

REVIEW

Angiogenesis-Dependent Diseases and Angiogenesis Therapy

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The discovery of the molecular mechanisms of physiological vasculogenesis and pathological angiogenesis helped to recognize two classes of diseases: one where the therapeutic angiogenesis can repair the tissue damages (arteriosclerosis, myocardial infarction, limb ischemia) and the other one where inhibition of pathological angiogenesis can cure the disease or delay its progression (retinopathies, benign and malignant angiogenic tumors, progression of

malignant tumors). Although there are an exponentially growing number of new synthetic molecules characterized mainly by antiangiogenic properties, the discovery of a large battery of natural pro- and anti-angiogenic factors suggests that this may provide a more physiological approach to treat both class of angiogenesis-dependent diseases in the near future. (Pathology Oncology Research Vol 7, No 2, 85–94, 2001)

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Introduction

In the global database of PubMed/Medline, nearly 10.000 articles can be found using the keyword search "angiogenesis". In 2000 around 500 new items were introduced into the PubMed/Medline, while in the first 5 month of 2001 the number of new items reached 400 indicating an exponential growth of articles on this subject. Therefore one can declare angiogenesis as one of the hottest area of biomedical research nowadays. Due to this enormous increase of data on this topic our knowledge of the molecular biology and pathology of angiogenesis increased considerably. We do not address the molecular biology of angiogenesis in physiologic conditions here, which can be found in several excellent millennium-reviews elsewhere. Rather, this paper intends to summarize our current knowledge of angiogenesis-dependent diseases and to demonstrate, that a new family of therapeutics, called angiogenesis-agents, are already available to treat – in most cases as yet experimentally – these diseases.

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Angiogenesis-dependent diseases

Genetic disorders

VON HIPPEL-LINDAU DISEASE (VHL). In a rare, autosomal recessive genetic defect, multiple hemangiomas develop in the cerebellum, brain stem and the retina, called von Hippel-Lindau disease (VHL). This highly vascularized tumor is composed of dilated capillaries frequently forming cavernous vessels, where the endothelial cells are not fully matured and rarely produce vWF. The genetic disorder is a mutation of the VHL gene which produces pVHL which binds to HIF1 α . The role of pVHL is to bind HIF1 α , and promote ubiquitination and degradation. However, the mutated pVHL unable to bind HIF1 α , which accordingly is accumulated, resulting in the transcriptional activation of genes carrying HRE elements such as VEGF, VEGFR, EPO etc. As a result hemangiomas develop in the brain of these patients. Interestingly, the serum level of EPO is also elevated. Due to its function VHL gene is now considered as tumor suppressor gene for endothelial tumors.⁴

HEREDITARY HEMORRHAGIC TELEANGIECTASIA (HHT). This is an autosomal dominant genetic disorder (Osler-Weber-Rendu disease) resulting in vessel proliferation but more specifically arterio-venous dilatations in various organs and tissues. It is the disease of the vessel enlarge-

ment phase. The gene responsible for those alterations is endoglin, a signaling element of the TGF β receptor system. TGF β signals through its receptor, TGFRI and II. Type II phosphorylates Type I upon binding TGF β , activin of BMP followed by intracytoplasmic phosphorylation of SMADs. Two receptors cooperate with TGFs, betaglycan and endoglin providing the appropriate conformation of TGF β for the signaling receptor (HHT1). Furthermore, it is also suggested that endoglin cooperates with the VEGFRs in their signaling. ALK-1 is an accessory molecule for both TGF/TGFR-II as well as activin/activin II receptors and is preferentially expressed by vascular endothelium. Missense, and nonsense mutations, deletions, insertions in ALK-1, another TGF β receptor is responsible for the HHT2.¹⁸

REMODELING DISORDERS. The large thoracic vessels undergo considerable remodeling during development. Genetic analysis has shown that loss of MFH-1, dHand or Msx1, Pax-3, Prx1, retinoid acid receptors, the neurofibromatosis type-1 gene product, Wnt-1, connexin-43 or endothelin-1 induce aortic arch malformations. Prostaglandins mediate closure of the neonatal ductus arteriosus. Signals involved in neuronal patterning also seem to be involved in vascular patterning. In the avian heart, there is a close spatial juxtaposition between coronary arteries and Purkinje cells of the myocardial conduction system. Endothelin-1, locally generated in the coronary artery, is an instructive cue for the differentiation of cardiomyocytes into Purkinje cells. Loss of semaphorin-3C or of neurophilin-1, a receptor for neurorepulsive semaphorins, induces abnormal patterning of the large thoracic vessels. Arterial rarification also occurs during pulmonary or systemic hypertension. An imbalance between endothelin-1 and nitric oxide initially induces vasospasms, but when sustained, this progresses to irreversible vascular loss. Loss of PLGF or u-PA protects against pulmonary vascular remodeling.⁴³

Arteriosclerosis

The deficiency of functional blood vessels in cardio- and cerebrovascular syndromes contributes to a variety of ischemic symptoms, including angina, intermittent claudication and loss of mental function in transient ischemic attacks. Poor perfusion in diabetes is also the leading cause of diabetic limb amputation.

A pathological type of arteriogenesis is the 20-fold enlargement of preexisting collateral arterioles after occlusion of a supply artery. As a result of the increased collateral flow, endothelial cells express monokines (monocyte chemoattractant protein-1) and monocyte adhesion molecules (such as intracellular adhesion molecule-1). The recruited monocytes infiltrate the vessel wall and destroy the media,

using proteinases and death factors (TNF- α). Activated endothelial cells then upregulate bFGF, PDGF-B and TGF β -1, thereby inducing the re-growth of smooth muscle cells and vessel enlargement.¹⁴

INVOLVEMENT OF HYPOXIA AND NUTRIENTS. Hypoxia-inducible transcription factors (HIF1 β , HIF1 α and HIF2 α) trigger a coordinated response of angiogenesis and arteriogenesis by inducing expression of VEGF, VEGFR1, VEGFR2, neurophilin-1, Ang2, nitric oxide synthase, TGF β -1, PDGF-BB, endothelin-1, interleukin-8, IGF-II, TIE1, cyclooxygenase-2 and so on. The von Hippel-Lindau tumor suppressor gene product inhibits the expression of hypoxia-inducible target genes during normoxia by promoting HIF1 α degradation. Gene inactivation studies have shown that angiogenesis, not vasculogenesis, is regulated by hypoxia. Tumors lacking HIF1 β or HIF1 α fail to develop vascularization and lack hypoxic induction of VEGF expression, whereas stabilization of HIF1 α by peptide regulator-39 induces angiogenesis in the severely hypoxic myocardium. Hypoxia-inducible factors and hypoxia-response elements are now being tested for angiogenic (gene) therapy of tissue ischemia. Metabolic stimuli, including hypoglycemia and low pH, also stimulate vessel growth, but their mechanisms remain to be determined.³⁷

INVOLVEMENT OF MECHANICAL FACTORS. Vasculogenesis occurs mostly independently, whereas angiogenesis coincides with its onset of and is influenced considerably by flow. As a result of the higher blood pressure in the capillaries proximal to the aorta, coronary arteries become covered by smooth muscle cells at earlier times than do veins. Remodeling of the developing thoracic arteries or of collateral vessels after arterial occlusion also depends on flow. Gene inactivation studies have shown that shear-stress-induced vascular remodeling is affected by nitric oxide and P-selectin, that the response of resistance arteries to flow is determined by vimentin, and that vascular tone is affected by bFGF. Mechanical forces vascular function through shear-stress-responsive gene transcription.⁶

Retinopathy

The retina of the eye is supported by retinal and chorioidal vessels. Cornea and vitrous fluid contain a unique angiogenesis inhibitor, PEDGF, which is considered to be responsible for the angiogenic inactivity of the adult retina. Damage to these vessels result in ischemic lesions of the retina manifested in retinopathies of the newborn, diabetic retinopathies and retinal venous occlusions. Neoangiogenesis in the retina is initiated by the ischemic injury or hyperglycaemia induced PKC activation. All of these factors lead to the initiation of VEGF expression in retinal pigment epithelium. The developing new vessels

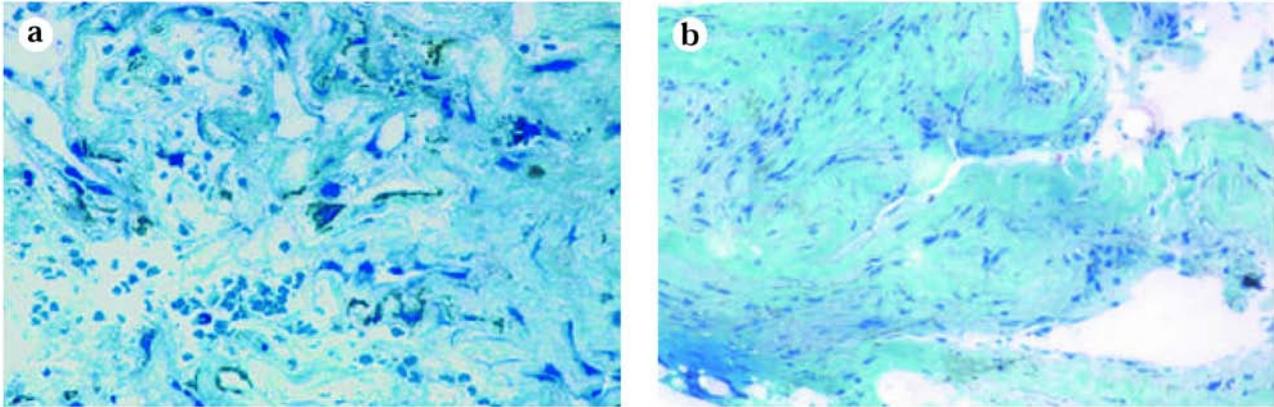


Figure 1. Morphology of pre-retinal membrane in diabetic retinopathy. (a) Early, proliferative vascular patterns are characterized by new vessels (V) as well as mesenchymal matrix. (b) Late phase of retinopathy characterized by massive accumulation of connective tissue stroma. Epon embedding, semithin sectioning, toluidine blue staining.

cross the entire retina and start to grow on the vitreous interface (*Figure 1a*). Cytokines produced initially by the injured pigment epithel followed by the activated endothelial cells attract inflammatory cells and mesenchymal ones which would destroy existing anatomic borders as well as produce a new pathologic extracellular matrix (*Figure 1b*). These alterations ultimately will lead to the detachment of the retina and blindness. In diabetic retinopathies the moderate expression of VEGF primarily induces increased permeability and only in a later stage initiates endothelial proliferation. Glial cells in the hypoxic retina not only produce VEGF, but also IL-8 and HGF as well and pericytes start to express bFGF all responsible for the neoangiogenesis.²¹

AIDS/Kaposi sarcoma

It was recognized early on the AIDS epidemic, that the chronic infection with HIV results in unique vascular disorders and later development of vascular tumors i.e. Kaposi sarcoma. However, the pathomechanism of this complication of HIV infection was not known for a long time. HIV-TAT is the transactivating protein of the AIDS virus responsible for the viral transcription. Meanwhile this protein has several biological activities based on the multimodular nature of the protein: RGD, heparin-binding domain and chemokine-like sequences. Therefore, TAT is able to induce proliferation and migration of endothelial as well as transformed endothelial cells (Kaposi), induces uPA expression but only in concert with other inflammatory cytokines such as IL-1, TNF α , IFN γ .¹ Interestingly, heparin and heparin-like carbohydrates enhance the angiogenic potential of TAT. Accordingly, one can consider TAT as a viral heparin-binding growth factor which is further supported by the observation, that TAT binds the VEGFR, KDR. Meanwhile TAT is not a viral oncogene,

since it is unable to transform endothelial cells: this is achieved by the transforming effect of HSV8 virus in Kaposi sarcoma.⁵

Cancer

Vascularization is the hallmark of malignant tumors without which solid tissue cannot grow beyond 1-2 mm². One, of the most studied and documented form of vascularization is tumor-induced neoangiogenesis, however there are clearly other options available for the growing tumor such as vessel cooption or recapitulation of an embryonic genotype: vascular mimicry. These options are based on the tumor-type and the genetic changes concurred during carcinogenesis.

TUMOR-INDUCED NEOANGIOGENESIS (FIGURE 2A). The genetic background of the “angiogenic switch”¹³ during tumor progression is not fully understood, but recent discoveries of the main angiogenic factors, VEGF, bFGF, PDGF, suggest that the switch is able to turn on the expression of the genes of these factors in tumors. Later studies identified several anti-angiogenic factors as well, suggesting that the “angiogenic switch” might also control the expression of these factors. Tumor-induced neoangiogenesis therefore means the predominance of pro-angiogenic- over anti-angiogenic machineries in cancer. It is now accepted that cancer cells may use physiological pathways to turn on the pro-angiogenic genotype.^{10,11} In this case hypoxic tumor cells in the growing tumor tissue, which express wt-HIF1 gene, activate the expression of HRE-containing angiogenic factor genes including the main angiogenic mitogen, VEGF. Amplification of several oncogenes in cancers also leads to the overexpression of pro-angiogenic factors. It is a characteristic genetic alteration in a considerable proportion of human cancers that wtP53 expression is lost. An angiogenic

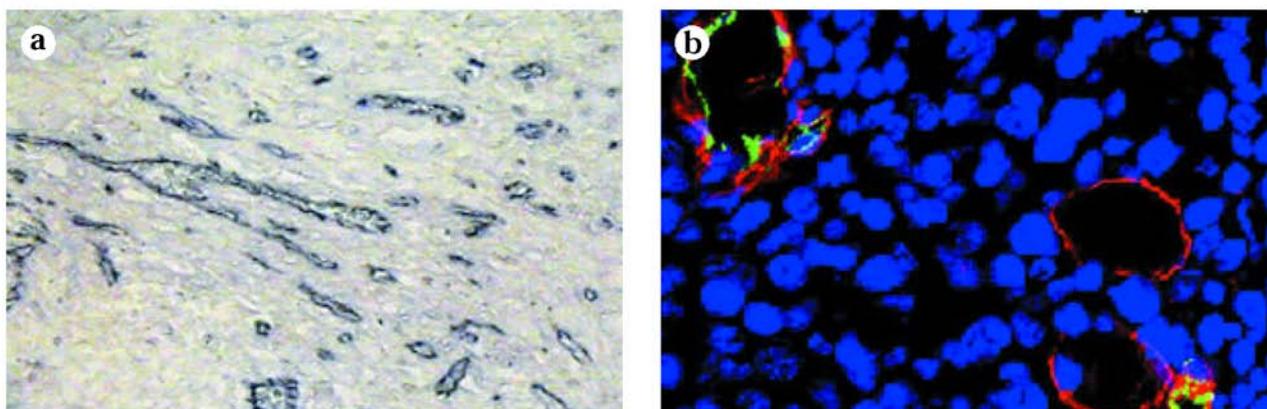


Figure 2. Immunohistochemistry of tumor vasculature. (a) Tumor-induced neoangiogenesis in glottic cancer. Proliferating blood vessels are labelled with CD31/PECAM marker. Paraffin embedding, antigen retrieval, CD31 labeling, peroxidase/DAB reaction (brown). (b) Intratumoral coopted vessels (V) and vascular channels (C) in experimental melanoma. Note laminin-positive, CD31-negative sinuses. CD31: green, laminin: red, nuclei: blue. Frozen section, triple-labeling immunofluorescence, confocal microscopy.

consequence of this fact is that such cells would not be able to express thrombospondin, one of the most significant physiological angiogenesis inhibitors. The result of these genetic alterations is the imbalance in the gene expression of pro- and anti-angiogenic factors facilitating the development of the angiogenic switch in cancer.²⁰ The first step of tumor-induced angiogenesis is the alteration of the basement membrane over the entire circumference of venules near the tumor tissue, characterized by loss of electron density and called as gel-sol transition, probably mediated by matrix metalloproteinases or plasminogen activators, secreted by the tumor cells. This alteration must be partly responsible for the initiation of endothelial cell division and migration. On the other hand, angiogenesis factors such as bFGF or VEGF, produced by the tumor cells or host cells, can be liberated from the endothelial basement membrane during this process. Loosening of interendothelial cellular contacts does not occur, suggesting that loss of contact inhibition is not responsible for initiation of cell division and migration. Only the tips of the emigrating endothelial cells are free of basement membrane. During migration, endothelial cells are arranged parallel, maintaining their polarity (basal-luminal), and consequently a slit-like lumen is formed between the endothelial cells. This lumen is continuous with the lumen of the original vessel and is sealed by intact inter-endothelial junctions. According to this model no extra stimulus is necessary to induce lumen formation and the retained polarity of endothelial cells allows the continuous deposition of the basement membrane. Numerous studies suggest that the growth of the new capillary sprouts is not oriented towards the tumor, instead the process yields a high density anastomosing network of capillaries at the periphery of tumor cell islands, a process leading to the observed phenomenon that vessel density is much higher around the tumor than inside. A possible explanation is that the continuously growing

tumor island incorporates the vessels at the periphery of tumor nests, thereby thinning out the network, whereas the network continuously develops at the advancing tumor-connective tissue interface. The intussusceptive-type growth of vessels yields a high number of large caliber vessels situated outside the area of the active capillary growth – it probably does not contribute to the nutrition of the tumor significantly, but rather provides more sites for sprouting.³²

VASCULAR MIMICRY. Recently, a new form of tumor vascularization was suggested based on the findings that tumor cells themselves are able to form channels in vitro and the existence of such tumor sinuses was suggested in vivo in various human cancers.²⁶ Furthermore, the genetic background of such channel formation was also discovered: tumor cells can ectopically express endothelial-specific genes such as TIE1, uPA, HGF/c-met, thereby recapitulating an embryonic geno- and phenotype. This mechanism suggests that tumor cells can form channels, which are connected accidentally to the existing intratumoral microvessels in an undiscovered manner. However, there are other theoretical pathways, which may lead to the formation of such sinuses such as programmed cell death of the incorporated normal vessels (*Figure 2b*)¹⁶ or the active infiltration of intratumoral vessels by tumor cells resulting in the so-called mosaic-vessels, where endothelial cells and tumor cells both contribute to lumen formation.⁷

VESSEL COOPTION. It is an alternative mechanism for the blood supply of malignant tumors when the growing tumor tissue incorporates the preexisting host vessels, called “cooption”.¹⁵ It was demonstrated that such tumors produce pro- and anti-angiogenic factors, the balance of which fundamentally influencing both the angiogenic process as well as the remodeling of the coopted vascula-

Table 1. Physiological pro-angiogenic agents ^{5,11,20,23,25,40}

<i>Growth factors</i>	<i>Cytokines</i>	<i>Chemokine</i>	<i>Hormones</i>	<i>Bioactive lipids</i>	<i>Matrix proteins</i>
ANG-1	IL-1b	PBSF/SDF1	androgen	PAF	CYR61, CTGF
EGF	IL-6		estrogen	PGE1,2	Fibrin
FGF1-9	IL-8		leptin	TXA2 ²⁸	Thrombin
G-CSF	TNF α			12-HETE ²⁹	
HGF					
IGF-1,2					
PD-ECGF (thymidine phosphorylase)					
PDGF					
PLGF-152/131					
VEGF165					
VEGF121					
VEGF189					
VEGF206					

Footnotes: EGF: epidermal growth factor, FGF: fibroblast growth factor, G-CSF: granulocyte-colony-stimulating factor, HGF: hepatocyte growth factor, IGF: insulin-like growth factor, PAF: platelet-activating factor, PDGF: platelet-derived growth factor, PD-ECGF: platelet derived endothelial growth factor, PGE1,2: prostaglandin E1,2, PLGF: placental growth factor, TXA2: thromboxane A2, VEGF: vascular endothelial growth factor

ture. It was observed that the predominance of anti-angiogenic factors (such as Ang-2) in the center of the tumors results in apoptotic death and degeneration of endothelial cells of the incorporated vessels¹⁹ which can lead to the formation of sinuses lined by tumor cells and still containing red blood cells. These channels theoretically might maintain their connections to the “surviving” intratumoral microvessels by the surviving subendothelial matrix network. If such a connection exists, tumor cells lining such sinuses can get an easy access to the microcirculation (the primary event of the hematogenous spread) without the struggle of the complex process of intravasation.⁴¹

Angiogenesis therapy

Therapeutic angiogenesis

Therapeutic angiogenesis can be defined as the use of biological agents, bioactive materials or environmental conditions to stimulate growth of new vessels, to restore or augment circulatory perfusion of tissues, to reverse ischemia or to accelerate healing. This approach is now used clinically in cases of heart- or cerebrovascular diseases, in critical limb ischemia, delayed wound healing and in peptic ulcer disease. Despite of the existing various angiogenic mechanisms, most ischemic tissues are generally unable to produce appropriate response to reverse the disease process including heart, brain, limb, skin, although there are several natural pro-angiogenic factors available in various tissues (*Table 1*).

ANGIOGENIC GROWTH FACTORS. Among the several angiogenic growth factors several are now under clinical trials including the most potent and universal ones such

as VEGF, a/bFGF, PDGF and G-CSF. These studies employ human recombinant proteins as well as exploiting the gene technology for delivering naked DNA or using adenoviral vectors to transfect endothelial cells. Clinical application of G-CSF is based on the observation, that contrary to what was thought, the embryonic type of angiogenesis (mobilization of bone marrow endothelial stem cells to the site of neoangiogenesis) is possible in adults as well, since endothelial stem cells are continuously present, though in a low number, in the circulation.^{6,17}

BIOACTIVE SYNTHETIC MATERIALS. In case of major wounds and skin losses such as burning, biocompatible materials containing matrices and angiogenesis modulators can be used to promote the healing process. A wound dressing containing a synthetic copper-peptide which can accumulate FGF can promote healing. An alternative for this is the use of a tissue-engineered biological coat containing cells which produce large quantities of angiogenic factors such as FGF or VEGF.²²

LASER THERAPY. In case of heart ischemia transmural revascularization can be stimulated by a laser beam. Following photoacoustic injury, myocytes, inflammatory cells and platelets provide a rich source of angiogenic factors. This device is now designed for intraoperative use (bypass surgery) as well as a transcatheter device.²²

OXYGEN THERAPY. Hyperbaric oxygenation is primarily designed to treat surface wounds. Patients are placed into a chamber, where the oxygen pressure is gradually increased

Table 2. Physiological anti-angiogenic agents ^{5,11,20,23,25,40}

Growth factors	Cytokines	Proteases	Protease-inhibitors	Glycosidase
ANG-2	IFN- α , β , γ	cleaved AT-III	TIMP1,2,3	heparinase-I,III ³⁴
NK1,2,4 (HGF)*	PF-4	collagen XVIII	maspin ⁴⁵	
TGF β	PR-39	fragment (Endostatin)	PAI-1	
		HmwKallikrein-d5	PEDF	
		plasmin fragment		
		(Angiostatin)		
		prothrombin- F1-2		
		TSP-1		

Footnotes: * see Figure 3a,b.

HSPG: heparan sulphate proteoglycan, IFN: interferon, TGF: transforming growth factor, THR: thrombin receptor, TIMP: tissue inhibitor of metalloprotease TSP: thrombospondin, uPA: urokinase-type plasminogen activator, AT-III: antithrombin-III, Hmw: high molecular weight, PAI: plasminogen activator-inhibitor, PEDF: pigment epithelium derived factor, PF-4: platelet-factor 4, TGF β : transforming growth factor- β , TIMP: tissue inhibitor of metalloprotease

to a much higher level than the physiological. At 2.0ATA blood plasma begins to carry increased amount of oxygen providing a systemic oxygen burst. It is proven that high oxygen tension stimulates proliferation of endothelial cells and induces angiogenesis in ischemic tissues.²²

Pharmacological inhibition of angiogenesis

The recognition of the angiogenesis dependence of diseases such as malignant tumor growth and progression as well as has retinopathies stimulated translational research activity over the past decade. Data has now accumulated on the major molecular pathways controlling angiogenesis in adult and in these diseases. It became evident that successful interference with the pathological neoangiogenesis can be achieved in several ways, but may require combination of various antiangiogenic factors or drugs. The potential for successful interference is based on our growing understanding of natural angiogenesis-inhibitors (*Table 2, Figure 3a,b*) which provide biological examples for such new therapies.

CONVENTIONAL THERAPEUTIC AGENTS. It is an interesting development of the field, that conventional therapeutic agents designed to treat angiogenesis-related or -unrelated diseases have been found to have “side effects”, i.e. antiangiogenic potential. These include most anticancer drugs, but others are also becoming members of this “club”. This continuously growing battery of natural and synthetic antiangiogenic compounds provided new agents for clinical trials for the treatment of advanced cancer as well as some ocular diseases.³⁵ A selected list of such compounds are summarized below (*Table 3*).

ENDOTHELIAL GROWTH FACTOR LIGANDS (*TABLE 4*). Since research in the past years identified the major endothelial mitogenic growth factors (VEGF, FGF, PDGF, HGF), these ligands and their molecular interactions with their signaling or accessory receptors have become a major pharmacological target. The recognition of the common chemical nature of these endothelial growth factors i.e. their heparin-binding potential, provides an easy though

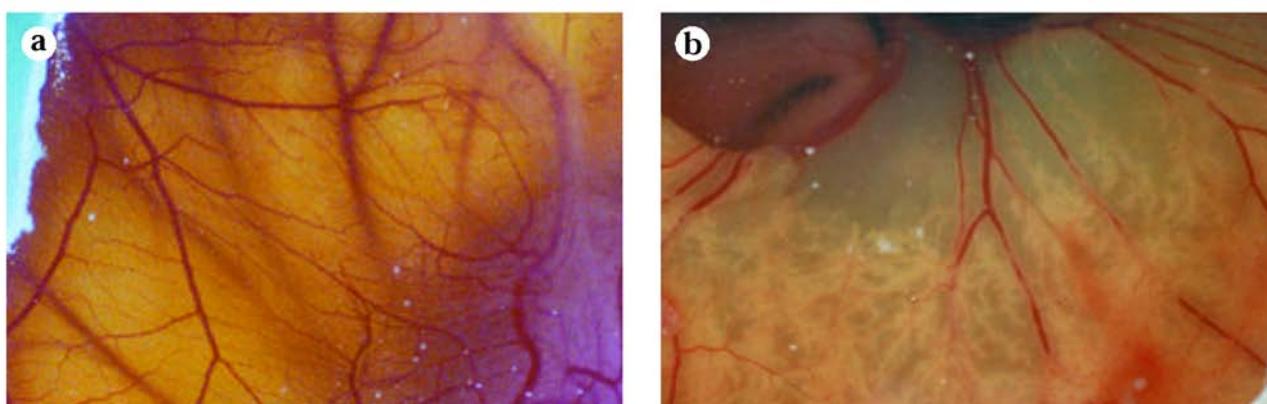


Figure 3. Inhibition of angiogenesis in chicken CAM assay by HGF peptide HHRGK₆₄₅₋₆₄₉. (a) Control 11 day old CAM. (b) HGF-peptide treated 11 day old CAM. (HHRGK treatment: 4 days, 100 μ g/ml). Note the significantly reduced arborization of blood vessels. CAM: chorioallantoic membrane

Table 3. Classical drugs with antiangiogenic potential

	Primary indication	Molecular target	Angiogenetic target
vinblastine	cancer	microtubules	endothel permeability
taxol	“	“	endothel proliferation
dolestatin	“	“	“
Combrestatin A	“	“	“
flavone acetic acid	“	cytokine expression	angiogenesis
low dose 5-FU	“	TS	endothel proliferation
low dose MTX	“	anti-folic acid	endothel proliferation
irsogladine	gastric ulcer	?	angiogenesis
radicicol	bacterial infection	Hsp90	angiogenesis
cyclosporine	immunosuppression	signal transduction	angiogenesis
captopril	hypertension	ACE	vascular development
celecoxib	inflammation	COX-2	endothel proliferation ¹²

Footnote: ACE: acetylcholine estherase, TS: thymidilate synthase

non-specific anti-angiogenic target. The earliest inhibitors were sulphated polysaccharides (D54152), cationic-peptides (protamine), or -molecules (Suramin). Identification of the peptide sequences of the major angiogenic mitogens facilitated the identification of heparin binding cationic peptides of VEGF or HGF. These usually small molecular inhibitors act as competitive antagonists for the heparin-binding ligand to prevent endothelial receptor activation. Although their concentration has to be much higher in vivo than of the physiological ligand, they can be safely administered since they are relatively non-toxic compounds. Meanwhile the most specific approach here is to target the major and the most specific endothelial mitogen, VEGF165. The recent discovery of the secondary and tertiary structure of VEGF165 provides new information to design ligands, monoclonal antibodies or peptidomimetics which can eliminate the effect of VEGF. The humanized anti-VEGF165 monoclonal antibodies now entered clinical trials following the success in various experimental angiogenesis models including tumor progression ones. An old clinically introduced cytokine, interferon (α/β) is now used clinically to down-regulate FGF expression both in endothelial as well as in stromal and tumor cells.^{2,20,25,35,36,40}

antibodies such as DC101 is able to interfere with the signaling of KDR similar to some anti-c-erb2 monoclonals. Although it is not a very promising single agent for anti-angiogenic therapy of tumors, combination with low dose conventional cytostatic agents (such as vinblastin) make it possible to reduce unwanted side effects of the cytostatic drug while maintaining the antitumoral effect. PF-4 is a natural inhibitor of angiogenesis targeting endothelial cells and the recombinant variant of it can now be used to inhibit ligation of the FGF-receptor.²⁰

ANGIOGENIC (MITOGENIC) SIGNALING (TABLE 5). Identification of the receptors of the major angiogenic mitogens revealed that the majority of these receptors fall into the category of tyrosine kinase growth factor receptors (flt1, KDR, c-met, c-kit etc). Accordingly, the tyrosine kinase activity of proliferating endothelial cells is a natural drug target. In the early phase of such investigations relatively non-specific kinase inhibitors, such as staurosporine, flavonoids, and genistein have been used successfully to cope with the signaling activity of endothelial cells. Later on, more specific TK-inhibitors became available which can specifically inhibit signaling of the KDR- (SU5416) or the PDGF-receptor (isoquinolines). These TK-inhibitors are highly specific

ENDOTHELIAL MITOGEN RECEPTORS. Recognition and identification of the major endothelial growth factors made it possible to identify their receptors as well (flt1, KDR). The VEGF165 receptor (KDR) is almost exclusively expressed in endothelial cells providing a highly specific endothelial target. Monoclonal antibodies are now available which recognise various epitopes on the extracellular domain of KDR. Some

Table 4. Angiogenesis inhibitors-mitogens

	Target	Clinical trial
D54152-sulphated polysaccharide	FGF	
cationic protein (Protamine)	heparin-binding factors	
cationic peptide-VEGF	VEGF	
Suramin		
(polysulphonated naphtyl urea)	FGF, VEGF, HGF	+
anti-VEGF antibody	VEGF165	
DistamycinA	bFGF, PDGF-B	
interferon α and β	cytokine expression	+
Angiozyme (ribozyme)	VEGF	+

Table 5. Angiogenesis inhibitors-mitogen signaling

Target	Clinical trial	
Isoflavonoids	Non-spec. PTK-inhibitors	+
Staurosporine derivatives	Non-spec. PTK-inhibitors	
Genistein	Non-spec. PTK-inhibitor	+
SU5416 (adenine-mimetic)	VEGFR2-PTK-inhibitor	+
tyrphostins	VEGFR2-PTK-inhibitor	
isoquinolines	PDGFR-PTK-inhibitor	+
retinoic acid	PKC-inhibitor	+
carboxyamidotriazole	Ca ²⁺ +channel-blocker	+
TNP-470	cell cycle control	+
octreotide	signaling	+

PTK= protein tyrosine kinase
PKC= protein kinase C

antiangiogenic agents which now entered clinical trials to treat advanced cancer. Some downstream steps of angiogenic signaling such as PKC-activation or Ca-signaling provide powerful though less specific anti-angiogenic targets (retinoic acid or other PKC inhibitors and carboxyamidotriazole, respectively). Since in cases of tumor progression, tumor cells as well as endothelial cells are both targets for therapeutic intervention due to the involvement of various mitogenic signaling pathways, the use of such broad spectrum TK inhibitors may provide a rational alternative for antitumor therapy.^{20,24,25,36}

The discovery of the expression of estrogen receptor- β in endothelial cells ultimately made it possible to interpret earlier data that sex hormones are angiogenic factors (Table 1)¹² However, it turned out that an estrogen-metabolite 2-methoxy-estradiol, has strong antiangiogenic activity both in vitro and in vivo³³ suggesting an endocrine form of antiangiogenic therapy in the future.

ENDOTHELIAL CELL-EXTRACELLULAR MATRIX INTERACTION (Table 6). Neoangiogenesis is characterized by changes in the endothelial cell-ECM interactions. Mitogenic stimuli introduced by the angiogenic factors (bFGF, VEGF, HGF PDGF) induce expression of new adhesion molecules such as $\alpha\beta3$ or $\alpha\beta5$ integrins, characteristic for the proliferating and migrating endothelial cells.⁹ On the other hand, the permeable vessel wall leaks soluble matrix ligands such as vitronectin, fibrinogen/fibrin or tenascin which would be key matrix components of

the remodeling vessel.³ Mitotically activated endothelial cells have to degrade, at least partially, their subendothelial basement membrane, therefore mitogens induce protease secretion as well (uPA and MMP-2, MMP-9). All of these events are critical for the initiation of angiogenesis and therefore can serve as drug targets.

Ligand competition for the $\alpha\beta3$ integrin is enough to immobilize and detach endothelial cells from their matrix, inducing their programmed cell death.⁹ This can be achieved in several ways: using cyclic RGD-peptides,⁸ chemical RGD-mimetics²⁷ or snake venom ligands (disintegrins with $\alpha\beta3$ specificities⁴⁴). However, the most powerful matrix ligands to prevent or inhibit endothelial cell proliferation, migration and neovascularization are degradation products of the perivascular matrix proteins, plasmin and collagen XVIII, angiostatin³⁰ and endostatin³¹ respectively. These proteins are highly effective in vitro and in vivo. They inhibit both the tumor-induced angiogenesis as well as other types of neoangiogenesis such as retinopathies.^{20,25,35} Their therapeutic window is extremely wide, and they can be administered for a very long period without significant resistance developing in endothelial cells. Since recombinant human forms are

Table 6. Angiogenesis inhibitors: endothelial cell-extracellular matrix interaction

Target	Clinical trial
Matrix ligand	
Rh-Angiostatin	uPAR, ATP-synthase +
Rh-Endostatin	collagen receptors +
cyclic-RGD peptide	$\alpha\beta3$
accutin-disintegrin	$\alpha\beta3$, $\alpha\beta5$
benzodiazepines	$\alpha\beta3$
Matrix receptor	
Humanized anti- $\alpha\beta3$ Ab	$\alpha\beta3$ +
Protease	
Rh-PAI-2	uPA
amiloride	"
p-amidobenzamidine	"
anti-uPA Ab	"
anti-uPAR Ab	uPAR
L-phenylalanin-N-methylamides (Batimastat, Marimastat)	MMP-2, MMP-9 +
AG3340	MMPs +
Minocycline	MMPs

Footnote: MMP: matrix metalloprotease, PAI: plasminogen activator-inhibitor, PAR: plasminogen activator receptor

now available, both of these agents entered clinical trials to treat advanced and/or primary cancer.

The expression of $\alpha\beta3$ integrin is a hallmark of proliferating endothelial cells, therefore this integrin itself provides a feasible drug target. Some anti- $\alpha\beta3$ monoclonal antibodies have been proved to be antiangiogenic, partially because the receptor-ligation induces apoptosis of the endothelial cells, partially because of the block of integrin signaling. Development of humanized anti- $\alpha\beta3$ monoclonal antibodies (Vitaxin–MedImmune, and RheoPro–Centocor) made it possible to use such agents clinically, similarly to the anti-erbB2 antibody treatment of advanced breast cancer.²⁵ The $\alpha\beta3$ integrin on the surface of endothelial cells not only serves as a matrix ligand but also provides a surface site for protease binding and accumulation (MMP-2 and uPA), critical in the initial step of endothelial cell release from the pre-existing vessel.³⁹ Therefore, the dramatic in vivo and clinical effects of anti- $\alpha\beta3$ antibody treatments on angiogenesis might be partially due to these effects.

Migration of endothelial cells is impossible without the secretion and function of the key matrix degrading proteases, MMP2 and uPA. Recognition of this fact made these enzymes also targets for therapeutical interventions. Interestingly, although there are several various forms of therapeutic uPA inhibitors now available, (chemical inhibitors, anti-uPA antibodies, or anti-uPA-receptor antibodies), none of them have yet entered clinical trials.²⁵ On the other hand, metalloprotease inhibitors are the first antiangiogenic agents entered into clinical trials.³⁵ There are now available both broad-spectrum MMP inhibitors as well as MMP-2 and MMP-9-specific ones, the which enzymes are used primarily by migrating endothelial cells and invading tumor cells. This dual target theory is most probably behind the competition between companies to develop more and more MMP-inhibitor variants.

One of the most interesting (and promising) antiangiogenic agents to date is *thalidomide*, which was introduced a couple of decades ago as sedative agent but was withdrawn due to its teratogenicity. Later on it turned out, that the teratogenic effect of thalidomide is due to its powerful angiogenesis inhibitory potential. Although it is still not completely understood, it seems that thalidomide inhibits expression of genes with SP1 promoter sites including α and $\beta3$ integrins, the key adhesion molecules of the proliferating and migrating endothelial cells. Recently thalidomide became the first successful antiangiogenic agent applied clinically to treat retinal neovascularization as well as a broad range of cancers including, prostate, breast, Kaposi sarcoma, glioma and melanoma.^{35,38}

Conclusions

The discovery of the molecular mechanism of physiological vasculogenesis and pathological angiogenesis helped to

recognize two class of diseases: one where therapeutic angiogenesis can repair the tissue damages (ischemic diseases, arteriosclerosis etc) and the other one where inhibition of pathological angiogenesis can cure the disease or delay its progression (retinopathies, tumor progression, chronic inflammatory processes). Though there are an exponentially growing number of new synthetic molecules characterized mostly by antiangiogenic properties, the discovery of the large battery of natural pro- and anti-angiogenic factors suggest that this may provide a more physiological approach to treat these diseases in the near future.

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References

- ^{1,2}Albini A, Benelli R, Presta M, et al: HIV-tat protein is a heparin binding angiogenic growth factor. *Oncogene* 12:289-297, 1996.
- ^{2,3}Bae DG, Cho YS, Yoon WH, et al: Arginic-rich anti-vascular endothelial growth factor peptides inhibit tumor growth and metastasis by blocking angiogenesis. *J Biol Chem* 275:13588-13596, 2000.
- ^{3,2}Bowdler T, Folkman J, Pirie-Shepherd S: The hemostatic system as a regulator of angiogenesis. *J Biol Chem* 275:1521-1524, 2000.
- ^{4,2}Brown JM: Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Molecular Medicine Today* 6:157-162, 2000.
- ^{5,2}Bussolino F, Albini A, Camussi G, et al: Role of soluble mediators in angiogenesis. *Eur J Cancer* 32A:2401-2412, 1996.
- ^{6,2}Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine* 6:389-395, 2000.
- ^{7,2}Chang YS, di Tomaso E, McDonald DM, et al: Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 97:14608-14613, 2000.
- ^{8,2}DeNardo SJ, Burke PA, Leigh BR, et al: Neovascular targeting with cycle RGD peptide (cRGDf-ACHA) to enhance delivery of radioimmunotherapy. *Cancer Biotherapy Radiopharmaceuticals* 15:71-79, 2000.
- ^{9,2}Elicieri BP, Cheresh DA: The role of α v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J Clin Invest* 103:1227-1230, 1999.
- ^{10,2}Fidler IJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185-188, 1994.
- ^{11,2}Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27-31, 1995.
- ^{12,2}Gately S: The contributions of cyclooxygenase-2 to tumor angiogenesis. *Cancer Metast Reviews* 19:19-27, 2000.
- ^{13,2}Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353-364, 1996.
- ^{14,2}Hiltunen MO, Turunen MP, Laitinen M, et al: Insights into the molecular pathogenesis of atherosclerosis and therapeutic strategies using gene transfer. *Vasc Med* 5:41-48, 2000.
- ^{15,2}Holash J, Maisonpierre PC, Compton D, et al: Vessel cooption, regression and growth in tumors mediated by angiopoietins and VEGF. *Science* 284:1994-1998, 1999.

- 16.²*Holmgren L, O'Reilly MS, Folkman J*: Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* 1:149-153, 1995.
- 17.²*Isner JM, Asahara T*: Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J Clin Invest* 103:1231-1235, 1999.
- 18.²*Jacobson BS*: Hereditary hemorrhagic telangiectasia a model for blood vessel growth and enlargement. *Am J Pathol* 156:737-742, 2000.
- 19.²*Jiménez B, Volpert OV, Crawford SE et al*: Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nature Medicine* 6:41-48, 2000.
- 20.²*Kerbel RS*: Tumor angiogenesis: past, present and the near future. *Carcinogenesis* 21:505-515, 2000.
- 21.²*Lee P, Wang CC, Adamis AP*: Ocular neovascularization: an epidemiologic review. *Surv Ophthalmol* 43:245-269, 1998.
- 22.²*Li WW, Jaffe M, Li VW et al*: Lessons to be learned from clinical trials of angiogenesis modulators in ischemic diseases. In G.M. Rubanyi, (eds.), Marcel Dekker, Inc. New York, Basel, 2000, pp. 519-536.
- 23.²*Li WW*: Tumor angiogenesis: molecular pathology, therapeutic targeting and imaging. *Acad Radiol* 7:800-811, 2000.
- 24.²*Luzzi KJ, Varghese HJ, MacDonald IC, et al*: Arginine-rich anti-vascular endothelial growth factor peptides inhibit tumor growth and metastasis by blocking angiogenesis. *J Biol Chem* 273:373-379, 1998/1999.
- 25.²*Malonne H, Langer I, Kiss R, et al*: Mechanisms of tumor angiogenesis and therapeutic implications: angiogenesis inhibitors. *Clin exper Metastasis* 17:1-14, 1999.
- 26.²*Maniotis AJ, Folberg R, Hess A, et al*: Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 155:739-752, 1999.
- 27.²*Nicolaou KC, Trujillo JJ, Jandeleit B, et al*: Design, synthesis and biological evaluation of nonpeptide integrin antagonists. *Bioorg Med Chem* 6:1185-1208, 1998.
- 28.²*Nie D, Lamberti M, Zacharek A, et al*: Thromboxane A₂ regulation of endothelial cell migration, angiogenesis, and tumor metastasis. *Biochem Biophys Res Commun* 267:245-251, 2000a.
- 29.²*Nie D, Tang K, Diglio C, et al*: Eicosanoid regulation of angiogenesis: role of endothelial arachidonate 12-lipoxygenase. *Blood* 95:2304-2311, 2000b.
- 30.²*O'Reilly MS, Holmgren L, Shing Y, et al*: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79:315-328, 1994.
- 31.²*O'Reilly MS, Boehm T, Shing Y, et al*: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88:277-285, 1997.
- 32.²*Paku S*: Current concepts of tumor-induced angiogenesis. *Pathol Oncol Res* 4:62-75, 1998.
- 33.²*Pribluda VS, Gubish ER, LaVallee, TM, Jr, et al*: Methoxyestradiol: An endogenous antiangiogenic and antiproliferative drug candidate. *Cancer Metast Reviews* 19:173-179, 2000.
- 34.²*Sasisekharan R, Moses MA, Nugent MA, et al*: Heparinase inhibits neovascularization. *Proc Nat Acad Sci USA* 91:1524-1528, 1994.
- 35.²*Schiller J*: Early trials of antiangiogenic agents. *Anticancer Agents in Oncology* 15:3-10, 1999.
- 36.²*Schlaepfli J-M, Wood JM*: Targeting vascular endothelial growth factor (VEGF) for anti-tumor therapy, by anti-VEGF neutralizing monoclonal antibodies or by VEGF receptor tyrosine-kinase inhibitors. *Cancer Metastasis Rev* 18:473-381, 1999.
- 37.²*Semenza GL*: HIF-1: Using two hands to flip the angiogenic switch. *Cancer Metast Reviews* 19:59-65, 2000.
- 38.²*Stephens TD, Fillmore BJ*: Hypothesis: Thalidomide embryopathy-proposed mechanism of action. *Teratology* 61:189-195, 2000.
- 39.²*Stetler-Stevenson WG*: Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 103:1237-1241, 1999.
- 40.²*Thompson WD, Li WW, Maragoudakis M*: The clinical manipulation of angiogenesis: pathology, side-effects, surprises, and opportunities with novel human therapies. *J Pathol* 190:330-337, 2000.
- 41.²*Tímár J, Tóth J*: Tumor sinuses – vascular channels. *Pathol Oncol Res* 6:83-86, 2000.
- 42.²*Venkov CD, Rankin AB, Vaughan DE*: Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation* 94:727-733, 1996.
- 43.²*Xu C, Lee S, Singh TM, et al*: Molecular mechanisms of aortic wall remodeling in response to hypertension. *J Vasc Surg* 33:570-578, 2001.
- 44.²*Yeh CH, Peng H-Ch, Huang TF*: Accutin, a new desintegrin, inhibits angiogenesis in vitro and in vivo by acting as integrin $\alpha_v\beta_3$ antagonist and inducing apoptosis. *Blood* 92:3268-3276, 2000.
- 45.²*Zhang M, Volpert O, Shi YH, et al*: Maspin is an angiogenesis inhibitor. *Nature Medicine* 6:196-199, 2000.