to preserve normal tissue structure. However, a large body of evidence suggests that this regulation is lost during tumor growth and metastasis [20]. Excessive MMP activity has been detected in colorectal, lung, breast, gastric, cervical, bladder, prostate cancer, and malignant glioblastoma. Moreover, in a number

of these studies, a good correlation was found between the amount of MMPs and the aggressiveness/invasiveness of the tumor [21–23].

2.2. Endothelial cell migration and proliferation: angiogenic factors

Following proteolytic degradation of the ECM, "leader" endothelial cells start to migrate through the degraded matrix. They are followed by proliferating endothelial cells, which are stimulated by a variety of growth factors, some of which are released from the degraded ECM.

A variety of angiogenesis inducers have been described (Table 2), which can be divided into three classes [24]. The first class consists of the VEGF family and the angiopoietins, which specifically act on endothelial cells. The second class contains most direct-acting molecules, including several cytokines, chemokines [25], and angiogenic enzymes [26,27] that activate a broad range of target cells besides endothelial cells. The prototype member of this group, FGF-2, was one of the first angiogenic peptides to be characterized. The third group of angiogenic molecules includes the indirect-acting factors, whose effect on angiogenesis results from the release of direct-acting factors from macrophages, endothelial or tumor cells. The most extensively studied are TNF-α and TGF-β, which inhibit endothelial cell proliferation *in vitro*. *In vivo*, TGF-β induces angiogenesis and stimulates the expression of TNF-α, FGF-2, PDGF, and VEGF by attracted inflammatory cells [28,29]. TNF-α has been shown to increase the expression of VEGF and its receptors, IL-8 and FGF-2 by endothelial cells, thus explaining its angiogenic properties *in vivo* [30,31].

Only the characteristics of the most prominent angiogenic factors, such as VEGF and FGF-2, and the recently described angiopoietins will be addressed here.

2.2.1. VEGF

VEGF belongs to the VEGF family, which currently consists of six members: VEGF-A (or VEGF), PlGF, VEGF-B, VEGF-C, VEGF-D, and orf virus VEGF (VEGF-E) [32]. The loss of only a single VEGF allele leads to embryonic lethality, implying that this factor plays a unique role in the development of the vascular system [33].

VEGF is expressed in different tissues, including brain, kidney, liver, and spleen, and by many cell types [32]. *In vitro*, VEGF stimulates ECM degradation, proliferation, migration, and tube formation of endothelial cells and induces in these cells the expression of uPA, PAI-1, uPAR, and MMP-1 [34–37]. *In vivo*, VEGF has been shown to regulate vascular permeability, which is important for the initiation of angiogenesis [38].

Transcription of VEGF mRNA is induced by different growth factors and cytokines, including PDGF, EGF, TNF-α, TGF-β, and IL-1β [32,39,40]. VEGF may thus function as a mediator for indirect-acting angiogenic factors. VEGF levels are also regulated by tissue oxygen tension. Exposure to hypoxia induces VEGF expression [41,42] rapidly and reversibly, through both increased transcription and stabilization of the mRNA. In contrast, normoxia down-regulates VEGF production and even causes regression of some newly formed blood vessels. By these opposing processes, the vasculature exactly meets the metabolic demands of the tissue (or tumor) [43]. Alternative exon splicing of the VEGF gene results in different VEGF isoforms containing 121, 145, 165, 189, or 206 amino acid residues, $VEGF_{165}$ being the predominant form. While $VEGF_{121}$ does not bind heparin and is freely diffusible, the

Table 2
Endogenous angiogenesis inducers

Inducers[a]	EC			References
	Proliferation	Migration	Differentiation	
Heparin binding peptide growth factors				
VEGF	Yes	Yes	Yes	32,44
PlGF	Weak	Yes	?	59
FGF-1, FGF-2	Yes	Yes	Yes	59
Pleiotrophin	Yes	?	Yes	190
HIV-*tat*	Weak	Weak	Yes	59
PDGF	Yes	Yes	Yes	191
HGF/SF	Yes	Yes	Yes	59,192
Non-heparin binding peptide growth factors				
TGF-α	Yes	Yes	Yes	59,193
TGF-β	Inhibition	No	Yes	194
EGF	Yes	Yes	Yes	59,195
IGF-I	Yes	Yes	Yes	195,196
Inflammatory mediators				
TNF-α	Inhibition	No	Yes	193
IL-8	Yes	Yes	?	197
IL-3	Yes	Yes	Yes	198
Prostaglandin E_1, E_2	No	No	Yes	27,199
Enzymes				
PD-ECGF/TP	No	Yes	?	26
COX-2	No	Yes	Yes	170
Angiogenin	No	Yes	Yes	200
Hormones				
Oestrogens	Yes	Yes	Yes	201
Proliferin	?	Yes	?	202
Oligosaccharides				
Hyaluronan oligo's	Yes	Yes	Yes	203,204
Gangliosides	?	?	?	205
Hematopoietic factors				
Erythropoietin	Yes	?	Yes	206
G-CSF	Yes	Yes	?	207
GM-CSF	Yes	Yes	?	207
Cell adhesion molecules				
VCAM-1	No	Yes	?	79
E-selectin	No	Yes	Yes	79,80
Others				
Nitric oxide	Yes	?	?	193
Ang-1	No	Yes	Yes	208,209

[a] Induction of EC proliferation, migration, and differentiation as measured *in vitro*.

larger isoforms contain increasingly basic and heparin-binding residues and are bound to the cell surface or sequestered in the ECM [44].

Two high-affinity binding sites for VEGF have been identified on vascular endothelium: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Similarly to VEGF, regulation of VEGF receptor gene expression is regulated by hypoxia [45]. An additional member of this family, VEGFR-3 (Flt-4), is not a receptor for VEGF, but binds VEGF-C and VEGF-D [32]. During embryogenesis, expression of VEGFR-1 and VEGFR-2 is initiated at the time of blood island formation. Homozygous mutants inactivating VEGFR-1 or VEGFR-2 are lethal, implying that both receptors are essential for normal development of the embryonic vasculature [46,47]. Ligand binding triggers receptor dimerization and subsequent auto/transphosphorylation. Several studies have indicated that VEGFR-1 and VEGFR-2 have different signal transduction properties [44]: interaction of VEGF with VEGFR-2 is critical for VEGF-induced biological responses, whereas the function of VEGFR-1 in VEGF-mediated angiogenesis is still unclear.

Recently, neuropilin-1 (NP-1), a cell surface glycoprotein that binds semaphorin/collapsins, mediators of neuronal guidance, has been identified as a $VEGF_{165}$ receptor. NP-1 is expressed in endothelial cells and enhances the mitogenic effects of Flk-1 upon $VEGF_{165}$ stimulation [48].

Besides its function during embryogenesis, VEGF also plays a crucial role in angiogenesis in the adult. VEGF was detected in the ovary during corpus luteum formation [49] and in the uterus during growth of endometrial vessels and at the site of embryo implantation. Also, high VEGF levels were detected during the proliferative phase of wound healing [50]. VEGF is equally detectable in areas where endothelial cells are quiescent, such as heart, lung, and brain, pointing to the role of VEGF as a survival factor. Finally, VEGF is thought to play a role in several human cancers, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis [44,51].

2.2.2. Angiopoietins

Two other endothelial cell-specific receptors, called Tie-1 and Tie-2 (for "tyrosine kinase with immunoglobulin- and EGF-like domains"), were identified several years ago. Knockout experiments in mice have suggested a role for these receptors in blood vessel maturation. The ligands for Tie-2 have been discovered only recently: angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) both bind Tie-2, but only the binding of Ang-1 results in signal transduction and regulation of blood vessel maturation [52]. Therefore, Ang-2 is a natural antagonist of Ang-1 [53].

Recently, a model for the complementary roles of VEGF and angiopoietins in vascular development and angiogenesis was proposed [54]. During embryogenesis, VEGF promotes differentiation and proliferation of endothelial cells and the formation of immature vessels. Ang-1, acting through the Tie-2 receptor, induces the remodeling and stabilization of the blood vessels, which involves interactions with the ECM. In a normal adult vessel, Ang-1 is associated with Tie-2 to keep the vessels in a stable state. Up-regulation of Ang-2, by hypoxia or VEGF [55], for example in the ovary during corpus luteum formation or by tumor cells, disrupts the interaction between Ang-1 and Tie-2, resulting in destabilization of the vessels. Endothelial cells, which are no longer attached to the pericytes and the ECM, become responsive to angiogenic signals and, in the presence of VEGF, angiogenesis is promoted. The absence of stimulatory signals will cause regression of the vessels [54].

2.2.3. FGF-2

The FGF family consists of at least 19 members, which share 55% sequence identity at the amino acid level. All FGFs are 18- to 30-kDa proteins with high affinity for heparin. FGF-2 was one of the first angiogenic factors to be characterized [56] and has been studied extensively. The single-copy human FGF-2 gene encodes multiple FGF-2 isoforms with molecular weight ranging from 18,000 to 24,000 [57]. Both low and high molecular weight FGF-2 isoforms show angiogenic activity *in vivo* and induce cell proliferation, chemotaxis, and uPA production in cultured endothelial cells [56]. Also, FGF-2 was found to induce tube formation in collagen gels and to modulate integrin expression, gap junction intercellular communication, and VEGF, Flk-1, and uPAR up-regulation *in vitro* [58,59].

FGF-2 is expressed at low levels in almost all organs and tissues examined, with high concentrations reached in the brain and pituitary. It is found in many cultured cell types, including fibroblasts, endothelial, smooth muscle, and glial cells. Although FGF-2 lacks a leader sequence for secretion, data suggest that FGF-2 is secreted from FGF-2-producing cells by an alternative secretion pathway [60] and accumulates in the ECM.

At least four members of high-affinity tyrosine-kinase FGFRs [61] have been described. Low-affinity binding sites were identified as proteoglycans, including syndecan and perlecan, containing HS side chains (HSPGs) [62]. These HSPGs are found in the ECM, the basement membrane, and the cell surface. It has been suggested that binding of FGF-2 to HSPGs results in protection of FGF-2 from inactivation in the extracellular environment and in storage of FGF-2 in the ECM and basement membrane. Stored FGF-2 can be released by heparitinase and soluble heparin or after ECM breakdown [62].

A dual receptor model has been proposed for FGF-2 in which interaction of the growth factor with non-signaling HSPGs is required for its binding to the FGFR. Heparin would induce oligomerization of FGF-2, which might be important for receptor dimerization and activation. FGFR activation will then trigger an intracellular signal cascade leading to multiple biological responses, including endothelial cell proliferation and migration, differentiation, protease production, and angiogenesis [63].

FGF-2-deficient mice develop normally without any evident phenotype, i.e. organogenesis, animal growth, life span, and the female reproductive cycle are unaffected by the absence of FGF-2 [64]. Nevertheless, different reports have implicated FGF-2 in both physiological and pathological angiogenesis. Mice lacking FGF-2 show neuronal defects and delayed wound healing [64]. Furthermore, FGF-2 is produced by many tumor cell lines *in vitro* and is thought to play a role in the growth and neovascularization of solid tumors [65]. High levels of FGF-2 are present in endothelial cells of Kaposi's sarcoma [66] and in proliferating hemangiomas [67], and elevated amounts of FGF-2 have been detected in the serum and urine [68] of patients with advanced colorectal, breast, ovarian, and renal carcinomas [69] and soft tissue sarcoma [70].

2.3. Cell–cell and cell–matrix interactions: adhesion molecules

The processes of cell invasion, migration, and proliferation not only depend on angiogenic enzymes, growth factors, and their receptors, but are also mediated by cell adhesion molecules [71]. To initiate the angiogenic process, endothelial cells have to dissociate from neighboring cells before they can invade the underlying tissue. During invasion and migration, the interaction of the endothelial cells with the ECM is mediated by integrins. Also, the final phases of the angiogenic process, including the construction of capillary loops and the determination of the polarity of the endothelial cells, which is required for lumen formation, involve cell–cell contact and cell–ECM interactions [71].

Cell adhesion molecules can be classified into four families depending on their biochemical and structural characteristics. These families include the selectins, the immunoglobulin supergene family, the cadherins, and the integrins. Members of each family are implicated in neovascularization [71].

Integrins are a group of cell adhesion receptors, consisting of non-covalently associated α and β subunits, which can heterodimerize in more than 20 combinations. Endothelial cells thus express several distinct integrins, allowing attachment to a wide variety of ECM proteins [72]. Integrin $\alpha_v\beta_3$ was found to be particularly important during angiogenesis. $\alpha_v\beta_3$ is a receptor for a number of proteins with an exposed Arg-Gly-Asp (RGD) sequence, including fibronectin, vitronectin, laminin, vWF, fibrinogen, and denatured collagen. In addition, $\alpha_v\beta_3$ has been shown to bind MMP-2, in an RGD-independent way, thereby localizing MMP-2-mediated matrix degradation to the endothelial cell surface [72,73]. $\alpha_v\beta_3$ is nearly undetectable on quiescent endothelium, but is highly up-regulated during cytokine- or tumor-induced angiogenesis. In activated endothelium, $\alpha_v\beta_3$ suppresses the activity of both p53 and the p53-inducible cell-cycle inhibitor $p21^{WAF1/CIP1}$, while increasing the Bcl2:Bax ratio, resulting in an anti-apoptotic effect [74]. Consequently, $\alpha_v\beta_3$ was found to promote melanoma growth by regulating tumor cell survival [75]. Another receptor that has been implicated recently in angiogenesis is integrin $\alpha_v\beta_5$. Antibodies directed against $\alpha_v\beta_3$ were found to specifically block FGF-2- or TNF-α-induced angiogenesis, whereas antagonists of $\alpha_v\beta_5$ blocked VEGF-induced angiogenesis [76]. This implies that specific cytokines may stimulate angiogenesis by distinct signaling pathways that may be mediated by specific integrins.

Besides integrins, a number of other cell adhesion molecules are involved in angiogenesis. Vascular endothelial cadherin or VE-cadherin mediates calcium-dependent homophilic interactions between endothelial cells. Recently, knockout studies in mice demonstrated that a deficiency or truncation of VE-cadherin induces endothelial apoptosis and inhibits transmission of the endothelial survival signal by VEGF, leading to embryonic lethality [77]. Members of the immunoglobulin superfamily mediate heterophilic cell–cell adhesion. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are expressed on quiescent endothelium, but are up-regulated after stimulation with TNF-α, IL-1, or IFN-γ [78]. Furthermore, VCAM-1 can induce chemotaxis in endothelial cells *in vitro* and angiogenesis *in vivo* [79]. Also, members of the selectin family, in particular P-selectin and E-selectin, which promotes adhesion of leukocytes to cytokine-activated vascular endothelium, have been shown to play a role in angiogenesis [79]. E-selectin was found to induce endothelial migration and tube formation *in vitro* and angiogenesis *in vivo* [80]. However, little is known about the mechanism of action of these molecules, and mice deficient in both E-selectin and P-selectin are viable and fertile [81].

3. Physiological versus pathological angiogenesis

With respect to activated endothelium, an important distinction must be made between physiological and pathological settings [2]. Although many positive and negative regulators (Table 3) operate in both, endothelial cell proliferation is tightly controlled in the former, whereas in the latter, the uncontrolled growth of microvessels may lead to several "angiogenic diseases" (Table 4) in different tissues.

3.1. Physiological angiogenesis

Besides during embryogenesis, angiogenesis is also activated in the female reproductive system [2] during ovulation, corpus luteum formation, and embryo implantation. During these processes, angiogenesis is mediated mainly by VEGF [44,49]. Neovascularization also plays a critical role in successful wound healing that is probably regulated by growth factors such as FGF-2 [64] and VEGF [50]. Macrophages may contribute to the healing process by releasing these angiogenic factors [82].

Table 3
Endogenous inhibitors of angiogenesis

Inhibitor	Mechanism of action	References
Protein fragments		
Angiostatin (fragment of plasminogen)	↓ EC proliferation, ↑ EC apoptosis	99,122
Endostatin (fragment of collagen XVIII)	↓ EC proliferation, ↑ EC apoptosis	128
aaAT (fragment of antithrombin 3)	↓ EC proliferation, ↑ EC apoptosis	130
Prolactin (16 kDa fragment)	↓ EC proliferation ↓ FGF-2-induced angiogenesis	210
Soluble mediators		
TSP-1	↓ EC proliferation, ↑ EC apoptosis	118
Troponin I	↓ EC proliferation	211
IFN-α	↓ EC proliferation, ↑ EC apoptosis, ↓ FGF-2-induced angiogenesis	161
IFN-γ	↓ EC proliferation, ↑ IP-10	212
PEDF	↓ EC migration ↓ FGF-2-induced EC proliferation	213
IP-10	↓ EC proliferation ↓ FGF-2- and IL-8-induced migration	25
PF-4	↓ EC proliferation ↓ FGF-2- and IL-8-induced migration	25
IL-12	↑ IFN-γ, ↑ IP-10	214
IL-4	↓ EC migration	215
VEGI	↓ EC proliferation	216
TIMP-1, -2	↓ MMP activity	107
PAI-1	↓ uPA activity	19
Retinoic acid	↓ EC migration, transcription factor	217,218
Ang-2	↓ Blood vessel maturation, antagonist of Ang-1	53
2-Methoxyoestradiol	↓ EC proliferation and migration, ↑ EC apoptosis	219
Tumor suppressor genes		
p53	↑ TSP-1 synthesis, ↓ VEGF synthesis	93
VHL	↓ VEGF synthesis	95

3.2. Angiogenesis in tumor growth and metastasis

Tumor growth is often a multi-step process that starts with the loss of control of cell proliferation. The cancerous cell then begins to divide rapidly, resulting in a microscopically small, spheroid tumor: an *in situ* carcinoma [2]. As the tumor mass grows, the cells will find themselves further and further away from the nearest capillary. Finally, the tumor stops growing and reaches a steady state, in which the number of proliferating cells counterbalances the number of dying cells. The restriction in size is caused by the lack of nutrients and oxygen [83]. *In situ* carcinomas may remain dormant and undetected for many years, and metastases are rarely associated with these small (2–3 mm^3, avascular tumors [2].

Table 4
Clinical manipulation of angiogenesis

Therapeutic goal	
Inhibition of angiogenesis	Stimulation of angiogenesis
Hemangiomas	Induction of collateral vessel formation:
Psoriasis	Myocardial ischemia
Kaposi's sarcoma	Peripheral ischemia
Ocular neovascularization	Cerebral ischemia
Rheumatoid arthritis	Wound healing
Endometriosis	Reconstructive surgery
Atherosclerosis	
Tumor growth and metastasis	

Yet, several months or years later, an *in situ* tumor may switch to the angiogenic phenotype, induce the formation of new capillaries, and start to invade the surrounding tissue. The "angiogenic switch" depends on a net balance of positive and negative angiogenic factors in the tumor. Thus, the angiogenic phenotype may result from the production of growth factors, such as FGF-2 and VEGF, by tumor cells and/or the down-regulation of negative modulators, like TSP-1, in tissues with a quiescent vasculature [84]. In both normal and pathological angiogenesis, hypoxia is the main force initiating the angiogenic process. Hypoxia induces the expression of VEGF and its receptor via HIF-1α [85,86] and is also an attractant for macrophages. In a tumor, the angiogenic phenotype can be triggered by hypoxia resulting from the increasing distance of the growing tumor cells to the capillaries or from the inefficiency of the newly formed vessels. Also, several oncogenes such as v-*ras*, K-*ras*, v-*raf*, *src*, *fos* and v-*yes* [41,86–88] induce the up-regulation of angiogenic factors like VEGF and increase the production of cytokines and proteolytic enzymes [89]. Moreover, on-

Table 5
Anti-angiogenic therapy: compounds and their mechanism of action [24,83,112,168,220–225]

Compound	Mechanism of action
Inhibitors of ECM remodeling	
Batimastat, marimastat, AG3340, Neovastat, PEX, TIMP-1, -2, -3, -4	MMP inhibitors, block endothelial and tumor cell invasion
PAI-1, -2, uPA Ab, uPAR Ab, Amiloride	uPA inhibitors, block ECM breakdown
Minocycline, tetracyclines, cartilage-derived TIMP	Collagenase inhibitors, disrupt collagen synthesis and deposition
Inhibitors of adhesion molecules	
$\alpha_v\beta_3$ Ab: LM609 and Vitaxin, RGD containing peptides, $\alpha_v\beta_5$ Ab	Block EC adhesion, induce EC apoptosis
Benzodiazapine derivatives	Antagonist of $\alpha_v\beta_3$
Inhibitors of activated endothelial cells	
Endogenous inhibitors: endostatin, angiostatin, aaAT	Block EC proliferation, induce EC apoptosis, inhibit angiogenic switch
IFN-α, IFN-γ, IL-12, nitric oxide synthase inhibitors, TSP-1	Block EC migration and/or proliferation
TNP-470, Combretastatin A-4	Block EC proliferation
Thalidomide	Inhibits angiogenesis *in vivo*
Linomide	Inhibits EC migration
Inhibitors of angiogenic mediators or their receptors	
IFN-α, PF-4, prolactin fragment	Inhibit FGF-2, inhibit FGF-2-induced EC proliferation
Suramin and analogues	Bind to various growth factors, including FGF-2, VEGF, PDGF, inhibit EC migration and proliferation
PPS, distamycin A analogues, FGF-2 Ab, antisense-FGF-2	Inhibit FGF-2 activity
Protamine	Binds heparin, inhibits EC migration and proliferation
SU5416, soluble Flt-1, dominant-negative Flk-1, VEGF receptor ribosymes, VEGF Ab	Block VEGF activity
Aspirin, NS-398	COX inhibitors
6AT, 6A5BU, 7-DX	TP antagonists
Inhibitors of EC intracellular signaling	
Genistein	Tyrosine kinase inhibitor, blocks uPA, EC migration and proliferation
Lavendustin A	Selective inhibitor of protein tyrosine kinase
Ang-2	Inhibits Tie-2

cogene products may act directly as angiogenic factors. This is the case for the protein product of FGF-4/hst-1 [90]. In contrast, the tumor suppressor p53 has been found to cause degradation of HIF-1α [91], inhibition of VEGF production [92], and stimulation of the inhibitor TSP-1 [93]. Finally, the VHL gene product inhibits tumor growth and suppresses the expression of hypoxia-inducible genes [94,95]. Consequently, inactivation of the *VHL* gene, as seen in VHL disease, an inherited cancer syndrome characterized by extensively vascularized tumors, results in stabilization and activation of HIF-1α [96].

The final step in the progression of a tumor is metastasis [2,83,97]. Neovascularization of a primary tumor increases the possibility that cancer cells will enter the blood stream and spread to other organs and is also necessary for the growth of metastases in distant organs [98]. Most of the micrometastases have a high death rate and are not vascularized until they switch to the angiogenic phenotype [2]. Mice experiments have shown that for certain tumors, like LLC, this switch is dependent on the removal of the primary tumor, which releases an angiogenesis inhibitor: angiostatin [99]. However, this is not a general rule, as metastases of B16 melanoma are not affected by removal of the primary tumor [2].

4. Inhibition of angiogenesis

Considerable insight into the molecular and cellular biology of angiogenesis has been obtained by *in vitro* studies using endothelial cells, isolated from either capillaries or large vessels. Most steps in the angiogenic cascade can be analyzed *in vitro*, including endothelial cell proliferation, migration, and differentiation [100]. However, to discover and evaluate the potency of anti-angiogenic compounds, it is crucial to have suitable *in vivo* models. Classical angiogenesis assays include the chick chorioallantoic membrane (CAM), rabbit cornea assay, sponge implant models, matrigel plugs, and conventional tumor models (reviewed in Refs. [101–105]).

An increasing number of anti-angiogenic compounds (Table 5) have been identified, many of which have been shown to hold anti-angiogenic activity in a particular assay, such as the CAM. More recently, research has focused on the search for compounds with a specific effect on an individual step of the angiogenic process. As each step in the angiogenic cascade involves a great variety of enzymes, cytokines, and receptors, angiogenesis presents different possible targets for therapeutic intervention. In the following section, we will only discuss the more recently discov-

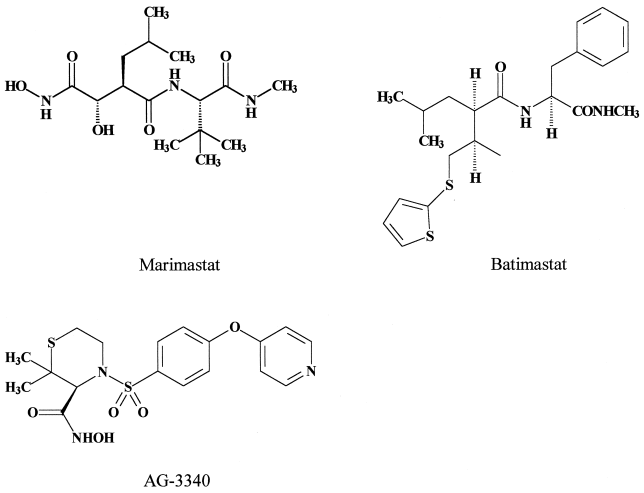

Fig. 1. Structural formulae of MMP inhibitors.

ered promising compounds or drugs undergoing clinical evaluation.

4.1. Inhibitors of cell invasion, motility, and adhesion

4.1.1. Inhibition of MMP activity

Recently, a naturally occurring non-catalytic fragment PEX of MMP-2 was found to prevent binding of the enzyme to the integrin $\alpha_v\beta_3$ receptor, leading to inhibition of enzymatic activity at the cell surface. A recombinant form of PEX was shown to block angiogenesis and tumor growth *in vivo* [106]. Despite the discovery of PEX and other endogenous MMP inhibitors (TIMP-1, -2, -3, -4) [107], research has focused on synthetic, orally available inhibitors. Several MMP inhibitors (MMPI) have been developed, from broad-spectrum inhibitors, which block most of the MMPs, to selective inhibitors, which interfere with the activity of a particular MMP. One general structural feature of MMPIs is the presence of a metal-binding group, often a carboxyl, thiol, or hydroxamate, that chelates the zinc atom in the active site of the enzyme [20].

Batimastat (BB-94, Fig. 1), a pseudopeptide hydroxamic acid with potent activity against most of the major MMPs (MMP-1, -2, -3, -7, -9), was the first synthetic MMPI evaluated in the clinic. The drug inhibits enzyme activity by reversible competition with the MMP substrate. Despite its ability to suppress or prevent the growth of various tumors in animal models [108,109], clinical studies with batimastat have been suspended because of its insolubility and, hence, low oral bioavailability. The related compound marimastat (BB-2516, Fig. 1) has an enzyme activity spectrum similar to batimastat, but with a more favorable pharmacological profile in humans, since it is orally available [110,111]. Marimastat has now entered phase III trials in patients with small cell lung, non-small cell lung, and breast cancer and is undergoing phase II studies for pancreas and brain cancer [112]. Other MMPIs in phase III of clinical development include AG-3340, a non-peptide, orally active hydroxamate (Fig. 1), designed on the basis of substrate structure [113], and Neovastat, an endogenous inhibitor isolated from cartilage [112].

4.1.2. Inhibition of cell adhesion molecules

The $\alpha_v\beta_3$ integrin, an adhesion receptor for extracellular matrix components with an exposed RGD sequence, is an attractive target for anti-angiogenic therapy since it is exclusively present on the cell surface of activated endothelial cells, but absent on quiescent endothelium or other cell types [72]. An RGD-containing peptide antagonist of $\alpha_v\beta_3$ and an anti-$\alpha_v\beta_3$ monoclonal antibody, LM609, were found to inhibit adhesion-dependent signal transduction by angiogenic factors, leading to apoptosis of the activated endothelium. Consequently, these compounds block endothelial tube formation *in vitro* and angiogenesis during development, arthritis [114], and in growing tumors [115,116]. Based on these convincing data in different animal models, the clinical potential of integrin antagonists is currently being evaluated in phase I and II trials for patients with late-stage cancer. Vitaxin, the humanized form of the anti-$\alpha_v\beta_3$ antibody (LM609), has successfully completed Phase I clinical trials [112].

4.2. Inhibitors of activated endothelial cells

4.2.1. Endogenous inhibitors

Several natural inhibitors of angiogenesis have been detected (Table 3). Among them, TSP-1 is considered to be the main physiological inhibitor of angiogenesis, being constitutively produced by normal cells. Its expression is inversely correlated with angiogenesis, i.e. during tumorigenesis, TSP-1 is down-regulated while the angiogenic activity is increased. Accordingly, it was shown that TSP-1 production is regulated by the tumor suppressor p53 [117]. Mutation of *p53* results in the loss of TSP-1 production and a switch to the angiogenic phenotype [93,118]. Consequently, overexpression of TSP-1 causes a decrease in angiogenesis and inhibition of tumor growth [119].

However, the most promising tumor-shrinking anti-angiogenic drugs thus far are derived from an unlikely source: the tumor cells themselves. Angiostatin and endostatin are examples of endogenous inhibitors that are generated by the proteolysis of inactive circulating precursors. Angiostatin, which encompasses the first four disulfide-linked kringle domains of plasminogen [99], was originally purified from the serum and urine of mice bearing LLC. These mice did not suffer from metastases until the primary tumor was removed, which resulted in rapid growth of the previously dormant lung metastases. The mediator of angiostatin production in LLC was identified as a tumor-infiltrating macrophage, expressing metalloelastase [120]. However, tumor cells themselves have also been shown to produce proteolytic activity that generates angiostatin from plasminogen, and this enzymatic activity was found to differ from that

released by tumor-infiltrating macrophages [121]. *In vivo* experiments in mice have shown that angiostatin suppresses the growth of a number of human tumors and their metastases [122]. Immunohistochemical analysis revealed that the rate of tumor cell proliferation was identical in growing and dormant metastases. However, the apoptotic rate was 3-fold higher in the dormant metastases. Thus, tumor dormancy may depend upon a balance between tumor cell growth and death [123]. A recent report showed that ATP synthase binds angiostatin, implying that angiostatin interferes with ATP production, resulting in the inhibition of endothelial cell growth [124]. Finally, several data suggest that different kringle domains may contribute to the overall anti-angiogenic function of angiostatin by their distinct anti-migratory and anti-proliferative activities [125,126].

Endostatin, a carboxy-terminal fragment of collagen XVIII, derived through elastase-mediated cleavage [127], was isolated from the conditioned media of hemangioendothelioma (EOMA) cells [128]. Endostatin specifically suppresses endothelial cell proliferation *in vitro* and increases the apoptotic rate in tumors 7-fold without affecting the proliferation rate of the tumor cells. *In vivo*, endostatin showed potent inhibitory activity against EOMA, Lewis lung, T241 fibrosarcoma, and B16F10 tumor cell lines. Interestingly, endostatin does not seem to induce drug resistance [129]. Moreover, repeated cycles of systemic endostatin administration in tumor-bearing mice caused sustained tumor dormancy in the absence of further treatment [129]. Its anti-tumor activity is now being evaluated in phase I trials for a variety of solid tumors. Recently, a third fragment with potent anti-angiogenic activity was purified from small cell lung cancer. aaAT (anti-angiogenic antithrombin) results from the cleavage of antithrombin by a yet unidentified enzyme [130].

4.2.2. Synthetic compounds

TNP-470 (AGM-1470), a synthetic derivative of the antibiotic fumagillin, is perhaps the most studied inhibitor of angiogenesis [131] (Fig. 2). However, its molecular target has been identified only recently. TNP-470 has been shown to bind, and subsequently inhibit, type 2 methionine aminopeptidase [132], resulting in the abrogation of amino-terminal processing of methionine, which may lead to the inactivation of as yet unidentified proteins essential for endothelial cell growth [133]. TNP-470 inhibits endothelial cell proliferation and migration *in vitro* [134,135]. In animal models, TNP-470 is effective in the treatment of a wide variety of tumors and their metastases [136–139]. Its anti-tumor activity together with its moderate side-effects has led to phase II-III clinical trials for a variety of solid tumors and phase I trials for lymphomas and acute leukemias [112].

Thalidomide (Fig. 2) is a well-known teratogen with anti-inflammatory and anti-angiogenic activity [140]. The exact mechanism of the drug is not yet known. Thalidomide was inactive in the CAM assay, but showed potent inhibitory activity upon oral administration in the FGF-2-induced

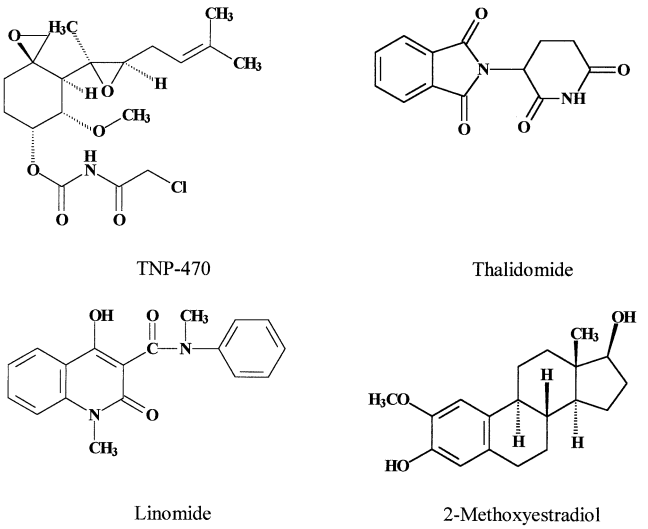

Fig. 2. Structures of inhibitors of activated endothelial cells.

cornea assay, which may reflect the need for metabolic activation in the liver [141]. The anti-tumor efficacy of thalidomide has been demonstrated in a large study comprising 84 patients with multiple myeloma. An oral dose of 200 mg thalidomide/day induced marked and durable responses in some patients, including those who relapsed after high-dose chemotherapy [142].

The tubulin-binding drug Combretastatin A-4 exhibits a selective toxicity for proliferating endothelial cells *in vitro* by induction of apoptosis [143]. *In vivo*, systemic drug administration causes vascular shutdown within experimental and human cancer models at doses that are 10% of the maximum tolerated dose. Histologically, the reduction in blood flow is associated with extensive necrosis of the tumor [144,145]. These actions against tumor vasculature and the broad therapeutic window demonstrate the clinical potential of this drug.

4.3. Compounds that interfere with angiogenic growth factors or their receptors

Of the long list of growth factors involved in the angiogenic process, VEGF and FGF-2 are considered the most important mediators of tumor angiogenesis. Consequently, different strategies have been developed to inhibit the production or release of these growth factors or to interfere with their receptor interactions. Specific targeting of VEGF using anti-VEGF antibodies, soluble VEGF receptors, or dominant negative Flk-1 [146–148] decreased the vessel density and reduced the growth rate of several tumors in animal models. Anti-VEGF antibodies and soluble Flk-1 and Flt-1 receptors have also proven successful in the treatment of, respectively, ischemia-associated iris neovascularization in primates and retinal neovascularization in a murine model for ischemic retinopathy [44]. Accordingly, a humanized anti-VEGF antibody that has completed phase I

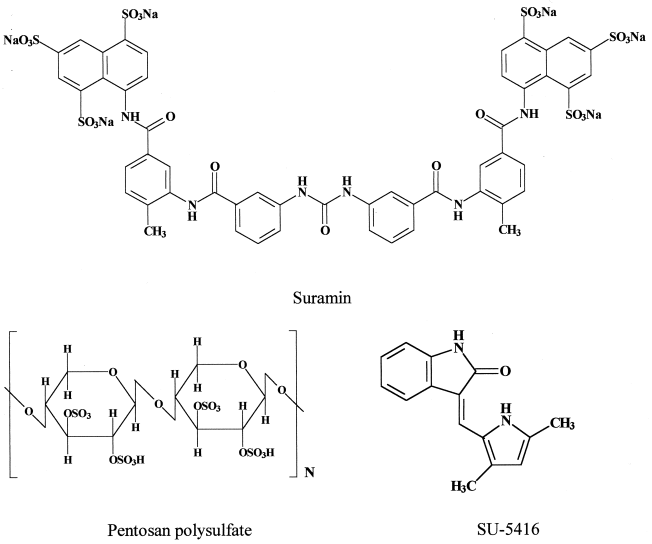

Fig. 3. Structures of growth factor antagonists.

trials without significant systemic toxicity is now being tested in phase II studies involving patients with metastatic renal cell cancer [112]. Similarly, a soluble recombinant extracellular domain of the Tie-2 receptor has been constructed that substantially impaired angiogenesis, tumor growth, and metastases [149].

To interfere with receptor signaling, synthetic low molecular weight inhibitors of tyrosine kinase receptors have been designed. The first receptor antagonist to enter clinical trials was SU5416, which selectively blocks VEGF-induced phosphorylation of Flk-1. SU5416 displayed potent antitumor activity in animal models and was found to induce endothelial cell apoptosis [150,151]. The efficacy of SU5416 is currently being evaluated in phase II trials for Kaposi's sarcoma, metastatic colorectal cancer, and VHL disease [112]. However, tumor cells may produce several cytokines and, due to their instability, they may switch from the production of one cytokine to another. Therefore, inhibition of one single growth factor may cause only partial control of tumor growth. This hypothesis has lead to the development of SU6668, a potent inhibitor of VEGF, FGF-2, and PDGF tyrosine kinase receptors [151], which has recently entered a phase I study for advanced tumors [112].

A number of drugs originally developed for their capacity to inhibit FGF-2-induced angiogenesis have been shown to interfere with the biological activities of several other heparin-binding growth factors [152]. These molecules may either mimic or bind heparin. Since the availability and biological activity of FGF-2 on endothelial cells strictly depend on the extracellular heparin concentration, the angiogenic activity of FGF-2 might be modulated *in vivo* by using exogenous heparin analogues [62]. These include among others suramin [153] (Fig. 3), pentosan polysulfate (PPS, Fig. 3) [154], polysulfonates [155], and carboxylated compounds [156]. Suramin [157] and PPS have been evaluated in patients with various tumors, including Kaposi's sarcoma [158], but the general observation is that high doses of these compounds are required to show activity and that their efficacy is limited by anti-coagulant side-effects. However, the introduction of minor structural changes to suramin was found to result in significantly less toxicity without loss of activity [159].

Promising results have also been obtained with IFN-α in the treatment of juvenile, life-threatening hemangiomas [160]. IFN-α was shown to down-regulate the expression of FGF-2 [161], which is abundantly present in hemangioma lesions and in the urine of patients suffering from proliferating hemangiomas [162].

4.4. Miscellaneous

Other possible targets for anti-angiogenic therapy include enzymes with important angiogenic properties such as PD-ECGF/TP and COX. PD-ECGF was found to stimulate endothelial cell migration *in vitro* and angiogenesis *in vivo.* Its angiogenic effect is mediated by the release of 2-deoxy-D-ribose, as a result of the reversible phosphorolysis of pyrimidine (deoxy)nucleosides by TP, to 2-deoxyribose 1-phosphate and their respective bases. 2-Deoxyribose 1-phosphate is rapidly dephosphorylated and transported out of the cell. However, the mechanism by which this molecule induces angiogenesis has not yet been elucidated [26]. TP is overexpressed in many solid tumors, including breast [163], ovarian [164], colorectal [165], and pancreatic cancers [166], and in the endometrium during the menstrual cycle [167], pointing to a role for this enzyme in both physiological and tumor vascularization. To date very few inhibitors of TP have been described, 6-aminothymine (6AT), 6-amino-5-bromouracil (6A5BU), and 7-deazaxanthine (7-DX) [168] being the most potent.

Recent reports have implicated COX-2, an enzyme that controls several cellular processes involved in colon cancer development, in the regulation of tumor angiogenesis [169]. COX-1, which is constitutively expressed and required to maintain the integrity of gut and kidney, and COX-2, which is inducible, mediate the conversion of arachidonic acid (AA) into prostaglandin G_2, which is subsequently converted to prostaglandin H_2, and eventually into a number of other prostaglandins and thromboxane A_2 [170]. Using a coculture of endothelial cells and colon cancer cells, COX-2 was shown to stimulate colon cancer cells to release angiogenic prostaglandins, which induce migration and tube formation of endothelial cells [169]. This was blocked by traditional non-steroid anti-inflammatory drugs (NSAID) like aspirin, which inhibit COX-1 and COX-2, and NS-398, a selective COX-2 inhibitor [171]. However, addition of exogenous prostaglandins could only partially reverse the inhibitory effect of these drugs, indicating that COX-2 may also exert additional prostaglandin-independent effects on angiogenesis [171]. In this context it should be noted that

overexpression of COX-2 in intestinal epithelial cell lines results in resistance to butyrate-induced apoptosis and down-regulation of the adhesion molecule E-cadherin [172]. Also, the COX-2 product thromboxane A_2 was identified as a mediator of COX-2-dependent endothelial cell migration and angiogenesis [170]. Since inhibitors of COX-1 are associated with gastrointestinal and renal toxicity, further studies should focus on specific COX-2 inhibitors for the treatment of malignant colon cancer.

Finally, experimental evidence suggests that a combination of anti-angiogenic drugs with different mechanisms of action may lead to synergistic anti-angiogenic effects. In addition, angiosuppressive therapy has been shown to increase the efficacy of classical chemotherapeutic agents in anticancer treatment [83]. Furthermore, exposure to angiostatin potentiated the antitumor effect of ionizing radiation [173]. Interestingly, radiation was found to induce VEGF, resulting in the protection of tumor blood vessels from radiation-mediated cytotoxicity and, hence, tumor radioresistance. Consequently, treatment of tumor-bearing mice with a neutralizing antibody to VEGF prior to irradiation was associated with a synergistic antitumor effect [174].

4.5. Vascular targeting

Vascular targeting aims at inhibiting tumor growth by destruction of the tumor vasculature. The main problem so far has been the lack of specific markers for activated, i.e. tumor, endothelium. Potential target molecules include the $\alpha_v\beta_3$ integrin, E-selectin, and VEGF and Tie receptors. Destruction of the tumor vessels may be achieved by the local delivery of peptides or antibodies with direct biological activity or conjugated to toxins. Accordingly, VEGF chemically linked to a truncated diphtheria toxin molecule (DT385) was found to specifically inhibit the proliferation of Flk-1 positive endothelial cells *in vitro* and angiogenesis *in vivo* [175]. Recently, an *in vivo* selection of phage display libraries was used to identify peptides that are present exclusively on blood vessels of specific organs [176]. This method was then applied to target tumor blood vessels. Therefore, phage peptide libraries were injected into the circulation of nude mice bearing human breast carcinoma xenografts. Recovery of phages from the tumor led to the identification of three main peptide motifs that target the phage to the tumors. When coupled to the cytotoxic drug doxorubicin, these peptides enhanced the efficacy of the drug against the mammary carcinomas in nude mice and reduced its toxicity [177].

Modulation of angiogenesis can be accomplished by the administration of single or multiple doses of angio-regulatory peptides or drugs or by means of gene therapy. Gene therapy offers a potential way to achieve sustained therapeutic release of active substances. For example, adenoviral and retroviral vectors that transduce the cDNA encoding angiostatin [178] or PF-4 [179] or antisense VEGF [180] have been used to inhibit endothelial cell growth *in vitro* and angiogenesis and tumor growth *in vivo*. Exact targeting can be attained by the use of endothelial specific (i.e. E-selectin, VEGF, or Tie) promoters.

5. Concluding remarks and perspectives

Currently, a large variety of chemotherapeutic drugs are being used to treat cancer. Unfortunately, many compounds hold limited efficacy, due to problems of delivery and penetration and a moderate degree of selectivity for the tumor cells, thereby causing severe damage to healthy tissues. However, the activity of these compounds is mainly restricted by the development of drug resistance. Tumor cells are a rapidly changing target because of their genetic instability, heterogeneity, and high rate of mutation, leading to selection and overgrowth of a drug-resistant tumor cell population [83,181].

Anti-angiogenic therapy, which targets activated endothelial cells, offers several advantages over therapy directed against tumor cells. First, endothelial cells are a genetically stable, diploid, and homogenous target, and spontaneous mutations rarely occur. Also, turnover of tumor endothelial cells may be 50 times higher than that of endothelium in normal quiescent tissues, and activated blood vessels express specific markers, like integrin $\alpha_v\beta_3$, E-selectin, Tie, and VEGF receptors. Because anti-angiogenic therapy is directed at activated endothelial cells, its target should be easily accessible by systemic administration. Finally, different tumor cells are sustained by a single capillary, and tumor-associated endothelial cells contribute to both endothelial and tumor cell growth by releasing autocrine and paracrine factors. Consequently, the activated endothelium presents a more specific target than the tumor cells, and inhibition of a small number of tumor vessels may affect the growth of many tumor cells [181].

The genomic instability and heterogeneity of tumor cells may also explain the clinical observations that the outcome of patients, with tumors in the same pathological or clinical stage, and their response to anticancer therapy vary considerably. This points to the importance of establishing an angiogenic profile in patients with cancer and other chronic angiogenic diseases. Indeed, the intratumoral blood vessel density (IVD) was found to be of prognostic value in a variety of solid tumors, including invasive breast [182], lung [183], malignant melanoma, gastrointestinal [184,185], and genitourinary cancers [84]. In these tumors a positive correlation was found between tumor angiogenesis and the risk of metastasis, tumor recurrence, or death. Furthermore, identification of the angiogenic cytokines or enzymes involved might make it possible in the future to specifically adjust anti-angiogenic therapy to the individual needs of a patient. In this context it should be noted that major progress has been made in the quantitative assessment of measurable parameters directly associated with angiogenesis. These in-

clude histological quantification of IVD, using specific markers for endothelial cells, like vWF, CD-31 (platelet-derived endothelial cell adhesion molecule/PECAM), and CD-34 and the measurement of blood flow *in vivo* by using color doppler or magnetic nuclear resonance [83]. Other possibilities comprise the quantification of angiogenic factors in serum, urine, or tissue extracts. To date, most studies have focused on measuring a single positive regulator like FGF-2 or VEGF. Elevated levels of both growth factors have been detected in the sera [186] and urine [68] of patients with a wide spectrum of tumors, and cytokine levels have been reported to serve as prognostic indicators [187]. Also, it will be worthwhile in the future to consider endogenous inhibitors because their loss may induce a switch to the angiogenic phenotype and, subsequently, disease progression.

Thus far, chemotherapeutic drugs are being used at high doses to kill cancer cells. This implies that infrequent dosing schedules are necessary to allow recovery from toxicity. However, these drugs are not specific, in that they also inhibit the proliferation of various other cell types, including intratumoral blood vessels. Therefore, it is hypothesized that long-term, regular administration of chemotherapeutics at low doses might result in the inhibition of endothelial cell proliferation, angiogenesis, and subsequent tumor growth. Finally, despite recent advances in angiogenesis research, many questions remain unanswered, whereas others emerge and it is becoming clear that a lot of work still needs to be done.

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