MEDICAL INTELLIGENCE UNIT 20

Shaker A. Mousa

Angiogenesis Inhibitors and Stimulators

Potential Therapeutic Implications

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Potential Therapeutic Implications

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Medical Intelligence Unit

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Dedication

I would like to specially thank my wife Mawaheb, my sons Ahmed and Adam, my daughters Shaymaa, Sarah, and Deena, my father and my mother for their love, encouragement and support.

This book is dedicated to my family.

Shaker A. Mousa

CONTENTS

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PREFACE

Angiogenesis is the formation of blood vessels. The regulation of blood vessel growth underlies many normal biological processes such as reproduction, development, repair and wound healing. It also is a critical feature of pathological processes such as cancer, diabetic retinopathy, macular degeneration, inflammatory arthritis and ischemic heart disease. What causes blood vessels to grow? What inhibits their growth? This book comprehensively describes the angiogenic proteins that enhance and inhibit normal and abnormal vascularization. The book describes the potential therapeutic applications of basic fibroblast growth factor, vascular endothelial growth factor, PECAM-1 and thrombospondin as well as other factors. It brings together information about new blood vessel formation in many organ systems and comprises and outstanding resource on the state-of-the art in angiogenesis.

Chapter 1 is an introduction; it describes the mechanisms of angiogenesis. Chapter 2 describes the relationship between angiogenesis and ischemia. Chapter 3 describes how vascular smooth muscle cells and pericytes interact in angiogenesis. Adhesion molecules and the extracellular matrix are critically involved in angiogenesis. This is discussed in Chapters 4, 5 and 6. Growth factors, particularly vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), are the subject of Chapters 7 and 8. Angiogenesis in wound healing and ocular neovascularization are discussed in Chapters 9 and 10, respectively. Inhibitors of angiogenesis, with particular emphasis on drug targets and clinical trials, are the subject of the last five chapters.

CHAPTER 1

Mechanisms of Angiogenesis:
Potential Therapeutic Targets

Shaker A. Mousa

The formation of new capillary blood vessels, a process termed "angiogenesis", is dependent upon coordinate production of angiogenesis promoters and suppressors. Angiogenesis is an important event in a variety of physiological angiogenesis processes including ovulation, embryonic development, wound repair and collateral generation in the myocardium. Angiogenesis promotion is driven either by production of proangiogenic cytokines (TNFα, IL-8) growth factors (FGF, VEGF) matrix proteins, (laminin, fibrin, hyoluronan), other endogenous mediators and/or deficiency in the production of endogenous angiostatic mediators such as certain matrix proteins (thrombospondin), retinoids, tissue inhibitors of metalloproteinases platelet factor 4, and certain growth factors.[1,2]

There are a number of key steps in the angiogenic cascade including the reactivation of endothelial cells (EC), rupture of basement membrane, adhesion, migration, proliferation, table formation, and sprouting off new capillary blood vessels of preexisting vessels. The rate of basement membrane (BM) synthesis and the increase in collagen type IV synthesis has been shown to directly correlate with the formation of new blood vessels. Several studies suggested that different BM play a pivotal role in angiogenesis. BM is not only an essential element of all blood vessels, but it also plays the role of a local hormone for activated EC. In that regard, BM biosynthesis might represent an ideal target for developing suppressors of angiogenesis.

In adult organs, the turnover of EC, isan extremely slow process which accelerates only in a few physiological situations such as embryogenesis, ovulation, and wound healing. Under these special circumstances angiogenesis lasts for a relatively short time (days-weeks) then return to a quiescent state in a self limited and well regulated both temporally and spatially through a well coordinated and balanced angiosuppressors and angiopromoters. In contrast, pathological angiogenesis can last for years and somewhat out of control due to 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 including (Figs. 1.1-1.3):

1. Ocular neovascularization-mediated diseases: diabetic retinopathy (retinal neovascularization) and age-related macular degeneration (choroidal neovascularization), DR & AMD, respectively.
2. Cancer: metastasis of solid tumor.
3. Chronic inflammatory disorders: rheumatoid arthritis (RA), psoriasis.
4. Wound healing: surgery, peptic ulcer,
5. Vascular diseases—ischemic heart diseases, atherosclerosis.

Angiogenesis is one of the most pervasive and fundamentally essential biological processes encountered in mammalian organisms. It is tightly regulated in both time and space. It is driven by a cocktail of growth factors and proangiogenic cytokines and tempered by an equally diverse group of inhibitors of neovascularization. Angiogenesis also plays a central role in the etiology and pathogenesis of a number of pathological processes. Based on recent work from several laboratories, it is now

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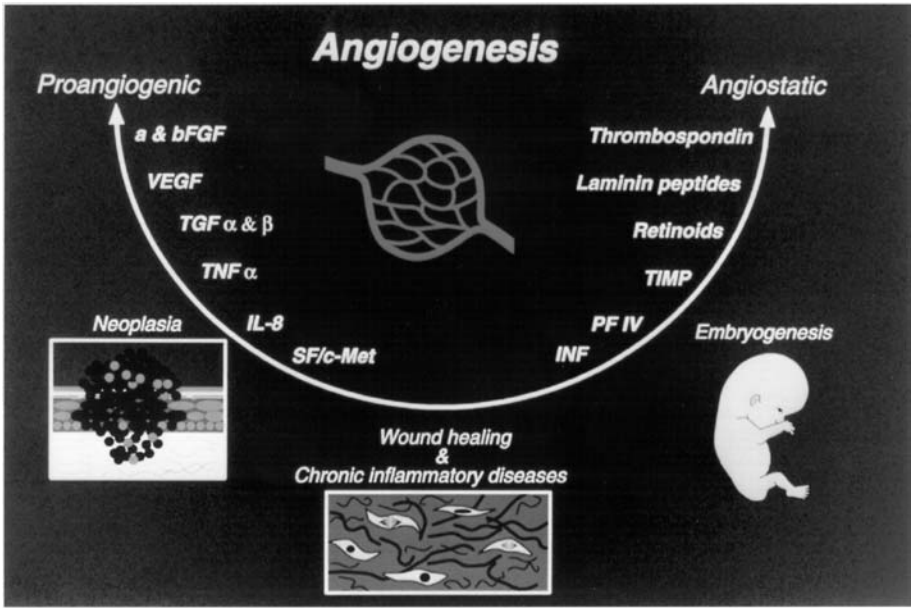

Fig. 1.1. A sketch showing the physiological and pathological implications of proangiogenic and anti-angiogenic mediators.

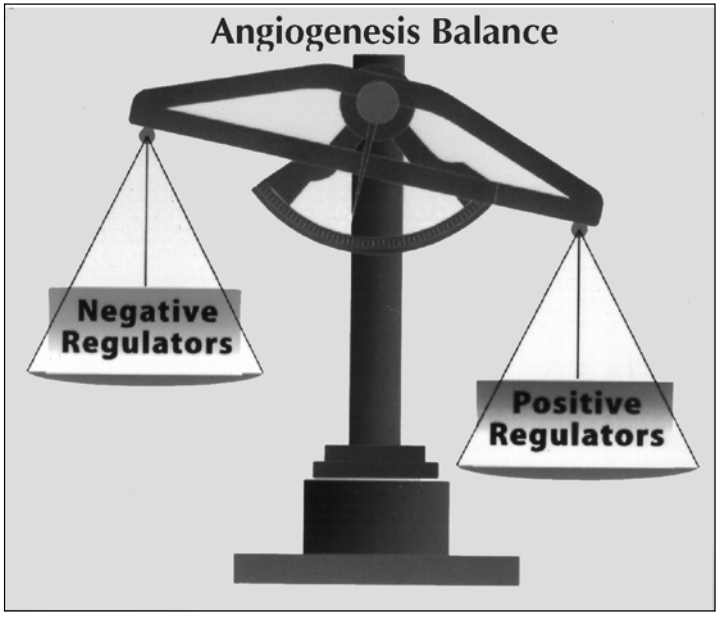

Fig. 1.2. An illustration showing the imbalance between positive and negative regulators of angiogenesis which would result in either accelerated or impaired angiogenesis.

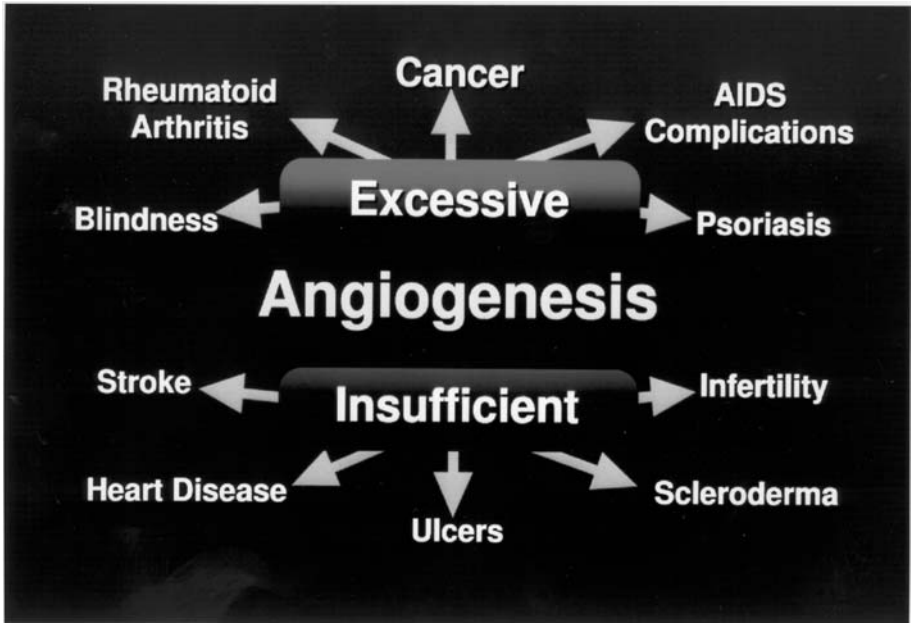

Fig. 1.3. A list of pathological disorders that could rise as a result of either excessive or insufficient angiogenesis.

eminently clear that most, if not all angiogenesis and vasoproliferative-dependent disease processes are a consequence of not only the unrestricted production of normal or aberrant forms of proangiogenic mediators but also the result of a relative deficiency in angiogenic inhibitory molecules.

A combined defect in the overproduction of positive regulators of angiogenesis and a deficiency in endogenous angiostatic mediators is a feature documented in tumor angiogenesis, psoriasis, RA and other neovascularization-mediated disorders. Hence understanding the mechanisms involved in the regulation of angiogenesis could have a major impact in the prevention and treatment of pathological angiogenesis processes.

Regulation of Angiogenesis

The organ microenvironment controls the extent of vascularization under physiological and pathological conditions. It has been demonstrated that in tissues, interferon-alpha (IFN-α) and -beta (-β) down-regulate the expression of the angiogenic factor, basic fibroblast growth factor (bFGF), and VEGF-induced angiogenesis which is sufficient to achieve therapeutic endpoints in animal models of coronary and limb ischemia. Conversely, inhibition of VEGF action by specific monoclonal antibodies or soluble receptors results in suppression of neovascularization associated with tumors and retinopathies. 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.

Angiogenic Mediators

A number of angiogenic growth factors have been purified and characterized. These growth factors appear to fall into two groups:

1. those that act directly on EC and
2. those that act indirectly to release specific EC growth factors.

Vascular Endothelial Growth Factor (VEGF)

One member of the first group is vascular permeability factor/vascular EC growth factor (VPF/VEGF) and placenta growth factor which is very similar in many aspects to VEGF. VEGF is a potent mitogen and angiogenesis-promoting factor in vivo. VEGF has been shown to be 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. Strong experimental evidence indicates that VEGF is a major mediator of angiogenesis associated with most human tumors and also ischemic retinal disorders. Anti-VEGF monoclonal antibodies or soluble receptors suppress neovascularization in a variety of animal models. Therefore, a humanized anti-VEGF antibody has therapeutic value for a variety of disorders where angiogenesis plays a significant role.

Human Basic Fibroblast Growth Factor (bFGF)

Basic fobroblast growth factor belongs to the family of heparin-binding growth factors. It induces a set of complex, coordinated responses in cultured EC, including cell proliferation, chemotaxis, and protease production. The identification of the functional domains of bFGF appears to be of critical importance in understanding the tight connections among various angiogenic stimuli. Interestingly, within the primary structure of bFGF the DGR (Asp-Gly-Arg) peptide sequence at positions 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 ($\beta 1$,$\beta 3$, $\beta 5$, . . .) and $\beta 3$ (αIIb, αv, . . .) that bind a variety adhesive proteins like fibronectin, fibrinogen, vitronectin, osteopontin, thrombospondin, and fibrin. Short peptides containing RGD or DGR sequence have been shown to inhibit the interactions of integrins with their ligand.[3]

Role of Kinases

Tyrosine Kinases

Two tyrosine kinases have been identified as putative VEGF receptors including FLT-1 and FLK-1/KDR tyrosine kinases. Both are located in EC. FLT-1 can be secreted and competitively inhibit VEGF-induced angiogenesis. Tie1 and Tie2 form a new subfamily of receptor tyrosine kinases and both are predominantly expressed in vascular endothelial cells during blood vessel formation and remodeling. Knockout mice for Tie1 and Tie2 genes demonstrated the critical role of Tie1 in vascular formation and Tie2 in sprouting of new capillary blood vessel from preexisting blood vessels.

FIK-1 Antagonists for Inhibition of Angiogenesis

A number of growth factor receptor tyrosine kinases have been implicated in angiogenesis including EGFR, FGFR, PDGFR, FlKl/KDR, Flt, Axl, Tek and Tie. The VEGF/FKl signaling pathway has been shown to be an important target for therapeutic intervention in tumor angiogenesis using several approaches including dominant-negative strategies. Therefore, a screening effort was undertaken to identify small molecule inhibitors of VEGF-mediated signal transduction. 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.

Identifying Inhibitors of Receptor/Ligand Interactions Involved in Angiogenesis

The tyrosine kinase receptors flk-1/KDR, flt-1, tie-1 and tie-2 are among the endothelial cell molecules implicated in angiogenesis. We have initiated a program to screen for antagonists of ligands

binding to these receptors, with flk-1/KDR: VEGF chosen as the model target. 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. Studies of receptor/ligand binding by real time surface plasmin resonance measurements utilizing Pharmacia's BiaCore instrument and data from a prototype large scale ELISA based screening assay will be presented.

Natural Anti-Endothelial and Neovascular Targeting Mechanisms of Platelet Factor-4 (PF4) Angiostatic Activity

PF4 is a natural component of the platelet alpha granule that is released under conditions of platelet activation. Although the physiological role of this protein is poorly defined, we have shown that the rPF4 possesses significant angiostatic activity that has led to its current clinical testing. Recently, we have demonstrated the ability of the protein to preferentially accumulate at sites of active angiogenesis in vivo, suggesting that it may play an important role in regulating endothelial cell function under pathophysiological conditions. Recent results with VEGF121 suggest that the ability of rPF4 to inhibit endothelial cell proliferation is not due to antagonism of growth factor/receptor binding, suggesting a more direct mechanism of endothelial cell interaction.

Role of Cell Adhesion Molecules and Extracellular Matrix

The Role of Integrin αvβ3 in Angiogenesis

Integrins αvβ3 and αvβ5 play a role in 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 exhibited high affinity for human αvβ3 and similar in vitro biological activities compared to LM609. In vivo studies demonstrated that Vitaxin significantly inhibited angiogenesis induced by either growth factors or tumors without affecting preexisting vessels. Recently, potential implications of αvβ5 in VEGF-induced ocular neovascularization has been suggested.[4] These findings indicate that antibody or peptide antagonists of integrin αvβ3, αvβ5 may have a profound therapeutic value in the treatment of diseases associated with angiogenesis.

Role of Laminin Receptors

Increased cell adhesion and metastatic propensity correlate with expression of the α2 subunit of laminin. Current studies demonstrated 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 α2-subunit of laminin (4X) compared to RE4 cells. Similarly, highly metastatic human melanoma cells secrete laminin containing significantly higher amounts of the α2-subunit compared to nonmetastatic melanoma or fibrosarcoma cells. Concomitantly, there is a ten-fold increase in α1-mRNA expression in metastatic cells. Laminin containing the α2-subunit has a greater adhesion promoting activity compared with laminin not containing the α2-subunit.

The Role of Matrix in Endothelial Cell Differentiation and Angiogenesis

Matrix plays a major role in the regulation of angiogenesis. Endothelial cells lining the inner vessel wall bind to a 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 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 promoted HUVEC attachment to a migration on several matrix components. The peptide also induced morphological changes in the cells on plastic and induced sprouting and tube formation into the Matrigel matrix. Analysis of endothelial cell 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 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.

Extracellular Matrix Proteins in Angiogenesis

Five members of the thrombospondin (TSP) family have been identified: TSP1, TSP2, TSP3, TSP4 and TSP5. TSP1 and TSP2 both block growth and migration of endothelial cells in response to 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 G1 and its ability to activate latent TGF-β.

Angiogenesis Models

In vivo angiogenesis occurs primarily at the level of the microvasculature (capillaries, arterioles, and 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 this is EC heterogenicity which might be related to the predominance of certain angiogenic stimuli and certain signaling cascades.[5,6] Angiogenesis can be studied both in vitro and in vivo with the net result of new vessel formation. However, there are several limitations to these models. Although heterogeneity of vascular endothelial cells is widely recognized, assays for angiogenesis still fail to take this heterogeneity 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 mouse model of corneal neovascularization utilizing basic fibroblast growth factor (bFGF) or vascular endothelial cell growth factor (VEGF) for the purpose of quantification and comparison of angiogenesis inhibitors has been developed. This model has the advantages of being inexpensive, reliable, reproducible and easily quantified. The amount of inhibition of the area of induced neovascularization per mole of drug administered is expressed in units of activity, which can be used to compare the potency of different angiogenesis inhibitors. A list of those models are shown in Table 1.1.

Angiogenesis and Ocular, Cancer, Inflammatory, Wound Healing and Cardiovascular Diseases

Angiogenesis and Ocular Diseases

Ocular neovascularization associated with diabetes, occlusion of the retinal vein, or prematurity and exposure to oxygen can lead to vitreous hemorrhage, retinal detachment followed by eventual blindness and neovascular glaucoma. Diabetic retinopathy is the leading cause of blindness in the western world, which is associated with retinal ischemia. Retinal ischemia releases into the vitreous diffusable angiogenic factors responsible for retinal and iris neovascularization. These include VEGF, IGF-1, bFGF and perhaps others. VEGF, by virtue of its highly diffusable nature and hypoxia inducibility might appear to be a more sensitive marker but not necessarily the most important angiogenic mediator. It still remains unclear which angiogenesis mediators are involved in the

Table 1.1. Angiogenesis models

I. In Vitro Models	II. In Vivo Models

- Cultured EC on different substrata
  - Matrigel
  - Collagen/Fibronectin
  - Laminin
  - Fibrin or Gelatin
- Sprouting from Aortic Rings
	- Matrigel in mice
	  - Chick chorioallantoic membrane (CAM Assay)
	  - Rabbit cornea
	  - Hypoxia/ischemia-induced retinal/iris NV in rats, mice, primate
	  - Laser-induced choroidal NV
	  - Human skin/human tumor
	  - Transplanted on SCID mice
	  - Tumor metastasis models in mice

mediator of choroidal NV, which leads to age-related macular degeneration (ARMD). Macular degeneration remains the leading cause of severe vision loss among older people. Histological and biochemical investigations have indicated changes in extracellular matrix composition of Bruch's membrane. The retinal pigment epithelium (RPE) is responsible for the maintenance of the normal integrity and barrier functions of Bruch's membrane. 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 AMD. Recently α-interferon has been tested in the treatment of choroidal NV. However, despite early signs of vascular regression, larger trials have failed to show a significant benefit.

Age-Related Macular Degeneration (ARMD)

Although the exact causes of age-related macular degeneration 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 the development of ARMD, but also will learn what needs to be done to prevent the process from starting. Several treatment modalities for ARMD are in various stages of investigation. One is digital indocyanine green (ICG) videoangiography. This technology allows the production of high-resolution ICG angiograms. Visual loss secondary to exudative ARMD—the occult form of choroidal neovascularization (CNV)—presents as a subpigment epithelial lesion presumed to be CNV, but it lacks the typical features of classic neovascularization. Since successful laser treatment depends on the accurate identification and localization of these aberrant vessels, this occult form is considered to be untreatable. ICG videoangiography is an important adjunctive technique to standard fluorescein angiography. It is especially useful in delineating occult neovascularization, neovascularization with overlying subretinal hemorrhage and neovascularization associated with pigment epithelia detachments. There is potential for some benefit from antioxidants. The evidence so far suggests a beneficial effect from foods rich in carotenoids, vitamins E and C intake. 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. Hence the antioxidant theory is entirely plausible. An upcoming study—the Age-Related Eye Disease Study—will be the first large trial to evaluate the effect of antioxidants on ARMD and cataract formation. Isolation of vascular endothelial growth factor has theoretical implications for the future of ARMD therapy. Increased levels in the eye when there is active vessel growth has been demonstrated. Thalidomide may get a second chance to prove some worth. Because of thalidomide's ability as a potent inhibitor of neovascularization in animal models and its safety in nonpregnant humans, it is being evaluated as an antiangiogenic agent in selected patients with neovascular ARMD, patients with subfoveal neovascularization and relatively good vision are randomized to thalidomide or placebo. The goal is to determine if thalidomide

can reduce the frequency of severe vision loss when compared with placebo in patients who do have these relatively small subfoveal neovascular membranes. The fact is that persistent leakage after laser treatment is a principal reason for vision loss. Hence in part two the study goal is to determine if thalidomide in conjunction with laser can reduce the likelihood of severe vision loss by reducing the rate of persistent or recurring leakage after initial treatment. The NIH-sponsored Choroidal Neovascularization Prevention Trial is evaluating whether laser treatment delivered to the perimeter of a macula with high-risk drusen can reduce the rate at which these eyes go on to develop neovascularization. This treatment will be compared to a control group who will not receive laser therapy. Laser photocoagulation, which offers a marginal benefit for treating neovascularization, has the limitation of causing concomitant damage to the overlying neurosensory retina. Surgical removal is an alternative means of eradicating subfoveal CNV with potentially less damage to neurosensory retina and potentially better visual function.

Angiogenesis and Cancer

Tumor progression and metastasis are classically conditioned where cells escape normal growth and adhesion controls and invade, migrate, attach and grow at inappropriate sites. Angiogenic factors including growth factors, cytokines, andcell adhesion molecules are known to control many of the above events. Substantial evidence has been accumulated over the part 25 years pointing to the dependency of solid tumors on angiogenesis which was first proposed by Folkman.[7,8] As the advancing edge of the tumor approach 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, (MVD) in breast and prostate carcinoma has been shown to correlate with malignant and metastatic potential and hence with a poor 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 this newfound understanding, 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 of clinical trials. Specific agents currently in clinical trials, as well as 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.

Angiogenesis and Chronic Inflammatory Diseases

Angiogenesis is required for the progression of chronic inflammation. 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 shown to have an imbalance in the production of positive and negative angiogenic mediators.

Rheumatoid arthritis is another example of chronic inflammatory diseases where the angiogenesis promotes the destruction of joints. Macrophages as a source of proangiogenic factors, are a constant feature of the inflamed rheumatoid synovium. Additionally, angiogenesis contributes significantly to diseases where there is persistent granulation tissue such as liver cirrhosis, pulmonary fibrosis and tissue fibrosis.

Reduction in VEGF Levels in Patients with Severe Rheumatoid Arthritis Treated with cA2 and Anti-TNF Monoclonal Antibody

The joint inflammation in rheumatoid arthritis (RA) is characterized by the presence of cytokines, inflammatory cells and pannus, a synovial cell hyperplasia that invades and destroys joint

cartilage and bone. Since the pannus is highly vascular, it is an "angiogenic" process. 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 post-therapy 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 with cA2. The data is consistent with the hypothesis that in patients with RA, the production of inflammatory cytokines, including IL-1, IL-6, GM-CSF, VEGF, RANTES, etc. is controlled by TNF secretion. Further, VEGF/VPR production may be responsible for fluid accumulation in the joint and may augment the growth of the highly vascular pannus.

Angiogenesis and Wound Healing

Wound healing is perhaps the most well-studied physiological angiogenic event that is strictly time-dependent upon the in-growth of new capillary blood vessels. Wound 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.

The macrophage plays a central role in controlling and sustaining balanced angiogenisis The macrophage can influence new capillary in-growth by several different mechanisms including the release of various mediators (IL8, TNF-α) that influence EC proliferation, migration and differentiation. Macrophages can also influence the composition of extracellular matrix (ECM) components such as thrombospondin or can produce proteases that alter the structure of ECM.

TNP-470 is a potent synthetic inhibitor of angiogenesis in vivo that suppresses tumor growth and is now in clinical trial. Treatment of mice with systemic TMP-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 post wound 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, post incision. In contrast, treatment with TNP-470 prior to wounding or treatment beginning on day 5 after wounding did not significantly affect wound healing. TNP-470 decreased the concentration of basic fibroblast growth factor, by 42% in the full thickness wounds. Histological changes also occurred. We conclude TNP-470 suppresses wound healing in a time-dependent manner and does not affect wound healing if given before or five days after the wound is made.

Angiogenesis and Vascular Diseases

Coronary occlusive disease is the leading cause of death in industrial nations and affects one in four adults. Although heart attacks are caused by occlusion of a coronary artery, some patients have total occlusions of new coronary artery without heart attack because of adequate collateral circulation. Many angiogenic promoters such as VEGF, FGF, and other factors can promote collateral vessel development in ischemic myocardium. An attractive possibility is that VEGF 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.

Potential Therapeutic Applications of Anti-Angiogenic Agents

Because of the unmet medical need and the market size of the previously listed pathological angiogenesis-mediated diseases, several pharmaceutical and biotechnology companies are heavily investigating various strategies in providing novel therapeutics (Table 1.2). Complete inhibition of angiogenesis should be well tolerated in most adults since under physiologic conditions angiogenesis is required only for wound healing and reproduction. Anti-angiogenic agents that bind to heparin

binding growth factors, inactivate matrix metalloproteinases, inhibit endothelial cell proliferating or target developing endothelium are currently being tested as single agents in clinical trials. Recombinant proteins such as interferon-α and Platelet Factor 4 may also have antiangiogenic activity through mechanisms that are not yet completely defined. Because they act through diverse mechanisms of action, antiangiogenic agents may achieve maximum biologic effect when administered together.

Critical Issues

What anti-angiogenic strategy?

- Cytokines/growth factor
- Cell adhesion molecules/matrix proteins
- Other angiostatic mechanisms

Is there a specific anti-angiogenesis strategy per specific angiogenic disease process?

Can a single mechanism anti-angiogenic agent be effective enough given the redundancy in the mediators?

Initially, angiogenic triggers may be different and specific for each disease. However, the final pathways and crucial steps are most probably regulated and modulated by similar mediators. It is possible that there is a final common pathway for the various pathological angiogenic-mediated diseases.

What disease target?

- Cancer
- Ocular
- Chronic inflammatory
- Cardiovascular

What animal model is clinically relevant?

What endpoints?

- Biochemical markers (Fluid phase angiogenic markers, microvascular density,..)
- Clinical endpoints (Cancer: tumor size, survival; ocular: vision acuity,..)

The first requirement of a new prognostic indicator is that it should possess a clear biological significance. Indeed, much evidence shows that tumor growth and metastasis are dependent upon neovascularization. Tumor angiogenesis (TA) refers to the growth of new vessels toward and within the tumor; and, unless tumor neovascularization occurs, cell proliferation reaches a steady state and the tumor grows no larger than about 2 mm in diameter. Moreover, for tumor cells to metastasize, they must gain access to the vasculature from the primary tumor, survive in the circulation, localize in the target organ and induce angiogenesis in that target organ. TA is necessary both at the beginning and at the end of the metastatic cascade of events. Recently, we showed a statistically significant correlation between incidence of metastases and microvessel density (MVD) of primary invasive breast carcinomas. Now, subsequent studies have shown that the association of prognosis with MVD exists not only in breast carcinoma but also in numerous other solid tumors. Numerous reports have suggested that microvascular density (MVD) within the tumor is an important, independent prognostic factor in a variety of solid tumors. Vascular density measurements clinical trials might be a valuable early clinical marker of angiogenesis inhibitors. In addition, the biological activity of angiogenesis inhibitors can be evaluated using patient samples in a variety of standard bioassays. Inhibition of tumor angiogenesis undergoing clinical testing. Documentation of efficacy of these novel agents requires carefully designed clinical trials where tumor shrinkage may not be the primary endpoint.

Table 1.2 Selected angiogenesis inhibitor strategies under preclinical and clinical investigation

Strategy/Compound	Company
I. Cytokine/Growth Factor Inhibitors	
• VEGF monoclonal antibody, soluble receptors	Genentech
• VEGF antisense oligonucleotide	Hybridon
• Soluble FLT-VEGF receptor	Merck
• VEGF receptor-tyrosine kinases	SUGEN
antagonists, Tie1, Tie2, . . .others	ImClone Systems
• CA2 Anti-TNFα monoclonal Ab	Centocor
• IL1β and TN Fα inhibitors	SmithKline Beecham
• GM1474, sulfated oligosaccharide (bFGF)	Glycomed
II. Cell Adhesion Molecules Molecules/Matrix Proteins	
• Vitaxin-humanized forms of LM609 (anti-avβ3)	IXSYS
• Cyclic peptide avβ3 ligands	Merck
• Small molecule avβ3 antagonists	Schering-Plough, Others
III. Other Angiogenesis Inhibitors	
• Platelet Factor-4	Repligen
• TNP470 (mechanism ?)	Takeda
• Thalidomide (mechanism ?)	EntreMed/BMS
• Angiostatin	EntreMed/BMS
• Receptor tyrosine kinase inhibitors (ZD1893)	Zeneca,..
• Matrix metaloprotineases (GM6001)	Glycomed
• Urokinase receptor sntagonist	Chiron
• Antioxidant- nitrone-related therapeutics	Centaur
• BB-94 (Batimastat),MMPI (Phase I/III)- Disc.	British Biotech/SK&F
• BB-2516,MMPI, BB-94 backup, Oral (I/II)	British Biotech
• DS-4152 (Tecogalan)- phase I Kaposi sarcoma	Daiichi
• Heparinase inhibitor, pentoxifylline, angiostatic steroids	

What duration of angiogenic inhibition is needed for optimal efficacy/safety?

Long-term Preclinical Studies of the Angiogenesis Inhibitor TNP-470 (AGM-1470):

The angiogenesis inhibitor TNP-470 (AGM-1470) is a synthetic analog if fumagillin that is one of eight angiogenesis inhibitors currently in Phase I/II clinical trials for the treatment of solid tumors. Our preclinical studies showed that: (1) a wide spectrum of tumor types were inhibited in vivo with a T/C (treated/control tumor volume) of approximately 0.35; (2) this inhibition was independent of immune status or sex of the mice; (3) drug resistance did not develop even after more than 200 days of therapy and (4) wound healing was delayed by approximately 12-40%, but only if the drug was administered in the first four days after wounding. These results, accompanied by a low level of toxicity, may be used as a model for the preclinical testing of other angiogenesis inhibitors.

Can an angiostatic agent be combined with other angiostatic, cytotoxic or radiation therapies?

Potentiation of Cytotoxic Cancer Therapies by Anti-Angiogenic Agents

Tumors are dynamic, complex, living tissues undergoing the varied processes of tissue growth under the guidance of aberrant malignant cells. Cytotoxic anticancer therapies have focused solely

on the eradication of the malignant cell, which is an absolute necessity; however, even the most heroic therapeutic strategies rarely achieve cure of many tumor types. The recognition that the growth processes of tumors are normal processes, that the invasion processes of tumors are normal processes and that it is inappropriate activation of the processes that comprises the morbidity of malignant disease allows the elucidation of a broad spectrum of new therapeutic targets in cancer. Anti-angiogenic agents are now available. The integration of anti-angiogenic agents into existing cancer treatment regimens has allowed the cure of established Lewis lung carcinoma and markedly increased tumor response to many standard cytotoxic therapies. Additionally, additive effects were demonstrated when an angiostatic agent such as TNP470 was combined with other angiostatic agents such as interferon-α or with cytoxic agents.

Conclusion

Recent evidence suggests that, in spite of the redundancy of angiogenic factors potentially involved in pathological angiogenesis, strategies aimed at antagonizing one specific endothelial cell mitogen at its release or receptor levels may form the basis for an effective and safe treatment of various angiogenic-mediated disease processes. Physiologic angiogenesis is fundamental to reproduction, development and repair. Pathological angiogenesis sustains the progression of many neoplastic and nonneoplastic diseases. The idea that tumor growth is angiogenesis-dependent was first proposed by Folkman et al (1966) and now is supported by extensive experimental evidence which led to the development of the field of angiogenesis research, from which prognostic, diagnostic and therapeutic applications have entered clinical trials.

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Chapter 2

Angiogenesis and Ischemia:
The Effect of SIKVAV and NPY in Revascularization

Derrick S. Grant and Zofia Zukowska-Grojec

The vascular supply to limbs and peripheral tissues is essential for normal physiological functions. Under certain pathologic conditions, however, vascular supply may be reduced to such an extent that it leads to necrosis of the tissue.[1] Angiogenesis, the process of new vessel growth, is necessary for many normal physiological (development, reproductive cycle) and pathological processes such as tumor growth, wound healing and ischemia.[2-4] Investigators have explored the use of angiogenic agonists to revascularize ischemic tissue.[2,5] We have recently examined in vitro and in vivo the ability of two potent angiogenic compounds, SIKVAV (a peptide derived from the alpha chain of laminin-1)[6,7] and Neuropeptide Y (NPY)[8] to revascularize ischemic tissue. In this Chapter, we review the structure and activities of SIKVAV and NPY as they pertain to ischemia-driven angiogenesis.

Normal Vascular Distribution and Angiogenesis in the Limbs

The peripheral microcirculation is comprised largely of an extensive network of arterioles, capillaries and postcapillary venules, which allow a rich distribution of nutrients to the muscles, cutaneous and subcutaneous tissues. Blood supply to the lower limb is primarily provided by the femoral artery and its branches (profundus, tibial and peroneal). Closely associated with these vessels are nerves, some of sensory origin and most of them being sympathetic, providing the vessels with resting vasoconstrictor tone. Sympathetic nerve activity is, in fact, the main regulator of limb blood flow at rest but it also contributes to redistribution of flow to the skeletal muscle during exercise. Blockage of arterial supply by a thrombus or constriction results in tissue hypoxia which release several angiogenic factors, including bFGF, VEGF and IL-8. Additionally nerve plexuses may secrete trophic factors that may stimulate vascular growth. In most cases, however, the endogenous supply of angiogenic factors is insufficient; it does not produce enough of an inductive effect to fully revascularize the ischemic tissues. Isner has shown that the application of exogenous growth factors to patent vessels surrounding ischemic regions can result in increased collateralization and a subsequent increase in blood flow.[9-14] Under this treatment, local arterioles and capillaries branch and become angiogenic especially with the use of angiogenic factors such as bFGF and VEGF.[12]

Ischemia in Diabetes

It has been shown that peripheral vascular, cerebrovascular and cardiovascular diseases are more prevalent in diabetics than in the normal population.[15] A prospective study using ankle-to-brachial arterial pressure ratios has also shown that occult peripheral arterial disease is much more common in patients with type II non-insulin controlled diabetes that in unaffected controls. The associated risk factors in diabetic ischemia included raised plasma triglyceride and glucose levels, reduced plasma concentrations of high-density lipoproteins, hypertension and increased systolic blood pressure.[15] It is surprising, however, that the exact etiology of vascular disease in the diabetic population is not

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known. Calcification is often associated with diabetic vessels but does not seem to be associated with the occlusive disease, nor is there any evidence for intimal involvement.[15] The thickened basement membrane in the vessels of the diabetic foot may also play a role in the disease state because of altered cellular nutrition due to reduced vascular permeability. Nevertheless, the distribution of macrovascular occlusive disease in diabetic patients seems to differ from that of non-diabetics. For instance, amputated specimens from the diabetic patients demonstrated that uncalcified occlusive artherosclerosis tends to involve the infrageniculate arteries between the knee and ankle[16] rather than inguinal branches. The fairly low number of native and functional collateral vessels found below the knee further exacerbates the problems associated with occlusive arteries in diabetics. In addition, existing collaterals often have dysfunctional shunting mechanisms due in part to diabetic peripheral neuropathy involving sympathetic nerves. In many patients infrainguinal bypass has been very successful and the literature contains significant data that suggest that an early aggressive surgical treatment of lower-limb occlusive arterial disease may reduce the frequency of ischemia or amputation in diabetic patients. It should be pointed out that although 80% of infrainguinal bypass surgical treatment is successful, another 10-15% of cases fail.[17,18] Therefore, an alternative treatment is needed for patients who may be at risk of losing patency in the bypass vessel and subsequently undergoing amputation.

Cardiovascular Risk in Diabetic Patients

Diabetic patients also have higher incidence of cardiovascular occlusion and myocardial infarct. The most common form of heart disease (80%) that results in heart failure is due to vascular insufficiency leading to ischemia and subsequent infarction. Coronary collateral vessels protect the myocardium from ischemia during coronary artery stenosis.[19] Most hearts have some intercoronary anastomoses which may be as large as 100 μm in diameter but these may be insufficient to provide appropriate perfusion during vascular occlusion. The changes in the cardiac ischemic tissue include: local hypoxia, edema, cardiac myocytic degeneration, pyknotic nuclei, and accumulation of granulation tissue and biochemically, a loss of lactic dehydrogenase and potassium. One final indication of cardiomyopathy is changes in mitochondrial oxidative phosphorylation and a decline in myocardial energy store.[1] Localized branching of coronary arterioles and capillaries usually helps to decrease the degeneration of the local muscle mass but may only result in sustaining 10-20% of the ischemic area. These features are also typically seen in ischemic skeletal muscle. Local angiogenesis is directly associated with increased amounts of several growth factors. For instance, canine myocardial infarct tissue contains more bFGF than noninfarcted tissue.[19] It is unclear, however, which cells are producing the angiogenic factors or if this local increase in angiogenesis can stimulate sufficient angiogenesis and blood flow to prevent long-term ischemia or infarctions leading to death of the animal.

A possible treatment for the prevention of tissue infarction as well as limb ischemia might involve the local administration of an angiogenic compound in the hypoxic area. This local administration would stimulate revascularization of the ischemic tissue and could rescue it from the irreversible damage caused by infarction. Several studies have investigated the efficacy of enhanced revascularization of ischemic tissue stimulated by local release of angiogenic factors. Two angiogenic growth factors, basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF), have been used successfully to revascularize ischemic tissue.[2,12,14,20] The mechanism of tissue ischemia in the heart, the angiogenic responses and the resulting expression of growth factors are very similar to the ischemic limb. Therefore, exogenous addition of growth factors has been investigated as a possible treatment for tissue ischemia. For example, bFGF has been shown to be a potent angiogenic factor.[5] Others[19] have examined the effect of localized administration of bFGF to ischemic canine myocardium and found an increase in coronary capillaries in ischemic hearts. However, bFGF has a short half-life (in the order of seconds to minutes) in vivo and under surgical procedures requires either an extremely large initial dose or repeated or continuous administration.[21] For these reasons, its use in a clinical setting might be cost-prohibitive. Finally, bFGF has been shown to have an effect on cell types other than vascular cells.[2] VEGF has shown some promise as to its specificity and has been shown to induce revascularization in the ischemic hindlimb of rabbits.[13,14]

These growth factors appear to require heparin, which plays an important role in the interaction with their receptor; this includes bFGF and (although to a lesser extent) VEGF.

While the in vivo potency and angiogenic efficacy of exogenous bFGF and VEGF are undisputed, it is still unclear what role they play endogenously and to what extent their actions involve interactions with other growth factors, proteases and extracellular matrix proteins (components of a complex network of systems participating in angiogenesis). It has been the intent of our laboratories to determine both in vitro and in vivo if new factors such as the laminin-1-derived peptide, SIKVAV, or the sympathetic nerve-derived neurotransmitter, neuropeptide Y, are angiogenic and could be used to revascularize ischemic tissue.

The Role of Laminin Peptides in Angiogenesis

The laminin-derived peptide SIKVAV[6,22,23] seems not to require heparin and has a direct effect on endothelial cell behavior. The SIKVAV peptide is smaller than the other growth factors and can be synthesized; therefore if proven to be effective in angiogenesis, it has potential clinical applications and may be practical to use alone or in combination with VEGF in myocardial revascularization.

The basement membrane is a biologically active structure in direct contact with the endothelium of the vessels and has a strong influence on the maintenance and stability of the endothelium,[24] as well as on the regulation of angiogenesis.[25,26] The basement membrane consists of numerous glycoproteins and proteoglycans, namely laminin, collagen IV, entactin, and perlecan.[27,28] The laminins are a family of large trimeric glycoproteins, several members of which have been shown to promote cell adhesion, migration, proliferation, and differentiation.[29-32] Currently, the laminin family consists of ten isoforms.[32] One predominant isoform, laminin-1 (EHS-laminin; 800,000- 1,000,000 Da) consists of three disulfide-linked chains, designated alpha 1 (Mr=400,000), beta 1 (Mr=210,000) and gamma 1 (Mr=200,000), all of which have been cloned and sequenced.[33] Specific biologically active regions of laminin-1 have been identified based on the activity of synthetic peptides corresponding to sequences in the protein produced by elastase digestion.[34] Previously, our studies showed that endothelial cells utilize the laminin-1 RGD sequence for attachment to laminin, while the YIGSR site is involved in both attachment and cell-cell interactions during morphogenesis. A third active sequence in the alpha 1 chain domain, designated PA22-2 (CSRARKQAASIKVAVSADR), promotes neurite outgrowth of PC12 cells and increases the number of metastatic lesions to the lung by melanoma cells.[23,34,35] The peptide also induces an increase in melanocyte motility, invasiveness into Matrigel, and most importantly, an increase in collagenase IV (gelatinase) activity, a key enzyme in basement membrane degradation.[34] The peptide also increases plasminogen activation.[6] These observations led to investigation of whether SIKVAV played a role in the stimulation of angiogenesis, since cell motility, invasiveness, and collagenase activity are all important mechanisms of neovascularization.

In vivo, SIKVAV has been found to be angiogenic in the Matrigel mouse assay[6,7,36] a useful model for the assessment of the in vivo angiogenesis.[36] Briefly, a bolus of Matrigel (4°C) containing the test factor is injected subcutaneously into the groin region of a C57/Bl6 mouse, where it gels. Seven to ten days post-injection, the plug is excised and sectioned and the number of vessels is quantified. SIKVAV increased mean vessel density in the Matrigel plugs in a dose-dependent manner as compared to control, vehicle-injected plugs and at doses as low as 10 μg/ml (2 μM).[7] It was therefore clear that SIKVAV had potential angiogenic capabilities in vivo. Additionally, to test the peptide's ability to induce collateralization, we examined the effect of SIKVAV in coronary ring assays. In this assay human internal mammary artery rings were cultured in fibrin in vitro. The rings were incubated in medium containing no peptide, control peptide, or SIKVAV itself at 100 μg/ml. Some capillary sprouts were seen with the rings containing the control peptide, however almost twice as many sprouts were observed with rings incubated with the SIKVAV peptide (Table 2.1). The next step was to determine whether the peptide plays a role in the revascularization of ischemic tissues.

Table 2.1 Capillary sprout formation (number)

Peptide(100 μ g/ml)	Day 2	Day 4	Day 5	Day 7
No peptide	7	25	75	200
Scrambled (SIKKV)	10	25	76	200
SIKVAV peptide	10	49	125	325

SIKVAV in Ischemic Hindlimb Revascularization

It has been the interest of the laboratory to investigate the use of this laminin peptide SIKVAV to revascularize ischemic hindlimb. We have used a rat model where longitudinal incision was performed, extending inferiorly from the inguinal ligament to a point 7 mm proximal to the patella of both limbs. Through this incision of one limb, using surgical loops, the femoral artery was dissected free, along its length in this region and ligated proximal to the adductor hiatus. A slow release LVAX pellet impregnated either with no peptide (placebo), a control peptide (100 μg/day), VEGF(VEGF-1, 165) (50 ng/day) or SIKVAV (100 μg/day) was placed in the popliteal fossa. These pellets were small, approximately 5 mm (containing calcium alginate), remain throughout the experiment and dissolved by 21 days. With this method, 50-100 μg of peptide per day was released during the first week. The incision was then sutured and the animal was allowed to recover for two weeks. Evaluation of angiogenesis in the ischemic limb was first done by opening the abdomen of each rat and inserting a catheter into the abdominal aorta and injecting radiopaque dye into the lower limbs. Radiographs were then taken to evaluate the degree of blood flow to the limb (Fig. 2.1). Limbs which did not have a ligated femoral artery (sham operated) showed normal blood flow to the adductor muscles and calf muscles. Additionally, the planter vessels of the footpads could clearly be seen (Fig. 2.1). In contrast limbs where the femoral artery was tied off showed markedly reduced blood flow to the thigh and calf and little or no flow to the foot. Limbs that contained a VEGF (VEGF-1, 165) or SIKVAV slow release pellet demonstrated remarkable revascularization of the limb including blood flow to the adductor and calf muscles (Fig. 2.1). Note, limbs with control peptide appeared similar to ligated untreated limbs; however, the control peptide had a slight increase in blood flow to the adductor compartment but little or no flow to calf and foot (not shown).

We used a second method to evaluate the degree of blood flow to the tissues and muscles of the hindlimb. Following the surgery and 14-21 day recovery, the rat's aorta was cannulated as above. However this time latex was injected into the vascular bed and allowed to harden. Tissue samples from the adductor and calf muscles and the footpad were excised and processed for H & E light microscopic histological sections. Evaluation of normal sections of the rat footpad (Fig. 2.2) showed the presence of latex in the capillary bed adjacent to the dermal papilla in the skin of the footpad. Little or no latex was seen in the limbs with ligated femoral artery indicating that little blood flow was reaching the skin in this area. This condition mimics the situation observed with diabetic patients. When SIKVAV or VEGF-treated limbs were examined, all the capillaries in the dermal region of the skin were filled with latex indicating blood flow to the region. We examined the adductor muscle from the normal limb and compared it to the limb treated with SIKVAV and found that there was little difference in latex content in the capillaries surrounding the muscle fibers (Fig. 2.2). These data indicate that SIKVAV has an effect on inducing revascularization of the hindlimb similar to VEGF. We do not know, however, whether the increase in blood flow to the ischemic limb is due solely to

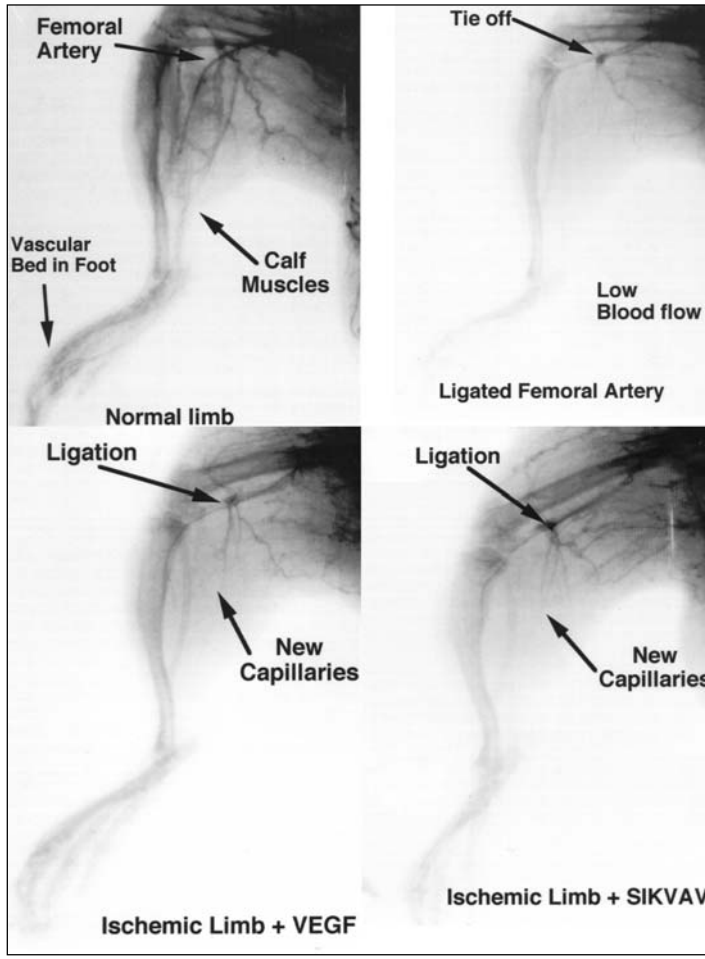

Fig. 2.1.Radiographs of 250 g rat hind limb following injection of contrasting dye in the abdominal aorta. Upper left panel shows normal rat limb whereas right panel shows a limb that has had the femoral artery ligated. Two lower panels show new capillaries forming in the adductor compartment and calf of the limb following femoral ligation and treatment with either VEGF or SIKVAV. Magnification = 500X.

neovascularization or a combination of new angiogenesis and collateralization. Secondly it would be interesting to determine the effect of SIKVAV on angiogenesis.

Since SIKVAV has been shown in vivo to attract polymorphonuclear leukocytes in the Matrigel/Murine plug assay[36] with subsequent enhancement of the angiogenic process, it will be important to examine the contribution and distribution of neutrophils, monocytes, and cytokines in the area of induced ischemic tissue. In fact monocytes and macrophages are known to express several growth factors including VEGF. This will help us determine what cells are involved in neovascularization and what cytokines help to promote the angiogenic activity of SIKVAV.

The Role of Neuropeptide Y in Angiogenesis

Sympathetic nerves have been considered to provide a trophic action on blood vessel development, and this action was believed to be mediated by catecholamines. However, catecholamines, at

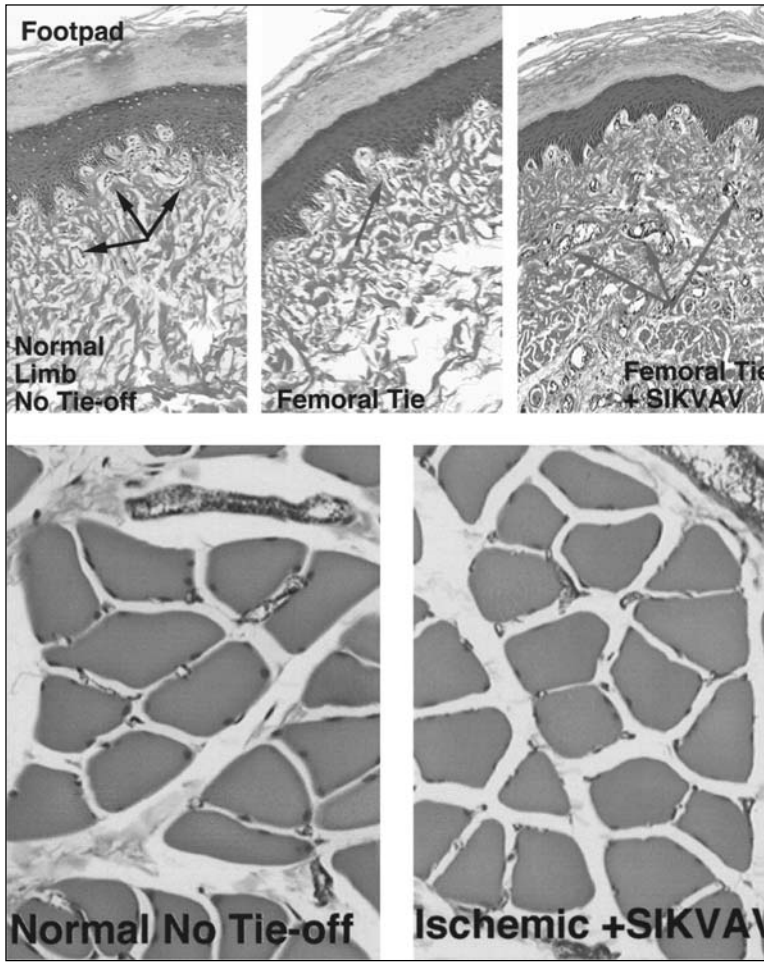

Fig. 2.2. Light micrograph of H&E histological section of the skin from normal rat footpad or footpad following femoral ligation. Latex was perfused through the abdominal aorta and can be seen in the capillaries within the dermal papilla (arrows). The lower panels show H & E histological sections of rat adductor muscle, normal (left) and ischemic (right) treated with SIKVAV. Magnification = 500X

physiological concentrations have weak or no direct mitogenic activities for either VSMCs or endothelial cells.[37,38] Neuropeptide Y (NPY), a sympathetic non-adrenergic transmitter, is the most abundant of all peptides in the heart and the brain (reviewed in ref. 39). It is released within cardiovascular tissues during exercise, stress and ischemia, and causes vasoconstriction by acting at Y1 receptor, one of the five of its Gi-coupled receptors.[39] More recently, our[40] and other[41] laboratories have discovered that NPY is also a potent mitogen for vascular smooth muscle cells. Unlike the NPY's vasoconstrictor activity (which is restricted to the Y1 receptor activation and requires a full molecule of the peptide at concentrations of at least 10-fold higher than the circulating levels) the mitogenic effect of NPY occurs over a wide range of concentrations beginning with sub-pM, and involves both Y1 and non-Y1-receptors (Fig. 2.3).[42] This has led us to propose that NPY's

physiological function is foremost that of a vascular growth factor and only in pathophysiological conditions would the peptide additionally become a vasoconstrictor .

To determine whether NPY fulfills the criteria of a trophic vascular factor, we undertook further studies of its potential angiogenic activity. In several in vivo and in vitro assays, NPY was found to be angiogenic. At low physiological concentrations, in vitro, NPY promoted vasodilation, adhesion, migration, proliferation and capillary tube formation by human umbilical vein endothelial cells (HUVECs), and enhanced sprouting of the rat aorta (Fig. 2.4), with potencies comparable to those of VEGF and bFGF.[42] In vivo, in murine and chick angiogenic assays, NPY was angiogenic and as efficacious as bFGF (Fig. 2.5). Under these conditions in vitro NPY's action involved activation of both Y1 and Y2 receptors. Of the two, the Y1 receptor responded with more immediate upregulation of expression within the first 6 hrs of capillary tube formation on Matrigel, but its expression subsided by 20 hrs and its activation was unable to fully mimic the NPY's effect.[42] Conversely, the Y2 receptor expression was initially less dramatic but increased to comparable levels and remained sustained for 20 hrs of cell differentiation on Matrigel. Additionally, the Y2 receptor agonist, NPY3-36, fully mimicked and Y2 receptor antagonist, T4 [NPY27-36]4), blocked the NPY's effect.[42] Thus, it is the Y2 which appears to be the main NPY angiogenic receptor (see diagram, Fig. 2.3).

When examined, the endothelium was found to express not only Y1 and Y2 receptors and be a site of peptide action, but also to be a source of the NPY's "converting enzyme", dipeptidyl peptidase IV (DPPIV).[42,43] This endothelial membrane-bound serine-protease is also present in activated T and B lymphocytes (as CD26)[43] and has been implicated in wound healing, AIDS[44] and metastasis formation either directly or in association with gelatinase,[45] another protease involved in angiogenesis. Interestingly, DPPIV terminates NPY's Y1 activity and cleaves a dipeptide Tyr_1-Pro_2 from NPY1-36 to form a Y2 agonist, NPY3-36 (Fig. 2.3).[43] Processed NPY3-36 loses its ability to activate vasoconstrictor Y1 receptor but is strongly angiogenic.[42] Our recent data indicate that blockade of DPPIV with inhibitors such as Diprotin A and aminoacylpyrrolidine-2-nitriles[46] or monoclonal antibodies which neutralize its enzymatic activity[45] attenuates growth-promoting effects of NPY1-36 in vascular smooth muscle cells[47] and abolish the NPY1-36- (but not NPY3-36-) induced endothelial cell migration in a wound healing assay (unpublished observation from our laboratory). Thus, DPPIV appears to be an important regulator of NPY's angiogenic activity, and may determine whether or not NPY release in the ischemic tissues such as the heart leads to vasoconstriction via Y1 receptors or angiogenesis via Y2 receptors (Fig. 2.3). This enzyme may also be a link between the two systems under study in our laboratories, laminin and NPY, via its association with other proteases (elastase and gelatinase) whose activation leads to the generation of angiogenic fragments from laminin such as SIKVAV. Thus, a proposed cascade of events initiated by angiogenic stimuli may involve activation of elastase, DPPIV and gelatinase, degradation of extracellular matrix and liberation of angiogenic laminin-derived peptides such as SIKVAV, and formation of NPY-derived fragment, NPY3-36, which became inactive as a vasoconstrictor but is potently angiogenic (Fig. 2.3).

Initially we have assumed all angiogenic activity of NPY to come from the sympathetic nerves. However, much to our surprise, we have found that human endothelial cells possess their own autocrine NPY, containing its mRNA and abundant intracellular stores of the peptide, colocalizing with DPPIV.[42] Thus, we have demonstrated the existence of two complete NPY systems: an autocrine endothelial and paracrine neuronal system, both of which may be important in angiogenesis during tissue repair.[48]

Neuropeptide Y and Its Role in Limb Ischemia

Sympathetic nerve activity is an important regulator of blood flow to the limbs by providing vasoconstrictor tone to blood vessels and this tone increases in states such as stress, exercise, and also ischemia. Sympathectomy has therefore been used to relieve the symptoms of peripheral vascular disease, the pain of claudication in the ischemic limb. The beneficial effect of sympathectomy, however, assumed to be due to vasodilation, is only temporary and the procedure has largely been abandoned. Considering that sympathetic neurotransmitter NPY is angiogenic and its release may be increased during ischemic states,[49,50] we postulated that the peptide plays a pivotal role in revascularization of

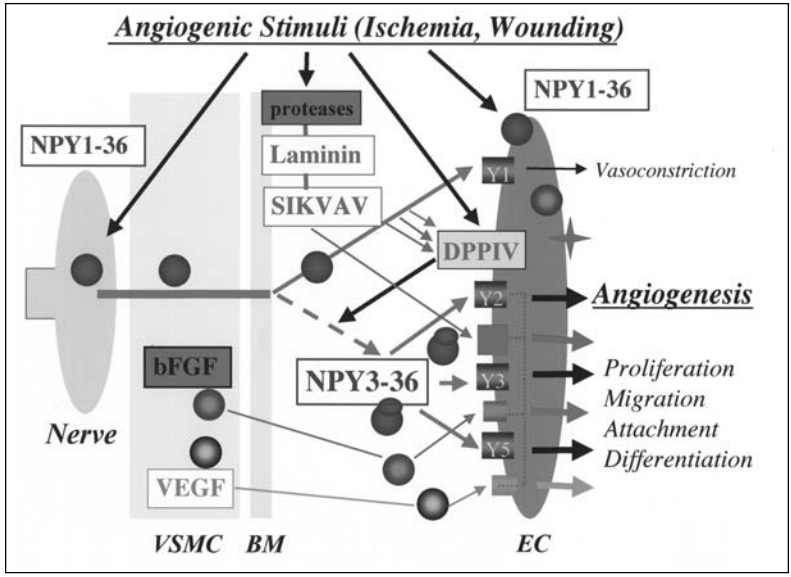

Fig. 2.3. Schematic diagram of the effect and mechanism of action of NPY and SIKVAV on the vessel wall during the induction of angiogenesis. VSMC= vascular smooth muscle; BM = basement membrane; EC = endothelial cell

the ischemic limb. However, its beneficial effects may be reduced by sympathetic vasoconstrictor tone primarily provided by catecholamines but presumably also by NPY's Y1 receptor-mediated vasoconstriction.

NPY has been found to be released from the heart during ischemic conditions such as angina pectoris, myocardial infarction, and congestive heart failure,[51] and from the skeletal muscle during exercise in humans and animals.[49,50] The exercise-induced elevation in circulating NPY levels is further augmented by hypoxia[50] but, interestingly, this response of NPY is lost in older patients.[50] Exercise is also implicated to stimulate angiogenesis in the heart and muscles, and the effect appears to be synergistic with ischemia.[52] Furthermore, the ability to respond with angiogenesis appears to be reduced with age. To test whether NPY could provide an angiogenic stimulus to the ischemic limb, we used the rat model of chronic (14 days) occlusion of the femoral artery (as above) with or without unilateral sympathectomy or local administration of NPY to the ischemic limb in a slow release pellet placed in the popliteal fossa.[48] At 14 days, femoral artery occlusion-induced ischemia increased venous output of NPY by 25% and markedly depleted NPY-immunoreactivity from the femoral artery in the ischemic compared to the non-ischemic limb, with no changes in NPY receptor expression in the skeletal muscle. Administration of NPY (1 μg/21 day in a slow release pellet, Innovative Research of America, Rockville, MD) into the ischemic limb increased venous NPY outflow three-fold, prevented vascular tissue depletion of NPY, and induced expression of NPY Y1, Y2 and Y5 receptors.[48] The slow release NPY pellet resulted in slight elevation of systemic plasma NPY levels and also induced expression of Y1 and Y2 but not Y5 receptors in the contralateral non-ischemic leg. Thus, NPY itself promotes the induction of its own receptors, independently of ischemia. Similar NPY-mediated induction of Y1 and Y2 receptor expression were observed in vitro in rat vascular smooth muscle cells, and implicated in promoting cell growth.[42] These receptors, therefore, may play an angiogenic role, in particular, the Y5 receptor since its expression was specific for ischemia. Indeed, along with its receptor induction, NPY markedly improved vascularization of the ischemic limb, increasing capillary density in the soleus and gastrocnemius muscles two-fold as compared to the untreated ischemic limb, and restored capillary density to the level present in the

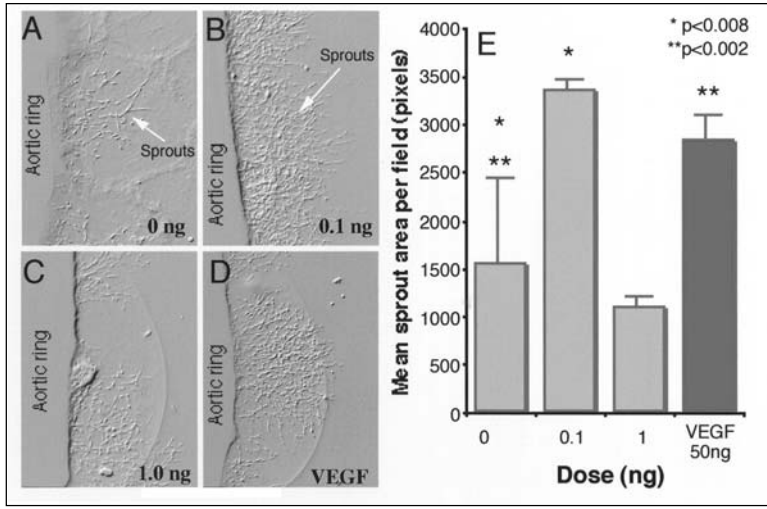

Fig. 2.4. The rat aortic ring capillary sprout assay. Left photomicrograph composite shows the edge of the aortic ring embedded in fibrin and sprouts extending into the matrix. To each well 0, 0.1 and 1.0 ng of NPY or 50 ng of VEGF was added. Graph on the right shows the quantitation of the relative density (area) of vessels extending from the cut edge of the rat aortic ring.

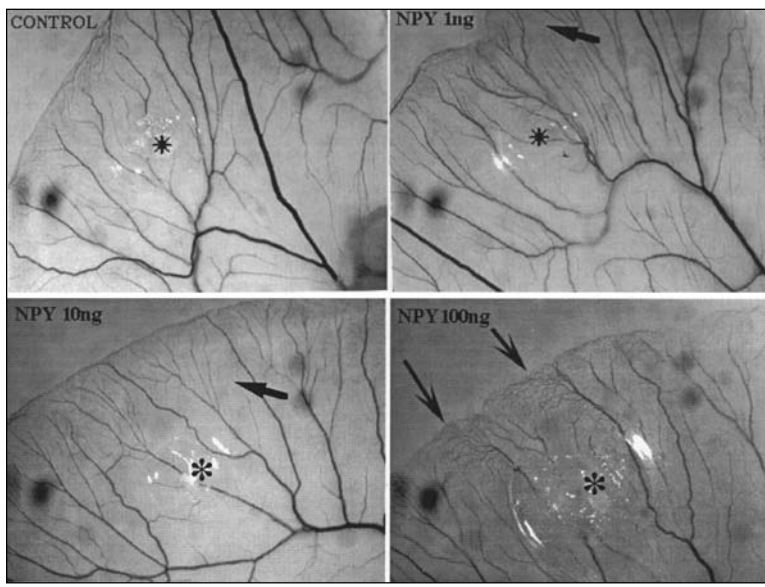

Fig. 2.5. Micrographs of the chicken yolk sac angiogenic assay. 40X magnification of the edge of a 7 day growing chicken yolk sac. The * indicates the position of the slow release pellet . Doses of 1 ng and 100 ng of NPY impregnated into the pellets show evidence of increased angiogenesis (see arrows). Note vasoconstriction of vessels around the 100 ng pellet.

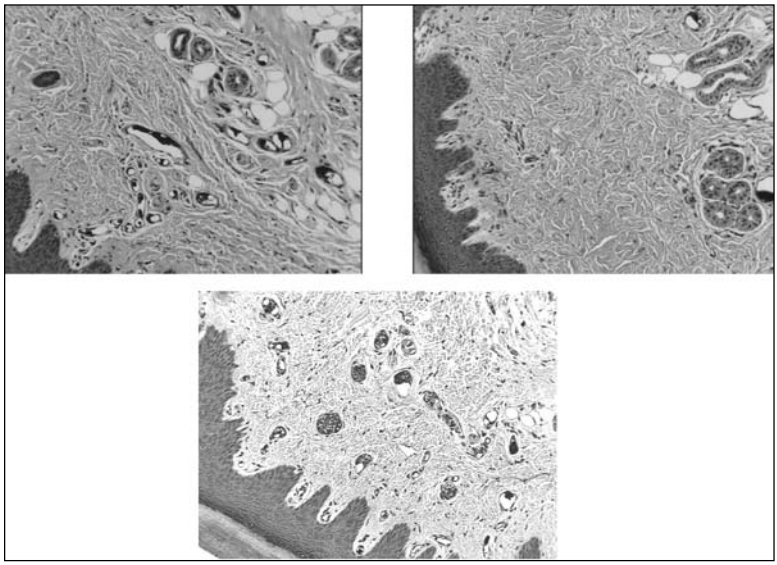

Fig. 2.6. Histological sections of the skin of rat footpad following latex perfusion. The upper left panel shows latex (dark spots) within the vessels of the dermal papilla whereas little or no latex is seen in the footpad of the ischemic limb. Latex is seen in the dermal capillaries of the ischemic limb treated with NPY. Magnification = 600X

non-ischemic muscles.[42] Similarly NPY was also able to restore blood flow to the rat footpad following femoral ligation and ischemia (Fig. 2.6). Interestingly, the effectiveness of NPY in a local delivery system both in terms of its ability to raise venous plasma NPY outflow and increasing capillary density in the ischemic limb was reduced in rats with streptozotocin-induced diabetes (unpublished observation from our laboratory) indicating that NPY processing to angiogenic fragments, detectable by our assay, is impaired in this condition.

Thus, our initial studies support the notion that the NPY system plays a role in angiogenesis in the ischemic limb. This is the first demonstration that NPY or NPY-derived peptides may be useful as angiogenic therapy for the peripheral arterial insufficiency and limb ischemia. Several factors position NPY favorably as compared to other angiogenic molecules such as VEGF and bFGF: it is a smaller molecule, thus being easier to deliver and less expensive to produce, is angiogenic at extremely low concentrations at which it is devoid of vasoconstrictor activities, it is endogenously released, thus amenable to pharmacological manipulations, and may be synergistic with other angiogenic factors and heparin. Also, by being a mitogen for both vascular smooth muscle and endothelial cells, similar to bFGF but unlike VEGF, NPY may be able to stimulate growth of not just capillaries but of fully developed vessels containing both cell elements (Fig. 2.4). We postulate that it is the Y5 and/or a combination of Y2 and Y5-type NPY receptors which are angiogenic. Thus, specific agonists activating these two receptors might be more beneficial than NPY itself, an agonist for the Y1-Y5 receptors, since they would be angiogenic but devoid of unwanted Y1 vasoconstrictive activity.

We are currently examining the effect of combining NPY and SIKVAV in the hind limb ischemic model to evaluate if the effect of these compounds is additive or synergistic. This may be an effective approach to revascularizing of chronic ischemic tissues by providing a local infusion to enhance angiogenesis. The current therapeutic options to improve tissue blood flow include surgical bypass, balloon angioplasty or endoluminal recanalization, treatments which are all hampered by significant failure rate. We hope to apply this approach to treatment of diabetic patients in an effort to alleviate ischemia and necrosis of limbs.

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CHAPTER 3

The Role of Smooth Muscle Cells and Pericytes in Angiogenesis

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The stages of angiogenesis have been defined on the basis of critical endothelial cell activities such as basement membrane proteolysis, cell migration, proliferation and tube formation.[1] Moreover, in vitro experiments have shown that endothelial cells alone contain all of the information necessary to create new capillaries.[2] Furthermore, one of the primary angiogenic growth factors, VEGF, has selective mitogenic activity for endothelial cells.[3] Thus, appropriate attention has been placed on the endothelium, while other cells, including smooth muscle cells (SMCs), are thought to have only an indirect or supporting role.

The importance of SMCs in the embryological development of blood vessels is apparent, however, since every vessel larger than a capillary is coated with layers of SMCs of variable thickness.[4] Recent studies of angiopoietin-1 and its receptor TIE-2 have demonstrated the importance of this system in the incorporation of SMCs into developing blood vessels. Mice that are deficient in angiopoietin-1[5] and TIE-2,[6] and human patients that have certain TIE-2 mutations,[7] exhibit vascular malformations that are due to a failure to recruit SMCs and pericytes to developing vessels. The platelet-derived growth factor (PDGF) B chain,[8] PDGF receptor β[9] and tissue factor[10] are also essential for the investment of vessels with SMCs and pericytes.

Another type of vascular development in which SMCs play an essential role is arteriogenesis, the expansion of pre-existing arteriolar connections into true collateral arteries. Arteriogenesis is essential to produce vessels of sufficient caliber to relieve ischemia of the myocardium, skeletal muscles and other vital organs. Ischemia-induced collateral formation in canine coronaries,[11-13] porcine pulmonary arteries,[14] and rabbit femoral arteries,[15] has demonstrated that SMCs as well as ECs proliferate during this process.

SMC proliferation has also been observed during therapeutic angiogenesis induced with growth factors. Isner and colleagues studied the ability of VEGF to induce revascularization in ischemic limbs of rabbits[16,17] and humans.[18] They found that an intravenous bolus of VEGF increased SMC as well as EC proliferation by about 2.7-fold in the rabbit ischemic hindlimb. Schumacher et al studied the effects of aFGF on angiogenesis in the ischemic hearts of rats and humans[19] and found that the new vasculature contained a three-layered wall with SMCs present in the intima and media. These data demonstrate the contribution of SMCs to vessel formation that occurs in response to ischemia and growth factors.

The Role of SMCs in Controlling Flow to the Capillary Bed

Smooth muscle cells are an important regulator of blood flow to capillary beds where angiogenesis is initiated. Not all of the vessels that form during angiogenesis develop into mature collaterals. The competition for blood flow causes variable shear stress forces in the vessel which can cause the over-proliferation of SMCs (intimal hyperplasia) and ultimately lead to the occlusion of the smaller

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collaterals.[20] It is believed that the over-proliferation of SMCs is caused by a failure of SMC apoptosis, inhibition of MMPs and/or an increase in fibronectin expression.[21,22]

SMCs also regulate capillary flow by their vasomotor function. Capillaries arise from both arterioles and metarterioles, vessels intermediate in size between arterioles and capillaries.[4] Precapillary sphincters, which are thickenings of smooth muscle cells at the origin of a capillary bed from an arteriole or metarteriole, control distal blood flow. The precapillary sphincter relaxes in response to reduced oxygen tension and constricts in response to norepinephrine. Since hypoxia is a major stimulus of angiogenesis,[23] the hypoxia-dependent flow of blood into the capillary zone is likely an important function of SMCs in angiogenesis. In support of this concept is the observation that vasodilation often occurs before endothelial cell sprouting is observed during angiogenesis.[24] Furthermore, it is interesting to note that several angiogenic factors, including VEGF[25] and bFGF,[26] induce NO synthesis by the endothelium and that the angiogenic effects of VEGF,[27] PGE1[28] and macrophages[29] are NO-dependent. It has been proposed that dilation of microvessels is an early and critical event leading to endothelial cell proliferation and migration.[24] Thus, the SMC, or perhaps the pericyte, which also exhibits vasomotor function,[30] may be critical sensors of NO production, hypoxia and other stimuli for angiogenesis.

Pericytes

Although SMCs do not cover the entire vascular tree and are notably absent from capillaries, SMC-like cells cover even the smallest vascular branches. There is a gradual transition from SMCs to pericytes on the arterial side of capillaries and then back to SMCs on the venular side.[4] This anatomical pattern has led some investigators to believe that pericytes are the "microvascular smooth muscle cell".[31] There are a variety of phenotypic similarities between SMCs and pericytes that support this concept.[31-33] Pericytes contain SMC isoforms of actin and myosin, SMC tropomyosin and cGMP-dependent protein kinase, all of which support a contractile function[31] which has been directly demonstrated.[30] The vasomotor function of pericytes may help control capillary blood flow as well as vascular permeability. Despite the similarities with SMCs, however, pericytes have a variety of distinguishing morphological and immunohistochemical characteristics.[31-33] Unlike SMCs, pericytes are enclosed by a basal lamina that is continuous with that of the endothelium and they possess non-muscle, as well as smooth muscle, actin and myosin.[31-33]

Although they are distinct cells, pericytes may be a progenitor of SMCs. It has been previously reported that pericytes can transform into smooth muscle cells.[34,35] Adventitial pericytes are a source of new medial SMCs for the injured rabbit aorta[36] and, under certain conditions, the intimal cells or arterial hyperplasia may originate from the pericytes of the adventitial microcirculation.[32] The metaplasia of SMCs to pericytes has also been observed. Nicosia et al showed that a subpopulation of intimal and subintimal (but not deep medial) SMCs could differentiate into pericytes in vitro.[37] These SMCs exhibit endothelial cell tropism and, upon differentiation into pericytes, coat microvessels that form from the endothelial cells grown in collagen gel culture. The pericytes were found to stabilize the microvessels, prolonging the lifespan of the cultured endothelium.[37]

Pericytes have a regulatory role in angiogenesis with the predominant experimental observation being an inhibitory effect. Antonelli-Orlidge and colleagues showed that when endothelial cells were cultured in direct contact with either pericytes or SMCs, their rate of proliferation was reduced.[38,39] This effect was mediated by the elaboration of activated TGF-β1 from pericytes and SMCs.[38,39] Additional observations supporting an inhibitory effect on angiogenesis is that vessels with the highest coverage by pericytes have the slowest EC turnover. Furthermore, there is a temporal association between cessation of microvessel growth and arrival of pericytes at the growing capillary. Conversely, fewer pericytes are associated with increased EC proliferation.[32]

Since TGF-β1 has mixed effects on EC proliferation with inhibition at high concentrations and stimulation at low concentrations,[40] it is possible that, under certain conditions, pericytes stimulate EC proliferation. Furthermore, TGF-β1 may act as an indirect stimulator of angiogenesis by augmenting the production of VEGF and bFGF from smooth muscle cells.[41] Under certain conditions, pericytes stimulate tube formation from microvascular ECs in vitro[42] and enhance capillary formation

in vivo.[43] There is also some in vitro evidence that SMCs can support angiogenesis. The growth of rat aortic SMCs preceded capillary growth, suggesting a role of SMCs in the migration of ECs and capillary growth.[44]

Detection of SMCs and Pericytes in Neovasculature

The importance of SMCs and pericytes in angiogenesis is supported by their detection in the neovasculature of tumors, wound healing and the endometrium. Pericytes, detected with antibodies to HMW-MAA (high molecular weight-melanoma associated antigen) and α-actin, or desmin, have been detected in the microvasculature of gliablastoma multiforme,[45] metastatic liver cancers[46] and experimental tumor models.[47] SMCs have been detected in the vasculature of glomeruloid microvascular proliferation in glial neoplasms and in intracerebral metastatic carcinomas.[48] The similarities between pericytes and SMCs often hinders a definite distinction between the two cells. Thus, cells with characteristics of SMCs and pericytes have been detected in the neovasculature of brain tumors[49] and brain metastases of small cell carcinoma of the lung.[50]

SMCs and pericytes have also been detected in the neovascularization that forms in wound healing. Ruiter et al detected pericytes in microvessels of granulation tissue from decubitus lesions, as well as wounds that were a result of 3 mm punch biopsies.[51] The distribution of HMW-MAA staining suggested that pericytes are present in the proliferating microvasculature.

Angiogenesis plays an important role in the endometrial and ovarian cycles of mammals. Myometrial coiled arteries develop in the endometrium in response to estrogen and progesterone of the menstrual cycle. Electron microscopy studies of primate endometrial vasculature during the postovulatory period of the menstrual cycle showed the presence of SMCs in the coiled arteries.[52] The corpus luteum experiences a sprouting of blood vessels as it grows, which regresses in luteolysis.[53] It is believed that the regression is a result of EC detachment from their basement membrane and SMC contraction and proliferation that leads to occlusion of the newly formed vessels.

SMCs As Sources of Angiogenic Growth Factors

Vascular Endothelial Growth Factor

The role of vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), has been well-established as a stimulator of angiogenesis primarily through its mitogenic activity on ECs.[54,55] SMCs are an important site of synthesis of VEGF, and through a paracrine effect, may control the proliferation of adjacent ECs. Kuzuya et al demonstrated that SMC-conditioned medium induced EC proliferation and migration.[56] The growth factor in the conditioned medium was identified as VEGF through heparin-Sepharose affinity chromatography and anti-VEGF immunoblotting. VEGF is known to be expressed by SMCs in vivo. In the human fetus, the peptide is localized to epithelial cells, myocytes and vascular SMCs.[57] In atherosclerotic arteries, VEGF is localized in the ECM, while in restenotic arteries, VEGF is detected in proliferating SMCs.[58] Similarly, VEGF gene expression is present in the medial SMCs in pig iliac arteries and balloon injury elevates its expression.[59] The presence of VEGF in healthy vessels shows the potential function of VEGF in maintenance and repair of the luminal endothelium, as well as being an available mitogen for angiogenesis. In ischemic hearts, VEGF expression is induced in SMCs of arterioles adjacent to the necrotic tissue, with a lower induction in the myocytes surrounding the necrosis.[60] VEGF is detected in SMCs and pericytes lining small and large blood vessels within the tube and hilum of the ovary, which is an area of intense cyclical angiogenesis.[61]

It is believed that several angiogenic factors act indirectly by stimulating VEGF expression by SMCs. For example, hepatocyte growth factor, platelet-derived growth factor and transforming growth factor-β stimulate VEGF and bFGF expression by human vascular smooth muscle cells in culture.[41,62] Similarly, Pedram et al found that endothelins (ET-1 and ET-3) stimulate VEGF production by vascular SMCs 3- to 4-fold.[63] These studies explain the mechanisms by which some "indirect" growth factors, which do not have an effect on EC proliferation directly, are able to modulate angiogenesis in

vivo. By expressing VEGF, SMCs are able to amplify angiogenic signals from other pleiotrophic growth factors, resulting in enhancement of the angiogenic response in vivo.[62]

Hypoxia is a potent stimulus of blood vessel growth. It is believed that its mechanism is through the induction of several angiogenic growth factors, including VEGF, PDGF-BB and TGF-β.[64] Hypoxia induces VEGF expression but not bFGF expression.[41] Stavri et al found that hypoxia and platelet-derived growth factor-BB have an additive effect on the upregulation of VEGF production by vascular SMCs.[65] The mechanism for the induction of VEGF production in SMCs is not fully understood. There are studies supporting a role for both cAMP[66]and cGMP[67] in signal transduction events that lead to VEGF expression.

Several factors are known to inhibit VEGF production by SMCs. Corticosteroids inhibit PDGF-induced transcription of VEGF mRNA and secretion of VEGF protein by human vascular SMCs.[68] Atrial natriuretic peptide and C-type natriuretic peptide inhibited VEGF production in SMCs.[63]

Fibroblast Growth Factors

The FGFs are angiogenic[69] with mitogenic activity established in several cells, including ECs[70,71] and SMCs.[72,73] Human vascular SMCs are known to express and respond to aFGF, also known as heparin-binding growth factor I and FGF-1. Winkles et al found that both human aortic and human umbilical vein SMCs express HBGF-I mRNA as well as HBGF-I-like polypeptide.[74] Human aortic SMCs in culture were shown to exhibit a concentration dependent increase in DNA synthesis in the presence of aFGF. In contrast, in primary canine carotid artery SMCs grown on fibrin glue, aFGF did not have an effect on proliferation except in the presence of low doses of heparin.[75] In vivo, the treatment of aFGF in the area of a subendocardial infarction in the dog via a sponge demonstrated a striking increase in SMC proliferation in the arteries and arterioles in the injured area.[76] In uninjured dogs, there was no evidence of SMC hyperplasia.[77] Similarly, the treatment of balloon injured rat carotid arteries with intravenous administration of aFGF resulted in a dose-dependent increase in intimal hyperplasia. Gene transfer of a vector for a secreted form of aFGF into porcine artery walls induced an increase in intimal thickening at 21 days, as well as an induction of angiogenesis in the expanded intima.[78] These experiments demonstrate the proliferative effect of aFGF for SMCs, when expressed in the presence of vascular injury.

It has been shown that vascular SMCs in vitro and in vivo produce basic fibroblast growth factor. In culture, bovine and human aortic SMCs are capable of expressing bFGF.[79,80] In freshly isolated bovine aortic endothelial and rat aortic smooth muscle cells, low levels of aFGF and bFGF were detected, whereas much higher amounts of the FGFs were detected in confluent cells.[81]

In situ hybridization studies on human placenta identified the presence of bFGF mRNA in SMCs surrounding mid-sized and large placental vessels, demonstrating a potential role for bFGF in angiogenesis of the placenta.[82] Cuevas et al found that infusion of bFGF onto the adventitia or into the injured media of the rat carotid artery stimulated the growth of the vasa vasorum and smooth muscle cells.[83] Lindner et al observe a fivefold response in smooth muscle cell replication after balloon catheter denudation and bFGF infusion, while there was no increase in SMC proliferation in arteries with an intact endothelium.[84]

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF), also known as scatter factor, binds to the proto-oncogene c-met,[85,86] and is a potent angiogenic factor in vivo.[62,87] HGF is secreted by rat and human ECs and SMCs in vitro[88] and in vivo,[89] as an inactive precursor that can be activated with urokinase[90] or plasmin.[91] The c-met receptor is also expressed by both SMCs and ECs, providing a mechanism for paracrine and autocrine vascular effects.[89] Rosen and Goldberg showed that HGF stimulated bovine aortic SMC and retinal pericyte proliferation approximately two-fold.[92]

Van Belle et al compared the ability of VEGF and HGF to induce collateral formation in the rabbit ischemic hindlimb model.[62] They found that HGF-treated rabbits had higher capillary density, higher maximum flow rates, higher flow reserves, increased muscle flow index and greater reduction

in muscle atrophy compared to VEGF-treated animals. HGF probably induces angiogenesis by both direct and indirect mechanisms. The indirect actions of HGF (and other indirect factors such as TGF-β1) could be mediated via the release of VEGF from SMCs.[62]

The Role of SMCs in the Production and Degradation of the Extracellular Matrix

The production, deposition, and degradation of the extracellular matrix (ECM) has an integral role in angiogenesis. The production and organization of the ECM is an important function of SMCs in the developing blood vessel and may supercede their contractible function at early developmental stages.[93,94] The vascular ECM is composed of collagens and other proteins, elastin and proteoglycans. SMCs are a major source of ECM under physiological and pathological states.[95] The deposition of collagen may be especially important in strengthening the developing blood vessel wall. Porcine aortic SMCs have been shown to express a variety of forms of collagen (types I, III, and V).[96] Other proteins, such as vitronectin[97] or osteopontin,[98] have haptotactic capacity for SMCs and ECs. Some ECM proteins that are synthesized by SMCs, such as thrombospondin[99] and vitronectin,[100] have recognized functions in angiogenesis.

The degradation of the ECM is also important in angiogenesis to provide a conduit for proliferating ECs and SMCs to form new vessels. The most important enzymes in degrading the ECM are the matrix metalloproteinases (MMPs), with their activation from the proenzyme form being an important early step in angiogenesis. Inhibitors of MMPs, including TIMPs, can inhibit angiogenesis.[101-103] Pericytes of the neovascularization of human breast cancer have been found to express MMP-9 (92 kD type IV collagenase, gelatinase B).[104] SMCs are also known to produce several MMPs.[105] Immortalized human aortic SMCs have been found to produce MMP-1 (tissue collagenase), MMP-2 (92 kD type IV collagenase, gelatinase B), and MMP-3 (stromelysin).[106] MMP-2 is thought to be required for SMC migration through the basement membrane, as was shown in a Boyden chamber assay with rat aortic SMCs.[107]

Several factors are known to modulate MMP expression by SMCs. Heparin inhibits MMP-3 and MMP-9 (92 kD gelatinase) induction but not MMP-2 or TIMP-1 in primate arterial SMCs.[108] Interleukin-1 and tumor necrosis factor-α (TNF-α) have been found to stimulate the synthesis of MMP-9, MMP-1, and MMP-3.[109,110] Stimulated T-lymphocytes have been found to induce the expression of MMP-1, MMP-3, MMP-9, and MMP-2 in human vascular SMCs, an increase mediated by CD40.[111] Vascular SMCs have been found to express a cell-surface protein that is responsible for activating pro-MMPs.[112]

The Role of SMCs in Plasminogen Activation

Smooth muscle cells are an important source of components of the plasminogen activator system. Plasmin has several potential roles in angiogenesis, including directly degrading ECM components,[113] activating growth factors (TGF-β1,[114] HGF[91]) and MMPs[115] and releasing growth factors from the ECM (bFGF[116] and VEGF[117]). Plasminogen is activated by tissue-type plasminogen activator (tPA) and urokinase (uPA), and inhibited by plasminogen activator inhibitors (PAI). The balance between these activators and inhibitors can control the angiogenic process.

Smooth muscle cells are known to express and secrete members of the plasminogen activation cascade. Urokinase (uPA) is expressed in mitogenic SMCs[118] and uPA levels have been correlated with microvascular density in tumors.[119] Tissue plasminogen activator (tPA) is detected in the extracellular space of SMCs[120] and is increased in migrating SMCs.[118] Plasminogen activator inhibitor-1 (PAI-1) is also expressed by SMCs[121] and plasminogen increases PAI-1 secretion, suggesting a negative feedback relationship.[115,122] Balloon injured rat aortic SMCs exhibited an increased expression of tPA, uPA, the receptor for uPA (UPAR), and PAI-1 which correlated with SMCs migrating into the intima.[123] Atherosclerotic plaques have also been found to have enhanced expression of tPA and uPA.[124,125] Plasmin proteolysis in atherosclerotic lesions could induce the formation of neovascularization in the plaque,[126,127] which can lead to plaque rupture and occlusion of the vessel.

Effects of Angiogenesis Inhibitors on SMCs

Smooth muscle cells, as well as endothelial cells, may be targets for inhibitors of angiogenesis. It has recently been found that several known angiogenesis inhibitors (octreotide, TNP-470, and angiostatin) inhibit the proliferation of both ECs and SMCs. Octreotide, a synthetic somatostatin analog, was shown to have anti-angiogenic activity in the CAM assay,[128] through the inhibition of the paracrine and autocrine effects of basic fibroblast growth factor (bFGF) and insulin-like growth factor-1 (IGF-1). Danesi and Del Tacca studied the effects of octreotide on human ECs, as well as a mixed population of vascular cells in the rat aortic ring.[129] They found that octreotide inhibited both EC and SMC proliferation with maximum effect between 10^{-9} and 10^{-7} M. The inhibitory effect of octreotide was thought to be mediated by the somatostatin receptor subtypes sst_2 and sst_5.[130]

The fumagillin analogue, TNP-470, has been shown to inhibit angiogenesis in vitro and in vivo.[131] The anti-angiogenic effects of TNP-470 have been shown to involve the inhibition of EC[132] and SMC proliferation.[133] Koyama et al demonstrated the inhibitory effects of TNP-470 on DNA synthesis in PDGF-BB and IGF-1 stimulated bovine aortic SMCs and found that the SMCs were as sensitive to TNP-470 as ECs, both with IC_{50}s of approximately 0.3 ng/ml.[133] In contrast, some cancer cell lines require 10^6 times higher concentrations of TNP-470 for the inhibition of proliferation.[132] As with endothelial cells, SMCs were arrested at G_o or G_1 of the cell cycle. It is thought that the inhibitory mechanism could be dependent on the decrease in the production and activation of cyclin-dependent kinase-2 (cdk2).

It has recently been discovered that angiostatin's effects may be partly directed towards SMCs. Walter and Sane demonstrated that angiostatin binds to both ECs and SMCs in the artery wall of human coronary arteries, as well as to human aortic SMCs in culture. Angiostatin was also found to inhibit hepatocyte growth factor-induced rabbit aortic SMC proliferation and migration in vitro.[134]

Conclusion

Smooth muscle cells and pericytes have a variety of important roles in angiogenesis. SMCs appear to sense appropriate conditions for angiogenesis and amplify the growth factors that initiate this process. They provide all of the necessary proteolytic enzymes for vascular reconstruction. Once angiogenesis has begun, SMCs or pericytes may also be involved in preventing uncontrolled vessel growth, through contact inhibition of EC proliferation, expression of new ECM components, and inhibition of matrix degrading enzymes. SMCs are involved in remodeling the neovasculature and regulating its blood flow. If the capillary network is insufficient for preventing organ ischemia, SMCs and pericytes participate in the growth of larger vessels (arteriogenesis). Finally, SMCs and pericytes, as well as ECs, may be targets for anti-angiogenic therapy.

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CHAPTER 4

Integrin αv in Health and Disease—Role of αvβ3 in Metastasis, Vascular Remodeling and Angiogenesis

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The invasion, migration, proliferation and survival of cells are now known to be regulated in part by members of the integrin family of cell adhesion proteins. The integrin family is composed of 15 alpha and 8 beta subunits that are expressed in over 20 different αβ heterodimeric combinations on cell surfaces. Integrins recognize extracellular matrix proteins or cell surface immunoglobulin family molecules through short peptide sequences present in their ligands. For example, several of the integrins (e.g., αvβ3, α5β1, aIIbβ3) interact strongly with the tripeptide Arg-Gly-Asp (RGD) within the context of specific extracellular matrix or cell surface proteins.[1-3] While some integrins selectively recognize a single extracellular matrix protein ligand (e.g., α5β1 recognizes only fibronectin), others can bind several ligands (e.g., integrin αvβ3 binds vitronectin, fibronectin, fibrinogen, denatured or proteolyzed collagen and other matrix proteins).[1,2] The unique combinations of different integrins on the surfaces of distinct cell types allow each cell type to recognize and respond differentially to a variety of extracellular matrix proteins.

Integrins mediate cellular adhesion to and migration on the extracellular matrix proteins found in intercellular spaces and basement membranes.[1,2] They also regulate cellular entry into and withdrawal from the cell cycle.[4-6] Ligation of an integrin by its extracellular matrix protein ligand induces a cascade of intracellular signals[7] that include tyrosine phosphorylation of focal adhesion kinase,[8-10] increases in intracellular pH;[11-13] in intracellular Ca^{2+},[14-16] inositol lipid synthesis,[17] synthesis of cyclins,[4] and expression of immediate early genes.[5] In contrast, inhibition of integrin-ligand interaction suppresses cellular growth or induces apoptotic cell death.[5, 18-20]

Inhibitors of integrin function include function-blocking monoclonal antibodies, peptide antagonists and peptide mimetics. Short, linear peptides containing the Arg-Gly-Asp moiety can inhibit the function of several integrins. Peptide antagonists that are specific for individual integrins have also been developed.[21-24] Cyclic RGD peptides selective for αv integrin,[21] for the αvβ3 integrin[24] and for the α5β1 integrin[22-23] have been described. A rigid structure generated by cyclization has proven to be a key feature of peptide antagonist potency.[21] Initial studies demonstrate that the interatomic distances between the C_b atoms of Arg and Asp in peptides selective for particular integrins may be in part responsible for peptide selectivity.[21]

Integrin αvβ3

A number of integrin family members are expressed on any given cell type, enabling cells to respond to an array of different extracellular matrix proteins. The integrin αvβ3, in particular, is a receptor for a wide variety of extracellular matrix proteins with an exposed tripeptide Arg-Gly-Asp moiety.[3] Integrin αvβ3, the most promiscuous member of the integrin family, mediates cellular

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adhesion to vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willibrand factor, osteopontin, and adenovirus penton base, among others.[3,25-27] Thus, expression of this integrin enables a given cell to adhere to, and migrate on many extracellular matrix proteins. Recent studies also indicate that the immunoglobulin superfamily molecules PECAM (CD31) and L1 are ligands for integrin αvβ3. This interaction may regulate lymphocyte emigration from the blood stream and may play a role in the process of angiogenesis.[28,29]

Integrin αvβ3 Expression

Despite its "promiscuous" behavior, αvβ3 is not widely expressed in normal tissues. It is not generally expressed on epithelial cells and is expressed only at low levels on resting vascular smooth muscle and endothelium.[30] This receptor is also expressed at low levels on certain activated leukocytes and on macrophages. It is expressed on osteoclasts, where it may play a role in bone resorption.[31-33] Its expression on endometrium during the putative window of embryo implantation suggests that it may play an important role in fertility.[34] Integrin αvβ3 is also expressed on certain invasive tumors including late-stage glioblastomas;[35] malignant ovarian carcinoma[36] and on malignant melanomas.[37-40] However, its expression appears most prominently on cytokine-activated endothelial or smooth muscle cells and on blood vessels in granulation tissue and tumors.[20,41-44] Since the integrin αv subunit is widely expressed on most cell types and associates with several different beta subunits, integrin αvβ3 expression is likely to be regulated by β3 transcription. Transcription of this subunit is modulated by substances such as TNFα, bFGF, GMCSF, retinoic acid and vitamin D_3.[45-47]

Integrin αvβ3 in Melanoma

Integrin αvβ3 is now considered a marker of the invasive phase of melanoma, as its expression is detected on malignant but not on benign melanoma lesions.[39,40,48,49] Integrin αvβ3 expression on melanoma correlates strongly with invasive and metastatic melanoma and with a poor clinical outcome.[39,40] Integrin αvβ3 contributes to the progression of melanoma by regulating melanoma cell proliferation[50,51] and survival,[19] as well as metastasis.[37,38,52] An NPXY sequence in the cytoplasmic tail of the β3 subunit has been shown to play a critical role in mediating the αvβ3 signaling events that are required for melanoma cell migration and metastasis.[53]

Expression of Integrins αvβ3 and αvβ5 in Angiogenesis

One of the most significant of the physiological roles played by integrin αvβ3 is its critical role in the process of angiogenesis. Integrin αvβ3 is minimally expressed on resting endothelium, but is significantly upregulated on vascular endothelium in human tumors,[44,54-56] in wound healing,[44,57-58] and in neovascular ocular diseases.[59] Integrin αvβ3 expression is also upregulated in response to experimentally applied angiogenic growth factors in vitro[43,45] and in vivo.[44,60] Basic fibroblast growth factor, but not TGF-beta or interferon gamma, significantly increases β3 mRNA and surface expression in cultured human dermal microvascular endothelial cells.[43,45] Basic fibroblast growth factor and tumor necrosis factor alpha stimulate αvβ3 expression on developing blood vessels in the chick chorioallantoic membrane (CAM)[20,60] and on the rabbit cornea.[60] Peak levels of integrin expression are observed on blood vessels 12-24 hours after stimulation with basic fibroblast growth factor. Integrin αvβ3 expression is also induced by human tumors cultured on the chick CAM[20,44] and by human tumors grown in human skin explants grafted onto SCID mice.[54] Integrin αvβ3 also is significantly upregulated on vascular cells in vivo during wound healing.[44,57,58]

The promoter for the integrin β3 subunit has been cloned. It contains a vitamin D response element, AP-1 and SP-1 transcription factor recognition sequences[61] as well as ets transcription factor recognition sequences. Ets is an endothelial cell-specific transcription factor that regulates a number of genes required for vasculogenesis and angiogenesis including uPA, stromolysin and collagenase. Ets is not expressed, however, in adult quiescent endothelium.[62] The exciting new finding that Hox D_3, a homeobox gene, can regulate the acquisition of the angiogenic phenotype from the

resting phenotype and increase integrin αvβ3 expression suggests that Hox D_3 either directly or indirectly regulates the β3 promoter.[63]

Antagonists of Integrins αvβ3 and αvβ5 Inhibit Angiogenesis

The enhanced expression of αvβ3 during angiogenesis suggests that it plays a critical role in the angiogenic process. In fact, recent experimental evidence supports this notion. Specifically, antagonists of integrin αvβ3 potently inhibit angiogenesis in a number of animal models. When angiogenesis is induced on the chick chorioallantoic membrane (CAM) with purified cytokines, αvβ3 expression is stimulated by four-fold within 72 hours.[44] Topical application of LM609, a monoclonal antibody antagonist of αvβ3, inhibit angiogenesis, but other anti-integrin antibodies do not inhibit angiogenesis.[44] Application of LM609 or cyclic RGD peptide antagonists, but not of other anti-integrin antibodies or control peptides, to tumors grown on the surface of CAMs reduces the growth of blood vessels into the tumor tissue. LM609 has no effect on pre-existing vessels.[44] In addition, LM609 also inhibits blood vessel formation in early embryonic development. When micro-injected into quail embryos, the antibody inhibits dorsal aorta formation.[64] In this case, the antibody prevents vessel maturation in the developing quail by preventing lumen formation. This finding suggest that αvβ3 plays a biological role in a late event of blood vessel formation that is common to embryonic neovascularization and angiogenesis.

Antagonists of integrin αvβ3 inhibit the growth of new blood vessels into tumors cultured on the chick chorioallantoic membrane without affecting adjacent blood vessels, and also induce tumor regression.[20] Up to fivefold differences in tumor sizes are observed between treated and control tumors. A single intravascular injection of 300 μg of LM609, but not of CSAT (an anti-β1 integrin antibody) halts the growth of tumors and induces the regression of tumors as determined by tumor weight.[20] Similarly, an injection of 300 μg of a cyclic RGD peptide antagonist of αvβ3 but not of an inactive control peptide induces tumor regression.[20] Histological examination of the anti- αvβ3 and control-treated tumors reveals that few if any viable tumor cells remain in the anti- αvβ3 treated tumors.[20] In fact, these treated tumors contained no viable blood vessels.

Antagonists of integrin αvβ3 also inhibit tumor growth in human skin. In exciting studies of the effect of these antagonists on human angiogenesis, Brooks and colleagues transplanted human neonatal foreskins onto SCID mice.[54] After permitting the skin to heal, they were able to demonstrate that the majority of the blood vessels within the human skin are human in origin. They then inoculated human αvβ3 negative breast cancer cells into the skin and began treating mice two weeks later with twice weekly intravenous injections of 250 μg doses of the αvβ3 antagonist LM609 and control substances. Tumor growth was either completely suppressed (8/12) or was significantly inhibited (4/12) when compared to mice treated with an irrelevant antibody control. Angiogenesis was significantly inhibited (by at least 75 %) in the LM609 treated animals.

Antagonists of integrin αvβ3 also inhibit angiogenesis in the rabbit cornea.[60] When angiogenesis is induced by TNFα or bFGF, LM609 and a cyclic peptide inhibitor of the integrin block corneal angiogenesis by at least 85%. Normal retinal angiogenesis in neonatal mice is also inhibited by 78% when mice are treated with cyclic peptide inhibitors of integrin αvβ3.[59] Integrin αvβ3 peptide antagonists also inhibit murine retinal neovascularization in an oxygen-induced model of ischemic retinopathy.[65]

In contrast, antibody inhibitors of integrin αvβ5, a related but non-identical integrin, but not inhibitors of integrin αvβ3, block angiogenesis induced by the growth factors VEGF and TGFα. A cyclic RGD peptide that inhibits both integrins is able to inhibit angiogenesis independent of the stimulus used. Chick chorioallantoic membrane angiogenesis induced by VEGF or TGFα is also inhibited by either the cyclic RGD peptide or the integrin αvβ5-specific antibody, but not by LM609.[60] Previous studies indicated that the integrin αvβ5, but not the integrin αvβ3, is activated by a protein kinase C dependent mechanism.[66] Thus, the phorbol ester PMA activates αvβ5 in a calphostin C inhibitable manner. It is interesting to note that PMA-induced angiogenesis is αvβ5- but not αvβ3-dependent. In fact, calphostin C also inhibits VEGF, TGFα and PMA induced angiogenesis.[60]

Since calphostin C does not inhibit αvβ3 dependent angiogenesis, these results suggest that two different signalling pathways mediate integrin dependent angiogenesis.

Integrin αvβ3 in Restenosis

The expression of these receptors on cytokine-stimulated blood vessels also suggests they may play roles in vascular proliferation and migration events associated with restenosis after angioplasty. Restenosis is thought to be caused by the activation of vascular smooth muscle cells to proliferate and migrate into the site of an arterial wound, such as occurs during angioplasty.[67] This process results in the reocclusion of blood vessels previously cleared by the angioplasty procedure. During restenosis, the healing of arterial wounds, or in atherosclerotic plaques, osteopontin, an extracellular matrix protein ligand for αvβ3 and possibly αvβ5, is significantly upregulated.[68-70] The calcification of atherosclerotic plaques may be induced by osteopontin expression, since osteopontin is a protein with a well characterized role in bone formation and calcification.[71] Vascular smooth muscle cell migration on osteopontin is dependent on the integrin αvβ3 and antagonists of αvβ3 prevent both smooth muscle cell migration[69,70] and restenosis in some animal models.[71,72] In fact, 7E3, a function-blocking anti-integrin β3 antibody that recognizes αvβ3 as well as platelet integrin αIIβ3 was recently approved for use in treatment of high risk angioplasty.[74]

Integrin αvβ3 and Apoptosis

αvβ3 antagonists block angiogenesis by selectively promoting unscheduled programmed cell death (apoptosis) of newly sprouting blood vessels. When bFGF stimulated CAMs are isolated from chick embryos which are treated by intravascular injection of CSAT (anti-β1), LM609 (anti- αvβ3) or saline, a significant increase in DNA laddering can be observed in LM609 treated CAMs but only after 48 hours of treatment.[20] CAMs are further analyzed by dissociation into single cell suspensions by collagenase treatment; DNA fragmentation is quantified by staining of cells with ApopTag an immunohistological stain that detects free 3′ OH groups of fragmented DNA.[75] Apoptag analysis reveals that a four-fold increase in apoptotic cells is induced on the CAM by either LM609 or cyclic RGD peptide treatment but not by control antibodies or peptides.[20] Furthermore, 25-30% of total CAM cells showed the morphological signs of apoptosis, including nuclear condensation and fragmentation, only in αvβ3 antagonist treated CAMs. Cryostat sections from CAMs treated for 2 days prior to analysis with either CSAT or antagonists of αvβ3 were analyzed for Apoptag and LM609 immunoreactivity to identify the cell types undergoing apoptosis in these tissues. In this case, CAMs treated with antagonists of αvβ3 exhibited intense ApopTag staining that colocalized with LM609 staining, on endothelial cells lining immature blood vessels. Minimal apoptosis was detected among non-vascular cells in these tissues. In contrast, CSAT treated CAMs exhibited minimal apoptosis of blood vessels suggesting that β1integrin antagonists had no effect on vascular cell survival or death.[20]

To further evaluate the effects of these antagonists on vascular cell events, single cell suspensions were prepared from CAMs treated with bFGF and in the presence or absence of LM609. These cells were then stained with the DNA dye, propidium iodide, to examine the content of DNA per cell. Cells with greater than one copy of DNA were presumed to have entered the cell cycle. These cells were then co-stained with ApopTag to evaluate their viability. This costaining procedure reveals that bFGF induces cells to enter the cell cycle and that LM609 induces programmed cell death of these same cells. These findings demonstrate that Mab LM609 can induce apoptosis of vascular cells that have responded to the cytokine.[20] These results indicate that LM609 promotes apoptosis among vascular cells at a point that is late in the cell cycle. More importantly, these findings demonstrate that antagonists of αvβ3 impact a late stage of angiogenesis perhaps during the process of vessel maturation. This is consistent with the studies by Drake et al,[64] showing that antagonists of αvβ3 block late stage development of new blood vessels in the quail by preventing lumen formation. Together, these findings are consistent with the notion that αvβ3 provides a survival signal to proliferative vascular cells during new blood vessel growth. Presumably, after new blood vessels are

fully mature, the vascular cells are refractory to antagonists of this integrin. These findings may explain why antagonists of αvβ3 selectively impact newly growing blood vessels.

Further analysis of the molecular basis behind this apoptosis induction indicates that interruption of αvβ3 signal transduction inhibits angiogenesis by stimulating p53 activity and increasing the expression of cell cycle inhibitor p21WAF/CIP1. In contrast, ligation of the integrin blocked p53 activity, p21 expression and increased the bcl2/bax ratio.[76] It is not currently known if integrin αvβ5 antagonists also induce apoptosis in angiogenic blood vessels or if its mode of action is significantly different.

Integrin αvβ3 and Proteinases

Cellular invasion is a key element in both angiogenesis and in tumor cell invasion and metastasis. Recent, exciting new studies show that integrin αvβ3 localizes the proteolytically active form of the matrix metalloproteinase MMP-2 to surfaces of cells expressing this integrin. This localization then enables cells to migrate through a locally degraded matrix. The interaction between integrin and protein depends on the C-terminus of the MMP-2 molecule.[77] The studies suggest that integrins and proteinases cooperate to promote invasion and imply that inhibition of the integrin-protease interaction can potentially prevent metastases.

Conclusion

In summary, angiogenesis depends on the stimulation of quiescent vascular cells by growth factors released from tumors or other diseased tissues and also on the interaction of the integrin αvβ3 with one of its ligands.[20,44] Stimulated endothelial cells depend on αvβ3 function for survival during a critical period of the angiogenic process, as inhibition of αvβ3/ligand interaction by antibody or peptide antagonists induces vascular cell apoptosis and inhibits angiogenesis.[20,44] These observations open the door for further analysis of the regulation of cellular function and cell signaling by integrins, as well as for new therapeutic strategies to treat angiogenic disease. These strategies have led to the development of antagonists to integrin αvβ3 and integrin αvβ5 which promote unscheduled programmed cell death of newly sprouting blood vessels. These antagonists cause regression of pre-established human tumors growing in laboratory animals and thus may lead to an effective therapeutic approach for most solid tumors in man. These include peptide inhibitors of individual integrins as well as peptides which inhibit both integrins; non-peptidic, organic inhibitors; and chimeric or humanized antibody inhibitors of integrin αvβ3. The first antagonist, a humanized form of the antibody LM609, has already entered phase I clinical trials and the first of the peptide antagonists are expected to enter initial clinical trials this year.

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CHAPTER 5

PECAM-1 and Angiogenesis

Horace M. DeLisser

Angiogenesis, the formation of new vessels from a preexisting vasculature depends critically on the ability of the capillary endothelial cells to sever their normal cell-cell associations, to migrate through extracellular matrix, and to reform their cell-cell attachments to generate new capillaries. In the initial stages, adhesive interactions between endothelial cells and the extracellular matrix are dominant thus facilitating endothelial cell invasion of and migration through the extravascular matrix. Later, adhesive interactions between endothelial cells are supreme thereby promoting the organization of vascular tubes. The adhesive interactions of endothelial cells with each other and with the extracellular matrix, along with the adhesion receptors that mediate these interactions, are thus of fundamental importance to this process.[1,2] This indicates important roles for endothelial cell-cell adhesion molecules and endothelial cell-matrix adhesion molecules (e.g., integrins) in the formation of new vessels. The participation $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in in vivo angiogenesis has been demonstrated and progress has made in understanding their role in vessel formation (discussed in Chapter 3). Recently, evidence has emerged to implicate another endothelial cell-cell adhesion molecule, PECAM-1, in angiogenesis.[3] In this chapter we will review the data that has established a role for PECAM-1 in in vivo angiogenesis as well as the emerging data which suggests that it may be involved at several of the stages of the formation of new vessels.

PECAM-1—A Multi-Functional Vascular Cell Adhesion and Signaling Molecule

The platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (reviewed in DeLisser et al 1997a[4]) is a 130 kD membrane glycoprotein of the immunoglobulin superfamily that concentrates at intercellular junctions of endothelial cells[5] (Fig. 5.1, arrows) but is also found on platelets and most leukocytes. The mature form of human PECAM-1, consists of a large extracellular domain of 574 amino acids, a single membrane-spanning region of 19 hydrophobic residues and a cytoplasmic tail of 118-amino acids.[6] The extracellular region is organized into six Ig-like homology domains. Of these, domains 1 and 2 appear to be directly involved in mediating ligand binding.[7-9]

Two important aspects of this molecule should be noted. First, PECAM-1 has the ability to mediate cell-cell adhesion through multiple ligand interactions. It is able to interact both with itself (homophilic adhesion) or with a number of non-PECAM-1 molecules (heterophilic adhesion), including the integrin $\alpha v\beta 3$,[10,11] an unidentified proteoglycan,[7] an unknown ligand on activated T-cells surface,[12] and human CD38.[13] Second, a variety of studies have established that engagement of surface PECAM-1 on leukocytes, platelets and COS cell transfectants expressing PECAM-1 may transduce intracellular signals that activate the adhesive function of integrins.[14-18]

The presence of PECAM-1 on platelets, leukocytes and endothelial cells led to early speculation that it was a multi-functional vascular cell adhesion molecule involved in leukocyte-endothelial and endothelial-endothelial interactions. These initial suggestions have been largely confirmed as recent studies have implicated PECAM-1 in an expanding number of functions. Data indicate that PECAM-1 may play important roles in the recruitment of leukocytes at inflammatory sites, cardio-

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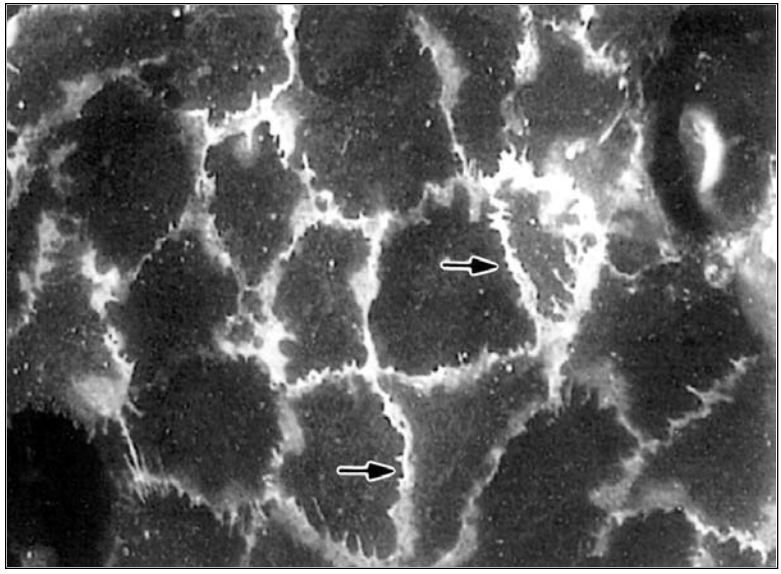

Fig. 5.1. Localization of PECAM-1 at the intercellular junctions of endothelial cells. Immuno-fluorescence staining of human umbilical vein endothelial cells stained with an anti-PECAM-1 monoclonal antibody. Adjacent endothelial cells show the characteristic concentration of PECAM-1 at intercellular borders (arrows).

vascular development, release of bone marrow leukocytes and T-cell mediated immune responses.[4] PECAM-1 may also contribute to the pathogenesis of malaria as it has been reported that PECAM-1 may function as an endothelial receptor for the binding of *Plasmodium falciparum*-infected red blood cells.[19] Recent studies by us have now provided evidence that PECAM-1 is involved in angiogenesis.[3]

The Involvement of PECAM-1 in In Vivo Angiogenesis

To directly investigate the possible involvement of PECAM-1 in in vivo angiogenesis, we studied the effect of inhibiting the function of PECAM-1, in rat and murine models of angiogenesis.[3] A polyclonal antibody to human PECAM-1, which cross-reacts with rat PECAM-1, was found to block cytokine (bFGF)-induced and chemokine (ENA-78)-rat corneal neovascularization. In mice, two monoclonal antibodies against murine PECAM-1 prevented vessel growth into subcutaneously implanted gels supplemented with basic FGF. The involvement of PECAM-1 in a biologically more complex context was studied using a well established tumor model in which the human non-small cell lung cancer cell line, A549, is grown subcutaneously in SCID mice.[20] Antibody (mAb 390 and Mec 13.3) that had been previously shown to prevent vessel growth into bFGF-containing subcutaneously implanted gels, also inhibited the growth and vascularity of A549 tumors in SCID mice, providing further evidence of PECAM-1's involvement in the formation of new vessels in vivo.[21] There is also evidence that PECAM-1 may participate in wound angiogenesis.[22]

The Involvement of PECAM-1 in Endothelial Tube Formation and Migration

Although the findings described above provide evidence that PECAM-1 is involved in the formation of new vessels, the precise mechanism(s) of its involvement in angiogenesis remains

undefined. With respect to endothelial cells, angiogenesis can be seen as composed of initial steps in which the cells are migrating into the surrounding tissue followed by latter steps in which these cells are reorganizing into vascular tubes. We therefore conducted initial studies to determine if PECAM-1 might play a role in endothelial cell migration and/or endothelial tube formation.[21]

PECAM-1 and Endothelial Tube Formation

A potential role for PECAM-1 in the formation of new vessels was initially suggested by studies of cultured endothelial cells.[16,23] Endothelial cells plated in the presence of anti-PECAM-1 antibodies adhered normally to the culture plate but failed to establish tight cell-cell contacts and the normal "cobblestone" appearance. This inhibition was reversible after removal of the antibody but was not seen if antibodies were added to intact monolayers. These findings suggested that PECAM-1-PECAM-1 interactions might be involved in the initial formation of endothelial cell-cell associations, such as those that might be required for the organization of endothelial cells into vascular tubes.

We therefore studied the effect of antibody against human PECAM-1 on the formation of tubes by human umbilical vein endothelial cells (HUVEC) plated on Matrigel.[21] Compared to BSA or anti-MHC-1 controls, the anti-PECAM-1 antibody significantly inhibited the formation of tubes by HUVEC on Matrigel (Fig. 5.2). Anti-PECAM-1 antibody (100 μg/ml), inhibited tube length by approximately 50% compared to BSA or anti-MHC-1 control (Fig. 5.3). Comparable studies with a murine endothelial cell line (H5V) plated on Matrigel[21] and rat endothelial cells cultured on collagen type I gave similar results.[3] Cell transfectants engineered to express PECAM-1 formed tubes but not control cells on a mixture of Matrigel and collagen type I.[21] Together these data provide evidence that PECAM-1 is involved in the in vitro formation of endothelial cell tubes. These data differ somewhat from those of Matsumura and associates[22] who reported that human endothelial tube formation on Matrigel was inhibited only when antibodies against human PECAM-1 and vascular endothelial (VE)-cadherin were given together but not when administered singly. The reasons for the differences between our studies and those of Matsumura et al[22] are not clear but may be due to differences in the antibodies used and the assay conditions. Our studies however do not exclude the possibility that PECAM-1 and VE-cadherin may have interactions with each other during angiogenesis or that these two intercellular molecules may have overlapping functions during this process.

PECAM-1 and Endothelial Migration

The concentration of PECAM-1 in endothelial intercellular junctions (Fig. 5.1) and the inhibition of in vitro endothelial monolayer and tube formation outlined above suggests that PECAM-1 may mediate endothelial cell-cell adhesive interactions required for the organization in vascular tubes. However the ability of PECAM-1 to mediate heterophilic adhesion and the observation that engagement of PECAM-1 transduces intracellular signals that activate the function of integrins suggests that it may participate in angiogenesis other ways. The early stages of angiogenesis involve the migration of endothelial cells into the surrounding perivascular matrix phenomena that is dependent on the integrin-mediated endothelial cell adhesion to extracellular matrix proteins.[24] A number of studies have established that engagement of surface PECAM-1 on leukocytes, platelets and COS cell transfectants expressing PECAM-1 may transduce intracellular signals that activate the adhesive function of integrins.[13-17] It is therefore possible that for endothelial cells, binding of endothelial PECAM-1 to one or more of its non-PECAM-1 ligands facilitates EC migration by augmenting integrin-dependent adhesion.

To determine if PECAM-1 might be involved in the migration of endothelial cells, the effect of anti-PECAM-1 antibodies on the closure of circular defects made in confluent monolayers of murine (H5V) and human endothelial cells (HUVEC) was investigated.[21] Compared to antibody controls, anti-PECAM-1 antibodies delayed the closure of wounds for both cell types in a dose-dependent manner (data shown for 100 μg/ml) (Fig. 5.4). During angiogenesis in vivo,

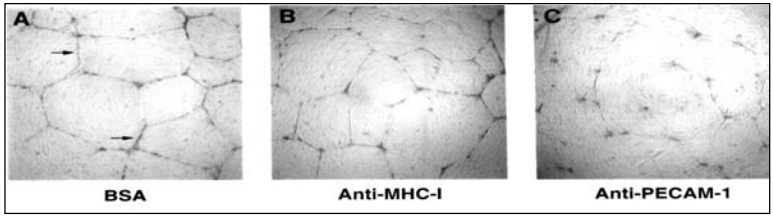

Fig. 5.2. Effect of anti-human PECAM-1 antibody on the formation of tubes by HUVEC. Shown are representative photographs of HUVEC plated on Matrigel. On this substrate, HUVEC were noted to organize into a network of tubes (arrows) in the presence of BSA (A) and antibody against MHC-I (B). However, inclusion of antibody against human PECAM-1 (C) impaired tube formation. (BSA and antibody concentration = 100 μg/ml).

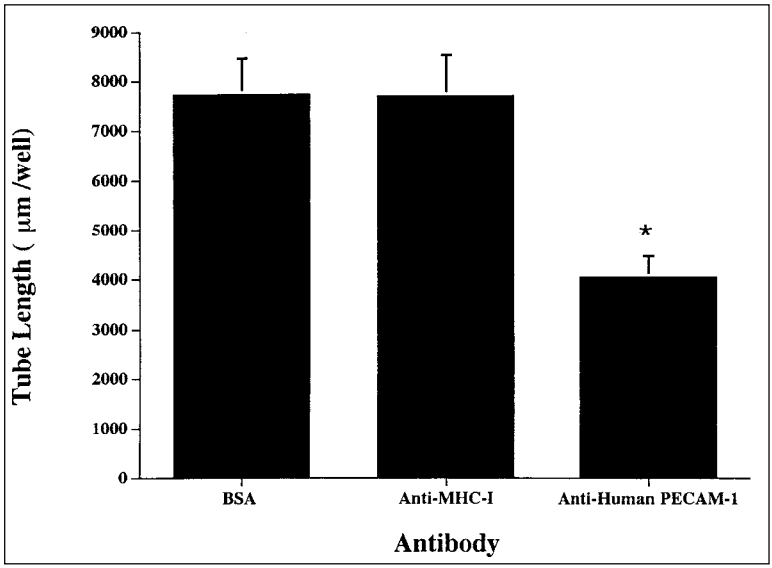

Fig. 5.3. Quantitation of the effect of anti-human PECAM-1 antibody on the formation of tubes by human and murine endothelial cells. HUVEC were plated on to Matrigel in 96 well plates in the presence of BSA, control antibody (anti-human MHC-I) or anti-PECAM-1 antibody (rabbit polyclonal against human PECAM-1) (BSA and antibody concentration = 100 μg/ml). Tube formation was subsequently assessed by determining the total length of tubes present per well (μm/well) using computer-assisted image analysis. For HUVEC, antibody against human PECAM-1 but not anti-MHC-I significantly inhibited tube formation. Data shown is representative of 4 experiments done in triplicate. (* $p < 0.01$ compared to BSA).

endothelial cell migration initially involves invasion of and movement through the matrix of the basement membrane. Therefore to further investigate the involvement of PECAM-1 in endothelial migration, we studied the effect of anti-PECAM-1 antibodies on the ability of endothelial cells to migrate through transwell filter inserts coated with Matrigel, a substrate composed of basement membrane components.[21] As shown in Figure 5.5, anti-PECAM-1 antibodies compared to BSA controls, inhibited by approximately 25 percent the passage of murine and human endothelial cells through Matrigel. Together these data provide evidence for a role for PECAM-1 in endothelial cell migration.

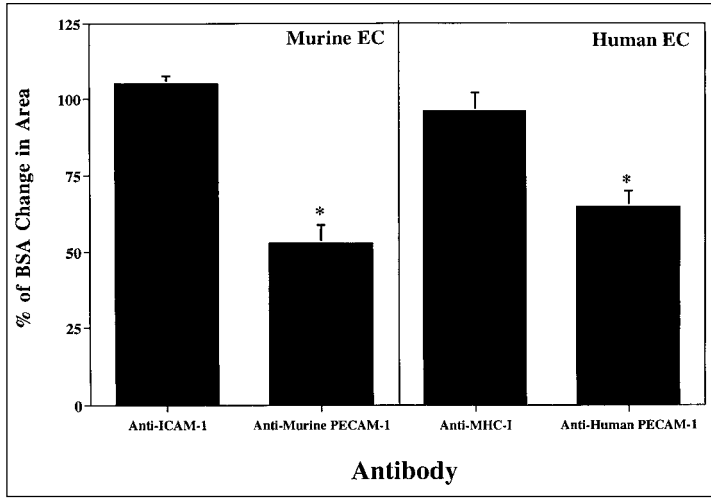

Fig. 5.4. Effect of anti-PECAM-1 antibodies on in vitro endothelial cell wound repair. Circular defects were made in confluent monolayers of human (HUVEC) and murine (H5V) endothelial cells which were subsequently cultured for 24 hours in the presence of BSA, control antibody (anti-human MHC-I or anti-murine ICAM-1) or anti-PECAM-1 antibody (rabbit polyclonal against human PECAM-1 or mAb 390 against murine PECAM-1) (BSA and antibody concentration = 100 μg/ml). Data are expressed as % of BSA Change in Area. Compared to control antibody, anti-human PECAM-1 antibody and anti-murine PECAM-1 antibody inhibited wound closure by 35% and 48% respectively. ($p < 0.01$ compared to control antibodies; n=3).

A Proposal for the Mechanisms of PECAM-1's Involvement in Angiogenesis

We have shown that TNF-α, a putative angiogenic factor, redistributes PECAM-1 out of intercellular junctions.[25] This phenomena may not be limited to TNF-α as Matthew Vadas and associates in a preliminary report have described similar findings for VEGF (The Hanson Centre for Cancer Research, 1996 Annual Report p. 92). In initial studies we too have observed that with immunoflourescene staining of HUVEC, VEGF decreased the concentration of PECAM-1 at intercellular junctions without decreasing the level of surface expression. Based on this and the data presented above the following hypothesis is presented for the mechanism of PECAM-1's involvement in angiogenesis. In quiescent vessels PECAM-1-PECAM-1 homophilic interactions in intercellular junctions provide signals that prevent endothelial cells from assuming a migratory phenotype. However, in the context of an angiogenic stimulus, angiogenic factors induce a redistribution of PECAM-1 out of EC junctions resulting in the loss of the inhibition of cell migration and increased surface PECAM-1 available to participate in heterophilic interactions. This could potentially involve interactions with αvβ3 on endothelial or stromal cells or with proteoglycans in the extracellular matrix. These ligand interactions in turn directly (if αvβ3 is the ligand) or indirectly trigger integrin-dependent phenomena such as endothelial cell migration. In the latter stages of angiogenesis as endothelial cells are reorganizing into vascular tubes, the re-establishment PECAM-1-PECAM-1 interactions suppresses the migratory phenotype of the endothelial cell thereby facilitating the stabilization of initial endothelial cell-cell associations and thus promoting the formation of vascular tubes.

Summary

Evidence is now emerging that indicates a role for PECAM-1 in tumor angiogenesis although its range of involvement is still being defined. Its mechanisms of involvement in the formation of

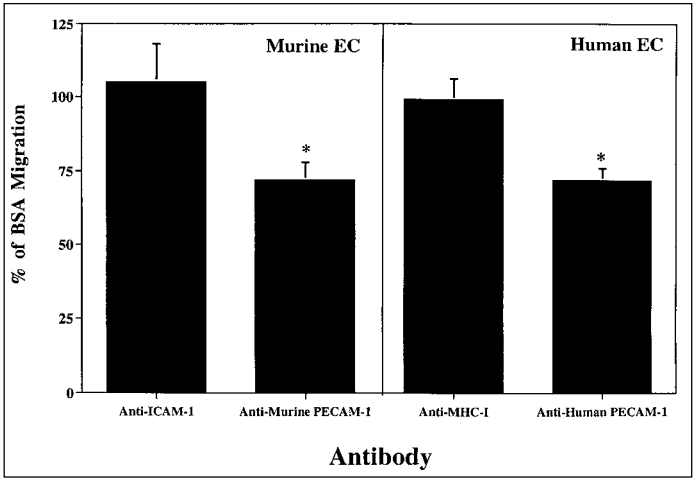

Fig. 5.5. Effect of anti-PECAM-1 antibodies on in vitro endothelial cell migration through Matrigel. Human (HUVEC) and murine (H5V) endothelial cells were allowed to migrate through transwell inserts coated with Matrigel in the presence of BSA, control antibody (anti-human MHC-I or anti-murine ICAM-1) or anti-PECAM-1 antibody (rabbit polyclonal against human PECAM-1 or mAb 390 against murine PECAM-1) (BSA and antibody concentration = 100 μg/ml). Data are expressed % of BSA migration. Anti-PECAM-1 antibody inhibited migration by ~ 25 % compared to control antibody. ($p < 0.01$ compared to control antibodies; n=3).

new vessels are not fully understood but data suggests that PECAM-1 may be involved at several stages of the process: early on when endothelial cells are migrating and later when endothelial cells are organizing into vascular tubes. This is consistent with its ability to participate in multiple ligand interactions. An increased understanding of the role of PECAM-1 in the formation of new vessels may lead to anti-angiogenesis strategies based on antagonism of this molecule.

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CHAPTER 6

Thrombospondin and Angiogenesis

Luisa A. DiPietro

Thrombospondin-1 (TSP1) is an extracellular matrix molecule that is produced by numerous cell types, including endothelial cells, fibroblasts, macrophages, monocytes, keratinocytes, and some tumor cells.[1,2] TSP1 was first described as protein associated with thrombin-activated platelets.[3] Subsequent investigations demonstrated that TSP1 is stored intracellularly in platelet α granules and is released upon platelet activation. TSP1 is also found in association with the cell surface or extracellular matrix, where it binds to heparan sulfate molecules.[4]

The demonstration that TSP1 is produced by endothelial cells initiated investigations into the role of TSP1 in angiogenesis.[5] Because many central observations have documented the importance of endothelial cell-extracellular matrix (ECM) interactions in the angiogenic process, the role of TSP1 in modulating endothelial cell function has been studied in great detail. Most investigations support a role for TSP1 as an anti-angiogenic molecule. However, many other investigations of the regulatory role of TSP in angiogenesis have produced opposing and apparently contradictory data. Depending upon the experimental system, TSP1 has been shown to be either inhibitory or stimulatory to endothelial cell functions associated with angiogenesis (Table 6.1). Thus, there exists considerable controversy regarding the in vivo function of TSP1 in the angiogenic process. This review will summarize the current evidence regarding the modulation of angiogenesis by TSP1, and will highlight the paradoxical aspects of TSP1 function in the angiogenic process.

The Molecular Structure of TSP1

TSP1 exists as a trimeric molecule of approximately 450 kD. Each of the three identical subunits is dumbbell shaped, containing two globular terminal domains joined by a central stalk.[9,10] The amino terminal globular domain of each subunit is heparin-binding while the COOH domain is involved in cell attachment. The stalk can be further divided into four regions:

1. a region possessing the cysteines involved in interchain disulfide bonding,
2. a region homologous to procollagen type I,
3. a region containing three type 1 properdin repeats, and
4. a region containing three EGF-like (type 2) repeats.

TSP1 is a member of a family of at least five ECM molecules. Four additional TSP1-related molecules that are encoded by four separate loci have now been described.[4,11] The members of TSP family of glycoproteins are designated as TSP1, TSP2, TSP3, TSP4 and TSP5. TSP1 and TSP2 are both trimeric proteins with similar structural motifs, and TSP2 has also been shown to inhibit angiogenesis.[12] However, TSP1 and TSP2 exhibit differential tissue distribution and thus may have distinct physiologic functions. TSP3, TSP4, and TSP5 are structurally less similar to TSP1, as they are pentameric molecules. TSP3, TSP4, and TSP5 also appear to have a more limited and specific tissue distribution. Interestingly, TSP5 has been shown to have no effect on angiogenesis.[12] Because nearly all of the published work regarding the role of TSP in angiogenesis has focused on TSP1, this review will center on this molecule.

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Table 6.1. Mechanisms by which TSP1 has been described to influence angiogenesis

	References
Effects on endothelial cell function in vitro	
Anti-proliferative activity (soluble)	13-16
Anti-adhesive activity	24-26
Adhesive activity (certain preparations)	14,27
Induction of apoptosis	28
Inhibition of chemotaxis	20,21
Induction of chemotaxis (high concentrations)	14,21
Inhibits in vitro tube formation	13,22
Effects on in vivo angiogenesis	
Anti-angiogenic (rat corneal assay)	20,29
Pro-angiogenic (rabbit corneal assay)	31
Effects on angiogenic factors	
Inhibition (FGF-2, scatter factor)	32,34
Activation (TGF-α)	36,37
Effects on non-endothelial cells	
Promotes myofibroblast growth	19
Enhances tumor cell growth or metastatic potential	35,65,67

TSP1 as a Regulator of In Vitro Angiogenesis

The effect of TSP1 on several parameters of endothelial cell function that are directly relevant to the angiogenic process has been examined in numerous in vitro studies. These observations have shown that TSP1 can directly influence endothelial cell growth, adhesion, chemotaxis, and differentiation, as well as the process of in vitro angiogenesis.

TSP1 and Endothelial Cell Proliferation

Several studies have shown that the addition of soluble TSP1 has an anti-proliferative effect on cultured endothelial cells.[13-16] For example, soluble TSP1 strongly inhibits the proliferation of lung capillary endothelial cells in response to either fetal calf serum or fibroblast growth factor 2 (basic fibroblast growth factor, FGF-2) in a dose dependent fashion.[14] The anti-proliferative effect of soluble TSP1 appears to be a general one, as soluble TSP1 inhibits the proliferation of many different types of endothelial cells.[15] Interestingly, proliferating endothelial cells actively synthesize and secrete endogenous TSP1.[17,18] In light of the inhibitory effect of soluble TSP1 on endothelial proliferation, the endogenous production to TSP1 by endothelial cells seems paradoxical. The answer to this incongruity may lie in the fact that the endothelial cell response to TSP1 is remarkably influenced by the solubility state of the molecule. In opposition to the effect of soluble TSP1, matrix bound TSP1 appears to be a permissive substrate for endothelial cell proliferation.[19] Thus, TSP1 that is directly incorporated into the ECM or bound to a cell surface might favor proliferation. The contrast between the effects of soluble and matrix bound TSP1 is worth noting, as this difference represents a potential answer to some of the conflicting data regarding the role of TSP1 and angiogenesis.

TSP1 and Endothelial Cell Chemotaxis

The effect of exogenous soluble TSP1 on endothelial cell chemotaxis is concentration dependent. The addition of low to moderate amounts of TSP1 to a modified Boyden chamber assay signifi-

cantly inhibits endothelial cell chemotaxis to FGF-2.[20,21] However, at high concentrations, TSP1 can stimulate, rather than inhibit endothelial migration.[14,21] The inhibitory effect of TSP1 on chemotaxis lies within the central stalk region of the molecule, while the stimulatory activity has been localized to the amino terminal heparin binding domain of the molecule.[14,21]

The effect of endogenously produced TSP1 on endothelial cell chemotaxis has also been investigated. Endothelial cells that have been engineered to produce reduced amounts of endogenous TSP1 exhibit enhanced chemotactic activity.[22] Therefore, endogenously produced TSP1 appears to be anti-chemotactic. Taken together, these studies indicate that local concentration of TSP1 may be a significant determinant of its vivo influence on endothelial cell chemotaxis.

TSP1 and In Vitro Angiogenesis

The effect of TSP1 on the formation of capillary cords and tubes in vitro has been described by several groups. The addition of anti-TSP1 antibodies enhances spontaneous in vitro capillary tube formation by endothelial cells.[13] In another study, cells in which TSP1 production was reduced by antisense methods exhibited notably increased formation of endothelial cords when plated on gelled basement membrane matrix.[22] These findings are in keeping with the previous observation that endogenous TSP1 synthesis decreases in endothelial cells when they form tube-like structures.[23] Thus, within the context of in vitro cord and tube formation, the effects of endogenous TSP1 appears to be inhibitory.

In other in vitro assay systems, TSP1 has been shown to promote rather than to inhibit the angiogenic process. TSP1, incorporated into matrix, promotes the in vitro capillary angiogenesis that occurs as an outgrowth of aortic explants.[19] In this system, the primary effect of TSP1 is not directly on the endothelial cells. Instead, TSP1 promotes myofibroblast growth, which in turn stimulates angiogenesis. This study demonstrates that TSP1 may influence angiogenesis via its interactions with cell types other than endothelial cells. Conceptually, then, the in vivo effect of TSP1 relies not only on the influence of TSP1 on endothelial cells, but also upon the interactions of TSP1 with other cell types within the environment.

Other Effects of TSP1 on Endothelial Cells

The effects of TSP1 on several other endothelial cell characteristics has also been examined. Pertinent to the process of angiogenesis, several investigations have examined the effect of TSP1 on endothelial cell adhesion. Similar to investigations of the effect of TSP1 on endothelial cell proliferation, the effect of TSP1 on endothelial cell adhesion may depend upon the form of TSP1 that is utilized. Soluble TSP1 has been reported to modulate focal adhesions in endothelial cells,[24] and to interfere with endothelial cell attachment.[25,26] Corresponding studies suggest that soluble TSP1 saturates surface receptors and produces an anti-adhesive effect. By comparison, depending upon the particular preparation, bound TSP1 may either promote or inhibit endothelial cell adhesion and spreading.[14,27]The effect of TSP1 on endothelial cell adhesion rests upon its concentration, solubility, and the accessibility of specific binding domains.

Another effect of TSP1 on endothelial cells that has been recently documented is the ability of soluble TSP1 to induce apoptosis.[28] TSP1, or fragments of TSP1 from the type I repeat region of the molecule, induce apoptotic changes in bovine aortic endothelial cells. Interestingly, this effect was decreased as cells reached confluence and was also inhibited if the cells were grown on a fibronectin matrix. These results suggest that, in the absence of growth promoting signals, TSP1 may induce apoptosis. From this finding, a hypothesis may be derived that TSP1 readily acts as an apoptotic signal only when the active induction of angiogenesis is absent or withdrawn. This hypothesis is most appealing, as it supports the notion that effects of TSP1 on endothelial cell function are indeed dependent upon the overall angiogenic milieu.

TSP1 and In Vivo Angiogenesis

In resemblance to in vitro investigations, the effect of TSP1 on in vivo angiogenesis has been shown to be both positive and negative. Utilizing the rat corneal assay of angiogenic activity, Bouck and colleagues showed that TSP1 completely inhibits the angiogenic activity of FGF-2.[20,29] Similarly, TSP1 has also been shown to inhibit the angiogenic activity of conditioned media from tumor cells.[29,30] In contrast, Ben Ezra et al have shown that, in a rabbit corneal assay of angiogenic activity, TSP1 augmented, rather than inhibited, the angiogenic activity of FGF-2.[31] One significant difference in these conflicting studies involves the method of incorporation of test substances into the inert polymer pellets. In the studies of Bouck and colleagues, FGF-2 and TSP1 were mixed into a single pellet, which was then implanted into the cornea. In contrast, BenEzra and colleagues implanted two separate pellets, one containing FGF-2 and one containing TSP1, at the same site. Because TSP1 has been shown to bind growth factors,[32-34] it may be that in a single pellet, TSP1 sequesters FGF-2 and thus inhibits angiogenesis. In contrast, when the two proteins are implanted in individual pellets, FGF-2 remains freely diffusible. Because the studies of BenEzra utilized much higher amounts of both TSP1 and FGF-2, Tuszynski and Nicosia have suggested that simple differences in the diffusibility of TSP1 and FGF-2 may explain the conflicting corneal assay results.[35]

Interactions of TSP1 with Angiogenic Factors

TSP1 has been shown to bind and influence the activity of several growth factors that play a role in in vivo angiogenesis. TSP1 both binds and activates transforming growth factor-β,[33,36,37] a growth factor which is potently angiogenic in vivo.[38] This finding suggests an additional complexity in the manner that TSP1 might influence endothelial cell growth and differentiation. Indeed, many cell types that can be found in areas of active angiogenesis produce both TGF-β and TSP1. For example, TGF-β and TSP1 are both produced by activated macrophages, a cell type known to be highly angiogenic and prominent in many sites of active angiogenesis.[39-41] In this scenario, TSP1, by activating latent TGF-β, would be expected to exert a pro-angiogenic effect.

TSP1 has also been shown to bind and influence the activity of FGF-2.[32] The binding of TSP1 to FGF-2 inhibited the effect of this growth factor on endothelial cell proliferation, suggesting that TSP1 might interfere with FGF-2 induced angiogenesis. Another angiogenic growth factor that directly binds to TSP1 is scatter factor (SF). SF is a potent inducer of angiogenesis, and the increased expression of this factor has been described in several tumor types.[42] TSP1 both binds SF and inhibits SF induced angiogenic activity both in vitro and in vivo.[34] Therefore, TSP1 can directly reduce the angiogenic capacity of at least two growth factors.

The documentation that TSP1 may influence angiogenesis by sequestering growth factors provides yet another mechanism by which TSP1 could down regulate angiogenesis. However, it seems clear that the anti-angiogenic activity of TSP1 goes beyond the inactivation of growth factors. TSP1 can inhibit the angiogenic response to many angiogenic factors to which it does not bind, suggesting that other mechanisms account for the anti-angiogenic activity of this molecule in many situations.

Interactions of TSP1 with Proteases

Recent reports have produced the unexpected finding that TSP1 can function as a protease inhibitor,[43] and thus may influence angiogenesis by affecting ECM turnover and composition. TSP1 inhibits several different types of proteases, including neutrophil elastase and cathepsin G, both serine proteases.[44,45] TSP1 also inhibits the proteolytic enzymes of the fibrinolytic pathway, including plasmin and urokinase plasminogen activator.[46,47] While the modulation of protease activity provides yet another way in which TSP1 might influence angiogenesis, the in vivo effects of TSP1 on the protease balance within tissues has not yet been examined.

TSP1 as a Regulator of Physiologic Angiogenesis

TSP1 has been shown to be involved in the regulation of physiologic angiogenesis in at least two circumstances. TSP1 production is modulated within healing wounds, a site of dynamic capillary growth and regression,[48-50] and TSP1 production also varies temporally during the endometrial cycle.[51]

The functional role of TSP1 production within wounds has been studied both in normal mice and mice that are genetically deficient for TSP1.[50,52] Inhibition of TSP1 synthesis in normal mice causes an inhibition of wound healing which includes a delay in reepithelialization and dermal reorganization.[50] The wounds of TSP1-deficient mice exhibited a similarly delayed initial healing response. However, during the resolution phase of repair, the wounds of TSP1-deficient mice fail to resolve, and exhibit instead increased granulation tissue formation, prolonged neovascularization, and sustained macrophage infiltration.[52] These findings suggest a dual role for TSP1 in wound repair. In the early phase of repair, TSP1 synthesis seems to facilitate the repair process, perhaps by activating TGF-β or through direct cellular interactions. As repair proceeds, soluble forms of TSP1 may serve as anti-angiogenic agents to effect the regression of the newly developed vasculature.

In the human endometrium, TSP1 production is modulated in a manner consistent with a role as an angiogenic inhibitor.[51] TSP1 is abundantly produced in endometrium during the secretory phase, while expression is low during proliferative phase. Thus, in the endometrium, TSP1 production is associated with periods of low capillary growth. The data from both wound healing and endometrial studies argue for an important role of TSP1 as an regulator of physiologic angiogenesis.

TSP1 in Pathologic Angiogenesis

TSP1 in Inflammatory Sites

Robust angiogenesis is a common characteristic of inflammatory diseases. In certain inflammatory lesions, a direct association of decreased TSP1 production with this enhanced angiogenesis has been noted. For example, psoriatic keratinocytes exhibit diminished TSP1 production. In psoriasis, the skin exhibits an excessive growth of keratinocytes with associated inflammation and neovascularization. The angiogenic component of this disease is mediated, at least in part, by psoriatic keratinocytes.[53] The increase in the angiogenic capability of psoriatic keratinocytes has been linked to both an increase in the production of the pro-angiogenic cytokine IL-8 concomitant with decreased TSP1 production. Within the context of the psoriatic lesion, then, the primary influence of TSP1 appears to be inhibitory.

In other inflammatory conditions, such as rheumatoid arthritis and atherosclerosis, an increased TSP1 production is observed.[54,55] In rheumatoid arthritis, TSP1 is present in endothelial cells, synovial lining cells, and macrophages of affected joints. Recent studies in a rat model of adjuvant-induced rheumatoid arthritis suggest that TSP1 exerts a biphasic modulatory effect on vessel formation in this disease.[56] As compared to control, the addition of TSP1 to rheumatoid joints caused initial enhancement of vessel growth followed by a decrease in vessel density. This effect is reminiscent of the dual effect of TSP1 that is observed in normal wound healing.

TSP1 and Tumor Angiogenesis

The importance of a decrease in TSP1 production to the development of a tumorogenic and angiogenic phenotype has been documented in numerous tumor systems.[20,30,57,58] These include tumor cells derived from melanoma, breast, and epithelial tumors. In many tumor models, TSP1 has been shown to be a major inhibitor of the angiogenic phenotype prior to malignant transformation, and the reduction in TSP1 production can be linked to the loss of a tumor suppressor gene.[29,30,59] Additionally, the artificial upregulation of TSP1 can cause substantially decreased tumor progression and angiogenesis in vivo.[58,60]

Despite striking evidence for the negative regulation of the angiogenic phenotype in tumor cells by TSP1, other studies suggest a positive role for TSP1 in tumor growth and progression. TSP1

has been localized in situ in tumors such as human breast carcinoma.[61] Cell lines derived from breast cancer, melanoma, squamous cell carcinoma, and osteosarcoma cells have each been shown to exhibit marked production of TSP1 in vitro.[62-65] Separate from its effects on angiogenesis, additional experimental evidence indicates that TSP1 can, at least in some cases, promote tumor growth and metastasis.[27,35,66,67]

Therapeutic Uses of TSP1

The described anti-angiogenic effects of TSP1, as well as the association of the loss of TSP1 production with tumor progression, have lent credence to the idea that TSP1 might be a useful anti-angiogenic treatment for tumor therapy. Specific TSP1-derived peptides have been shown to inhibit angiogenesis, suggesting a TSP1 based therapeutic approach could utilize peptides rather than intact TSP1.[21] The majority of the in vivo anti-angiogenic activity of TSP1 has been localized to the central stalk region of the molecule. Further analysis has more specifically localized the anti-angiogenic activity to several peptides from the procollagen like region of the stalk. Anti-angiogenic activity has also been described for the peptides derived from the properdin repeat region. Thus at least two different structural domains of TSP1 have therapeutic potential as possible anti-angiogenic agents.

The Regulation of Angiogenesis by TSP1

The multiple and complex roles of TSP1 as a regulator of angiogenesis seem difficult to reconcile. Nevertheless, the many studies of this molecule suggest that three considerations are extremely important to our understanding of this molecule. First, the effects of TSP1 are clearly and unequivocally dependent upon the solubility status of the molecule. The local concentration and predominant form of TSP1 can be dictated by

1. the presence of ECM molecules that bind TSP1,
2. local protease activity, and
3. the TSP1 turnover rate.

Soluble TSP1, and specific soluble fragments of TSP1, would be expected to be anti-angiogenic, while matrix bound TSP1 may promote the angiogenic response.

Secondly, the relative concentration of TSP1 may be extremely critical, as overall the angiogenic response in a tissue depends upon the balance of pro-angiogenic and anti-angiogenic factors. Thus, in the face of high levels of proangiogenic molecules and moderate TSP1 concentration, angiogenesis may proceed. Conversely, exceedingly high levels of TSP1 could overwhelm the effect of pro-angiogenic factors and result in an anti-angiogenic profile. In addition, the type of angiogenic factor is important, as TSP1 may work in concert with some angiogenic mediators (such as TGF-β), yet inhibit others.

Finally, because TSP1 can influence the function of not only endothelial cells, but also other cell types, the effect of this molecule will derive from its manner of interaction with the many cell types within any particular tissue. In some tissues, soluble TSP1 may positively influence angiogenesis through its interaction with non-endothelial cells such as myofibroblasts or tumor cells themselves. In other cases, if the primary interaction of soluble TSP1 is with endothelial cells, an anti-angiogenic effect may predominate.

These three considerations suggest that the actual effect of TSP1 in any tissue is unique to that particular environment. For example, in circumstances where soluble levels of TSP1 or its fragments are high, TSP1 would inhibit endothelial cell growth and capillary tube formation. In other situations, TSP1 might reside primarily as a bound molecule within the ECM or in association with TGF-β production. In such a setting, TSP1 would facilitate angiogenesis.

Although our understanding of the function of TSP1 as a regulator of angiogenesis has grown, an integrated model for the role of this molecule in the angiogenic process is not yet forthcoming. Perhaps, given the complexity of the in vivo angiogenic process, as well as the many functional domains present within TSP1, the conflicting functional reports should be seen as likely and

expected rather than unsettling.[68] Additional investigations, both in vitro and in vivo, will be necessary to fully grasp the precise mechanisms by which TSP1 influences the angiogenic process.

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CHAPTER 7

The Role of Vascular Endothelial Growth Factor in Angiogenesis

Napoleone Ferrara

The development of a vascular supply is a fundamental requirement for organ development and differentiation during embryogenesis as well as for wound healing and reproductive functions in the adult.[1] Angiogenesis is also implicated in the pathogenesis of a variety of disorders: proliferative retinopathies, age-related macular degeneration, tumors, rheumatoid arthritis, and psoriasis.[1,2]

The search for positive regulators of angiogenesis has yielded several candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc,[3,4] and more recently the angiopoietins, the ligands of the Tie-2 receptor.[5] The negative regulators so far identified include thrombospondin,[6,7] the 16-kilodalton N-terminal fragment of prolactin,[8] angiostatin[9] and endostatin.[10]

This chapter discusses the molecular and biological properties the vascular endothelial growth factor (VEGF) proteins. Over the last few years, several members of the VEGF gene family have been identified, including VEGF-B, VEGF-C, placenta growth factor and VEGF-D. For a review, see ref. 11. This focus of this chapter is on the original VEGF, referred to also as "VEGF-A". Work done by several laboratories over the last few years has elucidated the pivotal role of VEGF and its receptors in the regulation of normal and abnormal angiogenesis.[11] The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system.[12,13] Furthermore, VEGF-induced angiogenesis has been shown to result in a therapeutic effect in animal models of coronary or limb ischemia and, more recently, in humans.[11]

Biological Activities of VEGF

VEGF is a mitogen for vascular endothelial cells derived from arteries, veins and lymphatics but it is devoid of consistent and appreciable mitogenic activity for other cell types.[11] VEGF promotes angiogenesis in tridimensional in vitro models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures.[14] Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel.[15] VEGF also elicits a pronounced angiogenic response in a variety of in vivo models including the chick chorioallantoic membrane, etc.[16]

VEGF induces expression of the serine proteases, urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor 1 (PAI-1) in cultured bovine microvascular endothelial cells.[17] Moreover, VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts.[18] Other studies have shown that VEGF promotes expression of urokinase receptor (uPAR) in vascular endothelial cells.[19] Additionally, VEGF stimulates hexose transport in cultured vascular endothelial cells.[20]

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VEGF is known also as vascular permeability factor (VPF) based on its ability to induce vascular leakage in the guinea pig skin.[21] Dvorak and colleagues proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumors and wounds.[22] According to this hypothesis, a major function of VPF/VEGF in the angiogenic process is the induction of plasma protein leakage. This effect would result in the formation of an extravascular fibrin gel, a substrate for endothelial and tumor cell growth.[23] Recent studies have also suggested that VEGF may also induce fenestrations in endothelial cells.[24,25] Topical administration of VEGF acutely resulted in the development of fenestrations in the endothelium of small venules and capillaries, even in regions where endothelial cells are not normally fenestrated and was associated with increased vascular permeability.[24,25]

Melder et al have shown that VEGF promotes expression of VCAM-1 and ICAM-1 in endothelial cells.[26] This induction results in the adhesion of activated natural killer (NK) cells to endothelial cells mediated by specific interaction of endothelial VCAM-1 and ICAM-1 with CD18 and VLA-4 on the surface of NK cells.

VEGF has been reported to have regulatory effects on blood cells. Clauss et al reported that VEGF may promote monocyte chemotaxis.[27] Broxmeyer et al have shown that VEGF induces colony formation by mature subsets of granulocyte-macrophage progenitor cells.[28] These findings may be explained by the common origin of endothelial cells and hematopoietic cells and the presence of VEGF receptors in progenitor cells as early as hemangioblasts in blood islands in the yolk sac. Furthermore, Gabrilovich et al have reported that VEGF may have an inhibitory effect on the maturation of host professional antigen-presenting cells such as dendritic cells.[29] VEGF was found to inhibit immature dendritic cells, without having a significant effect on the function of mature cells. These findings led to the suggestion that VEGF may facilitate tumor growth also by allowing the tumor to avoid the induction of an immune response.[29]

VEGF induces vasodilatation in vitro in a dose-dependent fashion[30,31] and produces transient tachycardia, hypotension and a decrease in cardiac output when injected intravenously in conscious, instrumented rats.[31] Such effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived nitric oxide (NO), as assessed by the requirement for an intact endothelium and the prevention of the effects by N-methyl-arginine.[31] Accordingly, VEGF has no direct effect on contractility or rate in isolated rat heart in vitro.[31] These hemodynamic effects, however, are not unique to VEGF: other angiogenic factors such as aFGF and bFGF also have the ability to induce NO-mediated vasodilatation and hypotension.[32,33]

Organization of the VEGF Gene and Characteristics of the VEGF Proteins

The human VEGF gene is organized in eight exons, separated by seven introns. The coding region spans approximately 14 kilobases (kb).[34,35] The human VEGF gene has been assigned to chromosome 6p21.3.[36] It is now well established that alternative exon splicing of a single VEGF gene results in the generation of four different molecular species, having respectively 121, 165, 189 and 206 amino acids following signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{165}$ lacks the residues encoded by exon 6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Compared to $VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids; $VEGF_{189}$ has an insertion of 24 amino acids highly enriched in basic residues and $VEGF_{206}$ has an additional insertion of 17 amino acids.[34]

$VEGF_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the VEGF gene.[34] In contrast, $VEGF_{206}$ is a very rare form, so far identified only in a human fetal liver cDNA library.[34] The genomic organization of the murine VEGF gene has been also described.[37] Like the human gene, the coding region of the murine VEGF gene encompasses approximately 14 kb and is comprised of eight exons interrupted by seven introns. Analysis of exons suggests the generation of three isoforms, $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$.

Therefore, murine VEGFs are shorter than human VEGF by one amino acid. However, a fourth isoform comparable to $VEGF_{206}$ is not predicted, since an in-frame stop codon is present the region corresponding to the human $VEGF_{206}$ open reading frame. Analysis of the 3' untranslated region of the rat VEGF mRNA has revealed the presence of four potential polyadenylation sites.[38] A frequently used site is about 1.9 kb further downstream from the previously reported transcription termination codon.[39] The sequence within this 3' untranslated region reveals a number of sequence motifs that are known to be involved in the regulation of mRNA stability.[38]

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45,000 daltons.[40] These properties correspond to those of $VEGF_{165}$, the major isoform.[41] $VEGF_{121}$ is a weakly acidic polypeptide that fails to bind to heparin.[41] $VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$.[41] Such differences in the isoelectric point and in affinity for heparin may profoundly affect the bioavailability of the VEGF. $VEGF_{121}$ is a freely diffusible protein; $VEGF_{165}$ is also secreted although a significant fraction remains bound to the cell surface and the extracellular matrix (ECM). In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM.[42] However, these isoforms may be released in a soluble form by heparin or heparinase suggesting that their binding site is represented by proteoglycans containing heparin-like moieties. The long forms may be released also by plasmin following cleavage at the COOH terminus. This action generates a bioactive proteolytic fragment having molecular weight of ~34,000 daltons.[41] Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade. Thus, proteolysis of VEGF is likely to occur also in vivo. Keyt et al have shown that the bioactive product of plasmin action is comprised of the first 110 NH_2-terminal amino acids of VEGF.[43] These findings suggest that the VEGF proteins may become available to endothelial cells by at least two different mechanisms: as freely diffusible proteins ($VEGF_{121}$, $VEGF_{165}$) or following protease activation and cleavage of the longer isoforms. However, loss of heparin binding, whether it is due to alternative splicing of RNA or plasmin cleavage, results in a substantial loss of mitogenic activity for vascular endothelial cells: compared to $VEGF_{165}$, $VEGF_{121}$ or $VEGF_{110}$ demonstrate 50-100 fold reduced potency when tested in endothelial cell growth assay.[43] It has been suggested that the stability of VEGF-heparan sulfate-receptor complexes contributes to effective signal transduction and stimulation of endothelial cell proliferation.[43] Thus, VEGF has the potential to express structural and functional heterogeneity to yield a graded and controlled biological response. Recently, Poltorak et al have provided evidence for the existence of an additional alternatively spliced molecular species of VEGF.[44] A VEGF isoform containing exons 1-6 and 8 of the VEGF gene was found to be expressed as a major VEGF mRNA form in several cell lines derived from carcinomas of the female reproductive system. This mRNA is predicted to encode a VEGF form of 145 amino acids ($VEGF_{145}$). Recombinant $VEGF_{145}$ induced the proliferation of vascular endothelial cells, albeit at much lower potency than $VEGF_{165}$. $VEGF_{145}$ binds to the KDR receptor on the surface of endothelial cells. It also binds to heparin with an affinity similar to that of $VEGF_{165}$.

Recently, Muller at al solved the crystal structure of VEGF at a resolution of 2.5 A.[45] Overall, the VEGF monomer resembles that of PDGF, but its N-terminal segment is helical rather than extended. The dimerization mode of VEGF is similar to that of PDGF and very different from that of TGF-β.

Regulation of VEGF Gene Expression

Oxygen Tension

Among the mechanisms that have been proposed to participate in the regulation of VEGF gene expression, oxygen tension plays a major role, both in vitro and in vivo. VEGF mRNA expression is rapidly and reversibly induced by exposure to low pO_2 in a variety of normal and transformed cultured cell types.[46,47] Also, ischemia caused by occlusion of the left anterior descending coronary artery results in a dramatic increase in VEGF RNA levels in the pig and rat myocardium, suggesting the possibility that VEGF may mediate the spontaneous revascularization that follows myocardial

ischemia.[48,49] Furthermore, hypoxic upregulation of VEGF mRNA in neuroglial cells, secondary to the onset of neuronal activity, has been proposed to play an important physiological role in the development of the retinal vasculature.[50]

Similarities exist between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo).[51] Hypoxia-inducibility is conferred on both genes by homologous sequences. By deletion and mutation analysis, a 28-base sequence has been identified in the 5' promoter of the rat and human VEGF gene which mediated hypoxia-induced transcription.[52,53] Such sequence reveals a high degree of homology and similar protein binding characteristics as the hypoxia-inducible factor 1α (HIF-1α) binding site within the Epo gene.[54] HIF-1α has been identified as a mediator of transcriptional responses to hypoxia and is a basic, heterodimeric, helix-loop-helix protein.[55] HIF-1α is the only known mammalian transcription factor expressed uniquely in response to physiologically relevant levels of hypoxia. Inactivation of HIF-1α in mice resulted in developmental arrest and lethality by E11. Embryos manifested neural tube defects, cardiovascular malformations, and marked cell death within the cephalic mesenchyme. These results suggest that HIF-1α is a key regulator of cellular and developmental O_2 homeostasis.[56]

When reporter constructs containing the VEGF sequences that mediate hypoxia-inducibility were co-transfected with expression vectors encoding HIF-1α subunits, reporter gene transcription was much greater than that observed in cells transfected with the reporter alone, both in hypoxic and normoxic conditions.[57]

However, transcriptional activation is not the only mechanism leading to VEGF upregulation in response to hypoxia.[38,58] Increased mRNA stability has been identified as a significant post-transcriptional component. Sequences that mediate increased stability were identified in the 3' untranslated region of the VEGF mRNA.

Cytokines

Various cytokines or growth factors may upregulate VEGF mRNA expression. EGF, TGF-β or KGF result in a marked induction of VEGF mRNA expression.[59] EGF also stimulates VEGF release by cultured glioblastoma cells.[60] In addition, treatment of quiescent cultures of epithelial and fibroblastic cell lines with TGF-β resulted in induction of VEGF mRNA and release of VEGF protein in the medium.[61] Based on these findings, it has been proposed that VEGF may function as a paracrine mediator for indirect-acting angiogenic agents such as TGF-β.[61] Furthermore, IL-1α induces VEGF expression in aortic smooth muscle cells.[62] Both IL-1α and PGE_2 have been shown to induce expression of VEGF in cultured synovial fibroblasts, suggesting the participation of such inductive mechanisms in inflammatory angiogenesis.[63] IL-6 has been also shown to significantly induce VEGF expression in several cell lines.[64] IGF-1, a mitogen implicated in the growth of several malignancies, has also been shown to induce VEGF mRNA and protein in cultured colorectal carcinoma cells.[65]

Differentiation and Transformation

Cell differentiation has been shown to play an important role in the regulation of VEGF gene expression.[66] The VEGF mRNA is upregulated during the conversion of 3T3 preadipocytes into adipocytes or during the myogenic differentiation of C2C12 cells. Conversely, VEGF gene expression is repressed during the differentiation of the pheochromocytoma cell line PC12 into non-malignant, neuron-like, cells.

Specific transforming events also result in induction of VEGF gene expression. A mutated form of the murine p53 tumor suppressor gene has been shown to result in induction of VEGF mRNA expression in NIH 3T3 cells in transient transfection assays.[67] Likewise, oncogenic mutations or amplification of ras lead to VEGF upregulation,[68,69] Recently, Okada et al have provided more direct evidence for the hypothesis that VEGF-mediated angiogenesis is necessary for the in vivo malignant growth promoted by oncogenic ras.[70] Transfection of a $VEGF_{121}$ antisense expression vector into DLD-1 and HCT-116 colon carcinoma cell lines resulted in suppression of VEGF

production. The VEGF-deficient sublines, unlike the parental population or vector controls, were profoundly suppressed in their ability to form tumors in nude mice for as long as 6 months after cell injection. In contrast, in vitro growth of these sublines was unaffected, thus demonstrating the critical importance of VEGF as an angiogenic factor for HCT-116 and DLD-1 cells. Interestingly, expression of oncogenic ras, either constitutive or transient, potentiated the induction of VEGF by hypoxia.[71] Moreover, the von Hippel-Lindau (VHL) tumor suppressor gene has been recently implicated in the regulation of VEGF gene expression.[72-74] The VHL tumor suppressor gene is inactivated in patients with von Hippel-Lindau disease and in most sporadic clear cell renal carcinomas. Although the function of the VHL protein remains to be fully elucidated, it is known that such protein interacts with the elongin BC subunits in vivo and regulates RNA polymerase II elongation activity in vitro by inhibiting formation of the elongin ABC complex. Human renal cell carcinoma cells either lacking endogenous wild type VHL gene or expressing an inactive mutant demonstrated altered regulation of VEGF gene expression, which was corrected by introduction of wild type VHL gene. Most of the endothelial cells mitogenic activity released by tumor cells expressing mutant VHL gene was neutralized by anti-VEGF antibodies.[72] These findings suggest that VEGF is a key mediator of the abnormal vascular proliferations and solid tumors characteristic of VHL syndrome. Iliopulos et al[73] have shown that a function of the VHL protein is to provide a negative regulation of a series of hypoxia-inducible genes, including the VEGF, platelet derived growth factor B chain and the glucose transporter GLUT1 genes. In the presence of a mutant VHL, mRNAs for such genes were produced both under normoxic and hypoxic conditions. Reintroduction of wild type VHL resulted in inhibition of mRNA production under normoxic conditions and restored the characteristic hypoxia-inducibility of those genes.[73] In addition, Gnarra et al[74] have shown that VHL regulates VEGF expression at a post-transcriptional level and that VHL inactivation in target cells causes a loss of VEGF suppression, leading to formation of a vascular stroma. Interestingly, despite 5-fold differences in VEGF mRNA levels, VHL overexpression did not affect VEGF transcription initiation.

The VEGF Receptors

Two classes of high affinity VEGF binding sites were initially described in the surface of bovine endothelial cells, with K_d values of 10 pM and 100 pM, respectively.[75,76] Lower affinity binding sites on mononuclear phagocytes were subsequently described.[77] It has been suggested that such binding sites are involved in mediating chemotactic effects for monocytes by VEGF.[27]

Ligand autoradiography studies on fetal and adult rat tissue sections demonstrated that high affinity VEGF binding sites are localized to the vascular endothelium of large or small vessels in situ.[78,79] VEGF binding was apparent not only on proliferating but also on quiescent endothelial cells.[78,79] Also, the earliest developmental identification of high affinity VEGF binding was in the hemangioblasts in the blood islands in the yolk sac.[79]

The Flt-1 and Flk-1/KDR Tyrosine Kinases

Binding Characteristics

Two VEGF receptor tyrosine kinases (RTKs) have been identified. The Flt-1 (fms-like-tyrosine kinase)[80] and KDR (kinase domain region)[81] receptors bind VEGF with high affinity. The murine homologue of KDR, Flk-1 (fetal liver kinase-1), shares 85% sequence identity with human KDR.[82] Both Flt-1 and KDR/Flk-1 have seven immunoglobulin (Ig)-like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain.[82-84] Flt-1 has the highest affinity for rhVEGF$_{165}$, with a K_d of approximately 10-20 pM.[80] KDR has a somewhat lower affinity for VEGF: the K_d has been estimated to be approximately 75-125 pM.[81]

A cDNA coding an alternatively spliced soluble form of Flt-1 (sFlt-1), lacking the seventh Ig-like domain, transmembrane sequence and the cytoplasmic domain, has been identified in human umbilical vein endothelial cells.[85] This sFlt-1 receptor binds VEGF with high affinity (K_d 10-20

pM) and is able to inhibit VEGF-induced mitogenesis and may be a physiological negative regulator of VEGF action.[85]

An additional member of the family of RTKs with seven Ig-like domains in the ECD is Flt-4[86-88] which, however, is not a receptor for VEGF but rather binds a newly identified ligand called VEGF-C or VEGF-related peptide (VRP).

Recent studies have mapped the binding site for VEGF to the second immunoglobulin-like domain of Flt-1 and KDR. Deletion of the second domain of Flt-1 completely abolished the binding of VEGF. Introduction of the second domain of KDR into an Flt-1 mutant lacking the homologous domain restored VEGF binding. However, the ligand specificity was characteristic of the KDR receptor. To further test this hypothesis, chimeric receptors where the first three or just the second Ig-like domains of Flt-1 replaced the corresponding domains in Flt-4 were created. Both swaps conferred upon Flt-4 the ability to bind VEGF with an affinity nearly identical to that of wild-type Flt-1. Furthermore, transfected cells expressing these chimeric Flt-4 receptors exhibited increased DNA synthesis in response to VEGF or PlGF.[89] Recently, Wiesman et al have solved the crystal structure of a VEGF-Flt-1 domain 2 complex.[90] These studies have shown domain 2 in a predominantly hydrophobic interaction with the poles of the VEGF dimer.

An application of these structure-function studies is the generation of inhibitors of VEGF activity. The first three Ig-like domains of Flt-1 fused to a heavy chain Fc potently inhibits VEGF bioactivity across species. The Fc may confer sufficient half-life and stability when injected systemically.[91] Therefore this agent may a useful tool to determine the role of endogenous VEGF in several in vivo models.

Signal Transduction

VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells.[92] PLC-γ and two proteins that associate with PLC-γ were phosphorylated in response to VEGF. Furthermore, immunoblot analysis for mediators of signal transduction that contain SH2 domains demonstrated that VEGF induces phosphorylation of phosphatidylinositol 3-kinase, ras GTPase activating protein (GAP) and several others. These findings suggest that VEGF promotes the formation of multimeric aggregates of VEGF receptors with proteins that contain SH2 domains. These studies, however, did not identify which VEGF receptor(s) are involved in these events. Recently, it has been suggested that NO mediates, at least in part, that the mitogenic effect of VEGF on cultured microvascular endothelium isolated from coronary venules.[93] The proliferative effect of VEGF was reduced by pretreatment of the cells with NO synthase inhibitors. Exposure of the cells to VEGF induced a significant increment in cGMP levels. These findings suggest that VEGF stimulates proliferation of postcapillary endothelial cells through the production of NO and cGMP accumulation.

Several studies have indicated that Flt-1 and KDR have different signal transduction properties.[94,95] Porcine aortic endothelial cells lacking endogenous VEGF receptors display chemotaxis and mitogenesis in response to VEGF when transfected with a plasmid coding for KDR.[94] In contrast, transfected cells expressing Flt-1 lack such responses.[94,95] Flk-1/KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, while Flt-1 reveals a weak or undetectable response.[94,95] Also VEGF stimulation results in weak tyrosine phosphorylation that does not generate any mitogenic signal in transfected NIH 3T3 cells expressing Flt-1.[95] These findings agree with other studies showing that placenta growth factor (PlGF), which binds with high affinity to Flt-1 but not to Flk-1/KDR, lacks direct mitogenic or permeability-enhancing properties or the ability to effectively stimulate tyrosine phosphorylation in endothelial cells.[96] Therefore, interaction with Flk-1/KDR is a critical requirement to induce the full spectrum of VEGF biologic responses. In further support of this conclusion, VEGF mutants which bind selectively to Flk-1/KDR are fully active endothelial cell mitogens.[97] These findings cast doubt on the role of Flt-1 as a true signaling receptor. However, more recent evidence indicates that Flt-1 indeed signals, although our understanding of these processes is fragmentary. According to Waltenberg et al, members of the Src family, such as Fyn and Yes, show an increased level of phosphorylation following VEGF stimulation in transfected cells

expressing Flt-1 but not KDR.[94] Cunningham et al have demonstrated an interaction between Flt-1 and the p85 subunit of phosphatidylinositol 3-kinase, suggesting that p85 couples Flt-1 to intracellular signal transduction systems and implicate elevated levels of PtdIns(3,4,5)P3 levels in this process.[98] Activation of the PI3 kinase/Akt pathway has been implicated in the antiapoptotic/pro-survival activity of several growth factors,[99,100] including VEGF.[101] Surprisingly, however, administration of PlGF failed to promote endothelial cell survival in serum-free medium, whereas wild type VEGF or KDR-selective VEGF mutants had a strong survival effect.[101] Nevertheless, at least a biological response, the migration of monocytes in response to VEGF (or PlGF), has been shown to be mediated by Flt-1.[102]

Very recently, Soker et al have demonstrated the existence of an additional VEGF receptor[103] that binds $VEGF_{165}$ but not $VEGF_{121}$. This isoform-specific VEGF binding site is identical to human neuropilin-1, a receptor for the collapsin/semaphorin family that mediates neuronal cell guidance. When coexpressed in cells with KDR, neuropilin-1 enhanced the binding of $VEGF_{165}$ to KDR and $VEGF_{165}$-mediated chemotaxis. Conversely, inhibition of $VEGF_{165}$ binding to neuropilin-1 reduces its binding to KDR and its mitogenic activity for endothelial cells. These findings suggest that neuropilin-1 presents VEGF to the KDR receptor in a manner that enhances the effectiveness of KDR-mediated signal transduction. There is no evidence that neuropilin-1 itself signals following VEGF binding. Further studies are required to determine the role of neuropilin-1 in regulating the VEGF biological activities.

Regulation

The expression of Flt-1 and Flk-1/KDR genes is largely restricted to the vascular endothelium. The promoter region of Flt-1 has been cloned and characterized and a 1-kb fragment of the 5' flanking region essential for endothelial-specific expression was identified.[104] Likewise, a 4-kb 5' flanking sequence has been identified in the promoter of KDR that confers endothelial cell specific activation.[105]

As with VEGF, hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression. Exposure of rats to acute or chronic hypoxia led to pronounced upregulation of both Flt-1 and Flk1/KDR genes in the lung vasculature.[106] Also, Flk-1/KDR and Flt-1 mRNAs were substantially upregulated throughout the heart following myocardial infarction in the rat.[107] However, in vitro studies have yielded unexpected results. Hypoxia increases VEGF receptor number by 50% in cultured bovine retinal capillary endothelial cells but the expression of KDR is not induced but paradoxically shows an initial downregulation.[108] Brogi et al have proposed that the hypoxic upregulation of KDR observed in vivo is not direct but requires the release of an unidentified paracrine mediator from ischemic tissues.[109] Recent studies have provided evidence for a differential transcriptional regulation of the Flt-1 and KDR genes by hypoxia.[110] When human umbilical vein endothelial cells (HUVEC) were exposed to hypoxic conditions in vitro, increased levels of Flt-1 expression were observed. In contrast, Flk-1/KDR mRNA levels were unchanged or slightly repressed. Promoter deletion analysis demonstrated a 430-bp region of the Flt-1 promoter to be required for transcriptional activation in response to hypoxia. This region includes a heptamer sequence matching the HIF-1α consensus binding site previously found in other hypoxia-inducible genes. The element mediating the hypoxia response was further defined as a 40 bp sequence including the putative HIF-1 binding site. Such an element was not found in the Flk-1/KDR promoter. These findings indicate that, unlike the KDR/Flk-1 gene, the Flt-1 receptor gene is directly upregulated by hypoxia via a hypoxia-inducible enhancer element located at position -976 to -937 of the Flt-1 promoter.[110]

Negative inhibitors of KDR gene expression in cultured endothelial cells include TNF-α[111] and TGF-β.[112]

Role of VEGF and Its Receptors in Physiological Angiogenesis

Distribution of VEGF, Flk-1/KDR and Flt-1 mRNA

The proliferation of blood vessels is crucial for a wide variety of physiological processes such as embryonic development, normal growth and differentiation, wound healing and reproductive functions.

During embryonic development, VEGF expression is detected within the first few days following implantation in the giant cells of the trophoblast.[79,113] At later developmental stages in the mouse or rat embryos, the VEGF mRNA is expressed in several organs, including heart, vertebral column, kidney, and along the surface of the spinal cord and brain. In the developing mouse brain, the highest levels of mRNA expression are associated with the choroid plexus and the ventricular epithelium.[113] In the human fetus (16-22 weeks), VEGF mRNA expression is detectable in virtually all tissues and is most abundant in lung kidney and spleen.[114]

In situ hybridization studies have shown that the Flk-1 mRNA is expressed in the yolk sac and intraembryonic mesoderm and later on in angioblasts, endocardium and small and large vessel endothelium.[115,116] These findings strongly suggested a role for Flk-1 in the regulation of vasculogenesis and angiogenesis. Other studies have demonstrated that expression of Flk-1 mRNA is first detected in the proximal-lateral embryonic mesoderm, which gives rise to the heart.[117] Flk-1 is then detectable in endocardial cells of heart primordia and subsequently in the major embryonic and extraembryonic vessels.[117] These studies have indicated that Flk-1 may be the earliest marker of endothelial cells precursors. The Flt-1 mRNA is selectively expressed in vascular endothelial cells, both in fetal and adult mouse tissues.[118] Similar to the high affinity VEGF binding, the Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells, suggesting a role for Flt-1 in the maintenance of endothelial cells.[118]

VEGF expression is also detectable around microvessels in areas where endothelial cells are normally quiescent, such as kidney glomerulus, pituitary, heart, lung, and brain.[119,120] These findings raised the possibility that VEGF may be required not only to induce active vascular proliferation but, at least in some circumstances, also for the maintenance of the differentiated state of blood vessels.[119] In agreement with this hypothesis, Alon et al have shown that VEGF acts as a survival factor, at least for the developing retinal vessels.[121] They propose that hyperoxia-induced vascular regression in the retina of neonatal animals is a consequence of inhibition of VEGF production by glial cells. As previously noted, VEGF exerts anti-apoptotic effects on cultured HUVEC that are be mediated by the PI3 kinase/Akt pathway.[101]

The Flk-1/KDR, Flt-1 and VEGF Gene Knockouts in Mice

Recent studies have demonstrated that both Flt-1 and Flk-1/KDR are essential for normal development of embryonic vasculature. However, their respective roles in endothelial cell proliferation and differentiation appear to be distinct.[122,123] Mouse embryos homozygous for a targeted mutation in the Flt-1 locus died in utero between days 8.5 and 9.5.[122] Endothelial cells developed in both embryonic and extraembryonic sites but failed to organize in normal vascular channels. Mice where the Flk-1 gene had been inactivated lacked vasculogenesis and also failed to develop blood islands. Hematopoietic precursors were severely disrupted and organized blood vessels failed to develop throughout the embryo or the yolk sac, resulting in death in utero between days 8.5 and 9.5.[123]

However, these findings do not necessarily imply VEGF as being equally essential, since other ligands might potentially activate the Flt-1 and Flk-1 receptors and thus substitute VEGF action. Very recent studies[12,13] have generated direct evidence for the role played by VEGF in embryonic vasculogenesis and angiogenesis. Inactivation of the VEGF gene in mice resulted in embryonic lethality in heterozygous embryos, between day 11 and 12. The $VEGF^{+/-}$ embryos were growth retarded and also exhibited a number of developmental anomalies. The forebrain region appeared significantly underdeveloped. In the heart region, the outflow region was grossly malformed; the dorsal aortae were rudimentary, and the thickness of the ventricular wall was markedly decreased.

The yolk sac revealed a markedly reduced number of nucleated red blood cells within the blood islands. Also, the vitelline veins failed to fuse with the vascular plexus of the yolk sac. Significant defects in the vasculature of other tissues and organs including placenta and nervous system were observed. In situ hybridization confirmed expression of VEGF mRNA in heterozygous embryos. Thus, the VEGF+/- phenotype appears to be due to gene dosage and not to maternal imprinting.

While several heterozygous phenotypes have been described[124] this may be the first example of embryonic lethality following the loss of a single allele of a gene that is not maternally imprinted. Therefore, VEGF and its receptors are essential for blood island formation and angiogenesis such that even reduced concentrations of VEGF are inadequate to support a normal pattern of development. Interestingly, inactivation of the PlGF gene does not result in embryonic lethality, even in the homozygous state.[125] PlGF-/- mice are viable and fertile, although they may have some impairment of wound healing. These findings suggest that other members of the VEGF gene family may not be equally critical for vascular development.

Role of VEGF in Female Reproductive Tract Angiogenesis

The development and endocrine function of the ovarian corpus luteum (CL) are dependent on the growth of new capillary vessels.[126] Earlier studies suggested the release of angiogenic factors from developing CL.[127] The VEGF mRNA is temporally and spatially related to the proliferation of blood vessels in the rat, mouse and primate ovary and in the rat uterus, suggesting that VEGF is a mediator of the cyclical growth of blood vessels which occurs in the female reproductive tract.[128-131] Very recently, this hypothesis has been tested directly, thanks to the availability of effective inhibitors of rodent VEGF, such as truncated soluble Flt-1 receptors.[89,132] Treatment with Flt (1-3) IgG resulted in virtually complete suppression of CL angiogenesis in a rat model of hormonally induced ovulation.[132] This effect was associated with inhibition of CL development and progesterone release. Also, failure of maturation of the endometrium was observed, probably reflecting suppression of ovarian steroid production plus a direct inhibition of locally produced VEGF. Areas of ischemic necrosis were demonstrated in the CL of treated animals. However, no effect on the preexisting ovarian vasculature was observed. These findings demonstrate that, in spite of the redundancy of potential mediators, VEGF is essential for CL angiogenesis. Furthermore, they have implications for the control of fertility and the treatment of ovarian disorders characterized by hypervascularity and hyperplasia such as the policystic ovary syndrome.[133]

Role of VEGF in Pathologic Angiogenesis

Tumor Angiogenesis

Many tumor cell lines secrete VEGF in vitro.[119] In situ hybridization studies have demonstrated that the VEGF mRNA is markedly upregulated in the vast majority of human tumors so far examined. These include: lung,[134,135] breast,[136,137] gastrointestinal tract,[138,139] kidney,[140] bladder,[140] ovary,[141] endometrium[142] and uterine cervix[143] carcinomas, angiosarcoma,[144] germ cell tumors[145] and several intracranial tumors including glioblastoma multiforme[146-148] and sporadic, as well as VHL syndrome-associated, capillary hemangioblastoma.[149,150] In glioblastoma multiforme and other tumors with significant necrosis, the expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas.[146-148] A correlation exists between the degree of vascularization of the malignancy and VEGF mRNA expression.[143,149,150] In virtually all specimens examined, the VEGF mRNA was expressed in tumor cells but not in endothelial cells. In contrast, the mRNAs for Flt-1 and KDR were upregulated in the endothelial cells associated with the tumor.[138,151] These findings are consistent with the hypothesis that VEGF is primarily a paracrine mediator.[152] Immunohistochemical studies have localized the VEGF protein not only to the tumor cells but also to the vasculature.[138,147] This localization indicates that tumor-secreted VEGF accumulates in the target cells.[153] Interestingly, recent studies have suggested that the angiogenesis mediated by the HIV-1 Tat protein[154] requires

activation of the KDR receptor.[155] Tat induces growth of Kaposi's sarcoma (KS) spindle cells and has been implicated in the vascularity of the KS lesions.[155]

Elevations in VEGF levels have been detected in the serum of some cancer patients.[156] Also, a correlation has been noted between VEGF expression and microvessel density in primary breast cancer sections.[157] Postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of VEGF-poor, suggesting that expression of VEGF is associated with stimulation of angiogenesis and with early relapse in primary breast cancer.[158] A similar correlation has been described in gastric carcinoma patients.[159] VEGF-positivity in tumor sections was correlated with vessel involvement, lymph node metastasis and liver metastasis. Furthermore, patients with VEGF-positive tumors had a worse prognosis than those with VEGF-negative tumors.[159]

The availability of specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis in vivo and in vitro[160] made it possible to generate direct evidence for a role of VEGF in tumorigenesis. In a study published by Kim et al in 1993 such antibodies were found to exert a potent inhibitory effect on the growth of three human tumor cell lines injected subcutaneously in nude mice, the SK-LMS-1 leiomyosarcoma, the G55 glioblastoma multiforme and the A673 rhabdomyosarcoma.[161] The growth inhibition ranged between 70% and >95%. Subsequently, other tumor cell lines were found to be inhibited in vivo by this treatment.[162-165]

In agreement with the hypothesis that inhibition of neovascularization is the mechanism of tumor suppression, the density of blood vessels was significantly lower in sections of tumors from antibody-treated animals as compared with controls. Furthermore, neither the antibodies nor VEGF had any effect on the in vitro growth of the tumor cells.[161] Intravital videomicroscopy techniques have allowed a more direct verification of the hypothesis that anti-VEGF antibodies indeed block tumor angiogenesis.[166] Non-invasive imaging of the vasculature revealed a nearly complete suppression of tumor angiogenesis in anti-VEGF treated animals as compared with controls, at all time points examined.[166]

VEGF is a mediator of the in vivo growth of human colon carcinoma HM7 cells in a nude mouse model of liver metastasis.[162] Treatment with anti-VEGF monoclonal antibodies resulted in a dramatic decrease in the number and size of metastases. Similarly, administration of anti-VEGF neutralizing antibodies inhibited primary tumor growth and metastasis of A431 human epidermoid carcinoma cells in scid mice[163] or HT-1080 fibrosarcoma cells implanted in BALB/c nude mice.[164]

Recently, Borgstrom et al[167] have shown that a combination treatment that includes anti-VEGF monoclonal antibody and doxorubicin results in a significant enhancement of the efficacy of either agent alone and led in some cases to complete regression of tumors derived from MCF-7 breast carcinoma cells in nude mice.

Intravital fluorescence microscopy and video imaging analysis have been also applied to address the important issue of the effects of VEGF on permeability and other properties of tumor vessels.[168] Treatment with anti-VEGF monoclonal antibodies was initiated when tumor xenografts were already established and vascularized and resulted in time-dependent reductions in vascular permeability.[168] These effects were accompanied by striking changes in the morphology of vessels, with dramatic reduction in diameter and tortuosity. This reduction in diameter is expected to block the passage of blood elements and eventually stop the flow in the tumor vascular network. A regression of blood vessels was observed after repeated administrations of anti-VEGF antibody. These findings suggest that tumor vessels require constant stimulation with VEGF in order to maintain not only their proliferative properties but also some key morphological features.[168]

An independent verification of the hypothesis that the VEGF action is required for tumor angiogenesis has been provided by the finding that retrovirus-mediated expression of a dominant negative Flk-1 mutant, which inhibits signal transduction through wild-type Flk-1 receptor, suppresses the growth of glioblastoma multiforme as well as other tumor cell lines in vivo.[169]

Angiogenesis Associated with Other Pathological Conditions

Diabetes mellitus, occlusion of central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization.[2] The new blood vessels may lead to vitreous hemorrhage, retinal detachment, neovascular glaucoma, and eventual blindness.[2] Diabetic retinopathy is the leading cause of blindness in the working population.[170] All of these conditions are known to be associated with retinal ischemia.[171] In 1948, Michaelson proposed that a key event in the pathogenesis of these conditions is the release by the ischemic retina into the vitreous of diffusible angiogenic factor(s) ("factor X") responsible for retinal and iris neovascularization.[172] VEGF, by virtue of its diffusible nature and hypoxia-inducibility, was an attractive candidate as a mediator of intraocular neovascularization. Accordingly, elevations of VEGF levels in the aqueous and vitreous of eyes with proliferative retinopathy have been described.[173-175] In a large series, a strong correlation was found between levels of immunoreactive VEGF in the aqueous and vitreous humors and active proliferative retinopathy VEGF levels were undetectable or very low (<0.5 ng/ml) in the eyes of patients affected by non-neovascular disorders or diabetes without proliferative retinopathy.[173] In contrast, the VEGF levels were in the range of 3-10 ng/ml in the presence of active proliferative retinopathy associated with diabetes, occlusion of central retinal vein or prematurity.

More direct evidence for a role of VEGF as a mediator of intraocular neovascularization has been generated in a primate model of iris neovascularization and in a murine model of retinopathy of prematurity.[176,177] In the former, intraocular administration of anti-VEGF antibodies dramatically inhibits the neovascularization that follows occlusion of central retinal veins.[178] Likewise, soluble Flt-1 or Flk-1 fused to an IgG suppresses retinal angiogenesis in the mouse model.[179]

Neovascularization is a major cause of visual loss also in age-related macular degeneration (AMD), the overall leading cause of blindness.[2] Most AMD patients have atrophy of the retinal pigment epithelial and characteristic formations called "drusen". A significant percentage of AMD patients (~20%) manifest the neovascular (exudative) form of the disease. In this condition, the new vessels stem from the extraretinal choriocapillary.[2] Leakage and bleeding from these vessels may lead to damage to the macula and ultimately to loss of central vision. Because of the proximity of the lesions to the macula, laser photocoagulation or surgical therapy are of very limited value. Very recent studies have documented the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients.[180,181] These findings suggests a role for VEGF in the progression of AMD-related choroidal neovascularization, raising the possibility that a pharmacological treatment with monoclonal antibodies or other VEGF inhibitors may constitute a therapy for this condition.

Two independent studies have suggested that VEGF is involved in the pathogenesis of rheumatoid arthritis (RA), an inflammatory disease where angiogenesis plays a significant role.[182,183] The RA synovium is characterized by the formation of pannus, an extensively vascularized tissue that invades and destroys the articular cartilage.[184] Levels of immunoreactive VEGF were found to be high in the synovial fluid of RA patients while they were very low or undetectable in the synovial fluid of patients affected by other forms of arthritis or by degenerative joint disease.[182,183] Furthermore, anti-VEGF antibodies significantly reduced the endothelial cell chemotactic activity of the RA synovial fluid.[182]

It has been shown that VEGF expression is increased in psoriatic skin.[185] Increased vascularity and permeability are characteristic of psoriasis. Also, VEGF mRNA expression has been examined in three bullous disorders with subepidermal blister formation, bullous pemphigoid, erythema multiforme and dermatitis herpetiformis.[186]

Angiogenesis is also important in the pathogenesis of endometriosis, a condition characterized by ectopic endometrium implants in the peritoneal cavity. Recently, elevation of VEGF in the peritoneal fluid of patients with endometriosis have been reported.[187,188] Immunohistochemistry indicated that activated peritoneal fluid macrophages as well as tissue macrophages within the ectopic endometrium are the main source of VEGF in this condition.[187,188] VEGF upregulation has been also implicated in the hypervascularity of the ovarian stroma that characterizes polycystic ovary syndrome.[189]

Moreover, Sato et al proposed that VEGF may be responsible for the characteristic hypervascularity of Graves' disease.[190] TSH, insulin phorbol ester, dibutiryl cAMP and Graves' IgG were found to stimulate VEGF mRNA expression in cultured human thyroid follicles.[190]

Therapeutic Applications of VEGF-Induced Angiogenesis

The availability of agents able to promote the growth of new collateral vessels would be potentially of major therapeutic value for disorders characterized by inadequate tissue perfusion and might constitute an alternative to surgical reconstruction procedures. For example, chronic limb ischemia, most frequently caused by obstructive atherosclerosis affecting the superficial femoral artery, is associated with a high rate of morbidity and mortality and treatment is currently limited to surgical revascularization or endovascular interventional therapy.[191] No pharmacological therapy has been shown to be effective for this condition. It has been shown that intra-arterial or intramuscular administration of $rhVEGF_{165}$ may significantly augment perfusion and development of collateral vessels in a rabbit model where chronic hindlimb ischemia was created by surgical removal of the femoral artery.[192] These studies provided angiographic evidence of neovascularization in the ischemic limbs. Arterial gene transfer with cDNA encoding VEGF also led to revascularization in the same rabbit model to an extent comparable to that achieved with the recombinant protein.[193,194] In addition, the hypothesis that the angiogenesis initiated by the administration of VEGF improved muscle function in ischemic limbs was tested by Walder et al.[195] A single intra-arterial injection of $rhVEGF_{165}$ augmented muscle function in this rabbit model of peripheral limb ischemia. This exercise-induced hyperemia was significantly improved in ischemic limbs treated with $rhVEGF_{165}$.[195] Such improvement in perfusion was, however, not seen in other non-ischemic tissues including the contralateral limb. Similarly, Bauters et al[196] have shown that both maximal flow velocity and maximal blood flow are significantly increased in ischemic limbs following VEGF administration. Other studies have shown that VEGF administration also leads to a recovery of normal endothelial reactivity in dysfunctional endothelium. Following obstruction of a large artery and development of collateral vessels, the increase in blood flow which normally follows acetylcholine infusion is severely blunted; serotonin paradoxically leads to a decrease in blood flow.[197] Thirty days after a single intra-arterial bolus of $VEGF_{165}$, restoration of normal increase in blood flow in ischemic rabbit hindlimb following acethylcholine or serotonin infusion was demonstrated.[197]

Banai et al have shown that VEGF administration results in increased coronary blood flow in a dog model of coronary insufficiency.[198] Following occlusion of the left circumflex coronary artery, daily intraluminal injections of rhVEGF distal to the occlusion resulted in a significant enhancement in collateral blood flow over a four week period. In addition, Harada et al[199] have demonstrated that extraluminal administration of as little as 2 μg of rhVEGF by an osmotic pump results in a significant increase in coronary blood flow in a pig model of chronic myocardial ischemia created by ameroid occlusion of the left proximal circumflex artery. Also, magnetic resonance imaging provided a non-invasive assessment of the benefits secondary to VEGF administration in the porcine model.[200] Image series converted to a space-time map demonstrated reduction in the size of the ischemic zone and decreased delay in contrast arrival after VEGF treatment. These findings demonstrated improvement in cardiac global and regional function and reduced infarct size, resulting from enhanced collateral blood supply.[200,201]

A further potential therapeutic application of VEGF is the prevention of restenosis following percutaneous transluminal angioplasty (PTA). Between 15% and 75% of patients undergoing PTA for occlusive coronary or peripheral arterial disease develop restenosis within six months.[191] It has been proposed that damage to the endothelium is a crucial event triggering fibrocellular intimal proliferation.[202] Therefore, the induction of rapid re-endothelialization may be an effective strategy to prevent the cascade of events leading to neointima formation and ultimately to restenosis in patients. Recent evidence shows that VEGF accelerates re-endothelialization and also attenuates intimal hyperplasia in balloon-injured rat carotid artery or rabbit aorta.[203,204]

Recently, the hypothesis that VEGF may result in therapeutically significant angiogenesis in humans has been tested by Isner et al in a gene therapy trial in patients with severe limb ischemia.[205]

A case report of an interim analysis of this trial has been published.[206] Arterial gene transfer of 2000 μg of naked plasmid DNA encoding $VEGF_{165}$, applied to the hydrogel polymer coating of an angioplasty balloon, resulted in angiographic and histologic evidence of angiogenesis in the knee mid-tibial and ankle levels four weeks after the transfer. Such effects persisted at a twelve-week view.[206] In a subsequent trial, the $VEGF_{165}$ cDNA was injected intramuscularly.[207] Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers and/or rest pain due to peripheral arterial disease. A total dose of 4000 μg of naked plasmid DNA encoding $VEGF_{165}$ was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved; newly visible collateral blood vessels were directly documented by contrast angiography in seven limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in eight limbs. Ischemic ulcers healed or markedly improved in four of seven limbs, including successful limb salvage in three patients recommended for below-knee amputation. These findings suggest that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia.

Perspectives

The recent findings that heterozygous mutations inactivating the VEGF gene result in profound deficits in vasculogenesis and blood island formation, leading to early intrauterine death, emphasize the pivotal role played by this molecule in the development the vascular system. Future studies, using inducible gene knock out technology[208] should help determine when the embryo is most vulnerable to VEGF deficiency.

The elucidation of the signal transduction properties of the Flt-1 and KDR receptors holds the promise to dissect the pathways leading to such fundamental biological events as endothelial cell differentiation, morphogenesis and angiogenesis. Furthermore, a more complete understanding of the signaling events involving other endothelial cell specific tyrosine kinases as well as cell adhesion molecules and their interrelation with the VEGF/VEGF receptor system should provide a more integrated view of the biology of the endothelial cell, both in normal and abnormal circumstances. In this context, recent studies have shown that VEGF-mediated angiogenesis requires a specific vascular integrin pathway, mediated by $\alpha v\beta 5$.[209] Furthermore, a ligand selective for the endothelial cell-specific tyrosine kinase Tie-2 has been recently identified and named angiopoietin (Ang)-1.[210] Gene knockout studies have shown that Ang-1 is required for the correct assembly of the vessel wall.[5] Ang-1 seems to play a crucial role in mediating reciprocal interactions between the endothelium and surrounding matrix and mesenchyme, and has a later role than VEGF in angiogenesis. Also, unlike VEGF, Ang-1 does not directly stimulate endothelial cell growth. Interestingly, very recent studies provide evidence for the existence of Ang-2, a natural antagonist for the Tie-2 receptor.[211] Transgenic expression of Ang-2 disrupted blood vessel formation. The interrelation between the VEGF and Ang systems is likely to be an area of intense investigation in vascular biology.

The paradoxical finding that, in spite of the redundancy of angiogenic mediators, inactivation of VEGF alone substantially suppresses angiogenesis in a wide variety of physiological and pathological circumstances could be, at least in part, explained if one assumed the VEGF is a mediator of other angiogenic pathways. This hypothesis has recently received direct support by studies on FGF4.[212] The addition of conditioned medium of transfected cells expressing FGF4 to HUVEC resulted in a dose-dependent stimulation of VEGF, accompanied by a proliferative response. The induction of the angiogenic phenotype in HUVECs were completely suppressed by adding a neutralizing anti-VEGF antibody to FGF4-containing conditioned medium. These results suggest an indirect angiogenic activity of FGF-4 through the autocrine induction of VEGF secretion. Whether this paradigm applies to growth factors other than FGF4 remains to be established.

An attractive possibility is that recombinant VEGF or gene therapy with VEGF gene may be used to promote endothelial cell growth and collateral vessel formation. This would represent a novel therapeutic modality for conditions that frequently are refractory to conservative measures

and unresponsive to pharmacological therapy. Clinical trials using rhVEGF$_{165}$ for the treatment of myocardial ischemia associated with coronary artery disease are ongoing.

The high expression of VEGF mRNA in human tumors, the presence of the VEGF protein in ocular fluids of individuals with proliferative retinopathies and in the synovial fluid of RA patients as well as the localization of VEGF in AMD lesions strongly supports the hypothesis that VEGF is a key mediator of angiogenesis associated with various disorders. Therefore, anti-VEGF antibodies or other inhibitors of VEGF may be of therapeutic value for a variety of malignancies as well as for other disorders, used alone or in combination with other agents. Very recently, a humanized version of a high-affinity anti-VEGF monoclonal antibody, which retains the same affinity and efficacy as the original murine antibody, has been generated[213] and is being tested in humans as a treatment for solid tumors, alone or in combination with chemotherapy.

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CHAPTER 8

Regulation of Angiogenesis by Fibroblast Growth Factors

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Polypeptide factors which directly influence the migratory and proliferative activity of the human endothelial cell in vitro are well recognized as mediators of human angiogenesis in vivo. It is also well recognized that members of the fibroblast growth factor (FGF) gene family are not only potent human endothelial cell mitogens, but the survival and proliferation of human endothelial cells in vitro is absolutely dependent upon the presence of extracellular FGF. Indeed, in the absence of exogenous FGF, human endothelial cells do not proliferate but rather undergo apoptosis in vitro. Although these observations may also apply to other biological activities regulated by FGF gene family members including skeletal and smooth muscle growth, neurogenesis and organ/tissue repair, this review will focus on the role of FGF gene family members in the regulation of angiogenesis. For a description of the role of the FGF gene family in these and other biological processes as well as an historical perspective of the role of polypeptide growth factors in the regulation of angiogenesis, the following citations are highly recommended.[1-7]

The FGF Gene Family

There are presently 18 members of the FGF gene family based upon structural similarities to the two FGF prototypes, FGF-1 (acidic) and FGF-2 (basic).[6,8-12] These features are diagrammatically represented in Figure 8.1 which also demonstrates the presence or absence of a classical peptide sequence to direct import into the endoplasmic reticulum (ER) for transport via the Golgi apparatus to the extracellular compartment. Although many of the FGF gene family members were identified as oncogenes (FGF-3, FGF-4, FGF-5 and FGF-6),[13] it has been demonstrated that the oncogenic potential of FGF-1,[14,15] FGF-2[16,17] and FGF-4[18] is directly related to the presence or absence of a classical signal peptide sequence. Indeed, ligation of a signal peptide sequence to the FGF prototypes yields a transforming gene whereas deletion of the signal peptide sequence from FGF-4 attenuates the transforming potential of FGF-4.[18] Thus, it is anticipated that those FGF gene family members which contain a signal peptide sequence (FGF-7, FGF-8, FGF-9, FGF-10, FGF-13,[19] FGF-15, FGF-16, FGF-17 and FGF-18) will ultimately be characterized as oncoproteins while FGF-11, FGF-12 and FGF-14 will not.

Although the biological targets of the individual members of the FGF gene family are not known, it is anticipated that there will be a considerable degree of functional redundancy and specificity within the FGF gene family. While FGF-1 is the only member of the FGF gene family capable of interacting with all known members and isoforms of the FGF receptor (R) gene family,[20] FGF-7 is elegantly specific as a functional ligand for a specific isoform of FGFR-2.[21-23] Given the diverse set of cellular targets modified by members of the FGF gene family including epithelial, endothelial, neuronal and other cell types[13,24] as well as the ability of the FGFs to directly influence cell survival,[25-28] differentiation,[29,30] migration and proliferation,[31-33] it is perhaps not surprising that the function of

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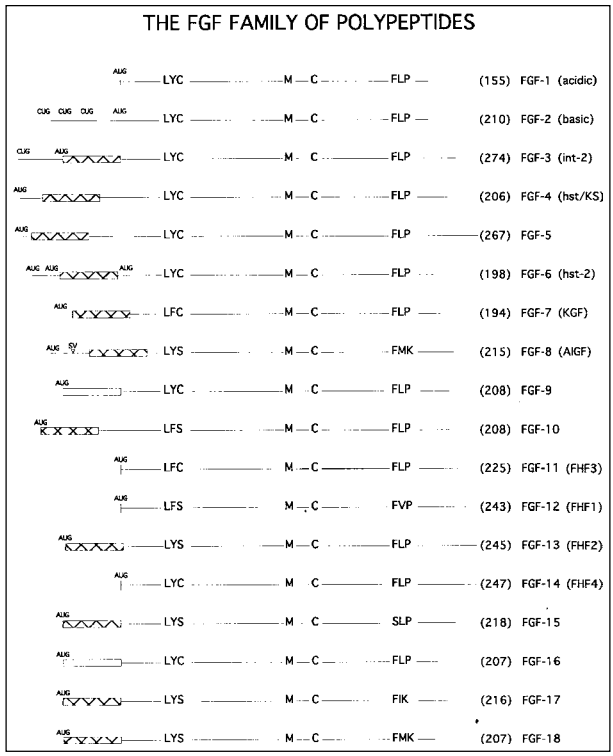

Fig. 8.1. The FGF gene family. The amino acid residues conserved among all know family members are shown. AUG indicates the position of conventional (Met-initiated) translation start sites and CUG the presence of alternative (Leu-initiated) translation start sites within the messenger RNA. The stippled boxes represent functional signal peptide sequences for conventional ER-Golgi mediated secretion. The open boxes indicate the proteins that are constitutively released but for which no typical signal peptide sequence has been predicted.

the individual members of the FGF gene family will be tightly regulated and will include transcriptional, translational, post-translational and trafficking elements of control. This is perhaps best illustrated by examining the differences between the FGF prototypes and FGF-3.

FGF-1 represents the least complicated translation product within the FGF gene family since unlike other members, the FGF-1 open-reading frame (ORF) is flanked by termination codons.[34,35] In comparison, the FGF-2[36-39] and FGF-3[40] transcripts exhibit multiple translation products directed by the presence of alternative 5'-CUG translational start sites (Fig. 8.2). While these alternative translation products alter the intracellular trafficking properties of FGF-2[41-43] and FGF-3[44,45] and may be cell- and tissue-specific with respect to their expression patterns, it is unclear how these intracellular trafficking events alter the functional properties of the individual translation product. However, some insight has been obtained from studies with FGF-1 since engineering FGF-1 to traffic to the nucleus as an endogenous translation product does not enable FGF-1 to exit the cell.[46] Thus, it is reasonable to anticipate that the expression of these alternative FGF translation products may be able to modify the functional activities of the conventional FGF translation product.

Whereas the FGF-1 transcript appears to present a single translation product, expression of the FGF-1 transcript appears to be quite complicated.[47-51] Indeed, the FGF-1 gene is transcribed into at lease six different FGF-1 transcripts each encoding the conventional (residues 1 to 154) FGF-1 translation product (Fig. 8.3). These FGF-1 mRNAs are a result of alternative transcription of a single FGF-1 gene with each FGF-1 transcript containing a constant and variable 5'-untranslated region (UTR) (Fig. 8.3). While the functional significance of these alternatively spliced transcripts is unclear, it is anticipated that cell- and tissue-specific FGF-1 transcript expression may be regulated by this feature.[49,51-54] Further, since it is known that the FGF-1 ORF exhibits considerable stem-loop structures which may be involved in translational control,[55] it is possible that the complexity of the large 5'-UTR in the FGF-1 transcript may be involved in the regulation of FGF-1 translation perhaps

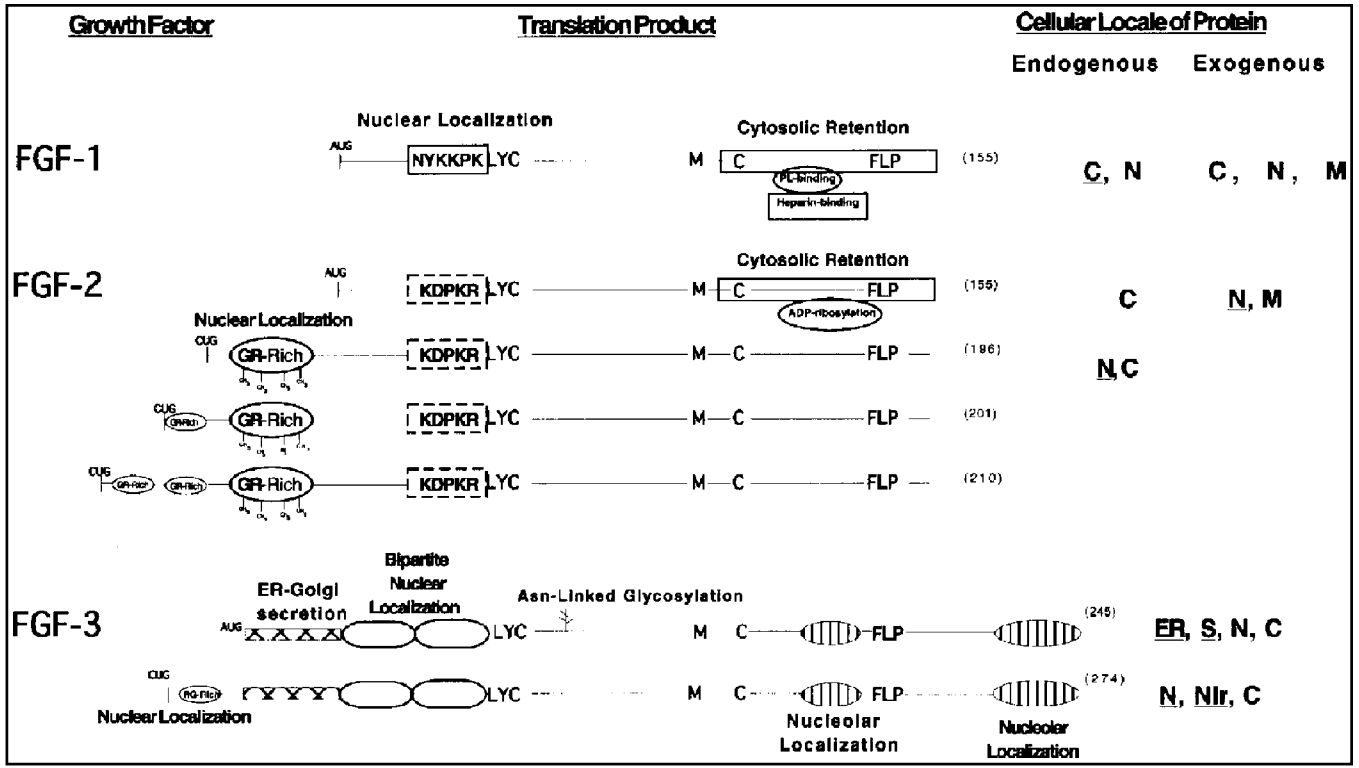

Fig. 8.2. Subcellular localization and posttranslational modifications of FGF-1, FGF-2, and FGF-3 proteins. C, cytosol; N, nucleus; M, membrane fraction-associated; ER, endoplasmic reticulum system; S, constitutively secreted; N1; nucleolus. The prominent localization is underlined. The different translation products are shown separately. Also indicated are the positions of some functional domains including the ones involved in phospholipid- and heparin-binding in vitro as well as the sites of extensive methylation in vivo (GR-rich) which are involved in determining of the subcellular localization of FGF-2 and FGF-3 proteins.

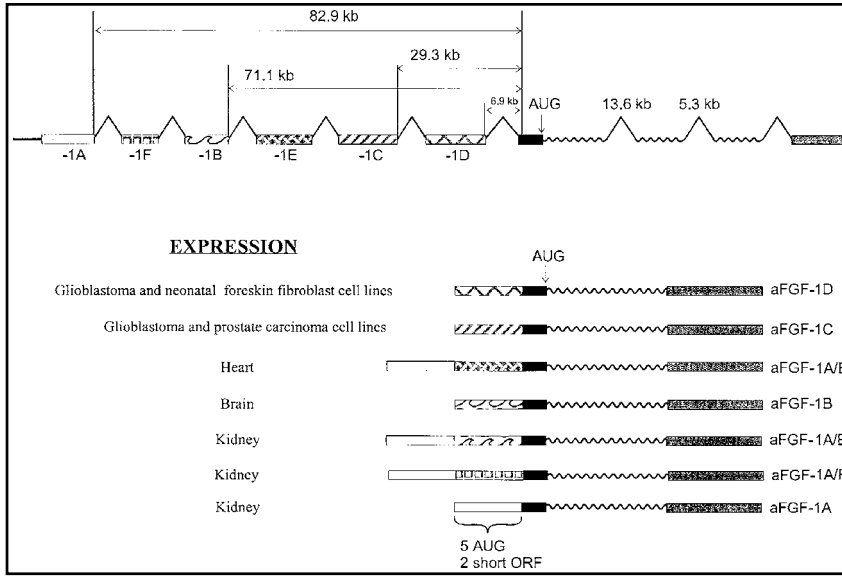

Fig. 8.3. Schematic of the structure of the human FGF-1 gene. The entire gene encompasses more than 100 kilobase pairs. The gene includes three protein coding exons and at least six untranslated exons that undergo alternative splicing to the first coding exon and generate at least seven different cytosolic messenger RNA species. The transcriptional start sites have not been defined for each exon. The expression of each FGF-1 messenger RNA species is regulated in a tissue-specific manner.

utilizing cell- and tissue-specific trans-acting factors which are able to recognize novel cis-acting sequences within the unique 5'-UTR of the FGF-1 transcript. Although there are examples of translational control regulated by trans-acting proteins,[56-58] there is currently no direct evidence to support this premise for FGF-1.

These complexities are also illustrated by the data obtained from murine gene deletion experiments. While deletion of the FGF-4 gene results in failure to develop beyond implantation,[59] the FGF-1 (Claudio Basilico, personal communication), FGF-2,[60] FGF-3[61] and FGF-5 null/null mice[62] only exhibit some physiologic and pathologic phenotypes, these observations are indeed surprising since the FGF prototypes are mesoderm inducers. However, given the level of structural redundancy within the FGF gene family, this may not be so surprising especially since the FGFR gene family null/null mice yield numerous abnormal phenotypes in both embryos and adults.[63-66] Thus, FGF gene redundancy may account for the lack of severe pathologic phenotypes in the embryo, and if this interpretation is accurate it would reflect the importance of FGF gene family members during embryogenesis. This concept is interesting since (1) FGF and FGFR gene family member expression is regulated in a temporal- and tissue-specific manner,[67,68] and (2) invertebrate FGF gene family members (*C. elegans*[69,70] and *D. melanogaster*[71-73]) do contain signal peptide sequences to direct their secretion through the conventional ER-Golgi-mediated secretion pathway and did not evolve a FGF gene which mimics the structural features of the FGF prototypes. Interestingly, the lack of a classical signal peptide sequence within a FGF gene first appears as a structural feature in the vertebrate *Xenopus*[74] and in all other vertebrate species examined to date.[6] Since invertebrate development does not appear to require the function of a signal peptide sequence-less FGF prototype, it is possible that vertebrate development may also not require the services of the FGF prototypes.

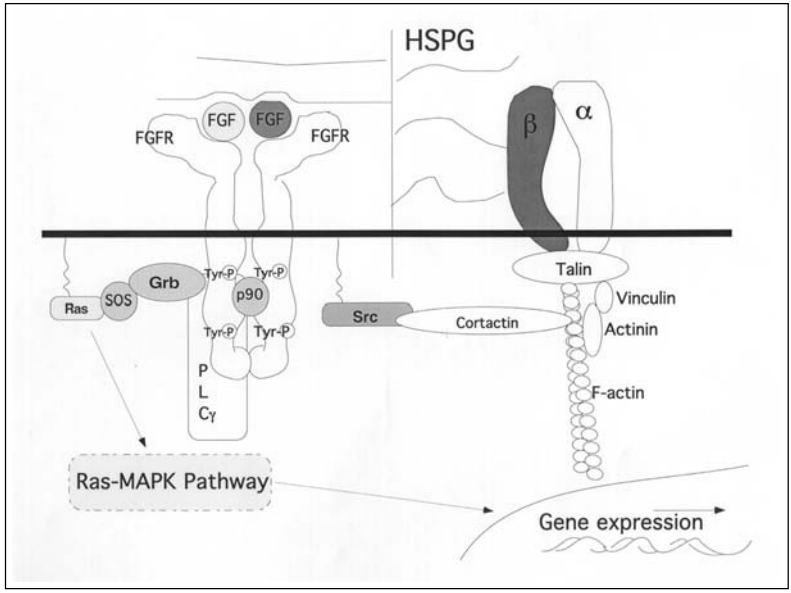

Fig. 8.4. Cell surface-mediated FGF signal transduction. The interaction of FGF ligands with their high affinity FGFR at the cell surface may be modulated by heparin sulfate proteoglycans and cell adhesion molecules such as integrins. The signalling cascade is initiated when noncovalent FGF dimers bind the FGFR and induce receptor dimerization, thus activating their intrinsic tyrosine kinase activity. Transphosphorylation of the two FGFR subunits exposes binding sites for several cytosolic proteins including phospholipase Cγ, a poorly characterized 90 kDa protein (p90) and members of the Src-family of tyrosine kinases. The cytoskeleton-associated protein cortactin is a substrate for Src-phosphorylation and is specifically phosphorylated upon FGFR activation. The extracellular FGF signal is further propagated to the nucleus by activation of the Ras-MAP Kinase pathways by interaction of Grb kinases with the SOS protein associated with the membrane protein Ras.

The FGFR Gene Family

The ability of FGF gene family members to regulate cell migration, proliferation, differentiation and survival is dependent on their ability to bind high and low affinity receptors present on the surface of target cells. These receptors include the four members of the FGFR gene family[67] which encode protein tyrosine kinases and cell-associated proteoglycans which include members of the syndecan gene family, glypican and perlecan.[75,76] While the function of these proteoglycans in FGF-dependent signaling remains controversial, it is likely that they act as co-receptors for FGFR-mediated signaling since they not only bind the translation products of the FGF gene family, but also protect FGF from modification by extracellular proteases during matrix remodeling. However, it is unlikely that intracellular signaling cascades are directly modified by the interaction between FGF and these proteoglycans.[77-79]

It is presently clear that the ability of the FGFR to regulate cell proliferation utilizes the ligand-activated Ras-MAPK pathway[1,80,81] and this is diagrammatically presented in Figure 8.4. Presumably, ligand activation of FGFR induces receptor dimerization and the activation of the intrinsic tyrosine kinase domain of the FGFR isoform which results in auto- and transphosphorylation of specific tyrosine residues on the receptor itself and on other intracellular substrate proteins.[2,82-84] These substrates are either non-enzymatic adapter proteins or intracellular enzymes which serve to amplify the signal generated by ligand-receptor binding. Indeed, there are differences between the four FGFR genes relative to the presence or absence of tyrosine phosphorylation sites within the

cytosolic domain of the FGFR isoforms and the ability to interact with intracellular signaling components such as phospholipase Cγ.[85] While these differences do exhibit differential abilities to signal the induction of cell growth, recent receptor chimera experiments suggest that while the tyrosine kinase domain of FGFR-1 is able to repress the differentiation of skeletal myoblasts, it is not sufficient for the stimulation of cell proliferation. In a series of rather elegant experiments, Kudla et al have demonstrated that a platelet-derived growth factor (PDGF)R:FGFR-1 chimera in which the extracellular domain of PDGF-R is fused to the FGFR-1 intracellular domain is able to regulate myogenesis but not the initiation of cell growth.[86] These data argue that the extracellular domain of FGFR-1 together with the FGF ligand and perhaps appropriate proteoglycan are required for the stimulation of cell proliferation.

The complexities of the FGFR-mediated signaling pathway are evident by the plasticity of the ligand-mediated intracellular signaling events. Indeed, the initiation of DNA synthesis by FGF requires the presence of the FGF ligand in the extracellular compartment during the entire G_1 phase of the cell cycle for the initiation of maximal DNA synthesis.[87] Removal of the ligand results in an attenuation of DNA synthesis and a reduction in Fos gene transcription and MAPK tyrosine phosphorylation. In contrast, removal of the FGF ligand does not appear to significantly effect the activation of the intracellular tyrosine kinase activity of Src,[88] its ability to phosphorylate its substrate, the F-actin-binding protein cortactin,[89] the transcription of the Myc gene nor the ability of cells to migrate in response to extracellular FGF.[81] Thus, the activation of the MAPK pathway by FGF-1 appears to correlate with the initiation of cell proliferation while the activation of the Src pathway appears to correlate with the ability of FGF-1 to stimulate cell migration. Interestingly, FGF-mediated mesoderm induction in Xenopus, an event which requires cell migration, also involves signaling mediated by the Src family tyrosine kinase, Laloo.[90]

The FGFR-mediated signaling pathway also appears to be integrated with cellular trafficking decisions. Although it is well described that the FGF prototypes are able to traffic in a FGFR-dependent manner to the nuclear compartment of a wide variety of cell types, the most direct evidence for FGF translocation across the plasma membrane came from Wiedlocha et al.[91] The addition of the lipid farnesyl group is a known post-translational and cytosol-specific event and the addition of the farnesylation sequence to FGF-1 results in the intracellular post-translational modification of the exogenous protein.[91] In addition, the ligation of the diphtheria toxin protein to FGF-1 results in the nuclear translocation of the chimera.[78] Although it is presently unclear how FGF is translocated across the plasma membrane, it is known that this process is FGFR-1-dependent and involves the trafficking of at least the three immunoglobin-like loop FGFR-1 translation product to the perinuclear region as a structurally intact and functional tyrosine kinase.[92] Additional studies using the two immunoglobin-like loop FGFR-1 isoform have also demonstrated that the first immunoglobulin-like loop is responsible for this trafficking event.[93,94] Although, the role of the FGFR in intracellular FGF traffic and the function of the FGF gene products within the nucleus remain unknown, the observations from immunohistochemical staining of a variety of tissue specimens in vivo imply that these trafficking events may be functionally significant.[95,96]

Mechanisms of FGF Release

Perhaps the most interesting observation which arose from the structural definition of the FGF prototypes was the absence of a classical signal peptide sequence to direct their secretion through the conventional ER-Golgi-mediated secretory pathway. Independent of any potential house-keeping function for the cytosolic and nuclear forms of the FGF prototypes, these polypeptide mitogens clearly act in an extracellular receptor-dependent manner to mediate their effects as survival, growth, migratory and differentiation factors.[1,5,13,32] Since the FGF prototypes require release from cytosol to initiate FGFR-dependent signaling events, a major issue within the FGF field has been to understand the mechanism of FGF prototype release. This mechanism also remains an issue within the interleukin-1,[97-101] annexin[102-107] and transglutaminase communities since these proteins are also known to act in an extracellular environment yet these structures lack a classical signal peptide sequence.

A priori, one would consider cell lysis as a result of cell death to be a contributor to the release of the FGF prototypes since their angiogenic, chondriogenic and neurogenic activities could significantly contribute to repair processes. Although it is possible that FGF-2 release may be regulated by apoptotic events,[110,111] the release of FGF-1 appears to be tightly regulated as an inducible event. Indeed, temperature stress has been well described as an inducer of FGF-1 release,[112-117] and it is anticipated that other forms of physical and biochemical stress including anoxia, hypoxia,[118] acidosis, and shear, as well as hormone depletion,[119] may also enable FGF-1 to be released from the cytosol. The release mechanism for FGF-1 in response to temperature stress requires gene transcription and translation,[112,113] is ATP-dependent[114] and occurs independent of the ER-Golgi apparatus since brefeldin A, a drug which interrupts the function of the Golgi apparatus does not impair the release of FGF-1.[112] Since ER-Golgi traffic regulates conventional secretion, the term "release" is used to describe the exit of cytosolic signal peptide-less proteins into the extracellular compartment by an ER-Golgi-independent pathway.[120]

The mechanism of FGF-2 release is also brefeldin A-insensitive although in the cell culture systems studied to date, FGF-2 release appears to be constitutive and not inducible.[121,122] Likewise, stress-mediated FGF-1 and constitutive FGF-2 release both appear to be independent of exocytosis and the multidrug resistance pathway since methylamine and verapamil do not repress their appearance as polypeptides in the extracellular compartment.[112,121-123] However, in contrast with the stress-induced release of FGF-1, the constitutive release of FGF-2 is sensitive to inhibition by cardenolides which implicate the function of the ion transport activity of the Na^+, K^+-ATPase in FGF-2 release.[48] Since similar agents are not able to repress the release of FGF-1 in response to temperature stress (Tarantini F, unpublished results), it is likely that the constitutive FGF-2 and stress-mediated FGF-1 release mechanisms have either diverged or are different among various cell types. Although it is difficult to exclude the latter, evidence suggests that cells that are able to release FGF-1 in response to temperature stress are not capable of mediating the release of FGF-2. Indeed, there appears to be a carboxy terminal domain in FGF-2 which is able to repress the release of FGF-1 in response to stress as a reporter gene chimera when the FGF-1 carboxy terminal sequence is replaced with the corresponding sequence from FGF-2 (Shi et al, 1997).[46] In contrast, FGF-1 is constitutively released when examined under conditions where FGF-2 release is also constitutive (Florkiewickz RZ, Baird A; personal communication). Thus, it is likely that while both FGF prototypes may be able to utilize the constitutive release pathway, the stress-induced pathway appears unique to FGF-1. However, there are reports of stress-induced FGF-2 release in diverse cell culture systems and as a result there may be a cell type-specific component to the stress-induced release pathway for both FGF prototypes.

While FGF-2 is constitutively released as a biologically active[112,113] polypeptide mitogen, FGF-1 is released in response to stress as an inactive protein with low heparin-binding affinity which requires activation by a reducing agent to generate both high heparin-binding affinity and biological activity. Biochemical and genetic evidence supports the hypothesis that FGF-1 is released as a latent homodimer and utilizes the cysteine residue at position 30 for homodimer formation.[113]However, the structural identity of the stress-induced heat shock chaperone which is involved in FGF-1 homodimer formation is presently not known. Since latent FGF-1 requires an extracellular reducing environment for activation, it is likely that in the absence of appropriate reducing equivalents, the FGF-1 homodimer would not be protected by heparan sulfate proteoglycans from extracellular proteases involved in matrix remodeling and as a result would be destroyed. This mechanism could also act as a safeguard to regulate inappropriate and/or the long-term consequences of FGF-1 signaling.

Recent studies have identified two components of the stress-induced FGF-1 release pathway and these include a proteolytic fragment of synaptotagmin (Syn)-1 and S100A13.[114,115,117] As described in Figure 8.5, Syn-1 is a transmembrane protein involved in the regulation of vesicular transport during conventional exocytosis.[124] While the Syn-1 gene encodes a p65 translation product, it is the p40 cytosolic domain of p65 Syn-1 which is involved in the regulation of FGF-1 release. Indeed, the FGF-1 homodimer is released in a stress-dependent manner as a detergent- and reducing agent-sensitive low heparin-binding affinity complex with the p40 fragment of Syn-1.[117] In addition, p40

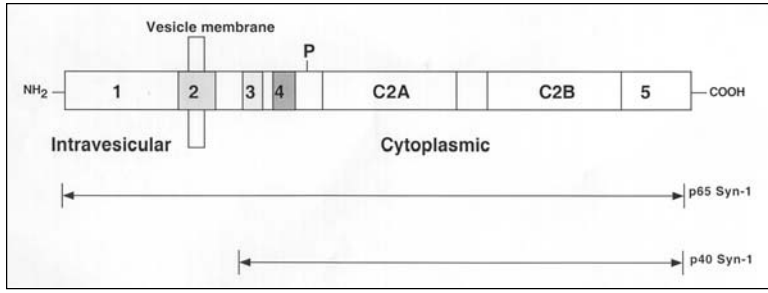

Fig. 8.5. The functional domains of Syn-1 (based upon Schiavo G et al, 1998). Syn-1 is a 65 kDa membrane protein with the following domains: 1 (residues 1-52) intravesicular portion is glycosylated in vivo; 2. (residues 53-78) single pass transmembrane domain; 3. (residues 90-112) protease sensitive site (e.g., trypsin) may be responsible for the generation of a p40 Syn-1 fragment; 4. (residues 102-119) the presence of an amphipatic alpha helice may cause stable dimerization of Syn-1; P. Thr residue 128 is phosphorylated in vivo by casein kinase II; C2A and C2B, this two internal repeats are highly homologous to the regulatory region of protein kinase C; C2A (residues 1350249) is associated with Ca^{2+}-dependent acidic phospholipid binding (e.g., PS) and C2B (residues 266-381) is associated with Ca^{2+}-independent binding to the clathrin receptor AP2 protein and binding of Syn-1 to immobilized heparin; 5. (residues 382-421), region highly conserved among members of the synaptotagmin gene family interacts with members of the neurexin family.

Syn-1 and FGF-1 have been isolated as a multiprotein denaturant-sensitive aggregate from ovine brain tissue extracts and this observation may reflect the biological significance of the stress-induced FGF-1 release pathway in vivo.[115]

The release of p40 Syn-1 with FGF-1 in response to stress anticipates the potential function of a proteolytic enzyme with the ability to recognize a hypersensitive proteolytic domain containing a basic amino acid-rich region near the interface of the Syn-1 cytosolic domain and the Syn-1 transmembrane sequence. Although the identity of the Syn-1 protease responsible for this cleavage is not known, it is anticipated that its enzymatic activity may be regulated by stress and possibly by cell cycle events. It is also not known whether the cleavage of p65 Syn-1 to p40 Syn-1 occurs on an intracellular exocytotic vesicle, at an intracellular site on the inner surface of the plasma membrane or within the extracellular compartment. However, the proteolytic cleavage of p65 Syn-1 as a component of the stress-induced FGF-1 release pathway assures that FGF-1 homodimer release is indeed an irreversible terminal event.

The presence of p40 Syn-1 and the FGF-1 homodimer as a denaturant- and reducing agent-sensitive complex in the extracellular environment suggests that the FGF-1 homodimer may have associated with p65 Syn-1 on the cytosolic side of a conventional secretory vesicle and utilize this intracellular traffic mechanism to gain access to the inner surface of the plasma membrane. Additional mechanistic insight into this pathway has recently been obtained by the purification of a multiprotein denaturant-sensitive aggregate from ovine brain which contained p40 Syn-1, FGF-1 and S100A13.[115] Interestingly, the S100 gene family encode calmodulin-like acidic signal peptideless proteins containing two calcium-binding EF-hand domains flanking a central hinge domain (Fig. 8.6).[125-127] Although the functions of the S100 gene family are not known, these proteins do interact with a variety of intracellular proteins including tubulin, annexin II, non-muscle myosin, vimentin, tau and microfibril-associated protein.[128,129] Interestingly, like FGF-1[116] and p40 Syn-1,[130] S100A13 is able to associate with acidic membrane phospholipids,[131] most notably phosphatidylserine, and the anti-inflammatory drug amlexanox, known to specifically interact with S100A13 and S100A2[132,133] is able to repress the stress-induced release of FGF-1 and p40 Syn-1.[115] Thus it is likely that S100A13 or perhaps other members of the highly conserved S100 gene family may play a role in the mechanism of stress-mediated FGF-1 release and it is also anticipated that the calmodulin-like properties of the S100 proteins may be functionally involved in the regulation of the intracellular traffic of the FGF-1 homodimer. These observations may be especially significant

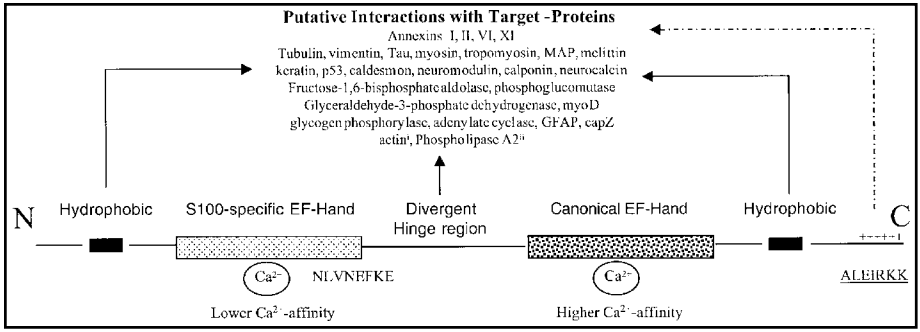

Fig. 8.6. The domain structure of S100 proteins (adapted from Kligman and Kilt, 1988; Schafer and Heizman, 1996). S100s are small (average 10 kDa Mr), acidic, soluble proteins that localize to different cellular regions including the cytoplasm, nucleus and ER-Golgi system. They may also be found extracellularly. Calcium modulates the interactions between S100s and a variety of target proteins the activities of which S100 are thought to modulate.

since the levels of the FGF prototypes[134,135] and some S100 proteins are elevated at sites of inflammation.[128,136]

It is also interesting that some S100 gene family members have been implicated as annexin-binding proteins.[137] Although annexin II does not contain a classical signal peptide sequence, it is well characterized as a cell surface-binding protein for plasminogen and tissue plasminogen activator[138,139] and as an acidic phospholipid-binding protein.[140] The interactions between annexin II and members of the S100 gene family may also be involved in the regulation of exocytosis in a variety of cell types including the endothelial cell.[141,142] Thus it will be of interest to determine whether annexin II also functions as a regulator of the stress-induced pathway of FGF-1 release possibly by its ability to interact with S100A13 (Fig. 8.7).

The mechanism by which the FGF-1 homodimer and p40 Syn-1 exit the cell during release is presently unknown. However, FGF-1 is known to exhibit molten globule character at temperatures consistent with temperature stress conditions in vitro and as a result is able to associate with and traverse acidic phospholipid-rich membranes.[143] In addition, cells stably transfected with the p40 Syn-1 fragment do not require stress to release p40 Syn-1 into the extracellular compartment; rather p40 Syn-1 release is constitutive.[114] This observation is particularly interesting. Indeed, although it is not known whether the p40 Syn-1 protein is also able to exhibit molten globule character, the interaction of Syn-1 with the plasma membrane may destabilize the lipid bilayer and facilitate membrane fusion or generate Syn-1-bound pores similar to the ones formed by the protein perforin.[144] Lastly, the release of the p40 Syn1:FGF-1 homodimer is ATP-dependent[114] and while the mechanism for ATP-dependence is not known, it is anticipated that this component of the stress-induced FGF-1 release pathway may be involved in flipping the FGF-1 release complex into the extracellular compartment. It is also anticipated that this process will involve the flipping of the acidic phospholipid, phosphatidylserine, which is already known to be ATP-dependent.[145] Interestingly, the gene encoding at least one component of this translocation machine has been identified and this gene termed scramblase,[146] encodes a signal peptideless membrane protein with a single site for potential phosphorylation by protein kinase C. Thus it is anticipated that post-translational events other than the proteolytic modification of p65 Syn-1 will be involved in the regulation of the stress-induced FGF-1 release pathway.

Future Directions

The FGF prototypes are well characterized as potent inducers of angiogenesis in vivo[147] and this is perhaps best illustrated by the recent observation that a single injection of recombinant human FGF-1 protein induces the formation of collateral blood vessels in humans with coronary

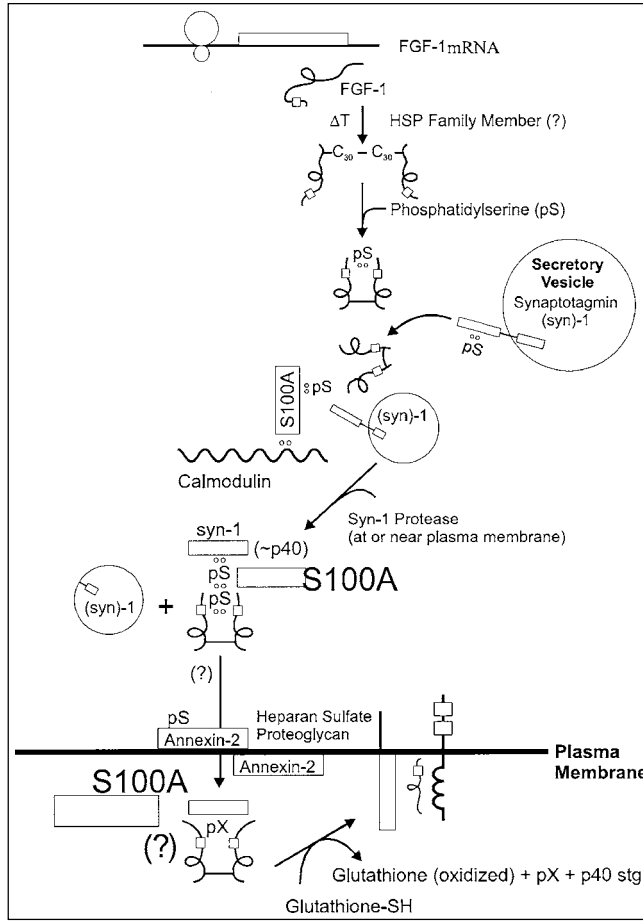

Fig. 8.7. A Working Hypothesis for FGF-1 Release. In response to temperature stress it is anticipated that a member of the heat shock protein (HSP) gene family forces FGF-1 to form a FGF-1cys30 homodimer. As a result, the FGF-1 homodimer may be able to recognize either S100A13 and/or the p40 domain of p65 Syn-1. While the stoichiometry of this complex is not known, these three phosphatidylserine-binding proteins traffic to a site where p65 is released from its transmembrane constraint by a proteolytic enzyme, and the p40 Syn-1 fragment together with the FGF-1 homodimer is released into the extracellular compartment. It is anticipated that S100A13 may also be released with the p40 Syn-1/FGF-1 homodimer complex, and we speculate that a phosphatidylserine-dependent flipping pathway may also be utilized for this purpose. Likewise, it is also possible that annexin II may be involved in the FGF-1 release pathway since it binds members of the S100A gene family and phosphatidylserine and is well represented on the outer surface of the plasma membrane as a receptor for plasma-derived proteases such as plasminogen. The reader is referred to the text for additional comments concerning this working hypothesis.

heart disease.[148] While this study is an important advancement in the treatment of the ischemic myocardium, other angiogenic growth factors such as other FGF gene family members[149,150] and members of the vascular endothelial growth factor (VEGF) gene family may also function in a similar manner.[151-155] Indeed, recent evidence suggests that there may be cooperativity between angiogenesis factors in the regulation of cell growth in vitro and in vivo.[156-158] Thus, one future direction will be to determine the mechanisms responsible for the regulation of these potentially

cooperative pathways and since stress appears to be a common denominator between the appearance of extracellular FGF-1 and the transcriptional activation of VEGF,[159-163] it is likely this insight into the mechanism of how cellular stress mediates angiogenic behavior will provide new insight into the regulation of this physiologic process in man. As a result, the demonstration of the ability of FGF-1 to mediate a coronary angiogenic event in man will be the first of many studies utilizing the ability of FGF to repair not only vascular events but also myogenic, neurogenic and chondrogenic events in man.

An additional direction will be to determine the extent of cooperativity between FGFR-dependent signaling cascades not only with other receptor and non-receptor tyrosine kinases but perhaps with non-tyrosine kinase receptor-mediated signaling events as well. These include receptors within the integrin,[164,165] cadherin, G protein-coupled[166,167] and Notch gene families.[168,169] Indeed, it is anticipated that these disparate receptor systems will be shown to communicate with each other and with members of the FGFR gene family to regulate decisions of cell fate, differentiation, migration, proliferation and death. Insight into the biochemical basis for these cooperative receptor-mediated signaling should ultimately lead to a new pharmacologic perspective from which antagonists and agonists may be designed.

Lastly, while there appears to be progress in the identification of novel anti-angiogenic materials including endostatin[170] and angiostatin,[171] the mechanisms by which these and other angiogenic effectors exert their regulatory signals is not known. Although it is possible that a general angiogenic antagonist may be forthcoming, it is also possible that all angiogenic events are not regulated by the same mechanism. Given our insight into the disparate behavior of endothelial cells derived from different anatomic sites of the same species, this hypothesis is reasonable. Thus, the definition of the similarities and dissimilarities of different forms or perhaps modes of angiogenesis are anticipated and it is likely that known and unknown members of the FGF gene family may be involved in the regulation of these disparate events.

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Chapter 9

Role of Angiogenesis in Wound Healing

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Angiogenesis, the formation of new blood vessels, is necessary for wound repair since the new vessels provide nutrients to support the active cells, promote granulation tissue formation and facilitate the clearance of debris.[1-3] Approximately 60% of the granulation tissue mass is composed of blood vessels which also supply the necessary oxygen to stimulate repair and vessel growth. It is well documented that angiogenic factors are present in wound fluid and promote repair while antiangiogenic factors inhibit repair.[1,4-5] Wound angiogenesis is a complex multipstep process. Despite a detailed knowledge about many angiogenic factors, little progress has been made in defining the source of these factors, the regulatory events involved in wound angiogenesis and in the clinical use of angiogenic stimulants to promote repair. Further complicating the understanding of wound angiogenesis and repair is the fact that the mechanisms and mediators involved in repair likely vary depending on the depth of the wound, type of wound (burn, trauma, etc.), and the location (muscle, skin, bone, etc.). The condition and age of the patient (diabetic, paraplegic, on steroid therapy, elderly vs infant, etc) can also determine the rate of repair and response to angiogenic factors. The sex of the patient and hormonal status (premenopausal, post menopausal, etc.) may also influence the repair mechanisms and responses. Impaired wound healing particularly affects the elderly and many of the 14 million diabetics in the United States. Because reduced angiogenesis is often a causative agent for wound healing problems in these patient populations, it is important to define the angiogenic factors important in wound repair and to develop clinical uses to prevent and/or correct impaired wound healing.

Wound Healing

Wound healing involves an initial clotting step followed by an inflammatory response.[3] The inflammatory period is very important with various studies demonstrating the key role macrophages have in repair.[6-8] For example, animals with low levels of macrophages exhibit delayed and impaired wound healing.[9] Likewise macrophage extracts and macrophages themselves have been used to accelerate repair in animal models and in elderly humans with impaired healing.[6,7,10,11] During debris removal by macrophages, the repair phase begins with cell proliferation and deposition of matrix. Susbsequently, there is a remodeling period that continues until wound repair is complete. The various phases of wound healing can overlap and last many days or even months.

Angiogenesis

The formation of new blood vessels in vivo involves various steps that may be regulated all or in part by angiogenic factors.[2] Initially the basement membrane underlying the endothelial cells is degraded. Several proteases, including metalloproteinases and the plasminogen system, have been show to affect tube formation in vitro. The cells then migrate through spaces in the basement membrane matrix and into the underlying connective tissue where they differentiate into tubes. Various agents including many growth factors (which are small diffusable molecules that are active at very low concentrations) have been found to trigger cell migration. Endothelial cells located in

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the original vessel proliferate to generate cells for migration. Proliferation is also induced mainly by growth factors, but these molecules may be active for proliferation at a different concentration than that required for migration. The final step in angiogenesis occurs when the basement membrane matrix is resynthesized by the endothelial cells. It is not clear what initiates this process but it may be related to the cessation of cell migration.[12] Inhibitors of collagen IV (basement membrane collagen) synthesis have been found to affect tube formation in vitro and angiogenesis in vivo demonstrating the importance of matrix synthesis in angiogenesis.[13]

Angiogenic Stimulators

A number of angiogenic stimulators have been identified in wound fluids and others are likely to exist and be important in repair (Table 9.1). The majority, but not all, of the stimulators in wound fluids are growth factors known to increase endothelial cell migration and proliferation in vitro.[4,5] Some of these factors act directly on the cells, such as VEGF, FGF and thymosin β4, whereas others, such as PGE_2, EGF, TGFβ and TNFα, regulate the inflammatory phase of wound healing and may not directly affect angiogenesis. These latter factors are not active in most in vitro assays with endothelial cells. In contrast, VEGF, FGF, and thymosin β4 promote endothelial cell migration. It is likely that the angiogenic stimulators act via different signalling mechanisms. For example, VEGF promotes proliferation also but has no effect on tube formation whereas thymosin β4 has no effect on proliferation but promotes tube formation.[14,15] FGF can promote both activities. A number of additional angiogenic factors have been identified, (Table 9.1) but their presence in wound fluid and role in repair have not been investigated. It is possible that these factors have not yet been detected as many are active in the nanogram range on endothelial cells.

The source of the angiogenic stimulators in the wound fluids is not known (Table 9.2). Some of the factors are released very early by the platelets involved in clotting and by the early inflammatory cells.[16] Serum has many migration, growth and angiogenic stimulators and thus can be considered as a source of repair molecules.[17] The surrounding tissues and cells, such as fibroblasts, also are fully capable of making and secreting certain angiogenic factors. Basement membrane matrix contains many angiogenic and potentially angiogenic factors and it can be assumed that these are released in the first step in angiogensis when the basement membrane is being broken down (Table 9.3). Basement membrane contains several known angiogenic stimulators such as FGF, EGF, TGFα and PDGF as well as proteases which could facilitate cell migration.[18] In addition, certain sequences from laminin have been found to be highly angiogenic and one (ser-ile-lys-val-la-val:SIKVAV) also induces protease activity.[19] With degradation of the basement membrane such active sequences may become more available and physiologically important. These factors may potentiate the external signal and sustain the angiogenic response. Lastly, the dead and dying cells in the wound site generate and release a number of angiogenic stimulators and protein fragments which are angiogenic. Thus, there are many sources of angiogenic factors present in the wound site. For example, FGF is present in platelets, macrophages, serum, fibroblasts and basement membrane. It is also released by endothelial cells when the cells are injured. For new blood vessel formation to occur, the release and activation of the angiogenic factors is highly regulated either by amount, type and/or level and affinity of cellular receptors. The activity of such a factor may also vary depending on whether it is bound or soluble in the wound and in the tissue. Given the high number of angiogenic stimluators described and present in the wound fluid, it is likely that the coordination of angiogenesis and repair is well regulated. Wound repair is a remarkably efficient process due, in part, to the large number of stimulators and their multiple actions on the cells.

Use of Angiogenic Stimulators in Wound Repair

Since angiogenic stimulators are present in wounds, many animal studies have determined whether adding wound fluids or the individual angiogenic molecules alone and in combination can accelerate repair (Table 9.4).[20-22] Because normal wound repair is a well ordered and efficient process, angiogenic stimulators have for the most part been found to be more effective in models of

Table 9.1. Angiogenic stimulators

Factors Present in Wounds	Other Angiogenic Factors
αFGF (FGF-1)	e-selectin
αFGF (FGF-2)	estrogen
TGF-α	haptoglobin
TGF-β	scatter factor/HGF
PGE_2	angiopoietin-1
TNF-α	angiogenin
VEGF	ESAF
IL-8	fibrin and fibrin degradation products
EGF	PDGF
thrombin	laminin peptide SIKVAV
thymosin β4	thyomsin α1 SPARC and fragments

Table 9.2. Sources of angiogenic stimulators in wounds

- early clotting and inflammatory cells: platelets, macrophages and leukocytes serum
- surrounding tissue and cells (fibroblasts, endothelial cells, etc)
- dead and dying cells release/generate factors
- oxygen levels

impaired healing. Studies of angiogenic factors in wounds have used topical, slow release pellets, injection, coatings on devices and even oral administration. Probably the most well-studied angiogenic factor used in wounds is bFGF. Basic FGF is widely distributed in tissues and contstitutes a family with at least 15 members.[23-25] Both FGF-1 and FGF-2 stimulate angiogenic activities in vitro including cell migration, tube formation, proliferation, collagenase production and plasminogen activator activity.[25] Both are also highly angiogenic in vivo in all models tested. Because of its broad angiogenic activity, bFGF is used as the positive control in most studies on angiogenic factors. When given topically, a significant increase in healing is observed in skin wounds in normal and diabetic animals.[26-29] It has also been used orally for the treatment of duodenal ulcers with some success.[30] In combination with other growth factors, such as PDGF, FGF can also stimulate wound healing.[31] Wound fluids and platelet-derived wound healing factors, both of which contain bFGF, are also effective in promoting repair in various models including spinal cord injury and non-healing cutaneous (chronic) wounds in humans.[20,21] Antibodies to bFGF reduce many of the angiogenic activities of early surgical wounds suggesting its important role.[1,27] In some injury models systems, bFGF is less effective. For example, bFGF has no effect on osteogenesis in a mandibular bone graft healing model although an increase in angiogenesis is observed.[32] FGF is present in wound fluid and is present at all of the stages of the repair process.[33] It is possible that the levels in certain wounds are maximal and additional FGF is not necessary. Likewise, a combination of growth factors may be more effective than individual factors.[29] Finally it should be noted that the timing of administration and mode of delivery of the angiogenic stimulator may be important. FGF not only promotes angiogenesis but also regulates fibroblast migration and proliferation. FGF binds to heparin and in certain wounds may exhibit different activities depending on whether it is soluble or bound.[34]

Many other growth factors are important in wound healing and have been tested in various model systems (Table 9.4). For example, acidic FGF (FGF-2) accelerates wound closure of punch

Table 9.3. Basement membrane components

Component	Possible Angiogenic Activity
Abundant components	
laminin	yes (fragments)
collagen IV	not known
perlecan	not known
entactin	not known
Growth factors	
TGF-α	yes
EFG	yes
FGF	yes
PDGF	yes
ILGF	not known
Proteases	
MMP-2	yes
MMP-9	yes
urokinase	yes
tissue type plasminogen activator	yes
Other components	
transferrin	not known
inhibin-like proteins	not known
amylase	not known
clusterin	not known

biopsies in rat and mouse skin.[35] Likewise, PDGF was very effective in promoting dermal healing in normal and diabetic animals with increased reepithelialization and vascularity observed.[36] EGF has been used in many wound model systems with considerable success in animals. Increased formation of healing tissue induced by EGF has been observed in the perforated mesentary of rats but it is not likely that the mechanism involved angiogenesis directly.[37] VEGF is expressed by epidermal keratinocytes during wound healing and mediates angiogenic activity during the proliferative phase of wound healing.[38,39] TGF beta has been shown to be more effective in promoting bone repair.[40] TGF beta has also been shown to influence connective tissue deposition and angiogenesis in a varity of tissues.[41] Thus, many in vivo studies have demonstrated accelerated wound healing in a variety of tissues using various factors either alone or in combination.

Angiogenic Thymic Peptides: Thymosin α1 and Thyomsin β4

When endothelial cells are plated on a basement membrane matrix (matrigel), they attach, migrate and form capillary-like structures with a lumen in 18 hours.[42] This in vitro tube assay has been used to define many angiogenic and antiangiogenic factors. Using subtractive cDNA cloning, we have identified a small 43 amino acid thymic peptide, thymosin β4, which is induced some five-fold at four hours after plating on matrigel.[43] Antisense oligonucleotides blocked tube formation and transfection or exogenous thymosin β4 resulted in increased and more rapid tube formation. Thymosin β4 promotes endothelial cell migration but does not influence the migration of smooth muscle cells, neutrophils, monocytes, fibroblasts, or tumor cells.[15] It is active at the ng level. In vivo it is highly angiogenic and is present in wound fluid.[44] It has not yet been tested for wound healing activity but another thymic peptide thymosin α1 is highly angiogenic and has been found to promote wound healing.[45] Thymosin α1 is a 28 amino acid N terminal fragment of the 113 amino acid prothymosin alpha 1. Thymosin α1 is an immune modulator used to treat infectious diseases.[46]

Table 9.4. Examples of angiogenic factors tested for wound healing activity

Factor	Model-Finding	Reference
Platelet-derived wound healing formula	cat spinal cord-increased survival of neurons	20,21
(growth factors) coated on teflon or in a collagen gel	human nonhealing wounds increased repair	19
bFGF topical	rodent full thickness & diabetic mice-increased healing	6,8,26-28
bFGF slow release pump	rabbit mandibular bone-osteogenesis not accelerated	32
aFGF	rodent dermal wounds– increased repair	35
PDGF-topical	diabetic mouse ear-increased healing	36
EGF-intraperitoneal injection	rat perforated mesentary-increased repair not by angiogenesis	37
TGF β-subcutaneous	increased granulation tissue in mice	41
thymosin β1-topical and intraperitoneal	rat full thickness skin-increased healing	44

Table 9.5. Angiogenic Activities of Thymosin α1

- promotes migration in the Boyden chamber and scratch wound assays
- promotes aortic ring sprouting
- promotes subcutaneous angiogenesis in matrigel implants
- promotes wound angiogenesis and repair in full thickness skin wounds in rats

Recently its role in angiogenesis and wound healing has been demonstrated. Thymosin α1 promotes endothelial cell migration with activity also in the ng range. The activity is highly specific with monocytes showing some response at certain doses but smooth muscle cells, neutrophils, fibroblasts and tumor cells show no response. It has no effect on cell proliferation. In vivo thymosin α1 promotes angiogenesis in a subcutaneous model.[44] When given topically (5 μg/50 μl) at day 0 and day 2 after wounding on 8 mm punch biopsies, repair was accelerated. A significant increase in epithelial gap closure, collagen content, wound contraction and wound angiogenesis was observed (Fig. 9.1). Similar findings were also obtained with intraperitoneal injections (60 μg/300 μl every other day). We conclude that small diffusable factors, such as the thymic peptides described here, are important modulators of angiogenesis and may have clinical uses in the treatment of patients with impaired healing. Their role in disease processes, such as impaired healing and tumor growth, is not known at this time.

Summary

Wound healing appears to involve a process more complex than simple angiogenesis and tissue growth. A variety of stimulators of inflammation, repair and angiogenesis are expressed temporally in wounds in a highly specific manner. Some stimulators in wound fluid have been identified and

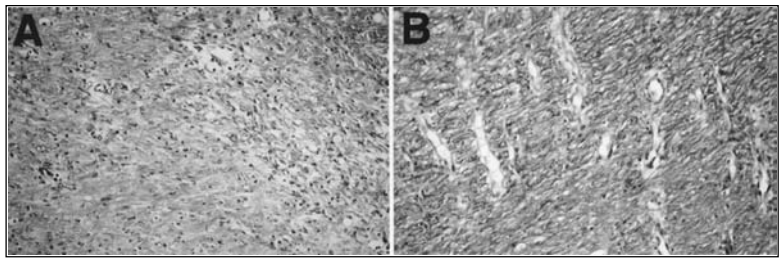

Fig. 9.1. Appearance of punch wounds after seven days with thymosin α1 topical treament. Wounds of 8 mm were made in 200 gm rats and treated topically at the time of wounding and 48 hours later. At seven days, the animals were sacrificed and histological sections were made of the wounds. A. Control, B Thymosin α1 treated.

show promise in studies where they are added to wounds. The problem of chronic wounds is significant and may require a combination of factors to initiate repair.

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Chapter 10

Ocular Neovascularization

Peter A. Campochiaro and Naba Bora

Angiogenesis plays a critical role in embryonic development, the menstrual cycle, and wound repair. Exaggeration of its role in wound repair can result in pathologies such as retinal and choroidal neovascularization. While these disease processes may share some features with neovascularization elsewhere in the body, they also have some unique features that set them apart. This is because while there is a general recipe that endothelial cells follow to make new blood vessels, it can be modified by surrounding cells resulting in tissue-specific aspects.

The general recipe for angiogenesis established from in vitro experiments has several key components:

1. alteration of the balance between stimulatory and inhibitory factors,
2. disruption of interactions between endothelial cells and their extracellular matrix (ECM),
3. proteolysis of surrounding tissue,
4. migration of endothelial cells,
5. proliferation of endothelial cells,
6. organization of endothelial cells into tubes, and
7. re-establishment of cell-cell and cell-ECM interactions.

The molecular signals involved in each of these steps can vary and must be determined for the tissue of interest, but it is likely that the cascade established for angiogenesis during embryonic development using knockout mice provides a general blue print for angiogenesis in adult tissues. Signaling through four endothelial cell-selective receptors, vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2, Tie-1, and Tie-2, has been implicated. Vascular endothelial growth factor is the ligand for VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Targeted disruption of these receptors results in death early in development with phenotypes that suggest that VEGFR-1 is necessary for differentiation of precursors into endothelial cells[1] and VEGFR-2 is needed for their organization into tubes.[2] The ligand for the Tie-1 receptor is unknown, but it seems to be involved in establishment of cell-cell interactions needed for the integrity of blood vessels.[3] Targeted disruption of Tie-1 results in death around embryonic day 14.5 (E14.5) to postnatal day 1 (P1), and there is widespread disruption of blood vessels resulting in edema and hemorrhage. The Tie-2 receptor has two ligands, angiopoietin 1 (Ang 1), an agonist that stimulates receptor phosphorylation, and angiopoietin 2 (Ang 2), an antagonist that blocks phosphorylation.[4,5] Ang1 promotes endothelial cell-ECM interactions and Ang2 blocks them. Tie-2 knockouts show immature vessels that lack branching and organization[3,6] and mice that overexpress Ang2 have a similar phenotype.[5] Angiogenesis in adult tissues is likely to involve these receptors and their ligands, but it is likely that a number of other stimulators, antagonists, or modifying factors are also involved and the specific molecular signals for each tissue must be determined to identify the best targets for pharmacologic intervention.

Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications, edited by Shaker A. Mousa.

Types of Ocular Neovascularization

Neovascularization can involve any tissue in the eye, but the tissues that are most commonly affected are the cornea, the iris, the retina, and the choroid. Corneal neovascularization occurs due to excessive wound repair after infection, trauma, or surgery. Retinal and iris neovascularization occur in the same disease processes, including diabetic retinopathy, retinopathy of prematurity, central retinal vein occlusion, and branch retinal vein occlusion. The most common of these, diabetic retinopathy, is a major cause of new blindness in developed nations,[7,8] but the other conditions also result in considerable visual morbidity. Choroidal neovascularization occurs in diseases in which there are abnormalities of Bruch's membrane and the retinal pigmented epithelium, and the most common disease of this type is age-related macular degeneration. This chapter will deal with retinal and choroidal neovascularization.

Ischemia (or Hypoxia) Plays a Central Role in the Development of Retinal Neovascularization

Our knowledge is more complete for the cascade leading to retinal neovascularization (Fig. 10.1) than that for choroidal neovascularization. One thing that has been established from numerous clinical and experimental observations is the central role of hypoxia or ischemia.[9-11] Occlusion of retinal vessels leading to retinal ischemia is a feature shared by each of the disease processes in which retinal neovascularization occurs and hence they are referred to as ischemic retinopathies.

VEGF is the Major Stimulatory Factor Involved in Retinal Neovascularization

Another well-established fact is that one of the stimulatory factors involved is VEGF. It is unlikely to be the only stimulatory factor because insulin-like growth factor I may also participate,[12] but there is strong evidence indicating that VEGF plays a central role. It is upregulated by hypoxia[13,14] and its levels are increased in the retina and vitreous of patients[15-18] or laboratory animals[19,20] with ischemic retinopathies. Increased expression of VEGF in retinal photoreceptors of transgenic mice stimulates neovascularization within the retina[21,22] and VEGF antagonists partially inhibit retinal or iris NV in animal models.[23-25]

Members of the fibroblast growth factor (FGF) family, particularly basic FGF or FGF2, have also been implicated in retinal neovascularization. However, when FGF2-deficient mice were compared to wild type mice in a murine model of oxygen-induced ischemic retinopathy, they developed the same amount of retinal neovascularization.[26] Also, transgenic mice with a rhodopsin promoter/Fgf2 gene fusion expressed high levels of FGF2 in retinal photoreceptors, but developed no retinal neovascularization or other abnormalities of retinal vessels; in the ischemic retinopathy model, they showed no significant difference in the amount of retinal neovascularization compared to wild type mice. These data argue against a major role for FGF2 in the development of retinal neovascularization.

Hypoxia Leads to Increased VEGF by Transcriptional Activation and Stabilization of mRNA

The details of how hypoxia leads to increased VEGF are not clearly known, but we are beginning to get a picture. A cytosolic heme protein is likely to be the sensor that detects decreased oxygen tension and generates reactive oxygen species which play a role in activating transcription factors. A transcription factor that plays a major role is hypoxia inducible factor-1 (HIF-1).[27] It was identified as a nuclear protein induced by hypoxia that binds to the hypoxia response element in the *erythropoietin* gene.[28] It has subsequently been demonstrated in several cell lines to stimulate transcription of multiple genes that are upregulated by hypoxia, including VEGF and possibly VEGFR-1.[29-31] HIF-1 is a basic helix-loop-helix protein with two subunits, HIF-1α and HIF-1β.[32] In the retina, HIF-1α is downregulated by hyperoxia and within 2 hours of the onset of hypoxia, it

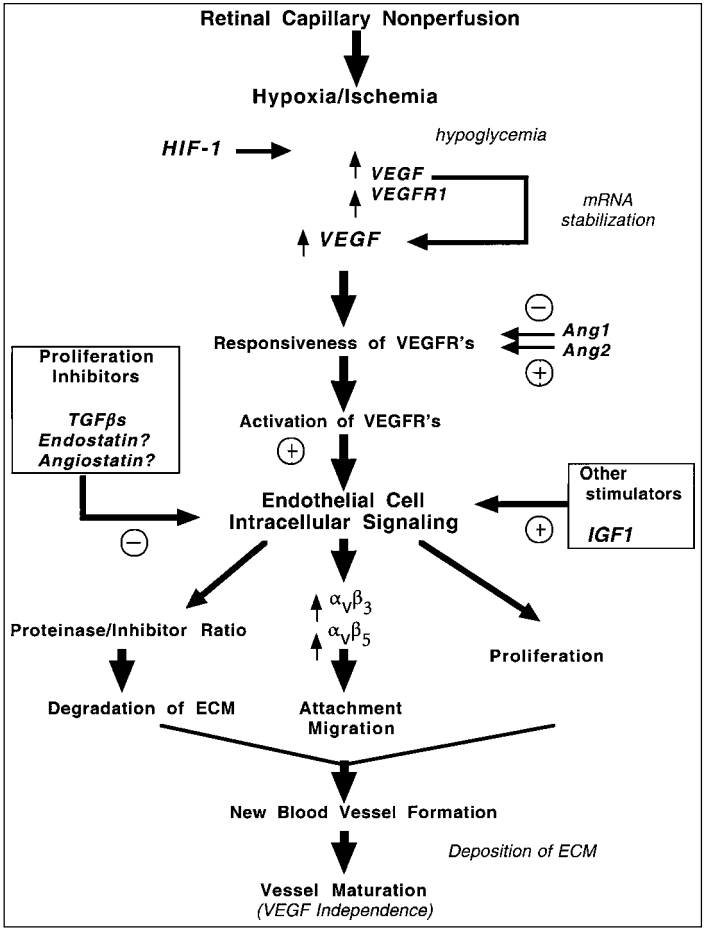

Fig. 10.1. A sketch showing the effects of positive and negative regulators of angiogenesis at different stages of new blood vessel formation.

is markedly increased.[33] Immunohistochemistry shows low baseline staining that is increased within 2 hours of hypoxia in cells of the inner retina, the region of the retina that is hypoxic. VEGF mRNA is decreased by hyperoxia and increased in cells of the inner retina within 6 hours of hypoxia. Therefore, HIF-1α levels in the retina are altered by changes in oxygen tension with a temporal and spatial pattern that is consistent with the hypothesis that HIF-1 plays a role in regulating VEGF expression in the retina. Hypoxia also helps to maintain elevated levels of VEGF by mRNA stabilization.

Permissive Factors may Increase Endothelial Cell Responsiveness to VEGF in Ischemic Retina

While VEGF plays a major role in the stimulation of retinal neovascularization, regulation of VEGF levels is unlikely to be the sole determinant of whether retinal neovascularization occurs. There are patients with nonproliferative diabetic retinopathy or other retinal diseases who have elevated levels of retinal VEGF, yet no evidence of retinal NV.[34,35] Also, it is difficult to stimulate

retinal neovascularization with exogenous VEGF alone and expression of VEGF in photoreceptors of transgenic mice stimulates growth of vessels from the deep, but not the superficial capillary bed of the retina nor from the choriocapillaris.[21] There are several possible reasons for this, but one thing that is different about the deep capillary bed is that it is still developing between postnatal day 7 (P7) and P10 when the transgene is turned on and there may be localized expression of a permissive factor that makes endothelial cells more responsive to VEGF. HIF-1 could play a role because there is a HIF-1 binding site on the VEGFR-2 promoter[31] and therefore HIF-1 may increase expression of VEGF receptors and make endothelial cells more responsive. Another potential permissive factor is angiopoietin 2. Angiopoietin 2 expression is increased in the retina during vascular development and in ischemic retinopathy, and it has been postulated that angiopoietin 2 blocks the interaction of endothelial cells with their ECM, making them more sensitive to the effects of VEGF and other soluble factors.[5]

Endogenous Inhibitors that Normally Antagonize Effects of VEGF may be Decreased in Ischemic Retina

Opposing VEGF and its collaborators are endogenous antagonists that may serve to balance transient fluctuations in stimulatory factors caused by inflammation. Members of the TGF-β family may play such a role. In vitro studies indicate that TGF-β produced by pericytes inhibits endothelial cell proliferation.[36] There is constitutive expression of TGF-β in the retina that is decreased by ischemia (our unpublished observations). Recently, we have demonstrated that rats with experimental autoimmune uveoretinitis have high levels of VEGF in the retina, but also have high levels of TGF-β and no evidence of neovascularization, while rats with ischemic retinopathy have high levels of VEGF, low levels of TGF-β, and develop neovascularization.[37] Other endogenous inhibitors, angiostatin and endostatin, have been identified in Dr. Judah Folkman's laboratory. Since these inhibitors are present in serum, it is likely that they are decreased in ischemic retina.

Intracellular Signaling Stimulated by VEGF is Complex and Overlaps with Other Growth Factors

Stimulatory factors act on cell surface receptors which act through a number of intracellular signaling pathways in endothelial cells to alter gene expression. Intracellular signaling is extremely complex. Many growth and trophic factors share the same pathways and there is considerable crosstalk between pathways. The manner through which specificity is maintained is not known, but presumably exists and when clarified could possibly be exploited for treatment development. It has been suggested that the β isoform of protein kinase C (PKC) is selectively activated by VEGF and may serve as a potential target.[38]

VEGF Signaling Promotes Expression of Genes Involved in Progression through the Cell Cycle, Proteinases, Proteinase Inhibitors and Integrins

Genes whose expression is altered by stimulatory factors include proteins involved in progression through the cell cycle, proteinases, proteinase inhibitors, and integrins. Their gene products affect how endothelial cells grow and interact with their ECM and can alter migration, proliferation, and survival. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are induced on endothelial cells participating in retinal neovascularization and provide a potential target for intervention.

Mature New Vessels are Less Dependent on VEGF

Once new vessels are formed, they re-establish interactions with ECM that makes them less dependent on soluble factors such as VEGF. Tie1 and Tie2 receptor signaling is likely to be involved in this process.

Strategies for Inhibiting Retinal Neovascularization

Since VEGF is a major stimulatory factor and acts early in the cascade, it is an appealing target for intervention. Several approaches have been used to neutralize its activity. Intravitreal injection of soluble VEGF receptors coupled to IgG heavy chains and antisense oligonucleotides for VEGF each decrease retinal neovascularization in murine oxygen-induced ischemic retinopathy by about 50%,[23,24] and intravitreal injection of an anti-VEGF antibody inhibits iris neovascularization in a monkey model of branch vein occlusion.[25] Newly developing retinal vessels are dependent upon VEGF for survival, but mature vessels are less dependent.[40] Therefore antagonizing VEGF has promise for treatment in patients with ischemic retinopathies.

Another approach that takes advantage of differences between new and established retinal vessels is antagonism of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, which are not detectable on normal retinal endothelial cells, but are present on endothelial cells participating in angiogenesis. In chick chorioallantoic membrane, blockade of $\alpha v\beta 3$ leads to apoptosis of endothelial cells and vascular regression. Studies from three laboratories have independently demonstrated that cyclic peptides that block $\alpha v\beta 3$ partially inhibit (again by about 50%) retinal neovascularization and therefore this approach also is encouraging.[39,41]

A drug that specifically inhibits the β isoform of PKC has been tested in a pig model of laser-induced branch vein occlusion and was found to partially inhibit the development of retinal neovascularization. Additional work is needed to determine the level of specificity afforded by this approach.

A preliminary report has suggested that a matrix metalloproteinase inhibitor partially inhibits retinal neovascularization in murine oxygen-induced ischemic retinopathy. Therefore, it has been demonstrated that antagonizing at several different points along the cascade can partially inhibit retinal neovascularization.[42] Perhaps attacking at multiple points simultaneously will provide greater efficacy.

The Pathogenesis of Choroidal Neovascularization is Poorly Understood

As noted above, the cascade leading to choroidal neovascularization is less well understood. It may share some steps with retinal neovascularization, but there may also be significant differences because of differences in the microenvironment. One major unanswered question is what is the primary stimulus for the development of choroidal neovascularization? Is it hypoxia as is the case for retinal neovascularization? There is some suggestion that choroidal blood flow may be altered in patients with age-related macular degeneration,[43,44] but it is not clear if this is sufficient to cause hypoxia. Another possible source of hypoxia that has been suggested is diffuse thickening of Bruch's membrane with lipophilic material decreasing diffusion of oxygen from the choroid to the RPE and retina, but there are no data to support this hypothesis. Also, hypoxia is unlikely to be present in other disease processes associated with choroidal neovascularization that occur in young patients, such as ocular histoplasmosis.

Another possible contributor to the development of choroidal neovascularization is inflammation. It has been hypothesized that deposits in and around Bruch's membrane may incite an inflammatory response and macrophages have occasionally, but not consistently been identified in pathologic specimens.[45,46] Inflammation has also been implicated in the development of choroidal neovascularization in ocular histoplasmosis and multifocal choroiditis, but there are many types of choroiditis that are rarely associated with neovascularization.

The most consistent pathologic finding in advanced AMD in which there is choroidal neovascularization is accumulation of abnormal ECM resulting in diffuse thickening of Bruch's membrane.[47] This and the presence of focal areas of thickening due to drusen suggest that there is disordered metabolism of the ECM. Breaks in Bruch's membrane and other abnormalities of the ECM are also consistently found in other diseases in which choroidal neovascularization occurs. Sorsby's fundus dystrophy is an autosomal dominant inherited disease that shares some phenotypic

characteristics with AMD, including extensive ECM deposits along Bruch's membrane, RPE and photoreceptor degeneration, and a high incidence of choroidal neovascularization.[48-51] Affected persons in two pedigrees with Sorsby's fundus dystrophy have a mutation in *the tissue inhibitor of metalloproteinase-3* (*TIMP-3*) gene,[52] whose product participates in regulation of ECM turnover.[53] This suggests that disordered ECM metabolism can lead to a phenotype like that seen in AMD and is associated with a high incidence of choroidal neovascularization.

Recently, the effect of ECM molecules on secretion of angiogenic growth factors by RPE cells was examined.[54] Addition of vitronectin, fibronectin, or fibrinogen to RPE cell cultures resulted in a small to moderate increase in VEGF and FGF2 in the media, while each of these growth factors was dramatically increased after addition of thrombospondin 1 (TSP1). Hypoxia resulted in increased VEGF, but not FGF2, in the media. RPE cells grown on TSP1-coated plates showed increased VEGF and FGF2 in their media compared to cells grown on plates coated with type IV collagen, laminin, vitronectin, or fibronectin. The TSP1-induced increase in secretion of growth factors was partially blocked by anti-$\alpha_5\beta_1$ or anti-$\alpha_v\beta_3$ antibodies indicating that it may be mediated in part by TSP1 binding to $\alpha_5\beta_1$ and/or $\alpha_v\beta_3$. These data suggest that alterations in the extracellular matrix of RPE cells can cause increased secretion of angiogenic growth factors which could contribute to the development of choroidal neovascularization.

Models of Choroidal Neovascularization

While there are animal models that closely mimic human ischemic retinopathy,[55] there are no models that closely mimic human choroidal neovascularization. Despite this, it has been possible to achieve choroidal neovascularization in animals and these models have provided useful information. The most reliable way to produce choroidal neovascularization is to rupture Bruch's membrane with laser photocoagulation.[56] The laser-induced model in primates has provided knowledge concerning the natural history of CNV,[56] the role of the retinal pigmented epithelium (RPE) in re-establishing the blood-retinal barrier (BRB),[57] and the participation of the RPE in the scarring and vascular regression;[57] it has also been used to demonstrate that steroids or nonsteroidal anti-inflammatory drugs partially prevent choroidal neovascularization.[58,59] Laser-induced damage to Bruch's membrane has also been used to create choroidal neovascularization in rats[60,61] and mice;[62] use of this model in genetically engineered mice provides a means of investigating the role of particular gene products in choroidal neovascularization. Implantation of devices that provide sustained release of growth factors in the subretinal space have demonstrated that the combination of surgical trauma and sustained release of FGF2[63,64] or VEGF can result in choroidal neovascularization.

Stimulatory Factors for Choroidal Neovascularization

While there is strong evidence indicating that VEGF and IGF-1 are stimulatory factors for retinal neovascularization, only circumstantial evidence exists implicating stimulatory factors for choroidal neovascularization. VEGF and FGF2 have been suggested to be involved because they are present in fibroblastic cells and transdifferentiated RPE cells of surgically removed choroidal neovascular membranes[65-68] and in a rat model of laser-induced choroidal neovascularization, increases in FGF2 and VEGF mRNA are seen in RPE-like cells, choroidal vascular endothelial cells, and fibroblast-like cells in the lesions.[69,70] Also sustained release of FGF2 in the subretinal space of minipigs[63] or rabbits[64] results in choroidal neovascularization. However, FGF2 knockout mice have the same incidence of choroidal neovascularization as wild type mice in a laser-induced model indicating that FGF2 is not necessary for choroidal neovascularization to occur.[62] Also, expression of FGF2 or VEGF in the photoreceptors of transgenic mice does not result in choroidal neovascularization,[21] indicating that increased expression of these growth factors in the region of the subretinal space, in the absence of damage to Bruch's membrane, is not sufficient to cause choroidal neovascularization. Additional work is needed to clarify how abnormalities in Bruch's membrane increase the risk of choroidal neovascularization and what role increased expression of VEGF, FGF2, or other growth factors play.

Strategies for Inhibiting Choroidal Neovascularization

As noted above, it has been demonstrated that anti-inflammatory drugs provide some protection from choroidal neovascularization in the laser-induced model in primates.[58,59] However, the relevance of this model to human choroidal neovascularization has been questioned, leading some investigators to suggest forgoing preclinical testing in models of choroidal neovascularization and going directly to clinical trials with potential antiangiogenic agents. For instance, interferon 2α was demonstrated to partially inhibit iris neovascularization in a primate model of branch retinal vein occlusion[71] and without additional preclinical investigations, it was tested in a multimillion dollar multicenter trial for treatment of choroidal neovascularization in which it was found to have a higher rate of visual loss than placebo.[72]

This suggests that the effect of a drug on primate iris neovascularization is not predictive of its effect on human choroidal neovascularization. While laser-induced models of choroidal neovascularization are not perfect, they are likely to be more predictive of possible effects in patients with choroidal neovascularization than models of iris or retinal neovascularization.

Future Applications and Anticipated Developments

We have a reasonably good understanding of the pathogenesis of retinal neovascularization and our current models are good. Identification of drugs that inhibit various steps in the cascade and testing them for efficacy in animal models alone or in combination should provide good candidate drugs to test in clinical trials.

As our understanding of the pathogenesis of choroidal neovascularization improves, it will be important to develop new animal models that more closely mimic the events that occur in humans. However our current models are likely to have some predictive value and should be used for preclinical testing of potential therapeutic agents. While it may be necessary to inhibit multiple steps in the pathway leading to choroidal neovascularization, it is likely that a beneficial drug regimen will be identified.

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CHAPTER 11

Anti-Angiogenesis Strategies
Potential Therapeutic Implications

Shaker A. Mousa

Angiogenesis is a process that is dependent upon coordinate production of angiogenesis stimulatory and inhibitory (angiostatic) molecules and any imbalance in this regulatory circuit might lead to the development of a number of angiogenesis-mediated diseases. Angiogenesis is a multistep process including: activation, adhesion, migration, proliferation and transmigration of endothelial cells across cell matrices to form new capillaries from existing vessels. In contrast, vessel rudiments may organize in place, a process termed vasculogenesis. Endothelial heterogeneity and organ specificity might contribute to differences in the response to different anti-angiogenic mechanisms (cultured EC vs. microvascular EC isolated from different tissues). Under normal physiological conditions, in mature organisms endothelial cell turnover or angiogenesis is extremely slow (months to years). However, angiogenesis can be activated for a limited time in certain situations such as wound healing and ovulation. In certain pathological states, such as metastasis (oncology) and ocular neovascularization disorders including diabetic retinopathy and age-related macular degeneration (opthamology) there is excessive and sustained angiogenesis. Hence understanding the mechanisms involved in the regulation of angiogenesis could have a major impact in the prevention and treatment of pathological angiogenesis processes. Additionally, endothelial cells play a major role in the modeling of blood vessels. The interplay of growth factors, cell adhesion molecules, matrix proteases and specific signal transduction pathways either in the maintenance of the quiescent state or in the reactivation of endothelial is critical in physiological and pathological angiogenesis processes.

Small Molecule Integrin Antagonists

The role of various integrins in angiogenesis-mediated disorders has been demonstrated. In addition to the well described role of $\alpha v\beta 3$ integrins (Chapter 4), a role for $\alpha 5\beta 1$ integrin in the modulation of angiogenesis was defined.[1,2] The role of $\alpha 5\beta 1$ integrin in angiogenesis was established through the use of $\alpha 5\beta 1$ integrin antagonist.[2,3]

Broad Anti-Cancer Effects of AG3340, Matrix Metalloproteinase Inhibitor

AG3340, a potent MMP inhibitor, dose dependently delay tumor growth, decreases angiogenesis and cellular proliferation and increases tumor necrosis and apoptosis across preclinical tumor models. In combination with cytotoxic agents, AG3340 produces enhanced antitumor activities. These data support ongoing phase III trials of AG3340 in frontline chemotherapy regimens against malignancies of the lung and prostate.

Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications, edited by Shaker A. Mousa.

Agents Modulating Fibrinolytic, Coagulation and Platelet Functions

Recent evidence points to the role of platelet and coagulation factors in the modulation of angiogenesis. A number of positive and negative regulators of angiogenesis are derived from both platelet and coagulation factors (Table 11.1).

CM101: An Anti-Pathoangiogenic Agent and Its Receptor

CM101 have been established as an anti-pathoangiogenic agent that might be beneficial in the treatment of cancer, wounds, rheumatoid arthritis and paralysis following spinal cord injury. CM101 when intravenously infused binds within minutes to a unique seven transmembrane protein (CM201) expressed only in pathologic vasculature.[7]

Kringle 5, a Naturally Occurring Inhibitor of Tumor Antiogenesis

Kringle 5 from human plasminogen is a potent inhibitor of endothelial cell migration and proliferation. It was also shown that Kringle 5 is a potent non-toxic inhibitor of in vivo angiogenesis based on models involving mouse cornea and tumor models. A small molecular weight peptide fragments from Kringle 5 displayed potent inhibition of angiogenesis.[7,8]

Anti-Angiogenesis Strategies Using Monoclonal Antibodies

Imclone is currently developing two monoclonal antibodies which block angiogenesis by different mechanisms. The first one, c-p1C11 directed against the extracellular domain of the VEGF receptor KDR blocks KDR-mediated signal transduction and endothelial cell proliferation. It inhibits angiogenesis in a dog model of oxygen-induced retinopathy and in the SCID mouse/human skin implant tumor model. An equivalent antibody, which reacts against the mouse VEGF receptor flk1, has been shown to effectively inhibit angiogenesis, tumor growth and metastasis in a wide variety of mouse models. The second antibody directed against VE-cadherin that is thought to be important in endothelial cell organization into vascular tubes potently blocks tube formation, angiogenesis, tumor growth and metastasis in a wide variety of mouse models. The second antibody directed against VE-cadherin that is thought to be important in endothelial cell organization into vascular tubes potently blocks tube formation, angiogenesis, tumor growth and metastasis, thereby validating the concept of targeting VE-cadherin.[7,8]

Angiogenesis Inhibitor Squalamine

Squalamine is a natural steroid from shark stomach and liver. Squalamine demonstrated broad activity in several tumor models (breast, lung, ovarian, prostate...) as well as models of angiogenic eye diseases. Squalamine demonstrated greater efficacy in inhibiting tumor growth in Lewis lung carcinoma model when combined with cytotoxic agents such as cisplatin. Additionally, squalamine (25mg/kg, QD x 5 days) demonstrated significant inhibition of oxygen-induced retinopathy in mouse neonatal model. Squalamine is in phase IIa trial for NSCLC at 100-400 mg/m2/day for 5 days in combination with carboplatin and paclitaxol.[7,8]

Angiostatic Steroid, AL-3789: Potential in Corneal Neovascularization

AL-3789 is an angiostatic steroid, which is devoid of glucocorticoid activity. Anecortave acetate (AL-3789) is a new angiostatic steroid that inhibits angiogenesis in a variety of preclinical models of neovascularization including: the chick embryo CAM, corneal neovascularization in rabbits, retinopathy of prematurity in kittens and rat pups, bFGF-induced subretinal neovascularization in rabbits and intraocular tumor growth in mice. Anecortave acetate does not have the ocular liability of a cortico-steroid because it lacks glucocorticoid activity. AL-3789 demonstrated potent inhibitory efficacy in inhibiting neovascularization in different models. Its effectiveness was demonstrated after topical or systemic administration. Recent clinical studies have shown that topical ocular anecortave

Table 11.1. Positive and negative regulators of angiogenesis derived from platelets and coagulation cascade[4-6]

Hemostatic System	Anti-Angiogenic Factors (Negative Regulators)	Pro-Angiogenic Factors (Positive Regulators)
Platelets	Platelet factor-4 (PF4)	Platelet Derived Growth Factor (PDGF)
	Thrombospondin (TSP-1)	Nitric Oxide (NO)
	Transforming Growth Factor-β (TGF-β)	
	Plasminogen Activator Inhibitor Type-1 (PAI-1)	
	Nitric Oxide (NO)	
Coagulation Cascade	Kininogen Domain-5	Fibrin
	Antithrombin III (AT-III)	Tissue Factor
	Prothrombin (Fragment 1 & 2)	
	Plasminogen Kringle (1-4) (Angiostatin)	
	Plasminogen Kringle-5	

acetate significantly inhibits the regrowth of ocular fibrovascular membrane in-patients with recurrent pterygium.[7,8]

Ocular Phamacokinetics of AG3340: Metalloproteases after Oral Administration

AG3340 is a potent inhibitor of matrix metalloproteases (MMPs). Since MMPs are involved in ocular angiogenesis, AG3340 levels were determined in ocular tissues at various times after oral administration. Therapeutic levels were measured for 24-48 hours after oral dosing, providing a rationale for its oral use in the treatment of angiogenesis-mediated ocular disorders.[8]

TNP-470 (AGM-1470)

The angiogenesis inhibitor TNP-470 (AGM-1470) is a synthetic analog of fumagillin currently in phase III clinical trials for the treatment of solid tumors. Preclinical studies showed that TNP-470 has a wide spectrum of efficacy against different tumor types, without any significant drug resistance, and with limited effect on wound healing.[7,8]

Potentiation of Cytotoxic by Anti-Angiogenic Agents

Tumors are dynamic, complex living tissues undergoing the varied processes of tissue growth under the guidance of aberrant malignant cells. Cytotoxic anticancer therapies have focused solely on the eradication of the malignant cell, which is an absolute necessity; however, even the most heroic therapeutic strategies rarely achieve cure of many tumor types. The recognition that the growth processes of tumors are normal processes, that it is in appropriate activation of the processes that comprises the morbidity of malignant disease allows the elucidation of a broad spectrum of new therapeutic targets in cancer. Anto-angiogenic agents are now available. The integration of anti-angiogenic agents into existing cancer treatment regimens has allowed the cure of established Lewis lung carcinoma and markedly increased tumor response to many standard cytotoxic therapies (Table 9.2).[7,8]

Table 11.2. Examples of different anti-angiogenesis strategies under investigation[7-9]

VEGF receptor-tyrosine kinases antagonists, Tie1, Tie2, (SU-5416)
Anti-VEGF antibody
Squalamine (MSI-1246)
Humanized form of LM609 (Vitaxin)
Small molecule αvβ3 antagonists
Small molecule integrin antagonists
TNP-470
2-Methoxyestradiol
CM-101
Interleukin-12
Platelet factor
Shark cartilage extract (AE-941)
Thalidomide and thalidomide analogs
Endostatin and angiostatin
High molecular weight kininogen domain 5
Matrix metalloproteinase inhibitors (AG-3340)
Urokinase receptor antagonist
Heparinase inhibitor, pentoxifyline, angiostatic steroids

Conclusion

Recent evidence suggest that, in spite of the redundancy of angiogenic factors potentially involved in pathological angiogenesis, strategies aimed at antagonizing one specific endothelial cell mitogen at its release or receptor levels may form the basis for an effective and safe treatment of various angiogenic-mediated disease processes.

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Chapter 12

Matrix Metalloproteinase Inhibitors in Angiogenesis-Mediated Disorders with Special Emphasis on Cancer

Henrik S Rasmussen

Matrix metalloproteinases (MMPS) are a family of enzymes that are secreted by connective tissue cells, inflammatory phagocytes and a number of different transformed cells. They are called metallo as they all contain a zinc atom at their active site. Under normal physiologic circumstances, they are involved in turnover and remodeling of the extracellular matrix (ECM) and collectively are capable of breaking down most, if not all, components in the ECM, including collagen, laminin, fibronectin, elastin, serpin, etc.[1,2] MMPs are also intimately involved in angiogenesis by a number of different mechanisms, including breakdown of basement membranes necessary for neovascularization as well as facilitation of vascular invasion and tubule formation.[3,4]

Increased activity of MMPs have been documented in a number of different diseases, and MMPs have consequently been implicated in the etiology and/or pathogenesis of diseases in which an increased breakdown of ECM or increased angiogenesis is believed to play a role, including progression of malignant tumors, certain inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease, periodontal disease, atherosclerosis, congestive heart failure, corneal disease, various fibrotic diseases and certain vascular abnormalities. Furthermore, treatment with inhibitors of MMPs (MMPIS) has been proposed/attempted for a number of these diseases. The results of these attempts are summarized below.

Matrix Metalloproteinases

Presently, 15 different MMPs have been identified, varying in their substrate requirement and potency (Table 12.1).[5-11] It is likely that more MMPs will be identified in the future. The activity of MMPS are under normal physiologic conditions tightly regulated, partly due to gene expression, partly through secretion as latent proenzymes which require modification of a I OkDa amino terminal domain for the expression of enzyme activity.[12] Once activated, MMPs are normally regulated by either general protease inhibitors or by a group of specific inhibitors known as tissue inhibitors of metalloproteinases (TIMPs).[13] So, normally the activity of MMPs are tightly regulated. However, in a number of different pathologic conditions, including cancer, certain chronic inflammatory conditions, etc. this regulation seems to get lost.

Role of Matrix Metalloproteinases in Various Pathologic Conditions

Cancer

Excessive MMP activity has been demonstrated during tumor growth and metastases for a range of different solid tumors, including colorectal cancer[14-16] cervical cancer[17] non-small cell

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Table 12.1. Matrix metalloproteinases

Enzyme	MMP number	Main substrate(s)
Interstitial collagenase	MMP-1	Fibrillar collagens
Gelatinase-A	MMP-2	Fibronectins, type IV collagens
Stromelysin- I	MMP-3	Nonfibrillar collagen, larninin, fibronectin
Matrilysin	MMP-7	Laminin, fibronectin, nonfibrillar collagen
Neutrophil collagenase	MMP-8	Fibrillar collagens, types I, II and III
Gelatinase-B	MMP-9	Type IV and V collagens
Stromelysin-2	MMP-10	Laminin, fibronectin, nonfibrillar collagen
Stromelysin-3	MMP-11	Serpin
Metalloelastase	MMP-12	Elastin
Collagenase-3	MMP-13	Fibrillar collagens
MT-MMP	MMP-14	Progelatinase A
MT2-MMP	MMP-15	Not defined
MT3-MMP	MMP-16	Progelatinase A
MMT4-MMP	MMP-17	Not defined

lung cancer,[18,19] breast cancer,[20] bladder cancer,[21] skin cancer,[22] brain cancer[23,24] and non-Hodgkin's malignant lymphoma.[25] Furthermore, a number of these studies showed a good correlation between the levels of MMPs and the aggressiveness/invasiveness of the tumor.[18,22-25] Hence, it has been speculated that MMPs are fundamental for tumor growth and spread, both local growth and formation of metastasis.[5] This has provided an attractive pharmacological target, and a number of specific inhibitors of the various MMPs are currently in development for treatment of cancer.

Arthritis

Elevated levels of MMP-9 and MMP-3 have been consistently demonstrated in the synovial fluid of patients with rheumatoid arthritis (RA),[27] as well as patients with osteoarthritis (OA).[28] Furthermore, these MMPs have been localized at the cartilage-pannus junction near the site of the active joint destruction.[27] MMP-9, which is produced by osteoclasts, plays a significant role in normal bone remodeling and pathologic bone resorption, and it has been hypothesized that excessive MMP-9 production contributes to the bone erosion, which is one of the hallmarks of RA and OA.

Inflammatory Bowel Disease

In the normal intestine, MMP activity is negligible, whereas excessive amounts of MMP-9 have been detected in the inflammatory infiltrate in Crohn's disease.[31] MMP-3 as well as MMP-9 has been demonstrated in high quantities in the lamina propria regions of inflamed mucosa in ulcerative colitis.[31]

Atherosclerosis

Recent studies have demonstrated the presence of MMPs in human vulnerable plaques[33] believed to be secreted by activated macrophages and to be in part responsible for the extracellular matrix degradation leading to a weakening of the plaques fibrous cap with subsequent plaque rupture.[34] The plaque disruption occurs most frequently at sites where the fibrous cap is the thinnest and most

heavily infiltrated with so-called 'foam cells.[33] Thus, it has been hypothesized that release of MMPs by these cells is a mechanism leading to plaque vulnerability and rupture.[34,35]

Excessive production of MMPs has also been demonstrated in pre-eclampsia,[26] bronchiectasis,[29] emphysema,[30] liver cirrhosis,[32] graft versus host disease,[36] periodontal disease,[37] although the potential clinical implications of this, if any, are unknown.

Matrix Metalloproteinase Inhibitors

General Considerations

One of the key issues in this area is whether broad-spectrum MMPIS, active against a range of different enzymes, or selective inhibitors, targeted against individual MMP enzymes, represent the optimal pharmacological therapy. A number of highly selective inhibitors have been identified and in principle should provide greater specificity, and consequently minimizing potential side effects. A prerequisite for this, however, is that a particular enzyme can be unequivocally identified as being responsible for progression of a certain disease entity. In cancer, for example, this would mean that a certain enzyme could be identified as being the cause of propagation in a certain cancer type. So far, this has not been demonstrated, partly because the field is still new and emerging, partly because there seems to be a significant overlap in substrate activity between the different enzymes".[38] Consequently, most companies have developed broad-spectrum inhibitors, although some companies have taken the more risky approach and developed more selective inhibitors. A number of different inhibitors are now in clinical trials (Table 12.2) and time will show which approach is the optimal one.

Most information has been accumulated so far with Batimastat and Marimastat, so most of the following review is based on these agents.

Batimastat is a broad-spectrum MMPI with potent activity against most of the major MMPS, that is, interstitial collagenase (MMP-1)(IC50=3 nM), stromelysin-I(MMP-3)(TC50=20 nM), gelatinase A (MMP-2)(IC50=4 nM), gelatinase B(MMP-9)(IC50=4 nM), and matrilysin (MMP7)(IC50=6 nM). There is also evidence that Batimastat is a potent inhibitor of progelatinase A(MMP-14)(unpublished observations). The molecular structure of Batimastat is shown in Figure 12.1. The molecule mimics the substrate of the MMPS so the drug works by competitive, potent, but reversible inhibition. Batimastat is almost completely insoluble and consequently, has a poor bioavailability when administered orally. Thus, the only way Batimastat can be administered is by direct injection into various body cavities (peritoneal and pleural cavities). Intra-peritoneal injection of Batimastat gives rise to elevated and sustained plasma concentrations with a half-life in humans of approximately 28 days, presumably because the drug is gradually absorbed from the peritoneal cavity into the bloodstream. While this pharmacokinetic profile is less suitable for human studies, it is a convenient administration schedule for rodent studies.

Marimastat (Fig. 12.2) is another broad-spectrum MMPI with an enzyme inhibitory spectrum very similar to Batimastat. However, Marimastat is soluble and thus is suitable for oral administration with an acceptable bioavailability, linear dose-plasma relationship, and a half-life of approximately 10-15 hours, justifying a twice daily dosage regimen. On the other hand, Marimastat is rapidly metabolized in rodents, undergoing a high first-pass effect, making testing of Marimastat in rodents difficult as sustained concentrations in this species are difficult to obtain. Consequently, most of the preclinical antitumor data have been generated with Batimastat, whereas Marimastat is being used in clinical trials. This approach, of course, is justified by the similarities in enzyme inhibitory activity between the two drugs.

Table 12.2. Matrix metalloproteinases in development

Drug	Company	Indication	Phase
Marimastat	British Biotech	Cancer	Phase III
Bay 12-9566	Bayer	Cancer	Phase 1/11 (Dropped)
CGS27023A	Novartis	Cancer	Phase I
AG3340	Agouron	Cancer	Phase III
OPB-3206	Otsuka	Cancer	Preclinical
KB 7785	Kanebo	Cancer	Preclinical
Bryostatin-I		Cancer	Phase I
Metastat	Collagenex	Cancer	Preclinical
Squalamine	Magainin Pharma	Cancer	Preclinical

Antitumor Activity

Preclinical Studies

In a *murine melanoma model*, in which human melanoma cells were injected subcutaneously in the foot pads of nude mice, Batimastat significantly reduced growth of subcutaneously implanted tumors; when Batimastat was administered from days 11-1 9 after tumor implantation, a 33% reduction in tumor growth was noted, whereas in animals in which Batimastat was administered from the day of tumor inoculation and up to day 19, a 58% reduction was detected.[39] In the same series of experiments, Batimastat also significantly inhibited spontaneous metastases formations. Animals were randomized to either Batimastat (30 mg/kg daily for 18 days after surgical removal of the primary lesson) or control (saline); a 76% reduction in metastatic tumor burden was detected in the Batimastat-treated animals ($p < 0.05$). The antiangiogenic activity of Batimastat has been assessed in a *murine End.I hemangioma model.*[40] End. 1 is a virus oncogene transformed mouse endothelial cell line that forms vascular lesions similar to hemangiomas. End. 1 tumors develop as they stimulate the host cell to create new blood vessels. This antiangiogenic activity can be quantified, and hence, represents a good model in which to assess antiangiogenic activity of various agents. Batimastat treatment resulted in a significant decrease in hemoglobin content (a quantitative measure for angiogenesis in this model) from 0.80-0.53 g/dL. These data indicate that Batimastat possesses potent antiangiogenic properties.[40]

In a *xenograft model of human ovarian carcinoma*, in which human ovarian carcinoma cells were implanted in the peritoneum of nude mice, animals were randomly allocated to receive Batimastat (30 mg/kg) or saline daily intraperitoneally from day 7 to day 20 after tumor implantation.[41] Batimastat significantly increased survival from 18 days in the control group to 105 days in the Batimastat-treated group ($p < 0.001$). Also, histological analysis demonstrated a significant increase in intratumoral, as well as peritumoral, fibrosis and a subsequent reduction in tumor cells.[42]

In a *xenograft model of human colorectal carcinoma,* human colorectal carcinoma cells were implanted in the intestinal wall of nude mice.[42] Batimastat resulted in a 50% reduction in tumor growth as well as a significant decrease in the incidence of local and regional invasion and distant metastases,[42] altogether resulting in a significant increase in median survival ($p < 0.05$). Similarly, in a *xenograft breast cancer model*, human breast cancer cells were implanted in the mammary fat pad of nude mice. Nine weeks after implantation, the primary tumors were resected and the mice were allocated to receive Marimastat or saline, administered intraperitoneally daily from week 9 to week 16. On week 16, all animals were killed and autopsied for lung metastases.[43] A significant reduction in local regrowth ($p=0.035$), as well as number of lung metastases (a 46% reduction from 27.5-14.9, $p < 0.0001$), volume of lung metastases (a 60% reduction from 179-74 MM3, $p < 0.0001$), and

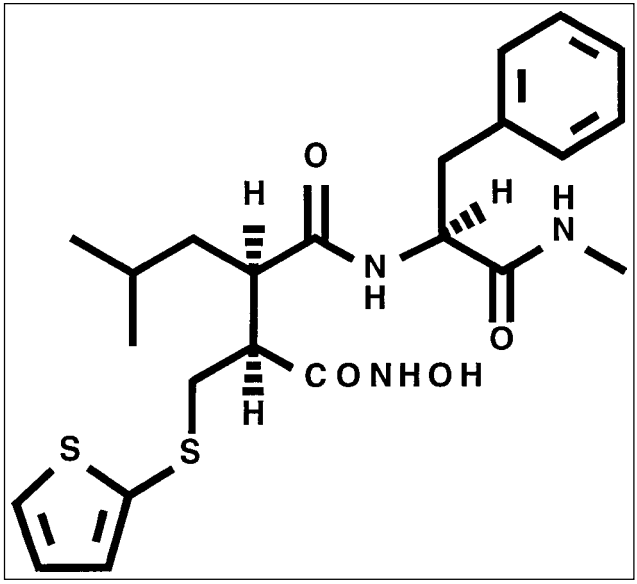

Fig. 12.1. Batimastat: Chemical structure

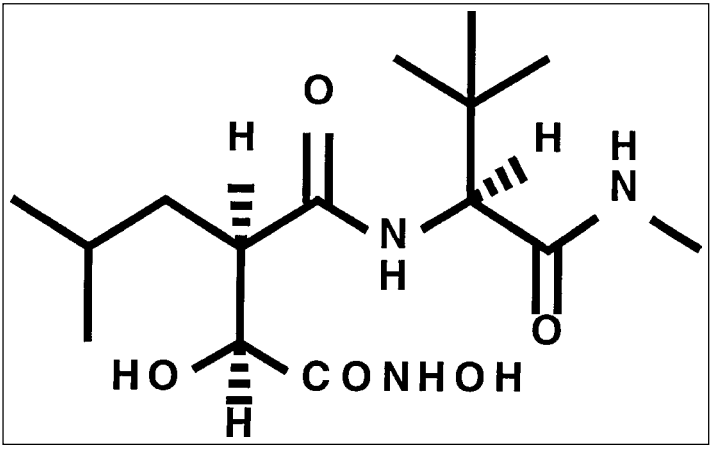

Fig. 12.2. Marimastat: Chemical structure

incidence of animals without any lung metastases (from 9% in the control group to 33% in the Batimastat group). In a *xenograft model in pancreatic cancer*, human HPAC cells were implanted in a nude mice*. The mice were then allocated to receive Batimastat or vehicle, starting 4 days prior to implantation and continuing until death or sacrifice on day 70. Batimastat resulted in a complete prevention of metastases from 20 in the control group to 0 in the Batimastat group ($p < 0.05$); furthermore, a significant reduction in total tumor weight (from 0.65-0.14g, $p < 0.05$) as well as tumor volume (from 0.60-01.21 cc, $p < 0.05$) was detected.

Clinical Studies

Design Considerations

Cancer: Designing a clinical trial program for the development of MMPIs in cancer is complex. As these drugs are not cytotoxic agents, and therefore do not kill tumor cells as such, a traditional cytotoxic development strategy would not only be inappropriate and a waste of time, it could also be potentially detrimental for the success of this class of agents. To illustrate this dilemma: Traditionally, when one develops a new cytotoxic agent, the purpose of the phase I program is to define the 'maximum tolerated dose (MTD)'. However, for chronically administered enzyme inhibitor, which may have to be administered for years, possibly in some cases for the rest of the patient's life, this is not appropriate. Following complete enzyme inhibition, no further activity is to be expected and it would be inappropriate to continue to escalate the dose just simply to induce toxicity. Consequently, the purpose of the phase I/II program with a drug of this class should be to identify the 'optimal biologic dose' rather than the MTD. Furthermore, tumor responses (complete response, partial response, stable disease and progressive disease) are cornerstones in the development of cytotoxic agents; however, MMPIs do not kill tumor cells. They work via prevention or reduction of further growth, not by killing the cancer cells. Consequently, applying standard tumor reductive criteria for efficacy evaluation in this class of agents would be inappropriate. The only way in which activity can be demonstrated is by randomized trials, demonstrating increases in survival and/or time to disease progression. This required large studies, including significant numbers of patients being treated for significant periods of time, which obviously is unsuitable for a phase II program. Consequently, one challenge for drug companies involved in this field is to identify biological markers which could be used during the phase III program to get an initial indication about the therapeutic potential of the drug, as well as being used to identify the 'optimum biological dose'. One such approach is described below, but undoubtedly a number of other strategies will be developed as more and more of these agents enter clinical trials.

Other indications: The special design problems described above are to a large extent unique for the development of MMPIs in cancer. In most other potential targets like rheumatoid arthritis and inflammatory bowel disease relatively reliable surrogate markers do exist which can be employed in the phase II program to help identify the optimal dose and get an indication of activity, so development in these indications, at least theoretically, should be somewhat simpler than in cancer.

Batimastat

Due to its pharmacokinetic limitations, Batimastat has only been studied in certain specific selected patient populations. In patients with malignant ascites,[44] as well as malignant pleural effusion, Batimastat was injected directly into the peritoneal and pleural cavities. After intraperitoneal injection of Marimastat, high and sustained plasma concentrations were achieved, detectable for up to 28 days after the injection, probably due to sustained release with subsequent absorption from the peritoneum.[44] In these patients Batimastat was well tolerated although some local pain was experienced. The observation that intraperitoneally administered Batimastat led to sustained plasma concentration levels within the predicted therapeutic range led to a phase I study in patients with advanced lung cancer.[46] However, in those patients who did not have ascites, Batimastat resulted in very substantial local toxicity. No systemic toxicity was noted.[46] Nonetheless, the insolubility of Batimastat necessitating intraperitoneal or intrapleural administration proved too much of a hurdle and the drug was abandoned.

Marimastat

Marimastat is a broad-spectrum MMPI with a pharmacokinetic profile suitable for chronic oral administration. It displays potent broad-spectrum inhibitory activity against most of the major

*Zervos E, Norman J, Gower W et al. Matrix metalloproteinase inhibition attenuates human pancreatic cancer growth in vitro and decreases mortality and tumorigenesis in vivo (unpublished data).

MMPs with IC50s against MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 in the nanomolar range. Consequently, Marimastat is very similar to Batimastat in terms of enzyme inhibitory spectrum, the only difference being that Marimastat is a weaker stromelysin-I inhibitor than Marimastat as the IC50 for Marimastat is approximately 230 nM as compared to 20 nM for Batimastat. Whether this weaker activity against stromelysin-I has any clinical implications is unknown. As Marimastat is not a cytotoxic agent, the initial phase I work was done in healthy volunteers.[47] That study showed a linear dose-plasma concentration relationship, a half-life of approximately 10 hours and a balanced excretion as 75% was metabolized in the liver, the remaining 25% excreted unchanged in the kidney. Plasma concentrations at all dose levels studied were well in excess of IC90 concentrations, indicating that oral administration of Marimastat produces pharmacologically active blood levels.

Marimastat has been tested in more than 500 patients in phase I/II studies in a number of different solid tumors in North America and Europe. In most of the studies, cancer-specific antigens (CSAs)(CEA in colorectal cancer, CA 19/9 in pancreatic cancer, CA 125 in ovarian cancer and PSA in prostate cancer) were measured as surrogate markers for biological activity.[48] Meta-analysis of these studies, as well as analysis of the individual studies, indicated that Marimastat treatment significantly reduced all four CSA rates of rise in a dose-dependent fashion. A good correlation between antigen rate of rise and survival, indicating that CSAs are potentially a useful surrogate marker for potential drug activity in patients with various cancers. It is noteworthy that these results were achieved in patients with advanced, rapidly progressive, treatment refractory cancer, a group that is traditionally virtually impossible to treat. It is conceivable, if not likely, that better results will be achieved in patients with earlier stage disease and lower tumor burdens, and presently, Marimastat is being tested in a number of studies in patients with earlier stage disease (including a true adjuvant study in patients with resectable pancreatic cancer).

In clinical trials, Marimastat has been well tolerated; the most common drug-related toxicity is a characteristic syndrome, consisting of musculoskeletal pain and stiffness, often commencing in the small joints in the hand, and if dosing continues unchanged, it will tend to spread to other joints as well. At very high and continuous dosages, the musculoskeletal toxicity can be very severe indeed, and resemble an inflammatory polyarthritis.[49] The symptomatology of the toxicity is very similar to what was seen in toxicological studies with the mice (unpublished data) and appears mainly to be tendinitis where the tendon is attached to the joint. The musculoskeletal side effects are dose-related with the incidence, rate, and severity of onset increasing with higher doses of Marimastat. At a dose of 10 mg bid, approximately 30% develop musculoskeletal symptoms after 3-6 months of treatment. However, implementing a short dosage holiday of 1-3 weeks, followed by dose reduction makes continued treatment possible. These encouraging phase I/II data have provided the foundation for a major phase III program and pivotal trials have been initiated in patients with advanced pancreatic cancer, gastric cancer, malignant glioblastoma, small cell lung cancer, non-small cell lung cancer, ovarian cancer and breast cancer. All of these trials are looking at hard clinical endpoints such as survival and disease progression and, when completed, should provide the first step towards identifying the potential clinical use of this class of agents.

Other Matrilmetalloproteinase Inhibitors

A number of other matrix metalloproteinase inhibitors are also in preclinical or clinical development for cancer. *AG3340* (Agouron Pharmaceuticals, Inc.) is a small synthetic MMPI. A phase I study in healthy volunteers demonstrated that the drug was well tolerated and rapidly absorbed following oral dosages between 10 and 200 mg. A phase I study in cancer patients has recently been initiated in the U.S.[50] *Bryostatins* are naturally occurring lactones derived from marine bryostrains, which inhibit MMP- 1, -3, -9, -1 0 and 11.[51] Bryostatin-I have been tested in phase I studies conducted by the NCI. So far, it has been well tolerated with myalgia being the principal toxicity. *CGS27023A* (Novartis) is an orally available broad-spectrum MMPI. A phase I study in 36 patients with various tumors demonstrated that the drug was well tolerated with myalgia/arthralgia and skin rashes being the major toxicity.[52] *BAY 12-9566* is the first more selective MMPI to be taken into clinical development. BAY 12-9566 is a selective inhibitor of MMP-2 and MMP-9, without

any significant influence on other MMPs.[53] So far, musculoskeletal toxicity has not been reported with BAY 12-9566, which may be due to its greater selectivity. Whether this greater selectivity reduces the clinical activity is an open question which will have to await comparative trials with a broad-spectrum inhibitor. OPB-3206 (Otsuka) is another MMPI, selectively inhibiting MMP-2 and MMP-9, which has shown antiproliferative and antimetastatic activity.[54]

Clinical Studies in Noncancer Indications

So far, clinical studies in indications other than cancer have not taken place. However, most of the companies with MMPIs in clinical development are considering starting up minor exploratory studies in indications like arthritis, inflammatory bowel disease, periodontal disease, graft-versus-host reaction, aortic aneurysm and congestive heart disease; in all of these indications, there is a strong clinical rationale and in some of the indications good preclinical evidence that a MMPI might be effective. For most of these indications, well validated surrogate markers exist which would greatly simplify the design of early studies, and there is no doubt that results from one or more of these indications will start to emerge within the next 1-2 years.

Conclusions

There is growing evidence that excess production of MMPs plays an important role for growth and spread of a number of malignant tumors, including colorectal, lung, breast, cervical and prostate cancer. Inhibitors of these enzymes have proven effective in a number of different models (ovarian, colorectal, brain, lung, pancreas, gastric, melanoma) in slowing the growth of the tumor as well as reducing the incidence of metastases, indicating that these agents represent a promising alternative approach in cancer treatment. Phase I/II studies in cancer patients have demonstrated that these agents are generally well tolerated without the toxicity, which characterizes traditional anticancer agents. Randomized clinical trials are now required to establish the potential activity of these agents. Theoretically, these agents could be promising in indications like arthritis, inflammatory bowel disease, periodontal disease, graft-versus-host reaction as well as certain cardiological diseases; however, experience in these areas are well behind the experience in cancer and substantial preclinical work as well as early exploratory clinical work is now required.

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Chapter 13

Pharmaceutical Development of an Antiangiogenic Drug-Candidate
Challenges and Opportunities

Marc Rivière, Violetta Dimitriadou, Gerald Batist, Éric Dupont

The development of antiangiogenic multifunctional drugs aims to make available to physicians and their patients a novel and effective therapy directly targeting angiogenesis-dependent pathologies for which current therapies are not optimal because of toxic effects or of only moderate efficacy. A variety of diseases can be targeted with the antiangiogenic approach, including cancer, inflammatory and cardiovascular diseases and autoimmune disorders (reviewed in refs. 1,2). In fact, more than 20 diseases have been identified as potential indication of this new approach.[1,3] Since the cancer application of antiangiogenic therapy is the most advanced indication in terms of pharmaceutical development, in this chapter we will focus on the challenges and opportunities the development of this new therapeutic class offers pharmaceutical developers.

New Therapeutic Area

In cancer, patients and clinicians are eager for new specific therapeutic pathways, alternative or complementary to classical chemotherapy. The use of cytotoxic drugs often appears limited by the modest degree of selectivity towards cancer cells they are intended to eradicate and by resistance mechanisms developed by these cells after only a few cycles of chemotherapy (reviewed in refs. 4,5). There is therefore a great need and interest to develop new drugs of high efficacy and low toxicity with the potential to increase survival without deleterious effects on quality of life due to toxicity of treatment. In this respect, antiangiogenic therapy is based on the interaction between different tissue compartments and specifically targets a cellular component which is essential for the survival and growth of tumor cells. As such, it represents an attractive therapeutic approach mostly because of its specificity for normal endothelial cells, which are known for their relative genetic stability that makes them much less susceptible to developing mechanisms of resistance than are tumor cells. Boehm et al[6] showed that chronic, intermittent therapy of three different mouse tumors with endostatin, an antiangiogenic agent,[7] did not lead to acquired resistance. In contrast, chemotherapy with cyclophosphamide resulted under the same conditions in partial resistance by the third cycle and even greater resistance by the fourth.

Angiogenesis inhibitors can be divided in to two categories as has been suggested by Voest.[8] The first group includes specific and semispecific inhibitors affecting proliferation and migration of endothelial cells only. Inhibitors of the second group show substantial effect both on endothelial and tumor cells (e.g., taxol). Here, we will focus only on specific angiogenesis inhibitors. Since endothelial cells and metalloproteinases are among the new primary targets of antiangiogenic treatment, this change in therapeutic focus requires a new conceptual approach in the development of drug candidates.

Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications, edited by Shaker A. Mousa.

Challenges

A new therapeutic concept, whatever its scientific focus or mechanism, must necessarily proceed through a series of clinical trials acceptable to both the regulatory agencies and the medical community. These are costly and take time. During this process, the antiangiogenic approach to the treatment of angiogenesis-related pathological conditions, has i) to prove that it is able to equal or exceed the therapeutic index of other available treatments and ii) to demonstrate that its benefits are worth a profitable price for the pharmaceutical developer as well as for the final payer.

Therefore, the major challenge for the pharmaceutical development of an antiangiogenic agent is to establish, in the absence of previous experience and published literature, the scientific foundations of a rationale to build clinical protocols. One must face the possibility that the optimal evaluation of the therapeutic potential of this novel class of drugs at both the preclinical and clinical levels might not use the same development paradigms that prevail in the currently available therapeutic approaches. This seems especially true in the oncology indications where chemotherapy development was based for decades on dose limiting toxicity and response rate in advanced disease.

The best starting option for a successful clinical investigation is to address the unique nature of these agents. The ideal candidate for antiangiogenic therapy, as outlined in preclinical studies, should exhibit a broad spectrum of activity (e.g., antitumor and anti-inflammatory) and have some degree of specificity for a particular step or component of the angiogenesis cascade in order to avoid side-effects. Angiogenic inhibitors act by blocking the evolution of the pathology, without directly targeting the primary etiology. The regression of a growing capillary bed might be a slow process, and will require long-lasting treatment without interruption, since microvessels may re-grow rapidly and also because tumor cells themselves are not affected directly and may continue to stimulate angiogenesis. Therefore, preclinical studies and early clinical trials have to demonstrate that the drug-candidate is not only efficient but can also be adapted to prolonged administration. In other words, it should be "resistant to resistance",[9] preferably orally bio-available, and must be well tolerated with an excellent safety/toxicity profile. In this perspective, a biologically active dose accompanied by no or only mild toxicity is the objective in the early dose-finding development of an antiangiogenic agent, rather than a maximal tolerated dose that will not be tolerated in long-term treatments.

Several antiangiogenic agents are being developed along these lines, including CM101,[10-12] CGS27023A[13] and Æ-941.[11,14,15] These three agents do not interfere with normal reproduction and embryogenesis in animal studies and there were no dose limiting toxicity identified in phase I trials. CM101 however is injectable while CGS27023A and Æ-941 are orally bio-available.

Antiangiogenic therapy may therefore be appropriately administered in many different clinical situations. Preclinical studies show activities for angiogenesis inhibitors regardless of tumor type. These inhibitors could be used as an adjuvant therapy with conventional treatment modalities, and this is enforced by biological data that suggest that they will likely be most effective in conditions of low tumor volume. Such patients could include for example, those who have been surgically completely resected and rendered "disease-free" but remain at high risk for relapse or recurrence (e.g., node-positive breast cancer, pancreas and lung cancer). Antiangiogenic strategy may also be used as a maintenance therapy in those patients with advanced, metastatic disease in whom tumor has been reduced by chemotherapy, radiotherapy or surgery but are at risk for tumor recurrence (e.g., lung cancer). A novel application could be the use of angiogenesis inhibitors as chemopreventive agents in cancer patients at high risk for developing other primary malignancies such as is the case of patients with head and neck cancer who are at significant risk for developing a second primary tumor.

Antiangiogenic drug-candidates could also be used to potentiate conventional anticancer or anti-inflammatory therapies as reported by Teicher et al who showed that the angiogenesis inhibitor TNP-470 does not compromise the anticancer activity of radiotherapy[16] or chemotherapy in experimental models.[17,18] It is possible that antiangiogenic agents could potentiate the optimal efficacy of these therapies. Similar beneficial effects of combinatory therapy have been reported in in vivo studies for a number of angiogenesis inhibitors such as SU101,[19] AG3340[20] and Æ-941.[14] These antiangiogenic agents are currently being tested in clinical studies. Although the mechanism of this synergy remains to be elucidated, the concept of combined modality therapies may offer the

best therapeutic prospect since it joins treatments with different mechanisms of action, toxicity and in fact cellular targets.

The greatest concern about the combinatory strategy in cancer therapy emerges from the heterogeneity of human tumors which are composed of genetically unstable sub-populations of cells showing different biological properties including the capacity to adapt and to circumvent anticancer therapies. Although the point has been made that targeting normal vascular cells may avoid the development of resistance,[6] there is also evidence that a variety of signals for neovascularization are derived from the tumors.[21] It is therefore theoretically possible that tumor cells could adapt to antiangiogenic treatment by altering the synthesis and secretion of these substances, such as an angiogenesis growth factor or a protease. In order to obtain a therapeutic tool that will avoid resistance, it may be preferable to combine a conventional anticancer treatment with one or more angiogenesis inhibitors, which target different aspects or steps of the angiogenic cascade and have limited toxicity.

Once proof of principle of the mechanism of action, safety, oral bio-availability and therapeutic positioning have been addressed, the next step in new therapy development is to test the efficacy of the drug candidate. A significant difficulty in designing clinical trials of angiogenic inhibitors is to choose the appropriate markers of biological activity and the clinical endpoints. Two issues arise at this point : the calculation of the effective doses to be administered without reaching toxic levels (range of the effective biologically dosage) and the duration of the antiangiogenic activity.

To date, no methods and markers are validated to evaluate antiangiogenic therapy during clinical trials to demonstrate the antitumor or anti-inflammatory efficacy of various angiogenic inhibitors. Clinical literature provides the suggestion of the correlation between the evolution of a tumor and the density of microvessels (MVD) in various types of neoplasias including breast cancer.[22-25] Studies on melanomas,[26] prostate,[27] lung,[28] ovarian,[29] gastric[30] and colon[31] carcinomas also support the potential value of this "angiogenesis index" as prognostic factor for the formation of metastases. Very recently, Obermair et al reported that high microvessel density adversely influences patient survival in clinical stage IB cervical cancer and identifies patients with negative nodes at risk for relapse.[32] However, several other studies challenge these observations.[33-35] The question remains whether MVD is a prognostic factor at the time of diagnosis only or whether it is also suitable as a marker of the therapeutic activity of antiangiogenic agents. Discordant results may be due to the fact that angiogenesis is just one of the multiple interdependent steps intervening in the process of tumor growth and evolution. For example, if a primary tumor despite angiogenic activity does not express other cellular or molecular factors (e.g., adhesion molecules), it will be unable to metastasize and in such case, microvascular density would be rather an unreliable index. Moreover, controversy also arises from the different methodologies currently chosen to identify and quantify capillaries. Problems associated to the quantification of tumor angiogenesis in tissue sections are the heterogeneity within the tumor, the vascular parameter measured, and the choice of the optimal method of quantification and the image analysis system (reviewed in refs. 36,37). There is an urgent need to identify morphological, biochemical or functional differences between normal and pathological vessels that could be used as selective therapeutic targets. These differences involve the vascular basal membrane (e.g., it appears thinner in neoplastic vessels) or the expression at the molecular level of distinct membrane antigens and adhesion molecules by the endothelium.[38] In fact, in recent years it has become evident that endothelial cells forming new vessels may over-express new antigens and receptors that distinguish them from endothelial cells from quiescent normal vessels. For example, a number of endothelial cell specific receptor tyrosine kinases known to be essential for embryonic development of the vascular system (flk-1/KDR, flt-1, tie-2/tek) are expressed preferentially by tumor associated endothelial cells.[39-42] The discovery of these and other specific markers for tumor[43-51] or inflammation-associated[46,49-52] vessels might refine this approach, some elegant animal models add to this intellectual approach. Transgenic mouse models of tumorigenesis are characterized by an angiogenic switch to new blood vessel growth beginning before the emergence of solid tumors (reviewed in ref. 53) which suggests an appropriate timing for this therapy. Antibodies or small peptides directed against these

specific endothelial antigens may be useful in defining efficacy since they can also be applied as specific markers or as neutralizing antibodies.[54]

Clinical trials seeking biological rather than clinical endpoints could also be guided by the molecular mechanism of action of the particular agent under investigation. Some surrogate points seem promising, such as the expression or regulation of known angiogenic factors, proteolytic enzymes, endothelial cells surface receptors and adhesion molecules which could be assessed in tumor tissue. Nevertheless, repeated measurements of such "markers" within tissue, serum or urine of patients (e.g., determination of enzymatic activity and circulating levels of metalloproteinases or angiogenic factors) might correlate with the activity of the angiogenic therapy. The duration of the antiangiogenic activity could be evaluated by assessing patients' plasma extract in various in vitro tests (e.g., zymographic essays, endothelial cells proliferation). Other potential markers include imaging techniques that permit assessment of the tumor blood flow and the tumor fluid permeability.

Several in vivo models for solid tumors (allografts, xenografts, transgenic mice) have been used to develop classical chemotherapy. Most of them are required by regulatory agencies, in preclinical studies, often demonstrated efficacy in a battery of in vitro tests, in order to evaluate the relative efficacy (and toxicity) of a cytotoxic agent. Specific endpoints have been established and are routinely used. In the case of antiangiogenic agents the use of the same endpoints to evaluate efficacy in preclinical and clinical studies may be inadequate. The fundamental differences in both mechanism of action and cell targets between the cytotoxic and angiogenic inhibitors, may modify the interpretation in these models. Conventional cytotoxic drugs are designed to respond to well-defined efficacy criteria such as reduction of tumor mass or decrease of metastatic nodules. Antiangiogenic therapy is cytostatic rather than cytotoxic. Complete regression of the tumor is not expected with monotherapy since mature vessels and tumor cells are theoretically not affected. Nevertheless, in some animal models, it has been demonstrated that some of the antiangiogenic agents like angiostatin alter the dynamic equilibrium between apoptosis and proliferation of tumor cells resulting in a state of relative dormancy of metastatic tumor growth.[55] Therefore, the parameters of efficacy could be for example the apoptotic index, the doubling time of the primary tumor, the time to relapse and as discussed, the degree of angiogenic evolution (e.g., endothelial cell proliferation or expression of specific molecular markers of angiogenesis), and the degree of alteration of factors or receptors related to the mechanism of action of each inhibitor. Translating this reasoning into clinical protocols, it is unlikely that classical phase II clinical trials in patients with bulky advanced disease will demonstrate significant tumor shrinkage. Therefore, an alternative paradigm for drug development is necessary and would aim at identifying clinical situations where very small volume disease is present in a comparative randomized study, or where "stable disease" represents a response in tumors documented to have been growing measurably.

The optimal duration of an antiangiogenic treatment is unknown. The rodent experiments conducted with angiostatin and endostatin[55] together with the reported anecdotal cases or various phase I studies, as well as biological reasoning have led to a consensus that antiangiogenic treatments should be administered for prolonged periods of time. The time interval between the "response" to the antiangiogenic therapy and its interruption, as well as the criteria for such an interruption, the type of follow-up and the criteria for resuming the therapy also have to be defined. The implication of these questions suggests further modification of clinical trial methodology. For example, in studying these agents it may be appropriate to continue therapy as long as possible, despite growth of measurable tumor, so long in the clinical status of the patient is stable and the tumor is not life-threatening.

Preclinical studies are the mandatory pathway to determine the mechanism of action of angiogenesis inhibitors, to evaluate the advantages and disadvantages of various antiangiogenic treatments and to compare them with traditional cytotoxic or anti-inflammatory strategies. By using the same models, different inhibitors can be compared for their relative efficacy, toxicity and for the development of resistance. They can enable testing the combination of different angiogenic inhibitors, or their combination with other therapies such as cytotoxic agents, radiation or immunotherapy, to provide prognostic information and to establish markers of efficacy for clinical trials. The major problem however, is how we choose the best preclinical assays since neither in vitro nor in vivo animal models

entirely mimic the clinical situation. In order to reconstruct the "human puzzle", the solution has been to test a combined panel of preclinical in vitro and in vivo assays, each of which provide complementary information and a different view emphasizing different aspects of the process.

In vitro assays offer the possibility to dissect the different steps of the angiogenic cascade and to identify some of the molecular events targeted by each angiogenic inhibitor. These assays can be used to screen antiangiogenic agent efficacy in inhibition of endothelial cell proliferation, migration, tube formation or extracellular matrix degradation in a semi-quantitative or quantitative manner. However, they cannot accurately reflect or predict the potency of the same inhibitors in vivo because of major differences of pharmacokinetics as well as other complex biologic processes involved in angiogenesis. It appears more fruitful to combine in vitro assays with other bio-assays such as the in vivo matrigel or rabbit cornea tests which offer a quantitative and precise means to screen angiogenesis inhibitors. These models have been extensively reviewed elsewhere.[56-60]

The in vivo efficacy of antineoplastic therapies is traditionally assayed by administering test compounds to mice bearing subcutaneous tumors produced by inoculating syngeneic or immunodeficient mice with cultured tumor cell lines or primary tumor fragments of various origins. In most cases, such an approach allows one only to predict within a broad range, the efficacy of each angiogenesis inhibitor for the different types of tumor tested. However, the value of such models is greatly affected by the fact that the target is a tumor growing in an ectopic site and endothelial cells from different sites exhibit morphological and functional heterogeneity.[61] Endothelial cells might also respond differently towards an angiogenic inhibitor based on their anatomical location.[62,63] Additional problems are related to the animal specificity (allografts), or to the absence of an essential homeostatic element (e.g., immune system[64,65]), as in the case of xenografts which were created to get closer to the clinical reality. Their huge disadvantage with respect to antiangiogenesis agents lies in the fact that they do not to take into consideration the potential major distinction between "natural" tumor-induced neovascularization and that observed after injection of exogenous cells. The potential value of endogenous tumors (carcinogen-induced or transgenics) represents possible responses to this dilemma but also could add confounding variables to be considered.

Independent of the model and endpoints used, substantial interspecies differences limit extrapolation of results obtained in isolated cells or animals to humans. For example, it is clear that the rate of tumor growth is different in transplantable tumors in mice versus their human counterparts. Endothelial cells growing in tumors in mice may also be less mature and consequently more vulnerable to antiangiogenic treatment in comparison to established capillaries of human tumors.[66] As far as inflammatory phenomena are concerned, the same precautions must be considered since immune cells from different species do not behave similarly under the same stimulus and often vary in their capacity to synthesize and secrete the same cytokines.

Despite their limitations preclinical studies using animal models remain the most appropriate means to define efficacy parameters and are indispensable in obtaining clues for the optimal design of clinical trials for cancer and inflammatory conditions.

Opportunities

The new therapeutic approach proposed by antiangiogenic therapy is a multifaceted opportunity: mechanistic, strategic and collaborative. From a mechanistic point of view, it involves a considerable amount of knowledge about the natural history of cancer and metastasis. The earlier simple models of cancer seen as a heterogeneous group of rapidly dividing cells is being replaced by a much more complex appreciation of a system in which at least three compartments are identified: the cancer cell compartment, the interstitium and the vascular compartment. Many interactions between compartments regularly occur and have begun to be better understood.

From a development strategy point of view, the opportunity includes the reassessment of the classical paradigms of anticancer therapy testing which has been focused on chemotherapy for decades. Chemotherapy has lead to substantial advances in many cancers, but its efficacy remains unfortunately limited in many others. A fundamental paradigm shift related to antiangiogenic agents involves a redefinition of cancer as a chronic disease that can be stabilized for very prolonged periods of time.

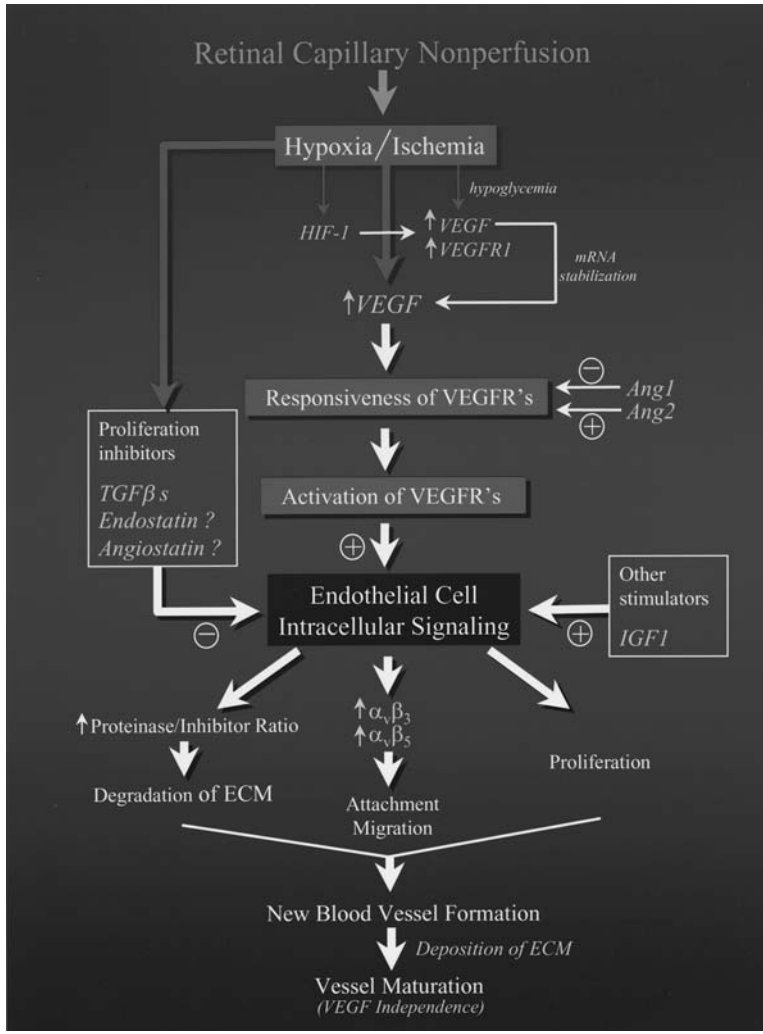

Fig. 13.1. Diagrammatic sketch showing the different factors, steps, and mediators involved in the initiation and propagation of neovascularization.

The objective of therapy is switched from a short term cytotoxic tumor response endpoint to a long term endpoint, including decreased recurrence of metastases or stabilization of actively expanding disease.

From a collaborative point of view, scientists and clinicians must establish close collaborations in order to better understand the different aspects of each compartment of the cancer system. Such collaboration should result in the design of new experimental models, in the reconsideration of optimal tolerated doses and in the definition of more appropriate clinical efficacy endpoints. It is vital for this novel therapeutic pathway to find the right balance between the optimal use of the preclinical information and the limited predictive value of this information for clinical trials. The identification of the mechanisms of action of each antangiogenic drug allows us to better identify

the important steps of the angiogenic cascade for each pathological condition, and should also lead to the generation of new active molecules.

Ultimately the greatest promise that the antiangiogenic therapy carries is a more efficient treatment strategy where patients are the great winners.

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Chapter 14

Surrogate Endpoints as a Measure of Efficacy in Clinical Trials of Angiogenesis Inhibitors

Przemek Twardowski, Stephen Gately and William Gradishar

It is now generally accepted that solid tumor growth and metastases are dependent upon the acquisition of an adequate blood supply.[1] Pharmacological targeting of tumor microvasculature in patients with malignant neoplasms represents an attractive therapeutic approach because inhibitors of angiogenesis are less likely to have the hematopoietic and gastrointestinal toxicity of standard antiproliferative therapies and appear not to induce acquired drug resistance.[2]

In the last decade numerous compounds with antiangiogenic activity were introduced into clinical trials (Table 14.1)[3] and others are currently in preclinical development (Table 14.2). The assessment of clinical efficacy of these agents presents unique challenges. The expected effect of a blockade of tumor neovascularization is the stability of the disease, not necessarily tumor regression. It is now widely believed that the most effective utilization of antiangiogenic therapy will be in the adjuvant setting of microscopic metastases, thereby preventing the emergence of a vascular supply and maintaining tumor dormancy. Therefore in clinical trials, the tumor regression (e.g., response rates) cannot be used as the sole marker of activity of the antiangiogenic compound. The discovery of an early indicator of biologic activity or surrogate marker would be extremely valuable in the clinical development plan of inhibitors of angiogenesis since the endpoints of survival and quality of life can take years to reach. The purpose of this chapter is to identify and discuss some putative surrogate markers of activity of antiangiogenic therapy.

Biomarkers in the Circulation

Angiogenic Proteins

The majority of malignant solid tumors overexpress at least one of the angiogenic growth factors, such as acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2),vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and others. Increased levels of growth factors and their receptors have been demonstrated in cancer cells, and elevated concentrations of these peptides have been detected in serum, cerebrospinal fluid and urine.[4,5] Elevated expression of growth factor receptors such as VEGFR-2 (KDR) has also been reported in different malignancies.[6] There is evidence suggesting a correlation between serum FGF-2 levels and prognosis.[7-16] In pediatric patients with hemangiomas treated with interferon alpha-2a, urinary FGF-2 levels decrease corresponding to tumor regression.[17] The measurement of circulating and urinary FGF-2 and VEGF and their response to antiangiogenic therapy may provide a useful surrogate marker of the activity of inhibitors of angiogenesis. It is important to remember that there are many factors influencing levels of angiogenic peptides. For example, VEGF is abundant in alpha granules of platelets, and plasma VEGF level is directly proportional to platelet count.[18,19] Also, the serum VEGF level is higher than plasma since separation of serum causes activation of platelets and release

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Table 14.1. Antiangiogenic agents introduced into clinical trials

Compound	Chemical Structure	Mechanism of Action	Clinical Trial Phase
Interferon alpha (Roche, Shering)	High molecular weight peptide	Inhibits endothelial cell proliferation and migration, inhibits production of bFGF	Phase III
TNP-470 (TAP Pharmaceuticals)	O-(chloroacetylcarbamoyl)-fumagillol	Inhibitor of endothelial cell proliferation	Phase I & II
Batimastat® (BB-94) (British Biotech)	Synthetic hydroxamate, peptidomimetic	Inhibitor of MMPs	Phase I & II
Marimastat® (BB-2516) (British Biotech)	Synthetic hydroxamate, peptidomimetic	Inhibitor of MMPs	Phase I - III
Tecogalan sodium (NCI)	29 kD sulfated polysaccharide	Inhibits binding of bFGF to endothelial cells	Phase I & II
Pentosan polysulfate (Baker Norton)	Sulfated polysaccharide	Binds and inactivates heparin-binding growth factors	Phase I & II
CM-101 (CarboMed)	Polysaccharide bacterial exotoxin	Induces inflammation in tumors, destroying growing capillaries	Phase I
Suramin (Park Davis)	Polysulfonated naphtylurea	Binds and inhibits angiogenic factors (a-FGF,b-FGF,IGF-1)	Phase II
Platelet Factor 4	7.8 kD polypeptide	Inhibits growth factor-induced endothelial cell proliferation	Phase I
CAI (NCI)	Carboxyamidotriazole	Inhibits calcium-influx regulated signal pathways	Phase I
Thalidomide (EntreMed)	N-phthaloylglutamimide	Inhibits TNF alpha, active metabolite is antiangiogenic	Phase I & II
MAb-4.6.1 (Genentech)	Monoclonal antibody	Binds to and inhibits VEGF receptor	Phase I
SU5416 (Sugen)	Low molecular weight compound	Binds to adenine-binding portion of FLK-1 (VEGF receptor)	Phase I

of VEGF. These factors need to be taken into account when considering using the levels of angiogenic growth peptides as surrogate markers of activity of antiangiogenic compounds.

Serum Tumor Markers

One of the most extensively evaluated antiangiogenic compounds is Marimastat®, the inhibitor of matrix metalloproteinases.[20-23] The investigators studying Marimastat® analyzed the effect of this agent on serum tumor markers as an early indicator of activity in patients with advanced malignancies. The tumor markers studied were CA-125 in ovarian cancer, PSA in prostate cancer, CEA in colorectal cancer, and CA 19-9 in pancreatic cancer. Eligible patients had to demonstrate at least a 25% increase in tumor marker level over a period of 4 weeks prior to entry into the trial. Three hundred and eighty-one patients were treated with Marimastat® at doses ranging from 5 mg per day to 50 mg twice a day for 4 weeks. A dose-dependent decrease in the rate of rise of tumor markers was noted in patients treated with Marimastat®. In the case of ovarian cancer, the median rate of increase of CA-125 was 51% in the 4 weeks preceding the initiation of treatment and 19% in the period of 4 weeks after the Marimastat® therapy was started. Combining data from the four evalu-

Table 14.2. Antiangiogenic compounds in preclinical and/or early clinical development

• 16 kDa prolactin fragment	• Linomide
• Amiloride	• Low molecular weight alpha v beta 3 inhibitor
• Angiostatin	• Minocycline
• Antibody to alpha v beta 3	• Neovastat-shark cartilage extract
• Antibody to bFGF	• Placental ribonuclease inhibitor
• Antibody to FLK-1 VEGF receptor)	• angiogenin inhibitor)
• Antibody to VEGF	• Protamine
• Captopril	• Retinoids
• Clo-3	• Somatostatin analogues (octreotide)
• D-penicillamine	• Steroids/heparin
• Endostatin	• Sulphated chitin derivatives
• Herbamycin A	• Thrombospondin
• Interferon-gamma	• TIMP-1, TIMP-2
• Interleukin-12	• Vitamin D analogues
	• YIGSR peptide

ated cancers, the median rate of increase of tumor marker was 52% in the 4 weeks before therapy and 14% after 4 weeks of treatment with Marimastat® at doses of 10, 25 or 50 mg BID. These differences were statistically significant, but there was no control arm that would confirm that these changes in tumor markers were the result of Marimastat® therapy. Though decreases in the levels of serum tumor markers will not constitute a surrogate marker of specific antiangiogenic activity, they may be helpful in early assessment of the efficacy of antiangiogenic compounds, particularly if these findings can be correlated with clinical endpoints such as disease-free survival (DFS) and overall survival (OS).

Serum Copper Levels

Serum copper levels are elevated in a variety of human[24-26] and experimental tumors.[27] Copper levels have been used to predict the onset of cancer by several years and levels of ceruloplasmin, the principal copper transporting protein, increase during tumor progression, often before tumors are palpable.[27] Copper is linked to angiogenesis;[28] copper ions stimulate endothelial cell migration, extracellular matrix production and corneal angiogenesis.[29,30] Additionally, angiogenic growth factors have been found to bind to copper with high affinity.[31] Recently copper ions were shown to enhance the binding of the proangiogenic factor angiogenin to endothelial cells, suggesting copper could regulate the biological activity of angiogenin.[32]

Lowering serum copper levels by imposing a copper deficient diet or administering D-penicillamine administration has been used as a treatment for experimental tumors. Decreased serum copper levels were associated with the significant inhibition of angiogenesis, tumor growth and neoplastic invasion in the brain.[33,34] Serum copper levels has been used to monitor for tumor recurrence following therapy.[35] Recently withdrawal of the angiogenesis inhibitor TNP-470 in experimental animals was followed by elevated serum copper levels that was correlated with rapid tumor growth.[36] When taken together, monitoring serum copper levels could provide useful information on the biological activity of angiogenesis inhibitors.

Histopathological Markers

Microvascular Density

Increased microvessel density in tumor tissue specimens has been shown to correspond with higher relapse rates and overall worse survival in breast cancer and other solid malignancies including melanoma, prostate, colon, bladder, head and neck and non-small cell lung cancers.[37-48] Tumor tissue specimens are immunostained to detect specific endothelial cell markers, usually von Willebrand factor (sometimes referred to as Factor VIII related antigen) or endothelial antigen, CD31. Vessels are then counted in three different "hot spot" regions that identify areas of high vascularity. This technique is laborious and subject to interobserver variation and poor reproducibility. However in certain easily accessible tumors, like cutaneous melanoma or gastric cancer, it would be possible to repeat biopsies after the initiation of antiangiogenic therapy. Decreased vessel counts could indicate an antiangiogenic effect. This approach is currently being investigated in clinical trials of Marimastat® but its value remains uncertain. In addition, the activity of tissue MMPs and other enzymes involved in angiogenesis and metastasis can be measured, after treatment with a compound (e.g., antiangiogenesis agent) targeting a specific enzyme is initiated.

Circulating and Bone Marrow Metastases

Since angiogenesis is a necessary step for extravasation of malignant cells into the circulation,[49,50] the number of circulating or bone marrow malignant cells may decrease after the initiation of antiangiogenic therapy. At this point the quantification of circulating cancer cells is difficult and its prognostic implications are uncertain. Therefore the impact of the inhibitors on angiogenesis on circulating tumor cells and its significance requires further investigation.

Clinical Response Rates

Some inhibitors of angiogenesis have been shown to prevent the growth[51] and even induce regression of solid tumors (i.e., angiostatin, endostatin).[2,52]

There is also compelling preclinical evidence indicating that the use of inhibitors of angiogenesis will be synergistic with chemotherapy and radiation.[53-55] Therefore it is possible that antiangiogenic agents, especially in combination with chemotherapy and/or radiotherapy, will improve the response rates in human malignancies thus providing evidence for clinical benefit or efficacy of antiangiogenic compounds. Trials of Marimastat® and other antiangiogenic compounds like carboxyamidotriazole (CAI) in combination with cytotoxic chemotherapy are currently underway, but no efficacy data is available at this time.

Imaging Studies

A variety of imaging studies including magnetic resonance imaging (MRI), Doppler ultrasound and positron emission tomography (PET) scanning can be used to evaluate tumor vessel morphology, density and blood flow.[56] There is a correlation of angiogenesis estimated by MRI with vascular density assessed pathologically in primary breast cancer.[57] Ferromagnetic compound conjugated with antibody to a receptor expressed on newly forming endothelial cells (alpha v beta 3 integrin) is being evaluated in an attempt to enhance the specificity and sensitivity of detection of neovasculature. These sophisticated imaging techniques may become increasingly useful in the assessment of activity of inhibitors of angiogenesis.

Wound Healing

One of the physiologic processes in which angiogenesis plays an important role is wound healing. Quantification of wound healing-associated neovascularization, utilizing subcutaneously implanted polytetrafluoroethylene (PTFE) tubes filled with collagen is being investigated in animal models. These PTFE tubes are removed several days after implantation, and vascularization is assessed by

measurements of hemoglobin concentration in the collagen matrix, or by microvessel density.[58,59] Ongoing studies are evaluating the effects of various inhibitors of angiogenesis on wound healing-associated angiogenesis using these models.

Specific Angiogenesis Inhibitor Biomarkers

It is important to remember that inhibitors of angiogenesis constitute a very heterogeneous group of compounds and that some surrogate markers may be specific for a particular agent. CM101 is a derivative of Group B Streptococcus (GBS) polysaccharide toxin. It induces a complement-mediated inflammatory response that targets newly developing blood vessels expressing E-selectin receptor. E-selectin is detectable in serum and its levels increase proportionately to the degree of inflammatory reaction. Therefore, measurement of E-selectin serum level may be considered a surrogate marker of antiangiogenic activity of CM-101.[60-64]

Pentosan polysulfate (PPS) is a semisynthetic heparinoid that has been in clinical use as an anticoagulant in several countries. Pentosan polysulfate has been shown to effectively block heparin-binding growth factor (i.e., bFGF) activities in in vitro and in vivo models. In a phase I trial of PPS in patients with advanced cancer, plasma samples were assayed for inhibition of heparin-binding growth factor-mediated cellular proliferation.(e.g., anti-HBGF activity). It was found that patients treated with PPS had prolonged high plasma levels of anti-HBGF activity suggesting the achievement of therapeutic concentrations of the drug.[65-68] Thus measurement of serum anti-HBGF activity constitutes a surrogate marker of possible therapeutic efficacy of PPS.

Serum of patients treated with inhibitors of antiangiogenesis can be tested for antiangiogenic activity in a variety of in vitro assays including endothelial cell proliferation This approach has recently been reported in a trial of suramin.[69] Even though it is costly and time-consuming, this method can provide an early indication of the "antiangiogenic state" achieved by an administration of an antiangiogenic inhibitor.

Conclusion

Currently there are no validated surrogate markers of activity of antiangiogenic compounds in humans and the search for them will undoubtedly become a focus of more intense investigation. The identification of a biomarker of antiangiogenic activity that is easily and reproducibly quantitated will be an extremely valuable tool in the clinical development of inhibitors of angiogenesis.

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Chapter 15

Lessons from Clinical Trials of Anti-Angiogenic Drugs in the Treatment of Cancer

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The process of angiogenesis plays a critical role in tumor growth and metastasis. Recently, there has been much interest in the use of anti-angiogenic drugs in the treatment of cancer. This chapter will review the results of phase I clinical trials of various anti-angiogenic agents, and will offer some suggestions for the future design of phase II and III studies.

Background

In the past few decades, researchers have become increasingly interested in the observation that tumor growth and metastasis are accompanied by significant new blood vessel formation i.e., angiogenesis. This work, pioneered by Dr. Judah Folkman, led to the hypothesis that tumors are "angiogenesis-dependent".[1] In the early prevascular phase, tumors are usually microscopic, and cell population is limited. With the onset of angiogenesis, the tumor cell population rapidly expands and metastasizes. This hypothesis is supported by multiple animal experiments in which tumors implanted in chicks, rodents and rabbits are restricted in growth during the avascular phase, but rapid growth and metastasis occur shortly after vascularization. In addition, angiogenesis inhibitors that are not themselves cytostatic to tumor cells in vitro inhibit tumor growth and metastasis in the animals in vivo.[2]

Multiple clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis-dependent. Angiogenesis, as determined by the amount of vascularity in the tumor specimen, was first shown to have prognostic significance in breast cancer by Dr. Folkman.[3] The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic value independent of other routinely used markers. Since 1991, an overwhelming majority of published reports have shown a significant correlation between the density of intratumoral microvessels in invasive breast carcinoma and the incidence of metastases and/or patient survival. Similar associations have now been reported for patients with melanoma, prostatic, testicular, ovarian, rectal and bladder carcinoma, central nervous system tumors, multiple myeloma, non-small cell lung carcinoma, and squamous carcinoma of the head and neck.[4] Furthermore, the levels of various angiogenic factors in bodily fluids have been demonstrated to correlate with prognosis in cancer patients.[5]

Multiple agents have been developed in order to inhibit this phenomenon of tumor-induced angiogenesis.[6-9] Many of these agents have completed phase I clinical trials and are currently in the beginning of phases II and III. This chapter will review the results thus far of the phase I studies and will offer suggestions for the future design of phase II and III clinical trials (Table 15.1).

Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications, edited by Shaker A. Mousa.

Table 15.1. Inhibitors of angiogenesis in clinical trials

	References
Compounds with unknown mechanisms:	
TNP-470	12, 37, 54, 64
Thalidomide	7, 17, 18, 47
Compounds that inhibit signal transduction:	
CAI	36
Compounds that target tumor vasculature:	
CM-101	11, 67
Compounds that inhibit angiogenic factors:	
bFGF:	
Pentosan polysulfate	39, 52, 60
Tecogalan	13, 65
Suramin	14, 32, 34, 44
VEGF:	
VEGF antibodies	16
SU-5416	
Cytokines:	
Interferons	3, 15, 26, 42, 55
Interleukin-12	8, 23, 61
Platelet factor-4	5, 28, 31, 48, 58
Compounds that inhibit matrix interactions:	
Batimastat	4, 49, 70
Integrin antibodies	24

TNP-470

TNP-470 is a derivative of fumagillin, a fungal product.[10] In tissue culture, TNP-470 seems to inhibit only endothelial cells and have little effect on tumor cells. In vivo, TNP-470 induced avascular zones in the chick chorioallantoic membrane (CAM) and suppressed blood vessel formation in the rat cornea assay and the rat sponge model. Its anti-angiogenic effect is probably due to the suppression of cyclin D1 expression in the mid G1 phase of the cell cycle.[11,12] In animal studies, this drug has been shown to be very effective against a wide number of tumor types.[13-15]

TNP-470 has completed seven phase I clinical trials. These trials enrolled adult and pediatric patients with solid tumors or leukemias. In one of these trials, the drug was administered to 18 patients with inoperable recurrent or metastatic squamous cell cancer of the cervix.[16] The starting dose was 9.3 mg/m^2. Grade 3 neurotoxicites consisting of weakness, nystagmus, diplopia, and ataxia were encountered in two patients receiving the 71.2 mg/m^2 dose. An intermediate dose level of 60 mg/m^2 was evaluated and found to be well tolerated by three patients. Only one patient experienced grade 3 nausea on this dose. One patient had a complete response, which continued for over 26 months, and three patients with initially progressive disease had stable disease for 5, 8, and over 19 months.

In another clinical trial, TNP-470 was given to 38 AIDS patients with Kaposi's sarcoma.[17] Grade 3 and 4 neutropenia were observed in two patients at doses of 10 mg/m^2 and 40 mg/m^2, respectively. These two patients were taking a concomitant medication that could have contributed to the neutropenia (zidovudine and bactrim). The patient at the 40 mg/m^2 dose also developed hematuria five weeks after study completion. One patient at the 20 mg/m^2 dose had grade 3 urticaria.

An intracranial bleed occurred within the solitary lymphoma mass of a patient at the 40 mg/m^2 dose, and a retinal hemorrhage was reported in a patient with newly diagnosed CMV (cytomegalovirus) retinitis at the 50 mg/m^2 dose. These two patients did not have any significant abnormalities in their platelet counts or coagulation profiles. Seven patients met the criteria for partial response, and five had disease stabilization.

In a third phase I trial, TNP-470 was administered to another 28 patients with AIDS-associated Kaposi's sarcoma.[18] In one patient receiving 76.3 mg/m^2, neurological symptoms developed including agitation, ocular and limb dysmetria, and mild nystagmus. Eight patients had disease stabilization. In the remaining clinical trials, grade 3 neurologic toxicities were again observed at high drug doses (two patients at 70.6 mg/m^2, one patient at 76.5 mg/m^2, and one patient at 105.9 mg/m^2).[19] These adverse neurologic effects were completely reversed with time. There was also one case of cataract development in a 69-year old patient. One patient who was administered TNP-470 at 76.5 mg/m^2 reported grade 1 hemoptysis. However, he had a history of multiple episodes of hemoptysis prior to drug treatment.

Currently, five phase II clinical trials have been initiated in breast, pancreas, cervical, kidney and brain tumors. The dose of TNP-470 is 60 mg/m^2 intravenously three times weekly. The results of these studies are pending. At least one partial response in a patient with metastatic breast cancer has been observed (Fig. 15.1).

Thalidomide

Thalidomide was developed in 1954 and marketed as a sleeping pill with no LD50 (lethal dose 50). As the number of fetal abnormalities linked with thalidomide increased, this drug was withdrawn from the market in 1961. In 1994, Dr. Folkman was able to demonstrate that the drug's teratogenicity correlated with its anti-angiogenic activity.[20] Over the years, other side effects have been well documented: drowsiness, constipation, peripheral sensory neuropathy, swelling and erythema of the limbs, fever, rash, amenorrhea, serostomia, increased appetite, loss of libido, nausea and pruritus. However, it has been difficult to study thalidomide in vitro and in mouse tumor models since this drug must be processed first by the liver of higher animal species into an active metabolite.

In one preliminary study, thalidomide was administered in conjunction with chemotherapy to seven breast cancer patients.[21] Side effects attributed to thalidomide were minimal and included constipation and a rash. In another study, thalidomide was given to 17 patients with Kaposi's sarcoma[22] Four patients developed a rash and one had Raynaud's syndrome. Five patients had a partial response, and one had stable disease. Twelve patients with prostate cancer have been enrolled in a third.[23] Four of these patients were reported to have decreased levels of PSA. In a fourth study, 32 patients with glioma were treated with thalidomide.[24] So far, two patients had minimal responses. Currently, phase II clinical trials are ongoing for patients with breast cancer, prostate cancer, Kaposi's sarcoma and glioblastoma.

Carboxyamido-Triazole

Carboxyamido-triaxole (CAI) is an inhibitor of non-voltage-gated calcium influx.[25]It inhibits endothelial cell proliferation and migration and creates inhibitory zones in the CAM assay. Carboxyamido-triazole has also been shown to be active against a number of tumor types in the mouse.

In a phase I clinical trial, 49 patients with refractory solid tumors were enrolled.[26] Toxicity of CAI most commonly consisted of dose-related grade 1-2 nausea, vomiting and anorexia. Reversible and rare sensory axonal neuropathy (grade 3, one patient) and neutropenia (grade 4, one patient) were dose-limiting toxicities observed at the 330 mg/m^2 dose. Disease stabilization and improvements in performance status were observed in 49% of evaluable patients who had disease progression prior to CAI. Currently, phase II clinical trials are ongoing for prostate cancer and ovarian cancer, with an oral dose of 300 mg/m^2/day.

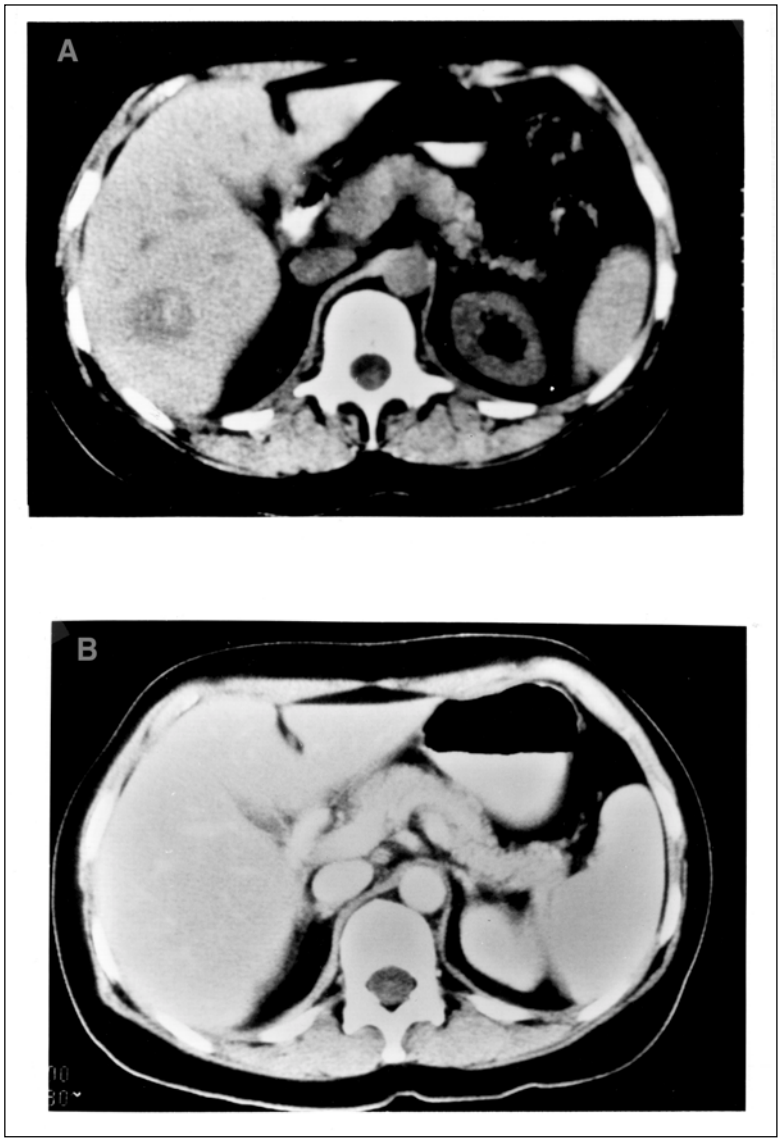

Fig. 15.1. CT image of the abdomen of a patient with metastatic breast cancer prior to the initiation of TNP-470 treatment (A); and after three months of TNP-470 treatment (B).

CM-101

CM-101 is a bacterial polysaccharide that induces neovascular inflammation in malignant tumors.[27] It appears that CM-101 binds selectively to the tumor neovasculature via endothelial cell receptors. In animal tumor models, administration of this drug resulted in a severe inflammatory response targeting the tumor, as evidenced by vasodilation, endothelial and tumor cell necrosis, invasion by inflammatory cells, and capillary thrombosis.

A phase I clinical trial was carried out with 15 patients with refractory malignancies.[28] Inflammation-related symptoms were universal in this group of patients, manifesting as fever, chills, tachypnea, tachycardia, and hypotension. Multiple inflammatory markers including TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6), IL-8 (interleukin-8), IL-10 (interleukin-10), and E-selectin were noted to be elevated, with a decline in circulating leukocytes.[29] All of these toxicities were reversible. One patient receiving 5 units/kg developed grade 4 arrhythmia and grade 3 hypotension. This patient had a history of supraventricular tachycardia. Another patient at the same dosage developed grade 4 dyspnea. Grade 3 dyspnea occurred in two patients at the lower dose levels of 1 and 2 units/kg. All three patients with dyspnea had pulmonary metastases and a carbon monoxide diffusion capacity of <76% of the predicted value. Pain at the tumor site was reported in half of the patient population. There were three patients with tumor shrinkage following CM-101 treatment (two patients at 2 units/kg and one patient at 3.3 units/kg). Currently, CM-101 is administered intravenously once weekly at 3.3 units/kg.

Inhibitors of Angiogeneic Factors

Inhibitors of bFGF (basic fibroblast growth factor) include pentosan polysulfate, Tecogalan, and suramin. Pentosan polysulfate is a semisynthetic pentasaccharide heparinoid used as an anticoagulant. Pentosan polysulfate has been shown to effectively block heparin binding growth factor activity, including bFGF, in in vitro and in vivo systems. In the first phase I study, 16 patients with Kaposi's sarcoma received pentosan polysulfate.[30] Dose-limiting toxic effects were characterized by anticoagulation and thrombocytopenia and were reversible. Three patients had stable disease. In another phase I clinical trial, 19 patients with various solid tumors were treated.[31] There was as expected an increase in anticoagulant activity as measured by aPTT (activated partial thromboplastin time) in most patients. Grade 3 thrombocytopenia occurred in one patient at the dose of 22.5 mg/m^2 and grade 4 thrombocytopenia in another at the dose of 30 mg/m^2. Grade 3 elevation of SGPT (serum glutamate pyruvate transaminase) happened in one patient at the dose of 30 mg/m^2. There were three cases of disease stabilization. In a third phase I study, pentosan polysulfate was administered to 13 patients with metastatic cancer at a dose required to maintain aPTT between 1.8 and 2.2 times the baseline value.[32] All patients experienced a prolongation of their PT (prothrombin time) values and at least grade I thrombocytopenia. One patient developed grade 3 elevations in SGPT and SGOT (serum glutamate oxaloacetate transaminase), and another grade 4 thrombocytopenia. One patient with colon cancer had stable disease.

Tecogalan is a sulfated polysaccharide isolated from the bacterium Arthrobacter. This compound inhibits angiogenesis by interfering with the binding of bFGF to cellular receptors. There were two phase I clinical trials that administered Tecogalan to patients with Kaposi's sarcoma and other solid tumors. Seventeen patients were treated in one trial.[33] Toxicities included fever, chills, headache, rigors, tachycardia, nausea, and mild elevation of aPTT. Six patients had stable disease. Another trial enrolled 14 patients.[34] Similar toxicities were observed, and one patient had disease stabilization.

Suramin is a polysulfonated napthylurea used to treat trypanosomiasis and onchocerciasis. Suramin has multiple effects on tumor cells including induction of tumor cell differentiation and inhibition of mitochondrial function, steroidogenesis, protein kinase C, glycolysis, membrane-associated ionic pumps, lysosomal enzyme function, and glycosaminoglycan synthesis. Suramin inhibits angiogenesis by antagonizing a number of growth factors including aFGF (acidic fibroblast growth factor), bFGF, and IGF-1 (insulin-like growth factor-1).[35] Suramin has a wide spectrum of serious toxicities, a narrow therapeutic window, a long half-life and complex pharmacokinetics. However, it has been shown to be effective in Kaposi's sarcoma, lymphoma, renal cell carcinoma, and particularly in prostate carcinoma.[36-39]

Inhibitors of VEGF (vascular endothelial growth factor) in clinical trials include antibodies against VEGF itself[40] or antagonists to VEGF receptors (SU-5416, Lee Rosen, personal communications). These drugs are at the completion of phase I trials, and are scheduled to begin phase II testing in the near future. For more details, please refer to the chapter in this book on VEGF.

Cytokines

Interferons are low molecular weight proteins produced by leukocytes, fibroblasts and T lymphocytes. They have a wide array of activities including increasing natural killer cell activity and inhibiting tumor cell proliferation and oncogene expression. Interferons suppress angiogenesis in vitro and in vivo primarily by inhibiting the production of bFGF. Interferon α-2a has been used successfully in treating hemangiomas of infancy.[41] The toxicities are primarily "flu-like" symptoms such as fatigue, fever and myalgia. Interferons combined with retinoids have been reported to have activity against lung cancer,[43] pancreatic cancer[43] and cervical cancer.[44] The combination of interferons and AZT (zidovudine) has also had some effect on Kaposi's sarcoma.[45]

Interleukin-12 (IL-12) is a cytokine that activates natural killer cells and Th1 T cells as well as induces the secretion of other inflammatory cytokines through induction of intermediary proteins. It inhibits angiogenesis in the mouse cornea assay, possibly via interferon-γ. Interleukin-12 has been tested in the context of phase I clinical trials.[46,47] Gene therapy with IL-12 has also been attempted.[48] Toxicities of IL-12 treatment included grade 4 leukopenia, thrombocytopenia, elevated liver function tests, fever, chills, fatigue, nausea and vomiting, mucositis and dyspnea. These approaches have yielded partial response or disease stabilization in a small number of patients.

Platelet factor-4 (PF-4) is released from the platelet alpha granules during platelet aggregation. It has many biological activities including inhibition of bone resorption and stimulation of fibroblasts, neutrophils and monocytes. PF-4 inhibits angiogenesis in in vitro assays and in the chick CAM assay. Phase I clinical trials were conducted with PF-4 in patients with colorectal carcinoma,[49] melanoma and renal cell carcinoma.[50] Minimal toxicity has been observed. However, there appeared to be no effect on the patients' tumors. Additional studies with Kaposi's sarcoma patients showed some tumor response.[51-53]

Inhibitors of Matrix Interactions

Inhibitors of matrix metalloproteinases including Batimastat and Marimastat[54-57] as well as antibodies against integrins[57] are addressed in other chapters in this book.

Discussion

As we embark upon phase II and III clinical trials of anti-angiogenic agents, some conclusions can be made from the results of the phase I studies. First, the published reports show that anti-angiogenic drugs in general have low toxicity. For example, in the case of TNP-470, all of the reported toxicities were considered by the investigators to be possibly, not directly, associated with drug administration. It appears that many of the observed toxicities affected the neurological system. One of the possible mechanisms involved was recently described in the identification of neuropilin-1 as a novel receptor for VEGF.[58] Furthermore, some of the success stories showed that anti-angiogenic drugs can be safely administered on a regular basis for a long period of time. TNP-470 was administered at 71.2 mg/m^2 for more than 26 months, and at 60 mg/m^2 for more than 19 months. However, since phase I trials are short-lived, one cannot reach any conclusions regarding potentially significant side effects from long term administration of anti-angiogenic drugs. It is reasonable to anticipate that certain toxicities will occur, since inhibition of angiogenesis may interfere with "good" processes such as wound healing, collateral formation, and reproduction.[59]

Although the major aim of these clinical trials was to determine drug toxicity, there were definite cases of disease stabilization observed during the short duration of drug administration typical of phase I trials. These studies determined the MTD (maximal tolerated dose) for the various anti-angiogenic drugs, and the dosage immediately below the MTD is usually used in phase II and III clinical trials. However, unlike the cytotoxic chemotherapeutic approach, it is not always clear with the anti-angiogenic drugs that the highest tolerated dose is the most effective at inhibiting blood vessel and tumor growth. In the case of TNP-470 administration to patients with cervical cancer, although there was one complete response at 71.2 mg/m^2, disease stabilization occurred in multiple cases at the lower dose of 47.3 mg/m^2. For Kaposi's sarcoma patients, partial response was

observed in one patient at 10 mg/m^2, another at 20 mg/m^2, two at 30 mg/m^2, two at 40 mg/m^2, and one at 70 mg/m^2. In the CM-101 clinical trial, there was clinical response in two patients at 2 units/kg and one patient at 3.3 units/kg. In the pentosan polysulfate study, clinical responses were observed in two patients at the 22.5 mg/m^2 dose and one at the 30 mg/m^2 dose. Finally, two out of the six cases with positive results in the Tecogalan study were administered the lowest dose of 30 mg/m^2.

The determination of the endpoints in phase II and III clinical trials of anti-angiogenic drugs poses a challenge for investigators. Whereas chemotherapy exerts its cytotoxic effects rapidly, resulting in tumor shrinkage, anti-angiogenic drugs are cytostatic to endothelial cells which may not translate into a rapid reduction in tumor size. Therefore, other endpoints such as prolongation of disease stabilization, quality of life and survival are more biologically relevant to this new approach at cancer treatment. It is important to have an untreated control group of patients in order to assess the results of the study. Furthermore, biologic markers of angiogenic activity may be used in order to monitor drug efficacy.[60] In contrast to the cytotoxic paradigm, more patience is required in order to assess the efficacy of an anti-angiogenic drug, and this may or may not translate into higher expenses for the clinical investigators.

Another complicating factor in the design of clinical trials for anti-angiogenic drugs involves the observation that multiple drugs that are already approved by the FDA for other uses, have been found to inhibit angiogenesis. These drugs include anti-estrogens such as tamoxifen,[61] taxol,[62] octreotide,[63] retinoids[64] and captopril.[65] It is possible that many commonly prescribed medications may have either a stimulatory or inhibitory effect on the growth of new capillaries. It is well known that many cancer patients also take alternative forms of medicine such as shark cartilage, which may also suppress angiogenesis.[66] Patients also may follow certain dietary regimens that contribute to angiogenesis inhibition.[67] It is therefore important to take these facts into consideration in designing the entrance criteria for a randomized study of anti-angiogenic drugs.

Further validation of anti-angiogenic therapy for cancer awaits the results of phase II and III clinical trials. The currently ongoing clinical trials represent the first generation of anti-angiogenic drugs that were developed in the laboratories in the late 1980s and early 1990s. More recent discoveries of many-fold more potent angiogenic inhibitors such as angiostatin[68] and endostatin[69] will open the door to a second generation of angiogenic inhibitors that probably will be tested in clinical trials at the turn of the century. In addition, there appears to be much in vitro and in vivo evidence supporting the use of various anti-angiogenic drugs together or in combination with commonly used chemotherapeutic or hormonal agents.[70] A recent approach at tumor vascular targeting using existing chemotherapy appears to have therapeutic potential.[71] In the context of a multi-disciplinary approach to cancer, an anti-angiogenic drug would most likely be given after surgery and then in conjunction with chemotherapy and/or radiation. In summary, while anti-angiogenic drugs pose formidable challenges to the clinical investigators, they hold great promise for the future therapy of many solid tumors.

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Index