Review Article

Mechanisms of angiogenesis in vascular disorders: potential therapeutic targets

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CONTENTS

Summary

Recent evidence suggests that, in spite of the redundancy of angiogenic factors involved in pathological angiogenesis, strategies aimed at inhibiting specific endothelial cell angiogenic factors at their release or receptor level may form the basis for effective and safe treatment of angiogenic-mediated disease processes. Physiologic angiogenesis is fundamental to reproduction, development and repair. Pathological angiogenesis sustains the progression of many neoplastic and proinflammatory diseases. The idea that tumor growth is angiogenesis-dependent was first proposed by Folkman *et al.* (1). This hypothesis is now supported by extensive experimental evidence from which a wide spectrum of diagnostic and therapeutic applications have been advanced.

Introduction

The formation of new capillary blood vessels, a process termed "angiogenesis", is dependent upon coordinate production of angiogenesis promoters and inhibitors. Angiogenesis is an important event in a variety of physiological processes including ovulation, embryonic development, wound repair and collateral vascular generation in the myocardium. Promotion of angiogenesis is driven by a production of cytokines such as TNF-α, IL-8, growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and matrix proteins such as laminin, fibrin, hyaluronan, or other endogenous mediators. Additionally, a deficiency in the production of endogenous angiostatic mediators such as thrombospondin, a matrix protein, or retinoids, tissue inhibitors of metalloproteinases, platelet factor 4, or other growth factors can also trigger angiogenesis (2, 3).
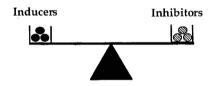
There are a number of key steps in the angiogenic cascade, including reactivation of endothelial cells (EC), rupture of basement membrane, adhesion, migration, proliferation of EC, tubule formation, and sprouting of new capillary blood vessels from preexisting vessels. The rate of basement membrane synthesis as a biochemical index of angiogenesis, measured as an increase in collagen type IV synthesis, has been shown to directly correlate with the formation of new blood vessels. Several studies suggested that different basement membrane play a pivotal role in angiogenesis. Basement membrane is not only an essential element of all blood vessels, but it also acts as a local hormone for activated EC. In this regard, basement membrane biosynthesis might represent an ideal target for developing inhibitors of angiogenesis.

In adult organs the turnover of EC is an extremely slow process (months to years) which accelerates only in a few physiological situations during embryogenesis, ovulation and wound healing. Under these special circumstances angiogenesis lasts for a relatively short time (days to weeks). It then returns to a quiescent state in a self-limited and well-regulated fashion. This process is controlled both temporally and spatially through a well-coordinated and balanced symphony of angiogenesis suppressors and promoters (Fig. 1). In contrast, pathological angiogenesis can last for years due to a chronic imbalance between angiogenic and angiostatic factors (overproduction of angiogenic factors and/or deficiency of angiostatic factors). This imbalance can result in various forms of pathological angiogenesis (Table I).

Regulation of angiogenesis

The microenvironment of individual organs controls the extent of vascularization under physiological and

Physiological Angiogenesis
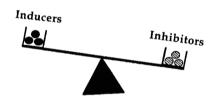

Pathological Angiogenesis

Fig. 1. Balanced or imbalanced levels of angiogenesis inducers (promoters) and inhibitors in physiological and pathological angiogenesis processes.

pathological conditions. In tissues, interferon-alpha (IFN-α) and interferon-beta (IFN-β) downregulate the expression of the angiogenic factors such as human basic fibroblast growth factor (bFGF) and vascular endothelium growth factor (VEGF). Growth factors and cytokines require receptor targets on endothelial cells to exert their influence. Clearly, endothelial cells play a major role in the modeling of blood vessels. The interplay of growth factors, cell adhesion molecules and specific signal transduction pathways, either in the maintenance of the quiescent state or in the reactivation of endothelial cells, is well coordinated.

Angiogenesis mediators

A number of angiogenic growth factors have been purified and characterized. These growth factors appear to fall into two groups: those that act directly on EC and those that act indirectly to release specific EC growth factors.

Vascular endothelial growth factor

Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is a potent mitogen and angiogenesis promoting factor *in vivo*. VEGF is secreted in response to hypoxic or ischemic insults, with the subsequent initiation and amplification of neovascularization. VEGF appears to be a crucial mediator of blood vessel growth associated with tumors and proliferative retinopathies. Anti-VEGF monoclonal antibodies or soluble receptors suppress neovascularization in a variety of animal models of ischemia-induced retinal angiogenesis and cancer. Therefore, a humanized anti-VEGF antibody may have therapeutic value for various disorders where angiogenesis plays a significant role.

1) Ribozyme inhibition of VEGF-mediated EC proliferation and neovascularization

Ribozymes are catalytic RNA molecules that are capable of highly specific transcleavage of target RNAs. Chemical modifications permit synthesis of hammerhead ribozymes which are resistant to nucleolytic degradation yet retain catalytic activity. These modified ribozymes have demonstrated utility in disease models of corneal angiogenesis, arthritis and restenosis. In cell culture, the exogenous delivery of ribozymes targeting several different transcription factors and signal transduction components have been shown to inhibit proliferation of aortic smooth muscle cells derived from rat, pig and human tissue. This phenotypic effect has been correlated with a reduction in target mRNA levels. These ribozymes are being tested in a pig coronary artery model of restenosis. Ribozymes targeting the metalloproteinase stromelysin inhibit stromelysin mRNA expression in cultured fibroblasts and in rabbit synovium following intraarticular administration. These and other ribozymes that alter metalloproteinase expression are being tested in a rabbit partial meniscectomy model of osteoarthritis. Ribozymes targeting the VEGF receptors Flt-1 and KDR were found to inhibit angiogenesis in a rat corneal model. These are also being tested in mouse models of tumor growth and metastasis. In each example, an inactive ribozyme control, in which the catalytic core has been altered to prevent cleavage, has been included. These inactive

Table I: Angiogenesis in physiological and pathological processes.

Physiological	Pathological
Ovulation and embryonic development	Ocular neovacularization-mediated diseases
	Diabetic retinopathy (retinal neovascularization), age-related macular degeneration-ARMD (choroidal neovascularization)
	Cancer: metastasis of solid tumor
Wound repair	Wound healing: surgery, peptic ulcer, *etc.*
Collateral vascular generation	Vascular diseases, *e.g.*, ischemic heart disease, atherosclerosis
	Chronic inflammatory disorders, *e.g.*, rheumatoid arthritis, psoriasis, *etc.*

ribozymes do not elicit the phenotypes seen with active ribozymes, indicating that the effects are mediated by ribozyme cleavage of the target RNA. Thus, it appears that exogenous delivery of synthetic hammerhead ribozymes has considerable therapeutic potential.

Human basic fibroblast growth factor

Human basic fibroblast growth factor (bFGF) belongs to the family of heparin-binding growth factors that induce a set of complex, coordinated responses in cultured EC, including cell proliferation, chemotaxis and proteinases production. Identification of the functional domains of bFGF is of critical importance in understanding the interconnections among various angiogenic stimuli. Interestingly, within the primary structure of bFGF, a DGR (Asp-Gly-Arg) peptide sequence at position 46-48 and 88-90 of bFGF has been located. This sequence represents the inverted sequence of the widespread recognition sequence RGD, a sequence that is specifically recognized by the family of the integrin receptors, αv (β1, β3, β5, *etc.*) and β3 (αIIb, αv, *etc.*) that bind adhesive proteins like fibronectin, fibrinogen, vitronectin, osteopontin, thrombospondin and fibrin. Short peptides containing RGD or DGR sequences have been shown to inhibit the interactions of integrins with their ligands (4).

Recent evidence suggests that a new family of angiogenesis-induced genes that may regulate FGF-induced EC migration, which, in turn, could play a major role in the early events of angiogenesis. The human homologue of the Jagged ligand for the Notch receptor from human EC exposed to fibrin has been identified. This ligand binds to a Notch-family receptor on EC leading to Jagged-induced Notch signaling through the FGF receptor, rather than the VEGF receptor signal transduction pathway.

Tyrosine kinases

Two tyrosine kinases have been identified as putative VEGF receptors, including FlT-1 and FlK-1/KDR, and have been located in EC. FlT-1 can be secreted and competitively inhibits VEGF-induced angiogenesis. FlK-1 and FlT-1 are also involved in development and assembly of vascular structures. Tie1 and Tie2 form a new subfamily of receptor tyrosine kinases and both of them are predominantly expressed in vascular EC during blood vessel formation and remodeling. Analyzing "knockout mice" for Tie1 and Tie2 genes demonstrated the critical role of Tie1 in vascular formation and Tie2 in sprouting of new capillary blood vessels from preexisting blood vessels.

1) FlK-1 antagonists

A number of growth factor receptor tyrosine kinases have been implicated in angiogenesis, including EGFR, FGFR, PDGFR, FlK-1/KDR, Flt, Axl, Tek and Tie. The VEGF/FlK-1 signaling pathway has been shown to be an important target for therapeutic intervention in tumor angiogenesis using several approaches including dominant-negative strategies. Screening efforts have been undertaken to identify small molecule inhibitors of VEGF-mediated signal transduction (5). Compounds that were potent and selective inhibitors of VEGF-mediated FlK-1 phosphorylation and DNA synthesis were tested for their ability to inhibit VEGF-mediated tumor growth. This strategy has proven successful for identifying FlK-1 antagonists. A monoclonal antibody to FlK-1 has been developed which neutralizes VEGF activation of receptor and inhibits *in vivo* tumor formation in a mouse model.

Cell adhesion molecules and extracellular matrix

1) Integrins

Integrins are a widely expressed family of cell adhesion receptors by which cells attach to extracellular matrices, to each other and to different cells. All integrins are composed of heterodimeric units expressed on a wide variety of cells, and most cells express several integrins. There are at least 8 known β-subunits and 14 α-subunits. Although the association of the different β- and α-subunits could in theory associate to give rise to more than 100 integrins, the actual diversity is much more restricted. The interaction of integrins with the cytoskeleton and extracellular matrix appears to require the presence of both subunits. The binding of integrins to their ligands is cation-dependent. Integrins appear to recognize specific amino acid sequences in their ligands. The most well-studied is the RGD sequence found within a number of matrix proteins including fibrinogen, vitronectin, fibronectin, thrombospondin, osteopontin, von Willebrand factor, *etc*. However, other integrins bind to ligands via a non-RGD binding domain, such as the 41 integrin receptors which bind and recognize the LDV sequence within the CS-1 region of fibronectin.

a) αvβ3 in angiogenesis

Integrins αvβ3 and αvβ5 play a role in the distinct pathway of angiogenesis. Integrin αvβ3 is preferentially expressed on blood vessels undergoing angiogenesis in various disease states. Antibody or peptide antagonists of this integrin block angiogenesis in response to human tumor cytokines in several preclinical models. These inhibitors of αvβ3 promote selective apoptosis of newly sprouting vessels preventing their maturation. The αvβ3 specific murine antibody LM609 was humanized utilizing Ixsys' patented codon-based mutagenesis technologies. The humanized antibody Vitaxin exhibits high affinity for human αvβ3 and similar *in vitro* biological activities compared to LM609. *In vivo* studies demonstrated that Vitaxin significantly inhibits angiogenesis induced by either growth factors or tumors without affecting preexisting ves-

sels. Recently, potential implication of $\alpha v \beta 5$ in VEGF-induced ocular neovascularization has been suggested (4). These findings indicate that antibody or peptide antagonists of integrin $\alpha v \beta 3$ and/or $\alpha v \beta 5$ may have a profound therapeutic value in the treatment of diseases associated with angiogenesis.

2) Laminin receptors

Increased cell adhesion and metastatic propensity correlate with expression of the α_2-subunit of laminin. Current studies demonstrate that ras-transformed rat fibroblasts (4R) are highly metastatic, whereas doubly transformed (ras +Ela) rat fibroblasts (RE4) are highly tumorigenic but not metastatic. The 4R cells express increased amounts of the α_2-subunit of laminin (4X) compared to RE4 cells. Similarly, highly metastatic human melanoma cells secrete laminin containing significantly higher amounts of the α_2-subunit compared to non-metastatic melanoma or fibrosarcoma cells. Concomitantly, there is a 10-fold increase in α_1-mRNA expression in metastatic cells. Laminin containing the α_2-subunit has a greater adhesion-promoting activity compared with laminin not containing the α_2-subunit.

3) Matrix proteins

Matrix plays a major role in the regulation of angiogenesis. Endothelial cells lining the inner vessel wall bind to laminin (a major component of the basement membrane). Two domains in the laminin A (CTFALRGDNP) and B1 (CDPGYIGSRC) chains block capillary-like tube formation on Matrigel®, a connective tissue matrix. A third cell binding site in the laminin A chain designated CSRARKQAASIKVSADR, was examined for angiogenic activity in human endothelial cells (HUVEC) in culture and in the CAM assay. The SIKVAV-containing peptide promotes HUVEC attachment to several matrix components. The peptide also induces morphological changes in the cells on plastic and induces sprouting and tube formation into the Matrigel® matrix. Analysis of EC-conditioned media indicated degradation of the Matrigel® and zymograms showed gelatinase (68 and 62 Kd) activity. Immunohistochemical and Northern blot analysis of HUVEC exposed to SIKVAV also showed an increased expression of cell surface integrins. The addition of the peptide to growing vessels in the CAM assays demonstrated increased capillary branching and formation of new capillaries from the parent vessels, a behavior which is observed *in vivo* in response to tumor growth or in the normal vascular response to injury.

a) Thrombospondin in angiogenesis

Five members of the thrombospondin (TSP) family have been identified: TSP1, TSP2, TSP3, TSP4 and TSP5 (8). Both TSP1 and TSP2 block growth and migration of EC in response to a variety of agonists. This activity is apparently a function of so-called "procollagen" and "properdin" modules that are present in TSP1 and TSP2 and absent in TSP3, TSP4 and TSP5. TSP1 and TSP2 share a common degradative pathway. TSP1 is unique in its enhanced expression in cells induced to enter G_1 and its ability to activate latent TGF-β.

Matrix metalloproteinases

Matrix metalloproteinase (MMP) enzymes are a target for a new class of inhibitory drugs and are highly expressed by many malignant tumors. The matrix metalloproteinase inhibitor (MMPI) Marimastat® (British Biotech Inc.) is beginning phase III studies for a variety of solid tumors such as lung, gastric, ovary and prostate. This drug is a broad-based inhibitor against all the major matrix metalloproteinases, particularly interstitial collagenase, 72 kDa gelatinase (MMP2) and 92 kDa gelatinase (MMP9). It is approximately 40-fold less potent against stromelysin than against interstitial collagenase. Marimastat® is well absorbed, with a bioavailability of 70% and an elimination half-life of 8-12 h in normal healthy volunteers. Twice-daily dosing leads to steady-state levels within 3 days. To achieve 90% inhibition of the enzymatic activity, plasma levels need to be approximately 40-80 ng/ml. The compound has shown efficacy against various solid tumors such as gastric cancer, has a continuing good safety profile, and is well tolerated. However, the efficacy of the drug has been related to optimal biologic dose using cancer antigens rather than maximum tolerated dose. It is unclear whether such antigens will really predict clinical outcomes. Musculoskeletal adverse events, such as local pain, stiffness and discomfort, have been reported. (One possible explanation for this side effect is the release of IL-1 by the matrix metalloproteinases.) The incidence of these events is 33% at 10 mg b.i.d. and disappears with discontinuation of treatment. Therefore, these side effects may be able to be managed by taking 1-2 week "drug holidays." This form of therapy may represent a viable strategy for tumors that are rapidly progressive, with high recurrence rates, and for which there is no available therapy.

Additionally, Chiroscience has selected two MMPIs, D-1927 and D-2163, for phase I clinical trials for cancer. The two compounds are orally bioavailable and are claimed to have superior profiles to other MMPIs in preclinical models. They are selective against specific MMP enzymes without affecting TNF or IL-1 release. In addition, the antiinflammatory MMP inhibitor, D-5410, is expected to enter phase II studies for arthritis. D-5410 has been shown in standard models of arthritis to be a potent, orally available, selective inhibitor of the key enzymes involved in arthritis as well as an inhibitor of TNF convertase, which contributes to its antiinflammatory effect.

Other natural products

1) Platelet factor 4

Platelet factor 4 (PF4) is a natural component of the platelet α-granule that is released under conditions of platelet activation. Although the physiological role of this protein is poorly defined, recombinant PF4 possesses significant angiostatic activity which has led to its current clinical testing. The protein has the ability to accumulate preferentially at sites of active angiogenesis *in vivo*, suggesting that it may play an important natural, physiological role in regulating EC function under pathophysiological conditions.

2) Neovastat® and AE-941

Neovastat® and AE-941 are extracted from marine biomass using an exclusive extraction and manufacturing technology. Neovastat®, an orally administered angiogenesis inhibitor, is in phase III trials in Canada for solid tumors refractory to standard therapies. The compound is also in phase I/II studies for first-line therapy as a single agent, or in combination with conventional chemotherapy, in lung, prostate and breast cancers.

AEterna Laboratories also plans to conduct phase I/II studies of AE-941 in rheumatoid arthritis and osteoarthritis and hopes to conclude a strategic alliance for these indications following the results of these studies. AEterna and an unnamed company specializing in ophthalmology will collaborate on development of AE-941 for ophthalmic indications such as age-related macular degeneration (AMD). In addition to the ophthalmology indication, AEterna may develop the compound as both an oral and topical agent for psoriasis. *In vivo* studies have demonstrated the antiinflammatory activity of AE-941. *In vitro*, the compound inhibits endothelial cell proliferation and has anticollagenolytic properties (prevents the action of collagenase enzymes which destroy collagen fibers).

3) Angiostatin

Angiostatin, a 38 kDa specific inhibitor of capillary EC proliferation, is a fragment of plasminogen. It contains at least three of the kringles of the parent protein, and as indicated above, is derived from malignant tumors (9, 10). Angiostatin has led to the maintenance of metastases in the dormant state through balancing the proliferation of tumor cells with the rate of apoptosis in three different types of primary murine tumors (10-12). A serine proteinase found in several human prostate carcinoma cell lines has been shown to cleave plasminogen and produce angiostatin (13). Recombinant human angiostatin protein has been described and inhibits the proliferation of bovine capillary endothelial cells *in vitro* and suppresses the growth of metastases in mice in the Lewis lung model. This protein is under development by EntreMed and Bristol-Myers Squibb.

4) Endostatin

Endostatin, a potent inhibitor of angiogenesis, was discovered by O'Reilly *et al.* (14). Endostatin has the potential to treat cancer in the same fashion as chronic diseases that are monitored and managed with drugs. Tumors cannot grow unless they stimulate the growth of new blood vessels to supply them with proteins, growth factors and oxygen. Primary tumor cells produce both stimulators and inhibitors of blood vessel growth; while the stimulators spark the formation of a network of blood vessels that bring fuel for a primary tumor's expansion, the inhibitors suppress the growth of metastases. Purified inhibitors have demonstrated the ability to suppress blood vessel growth and halt the growth of tumors in animal and human clinical trials. Data on endostatin reported by Folkman demonstrate that it may not be necessary to be on antiangiogenic therapy for a lifetime.

Endostatin is one of three endogenous antiangiogenic agents described so far generated by malignant tumors. The proteins are derived from within larger molecules, including fragments from thrombospondin (8), angiostatin (10) and endostatin, a 20 kDa C-terminal fragment of collagen XVIII (11). Endostatin specifically inhibits endothelial proliferation and thereby inhibits angiogenesis and tumor growth. In animal studies repeated doses of this protein at 20 mg/kg/day resulted in primary tumors regressing to dormant microscopic lesions. With increased dosing, tumor regression can occur. In the mouse Lewis lung tumor model, tumors stopped growing back after 250 days, and with the B16F10 melanoma cell line the "burn-out" of the tumors occurred in 80 days. Thus, the ability to inhibit specific EC proliferation suggests that combination therapy targeting both the proliferating EC and the proliferating tumor cell is better than treating each of these cell populations individually. While traditional cancer therapy may cause severe side effects because it slows cell division, especially in the gut and bone marrow where cells divide very rapidly, endostatin affects only EC division. Adding to its appeal as a cancer therapy endostatin does not appear to cause a buildup of drug resistance, leading to the speculation that antiangiogenic therapy could be administered whenever needed. Thus, Folkman and O'Reilly proposed that "the discovery of endostatin ... provides a pharmacological basis for dormancy therapy. Although it may take years for endostatin to travel through the sequence of clinical trials necessary to prove its safety and efficacy before becoming available to patients, its discovery, along with angiostatin, has changed our thinking and may yield improved therapies to cancer" (14).

5) TNP-470

The angiogenesis inhibitor TNP-470 (AGM-1470) is a synthetic analog of fumagillin that is one of eight angiogenesis inhibitors currently in phase I/II clinical trials for the treatment of solid tumors. Preclinical studies showed

that a wide spectrum of tumor types were inhibited *in vivo* with a T/C (treated/control tumor volume) of approximately 0.35% This inhibition was independent of immune status or sex of the mice and drug resistance did not develop even after more than 200 days of therapy. Wound healing was delayed by approximately 12-40% only if the drug was administered in the first 4 days after wounding. These results may be used as a model for the preclinical testing of other angiogenesis inhibitors. Treatment of mice with systemic TNP-470 on days 0, 2 and 4 affected wound contraction to an equivalent extent as treatment on days 0-16, that is, a 12% decrease in the percentage of the wound healed on day 12. Control wounds were 100% closed on postwound day 12 compared to TNP-470 wounds which closed by day 17. After linear wounds were made, TNP-470 also decreased the tensile strength of wounds on days 7 and 12 postincision. In contrast, treatment with TNP-470 prior to wounding or beginning on day 5 after wounding did not significantly effect wound healing. TNP-470 decreased the concentration of basic fibroblast growth factor by 42% in the full thickness wounds. Histological changes also occurred. It was concluded that TNP-470 suppresses wound healing in a time-dependent manner and does not effect wound healing if given before or 5 days after the wound is made.

TNP-470 may have utility in treating such pathologic reproductive processes as endometriosis, adenomyosis, dysfunctional uterine bleeding, choriocarcinoma and ectopic pregnancy. Recent evidence cited by D'Amato and colleagues (15) show that normal reproductive functions such as cyclical endometrial maturation and corpora lutea growth are dependent upon angiogenesis, and therefore inhibition of these processes could provide a new method for controlling fertilization. Mice had no successful matings after 4 cycles of treatment with TNP-470, but did mate successfully 6 weeks after treatment was stopped, thus demonstrating that treatment was reversible.

6) CM-101

CM-101 is in clinical trials against solid tumors. Phase I trials have been completed, and now the availability of material is the issue. This toxin is a polysaccharide exotoxin of 300,000 molecular weight that is composed of repeating epitopes that bind and cross-link critical tumor endothelial receptors rapidly after it is infused. The alternative complement pathway is activated, leading to a cytokine-driven inflammatory response targeting the tumor (16).

Angiogenesis models

Angiogenesis can be studied both *in vitro* and *in vivo* with the net result of new vessel formation. *In vivo* angiogenesis occurs primarily at the level of the microvasculature (capillaries, arterioles, venules), and yet most *in vitro* models have employed EC isolated from large vessels. This raises the question whether EC derived from different vascular beds can be used interchangeably to study different vascular angiogenesis events. Clearly, EC heterogeneity might be related to the predominance of certain angiogenic stimuli and signaling cascades specific to different tissues or sources of EC (5, 6). There are several limitations to these models. Although heterogeneity of vascular ECs is widely recognized, assays for angiogenesis still fail to take this into account. Many paradoxical results may be resolved by recognizing that this heterogeneity underlies many of the observed variable responses to angiogenesis inducers and inhibitors. Comparison of the potency of angiogenesis inhibitors has been difficult due to the myriad of different models used to assay for their inhibitory effects. A list of these models is given in Table II.

Potential therapeutic targets for angiogenesis

The angiogenesis process has been implicated in various disorders, including ocular, cancer, inflammatory, wound healing and cardiovascular diseases.

Ocular diseases

Diabetic retinopathy, occlusion of the retinal vasculature or premature retinopathy can lead to pathologic neovascularization on the surface of the retina. This, in turn, can cause vitreous hemorrhage, traction retinal detachment or neovascular glaucoma and resultant severe vision loss.

Diabetic retinopathy associated with retinal ischemia is the leading cause of blindness in the Western world.

Table II: Angiogenesis models.

In vitro models	*In vivo* models
Cultured EC on different substratum	Matrigel
Matrigel	Chick chorioallantoic membrane-CAM assay
Collagen/Fibronectin	Rabbit cornea
Laminin	Hypoxia/ischemia-induced retinal/iris NV in rats, mice, primates
Fibrin or gelatin	Laser-induced choroidal NV
Endothelial migration & proliferation assays	Human skin/human tumor transplanted on SCID mice
Sprouting from aortic rings	Tumor (metastasic) models in mice

Retinal ischemia releases into the vitreous diffusible angiogenic factors responsible for retinal and iris neovascularization. These include VEGF, IGF-1, bFGF and perhaps others. VEGF, by virtue of its highly diffusible nature and hypoxia inducibility, might appear to be a more sensitive marker but not necessarily the most important angiogenic mediator. Retinal vascular occlusive disease and premature retinopathy may also lead to neovascularization by a similar hypoxia-driven mechanism.

Blindness secondary to age-related macular degeneration (ARMD) is characterized by subretinal or choroidal neovascularization (CNV) in contrast to the preretinal neovascularization described above. ARMD is the leading cause of blindness in the elderly in the Western world. It still remains unclear which angiogenic mediators are involved in CNV, although there are some preliminary reports that VEGF may be involved.

Extracellular matrix alterations likely contribute to CNV. Bruch's membrane overlies normal choroid from which CNV emanates, and is a 5-layer basement membrane of the retinal pigment epithelium (RPE). The RPE, lying between the retina and Bruch's membrane, is responsible for the maintenance of the normal integrity of Bruch's membrane and of the overlying retinal photoreceptors. Histologic and biochemical investigations have indicated changes in the extracellular matrix composition of Bruch's membrane. Lipid infiltration and inflammatory cell (macrophage/monocyte) infiltration may contribute to the underlying pathogenesis of CNV. Further understanding of the role of RPE in the regulation of proangiogenic factors and extracellular matrix proteins would help in defining the ultimate therapeutic strategy for the treatment of ARMD.

Although the exact causes of ARMD remain elusive, many believe that as more is learned about the neovascular process, we will not only be able to develop new therapies to arrest its development but also will learn what needs to be done to prevent the process from starting. This is important because the current standard of therapy, laser photocoagulation, is unable to prevent blindness in the vast majority of patients with CNV. New laser treatment techniques are being investigated, including dye-enhanced laser treatment for selective ablation of CNV (photodynamic therapy) and alternative imaging techniques, such as ICG angiography-guided laser treatment. A recent novel pilot study, The Choroidal Neovascularization Prevention Trial, sought to determine whether light laser treatment of high-risk eyes without CNV could prevent the development of CNV. This study has been suspended because of preliminary results against laser treatment.

The Age-Related Eye Disease Study (AREDS) is a national multicenter study to determine whether there is potential prophylactic benefit from antioxidant vitamin supplementation. The data so far is based on observational studies and suggest a beneficial effect from foods rich in carotenoids and vitamins E and C. The risk was reduced by an estimated 43% in subjects whose dietary intake of carotenoid-rich foods was in the highest quintile for carotenoid intake. AREDS data should settle this issue by the year 2010.

Systemically administered medications are being investigated against CNV. Recently interferon-alpha (IFN-α) has been tested in the treatment of CNV. However, despite early sings of vascular regression, larger trials have failed to show a significant benefit. Because of its ability as a potent inhibitor of neovascularization in animal models (15) and its safety in nonpregnant humans, thalidomide is being evaluated as an antiangiogenic agent in selected patients with neovascular ARMD. This trial enrolls patients with subfoveal neovascularization and relatively good vision as well as patients with traditional laser photocoagulation treatment. The goal is to determine if thalidomide can reduce the severe vision loss, when compared with placebo, in patients who have relatively small subfoveal neovascular membranes and to determine whether it can reduce the nearly 60% CNV recurrence rate after laser photocoagulation.

Surgical CNV removal is an alternative means of eradicating subfoveal CNV with potentially less damage to neurosensory retina and potentially better visual function. The Submacular Surgery Trial is enrolling patients to answer the question as to whether surgery can play a beneficial role for these patients with subfoveal CNV. Other surgical techniques are now being investigated, including macular translocation to displace retinal fixation over areas not involved by CNV and RPE damage.

Neoplastic diseases

Tumor progression and metastasis involve cells that escape normal growth and adhesion controls and invade, migrate, attach and grow at inappropriate sites. Angiogenic factors such as growth factors, cytokines and cell adhesion molecules are known to control many of these events. Substantial evidence has accumulated over the past 25 years indicating the dependency of solid tumors on angiogenesis which was first proposed by Folkman (7, 8). As the advancing edge of the tumor approaches adjacent microvessels, proangiogenic factors are released from the tumor stimulating EC to grow and migrate toward the tumor and organize into a capillary network. This switch from the prevascular to vascular phase is accompanied by exponential growth of the tumor. An increase in the microvascular density in many solid tumors such as breast and prostate carcinoma has been shown to correlate with malignant and metastatic potential and hence the prognosis.

Tumor cells recruit new blood vessels via various angiogenic factors and are further amplified by the release of cytokines which attract and activate macrophages, mast cells and neutrophils. Research involving tumor-associated angiogenesis continues to yield new insights into the pathogenic mechanisms of this process. Based on a better understanding of the various mechanisms involved, innovative and novel therapeutic approaches targeting various steps in this process, as

well as the neovasculature itself, may be developed. In addition, as more is learned about the biology of angiogenesis, biological markers may be developed that can facilitate the performance of clinical trials. Specific agents currently in clinical trials and other approaches under development that act at various points in the complex process involved in neovascularization may soon have an impact on the treatment of neoplastic diseases.

Chronic inflammatory diseases

Angiogenesis is required for the progression of chronic inflammation, and agents that alter it affect the development of inflammation and the subsequent tissue destruction. Chronic inflammation following infection, trauma or immune response is an important trigger of angiogenesis. Psoriasis is a common skin disease characterized by excessive growth of epidermal keratinocytes, inflammation and microvascular proliferation. Psoriatic keratinocytes are known to produce a variety of proangiogenic factors as well as angiostatic factors such as thrombospondin. Psoriatic keratinocytes have been shown to have an imbalance in the production of positive and negative angiogenic mediators.

Rheumatoid arthritis is an example of a chronic inflammatory disease in which the role of angiogenesis is under active investigation. In the pathology of early rheumatoid arthritis, prominent microvascular changes occur. Hyperplasia of the synovial lining occurs in patients with rheumatoid arthritis. Although this hyperplasia is less in those patients with disease duration of less than 1 year, the measurements of synovial inflammation do not differ significantly between the two groups. Macrophages, as a source of proangiogenic factors, are one feature of the inflamed rheumatoid synovium. These cells and others have been implicated in the release of such angiogenic factors as matrix metalloproteinases and cytokines.

Recent studies have shown that blockade of TNF-α using cA2, a chimeric anti-TNF monoclonal antibody, rapidly reduces clinical and biochemical parameters of inflammation in patients with severe rheumatoid arthritis. MRI imaging of joints following cA2 therapy show a reduction in both joint fluid and inflammatory tissue; likewise, pre- and posttherapy synovial biopsies show a reduction in inflammatory cells. The clinical, MRI and biopsy observations correlate with a reduction in serum cytokines including IL-6 and VEGF following blockade of TNF-α with cA2. Further, VEGF/VPR production may be responsible for fluid accumulation in the joint and may augment the growth of the highly vascular pannus. Additionally, angiogenesis contributes significantly to diseases where there is persistent granulation tissue such as liver cirrhosis, pulmonary fibrosis and tissue fibrosis.

Wound healing

Wound healing is perhaps the most well-studied physiological angiogenesis event that is strictly time-dependent upon the ingrowth of new capillary blood vessels. Would healing is an essential biologic process that is driven by the cooperative interaction of a variety of cell types (vascular endothelium, monocyte-derived macrophage, connective tissue) and mediator systems. Macrophages play a central role in controlling and sustaining a balanced angiogenic level in its dual positive and negative regulation. Macrophages can influence new capillary ingrowth by several different mechanisms, including the release of various mediators (*e.g.*, IL-8 and TNF-α) that influence EC proliferation, migration and differentiation. Macrophages can also influence the composition of extracellular matrix components such as thrombospondin, or produce proteases that alter the structure of extracellular matrix.

Vascular diseases

Coronary occlusive disease is the leading cause of death in industrial nations and affects 1 in 4 adults. Although heart attacks are caused by occlusion of a coronary artery, some patients have total occlusion of a coronary artery without heart attack because of enough collateral vessels providing an alternate blood supply. Many angiogenic promoters such as VEGF, bFGF, *etc.* can promote collateral vessel development in ischemic myocardium. An attractive possibility is that the VEGF protein or gene therapy with VEGF may be used to promote revascularization in conditions of deficient tissue perfusion such as chronic limb ischemia, most frequently caused by obstructive atherosclerosis and associated with a high degree of morbidity and mortality. Additionally, there might be a link between angiogenesis and vascular remodeling.

Therapeutic applications of antiangiogenic agents

Because of the unmet medical need and the market size of the previously discussed angiogenesis-mediated diseases, many pharmaceutical and biotechnology companies are actively investigating various strategies for providing novel therapeutics (Table III). Complete inhibition of angiogenesis should be well tolerated in most adults since under physiologic conditions angiogenesis is required only for wound healing and reproduction. Antiangiogenic agents that bind to heparin binding growth factors, inactivate matrix metalloproteinases, inhibit endothelial cell proliferation or target developing endothelium are currently being tested as single agents in clinical trials. Recombinant proteins such as INF-α and platelet factor-4 may also have antiangiogenic activity through mechanisms that have not yet been completely defined. Because they act through diverse mechanisms of action, antiangiogenic agents may achieve maximum biologic effect when administered in combination. These agents are particularly attractive in the surgical adjuvant setting

Table III: Selected angiogenesis inhibitors under preclinical/clinical investigation.

Compound	Status	Company
Cytokine/growth factor inhibitors		
VEGF monoclonal antibody, soluble receptors	Phase I	Genetech
VEGF antisense oligonucleotide		Hybridon
Soluble FLT-VEGF receptor		Merck & Co.
VEGF receptor-tyrosine kinase antagonists, Flk-1, Tie1, Tie2, etc.		SUGEN; ImClone Systems; Regeneron
CA2 Anti-TNF-alpha monoclonal Ab	Preregistered	Centocor
IL1beta and TNF-alpha inhibitors		SmithKline Beecham
GM 1474, sulfated oligosaccharide (bFGF)	Preclinical	Glycomed
Cell adhesion molecules/matrix proteins		
Vitaxin-humanized forms of LM609 (anti-alphavbeta3)	Phase I	IXSYS
Cyclic peptide alphavbeta3 ligands		Merck KGaA
Small molecule alphavbeta3 antagonists		Schering-Plough; others
Other angiogensis inhibitors		
Platelet factor 4 (Iroplact)	Phase II	Repligen
TNP470 (Mechanism ?)	Phase II	Takeda
Thalidomide (Mechanism?)	Preregistered	EntreMed/BMS
Angiostatin, Endostatin		EntreMed/BMS
Receptor tyrosine kinase inhibitors (ZD1839)	Phase II	Zeneca
Matrix metalloproteinases (GM6001; Ilomastat)	Phase III	Glycomed
Urokinase receptor antagonists		Chiron
Antioxidant-nitrone-related therapeutics		Centaur
BB-94 (Batimastat), MMPI	Phase II	British Biotech/SK&B; Glaxo Wellcome
BB-2516 (Marimastat), MMPI, BB-94 backup, oral	Phase III	Britisch Biotech
DS-4152 (Tecogalan)	Phase II	Daiichi
ZD-0101/CM-101 (Group B Streptococcus polysaccharide exotoxin)	Phase II	Zeneca/CarboMed
AE-941 (Neovastat[TM])	Phase II	AEterma

due to potentially low risks for development of drug resistance and induction.

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