CANCER DRUG DISCOVERY AND DEVELOPMENT

Antiangiogenic Agents in Cancer Therapy

Edited by

Beverly A. Teicher

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Antiangiogenic Agents in Cancer Therapy

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PREFACE

The importance of normal cells and tissues to support the growth of tumors has been recognized for centuries. The observations of Van der Kolk *(1)*, Jones *(2)*, and Paget *(3)* more than 100 years ago documented this knowledge in the clinical science literature. Fifty years ago, Algire and Chalkey *(4)* reported that host vascular reactions could be elicited by growing tumors and described in exquisite detail the extent and tumor-specific nature of the induction of host capillaries by transplanted tumors. The central hypothesis of Algire and Chalkey was that vascular induction by solid tumors may be the major, and possibly, the only distinguishing factor leading to tumor growth beyond normal tissue control levels. By the late 1960s, Folkman and his colleagues *(5–7)* had begun the search for a tumor angiogenesis factor (TAF) and in 1971 in his landmark report in the *New England Journal of Medicine,* Folkman proposed "antiangiogenesis" as a means of holding tumors in a nonvascularized dormant state *(8)*.

Over the nearly 40 years since publication of that landmark paper, great strides have been made in understanding angiogenesis, blood flow, and tumor growth. Several angiogenic signaling molecules and angiogenic factors have been identified. Antiangiogenic agents from a wide variety of chemical classes, including steroids, polyanionic molecules, antibiotics, small molecule nutrients, synthetic small molecules, proteins, nucleic acid molecules (ribozymes and antisense DNA), and gene therapy agents have been identified. The significance of angiogenic activity as an important prognostic factor in many of the common solid tumors, and even in leukemia, is becoming more widely recognized.

Early clinical trials of antiangiogenic agents in cancer patients have been successful in that the toxicities observed with many of these new drugs have been mild. However, these early clinical trials have also highlighted the need to develop criteria by which to assess the clinical efficacy of these new agents. Finally, preclinical and early clinical studies have begun to incorporate antiangiogenic agents into combined modality regimens that are potentially curative.

Antiangiogenic Agents in Cancer Therapy describes our state of understanding of tumor growth and its dependence on vascular development as well as the present status of antiangiogenic agents on preclinical and clinical development and what is known about the mechanisms by which these molecules and treatment agents interfere with tumor vascular growth.

We are entering a potentially very exciting period in anticancer agent discovery where the therapeutic focus is expanding to include not only agents cytotoxic toward malignant cells, but agents that may be growth controlling, growth inhibitory, or activating or deactivating toward stromal cells or malignant cells, as well as agents that may alter signaling cascades from one cell type to another. At this impotant time in the development of cancer treatment, this volume takes stock of

what has been accomplished in the area of angiogenesis, where the experimental therapeutics of antiangiogenic agents is going, and the continuing evolution of the means and methods of cancer treatment and new drug development.

Beverly A. Teicher

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I ANTIAGIOGENIC FACTORS AND TUMOR VASCULATURE

1 Angiogenesis and Oxygen Transport in Solid Tumors

Zishan A. Haroon, Kevin G. Peters, Charles S. Greenberg, and Mark W. Dewhirst

CONTENTS

1. INTRODUCTION

Angiogenesis, the formation of new vessels from existing microvasculature, is a tremendously complex and intricate process, essential for embryogenesis and development of multicellular organisms *(1)*, but it occurs only rarely in adult tissues in a tightly controlled manner during normal wound healing and the female reproductive cycle (corpus luteum, placenta, and uterus) *(2)*. When these tight controls are breached, the result is unchecked angiogenesis, which has been implicated in the development and progression of a variety of diseases (Table 1). The prevalence of pathologic angiogenesis in human diseases, and the significant mortality associated with these disorders, underscore the importance and emergence of antiangiogenesis therapy as a major clinical tool. In the case of solid malignancies, the generation of proangiogenic substances is in part caused by the pathologic microenvironment that develops in response to uncoordinated vascular production.

A common consequence of the abnormal microvascular structure and function that exist in tumors is hypoxia, which is known to induce a number of factors involved in regulating angiogenesis. Thus, hypoxia may prove to be a common initial signal for tumor vessel formation. There are other microenvironmental factors that may also play a role in the process as well, including endogenous levels of nitric oxide (NO). This chapter presents an overview of the features of normal and pathologic angiogenesis, with an emphasis on the role of hypoxia and dysfunctional vasculature during the angiogenic process in tumors.

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Table 1
List of Major Diseases in Which Angiogenesis Plays a Role in Pathogenesis

Inflammatory diseases:
Arthritis, chronic inflammations, inflammatory bowel diseases, psoriasis
Neoplasms:
Breast, bladder, colon, glioblastoma, hemangioblastoma, lung, melanoma, neuroblastoma, pancreas, renal, uterine–cervix
Ocular diseases:
Age-related macular degeneration, proliferative retinopathy (diabetic)

2. NORMAL ANGIOGENESIS

Normal angiogenesis is a multistep, tightly orchestrated process that occurs predominantly during physiologic events involving tissue repair and/or remodeling (wound healing, placental development, and so on) *(3)*. Tissue repair and remodeling involves continuous feedback and interaction between endothelial cells and the extracellular matrix (ECM) in a process that has been termed "dynamic reciprocity" by Clark *(4)*. Similarly, vascular remodeling is accomplished by targeted apoptosis and proliferation, deposition of matrix and its stabilization, and organization by enzymatic crosslinking and proteolysis.

Normal angiogenesis involves an initial localized breakdown of the basement membrane in the parent vessel that is mediated by proteases *(3,5*; Fig. 1]. Endothelial cells then migrate into the perivascular space and adjoining matrix, and form a capillary sprout. These sprouts elongate by further endothelial migration at the tip, and proliferation at the base, to replace the migrated cells. Subsequently, remodeling occurs, and these cords anastamose to form a loop, basement membrane is laid out, and a patent vessel is formed.

2.1. Molecular Mediators of Angiogenesis

Although many angiogenic substances have been identified, polypeptide growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) seem to be the most potent ones (Table 2). It is interesting to note that the aforementioned growth factors signal through receptor tyrosine kinases *(6)*. VEGF, also known as vascular permeability factor, is a multifunctional cytokine that is upregulated in response to hypoxia, and is considered one of the most potent proangiogenic molecules *(7)*.

Apart from activating endothelial cells to proliferate and express matrix metalloproteinases, plasminogen activators, and tissue factor, the most prominent effect of VEGF is induction of vascular hyperpermeability. It has been consistently observed that hyperpermeability to plasma proteins is associated with both pathological and physiological angiogenesis *(8)*. This association may have two very important implications for angiogenesis: First, it leads to the formation of a provisional fibrin matrix, which provides the primary scaffold for assembly of elements necessary for neovascularization *(8)*. The provisional fibrin matrix probably provides a more fluid matrix that is supportive of the angiogenic process. Second, another suggestion for hyperpermeability's role in angiogenesis has been proposed by Folkman *(9)*, which relates to the observation that confluent endothelial cells are refractory to mitogenic stimuli. The vasodilation and hyperpermeability that precede angiogenesis may subject endothelial cells to stretch and decrease confluence, which increases reactivity to proangiogenic mitogens.

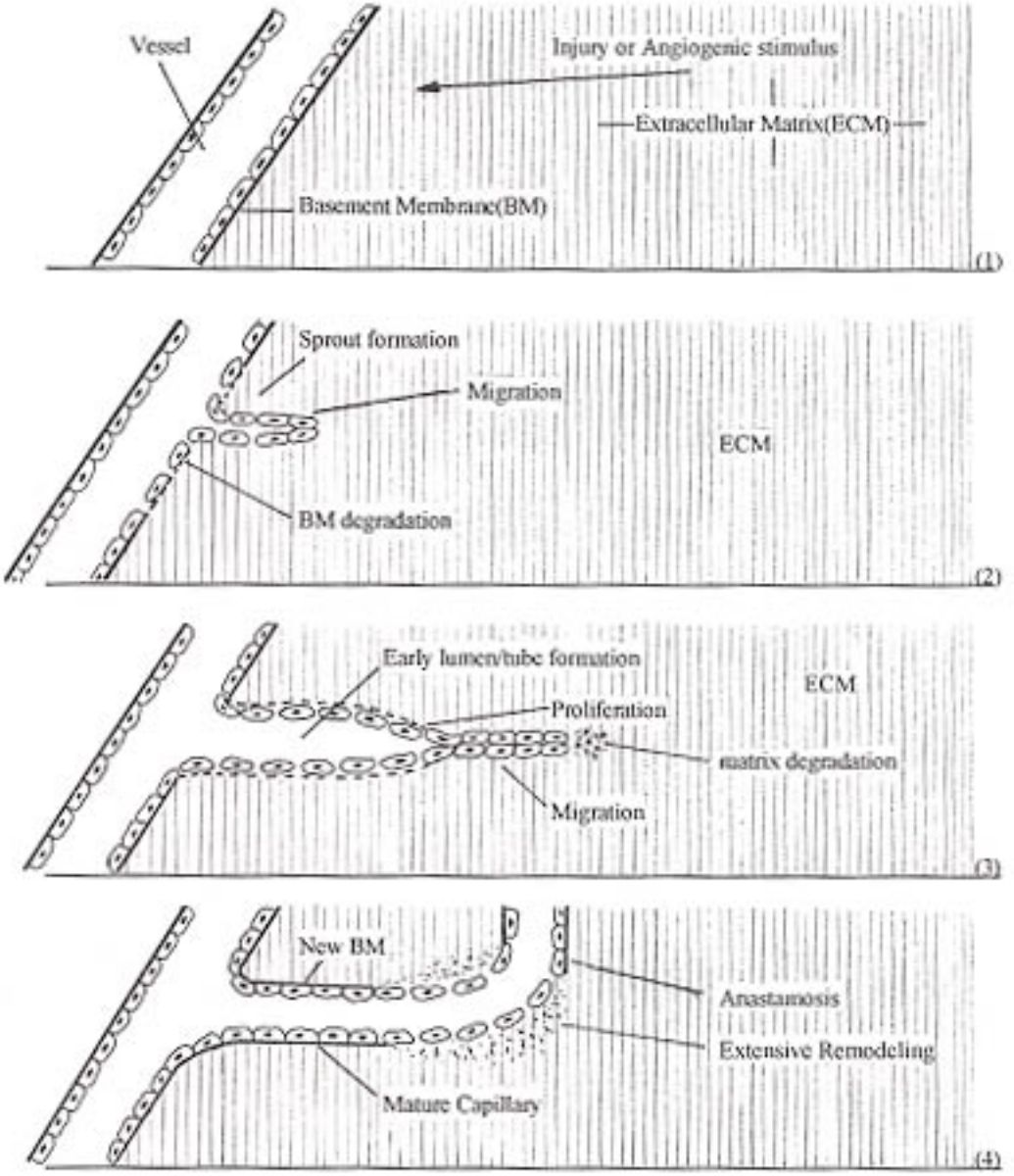

Fig. 1. A general mechanism of normal angiogenesis process. (1) Injury or tissue repair activates the endothelial cells. (2) Results in basement membrane degradation by proteases, and initial sprout formation by migration of endothelial cells. (3) Endothelial cells continue to migrate at the tip, with controlled matrix degradation. Proliferation occurs proximal to migration with formation of the primitive tube. (4) Extensive remodeling occurs all along the new capillary; new basement membrane is laid out to form a mature capillary as it anastamoses with other sprouts.

Normal angiogenesis involves complex interactions among endothelial cells, inflammatory cells, and ECM. These precisely controlled interactions involve ECM proteolysis during basement membrane degradation, invasion of the provisional fibrin matrix, and remodeling of the matrix and vessels *(10)*. These proteolytic activities also activate and/

Table 2
Prominent Molecules That Have Been Shown to Possess Proangiogenic Properties

Angiogenin
Fibroblast growth factors (acidic and basic)
Heparin
Hepatocyte growth factor (scatter factor)
Insulin-like growth factors
Interleukin-8
Platelet-activating factor
Platelet-derived endothelial cell growth factor
Platelet derived growth factor-BB
Transforming growth factor-α
Transforming growth factor-β
Tumor necrosis factor-α
Vascular endothelial growth factor

Table 3
Role of Balanced Proteolysis in Angiogenesis

Migratory path formation and remodeling:
Basement membrane degradation
Controlled ECM degradation involved during migration/invasion of endothelial and inflammatory cells into matrix
Anastamoses and capillary lumen/tube formation
Release of cytokines:
Release of bound basic FGF and VEGF
Activation of TGF-β from latent to active form
Degradation products with angiogenesis modulating capability:
Angiostatin (plasminogen)
Collagen derived peptides
Endostatin (collagen XVIII)
Fibrin and fibronectin fragments
16-kDa fragment of prolactin

or release important angiogenic cytokines, such as transforming growth factor-β (TGF-β), VEGF, and basic fibroblast growth factor (bFGF) *(11)*. In addition, biologically active degradation products of ECM, such as angiostatin from plasminogen, are generated, which regulate angiogenesis *(12)*. A balance of proteases and antiproteases, in a tightly regulated temporospatial pattern, is required for proper neovessel formation and remodeling/maturation (Table 3). Cytokines that regulate proteolytic activity during normal angiogenesis include bFGF, VEGF, TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) *(13)*.

Although endothelial cells provide the foundation for neovasculature, angiogenesis also involves complex interactions with fibroblasts and inflammatory cells, such as macrophages and mast cells. The role these inflammatory cells play during angiogenesis is further strengthened by the fact that many proinflammatory cytokines, such as interleukin-8 (IL-8) and TNF-α, induce angiogenesis *(14,15)*. Macrophages *(16)* and mast cells *(17)*

are involved during induction and propagation of the angiogenic cascade, and mediate their effects through secretion of cytokines and growth factors (including VEGF), release of proteases, and activation of fibroblasts. Fibroblasts are chiefly responsible for production of ECM and release of matrix metalloproteinases (MMPs) for selective degradation and organization of the ECM *(4)*.

Migration of endothelial and inflammatory cells forms an indispensable part of the angiogenic cascade. Migration is a multistep process that begins with a strong directional stimulus to migrate, and is followed by coordinate expression of receptors and matrix molecules to facilitate movement. TGF-β, VEGF, bFGF, and PDGF provide the chemotactic signals *(18)*, and chemokines provide chemokinetic signals *(14)* for both inflammatory and endothelial cells in induction of angiogenesis. The best-characterized receptors that are involved in migration during angiogenesis are integrins β_1, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ *(19)*. Although the evidence is still sketchy, it is becoming apparent that particular integrins are upregulated, and are required for the angiogenic effects of specific cytokines, such as $\alpha_v\beta_3$ for bFGF and $\alpha_v\beta_5$ for VEGF *(20)*.

The final steps in physiologic angiogenesis include transforming loosely associated endothelial cells and ECM into mature and patent vessels with intact basement membranes. Current evidence suggests that vessel maturation may be driven by novel molecular mediators, such as angiopoietins and their Tie family of receptors.

Angiopoietin-1 and -2 have recently been described as ligands for an endothelial-cell-specific tyrosine kinase receptor, Tie-2 *(21)*. Tie 2 receptors are essential for embryonic angiogenesis, since knock-outs of this receptor are embryonic lethal, with profound defects in assembly of microvessels, and it is proposed that the activity of the Tie-2 receptor is located downstream from VEGF on the angiogenic cascade *(22)*. The presence of the Tie-2 receptor on endothelial cells during angiogenesis in a variety of settings *(23)*, combined with evidence for constitutive angiopoietin 1 expression by vascular smooth muscle cells and/or pericytes, suggest that they may be involved in a regulated feedback system to modulate and steer the vessel maturation and organization process *(21)*. Meanwhile, angiopoietin-2 acts as a natural antagonist to Angiopoietin-1 and Tie-2, to provide a critical balance during induction of angiogenesis, vascular remodeling, and maturation *(21)*. Thus, angiopoietins and Tie-2 demonstrate a complex and intertwined relationship among themselves and their environment, and also exhibit the ability to regulate the angiogenic process.

3. TUMOR ANGIOGENESIS

Although developmental (embryonic) and disease-associated (pathologic) angiogenesis share many mechanistic features, as first suggested by Haddow *(24)* and later by Dvorak *(25)*, they probably differ regarding to regulatory controls *(26)*. As described above, tissue repair is normally a self-limiting process that occurs in response to hypoxia generated at the site of tissue injury. In contrast, progressive tumor growth creates ongoing hypoxia and acidosis, which do not recede as they would after injury (Fig. 2). Moreover, tumor vessels fail to mature into a normally functioning vasculature. In this manner, a positive feedback loop is created as continued tumor proliferation, handicapped by a disorganized vasculature, again outstrips its supply, which leads to continuing hypoxemia and angiogenesis (Fig. 3).

It is of interest to note that production of several of the proangiogenic compounds are regulated by hypoxia, including VEGF, bFGF, TGF-β, TNF-α, and IL-8 (Table 4).

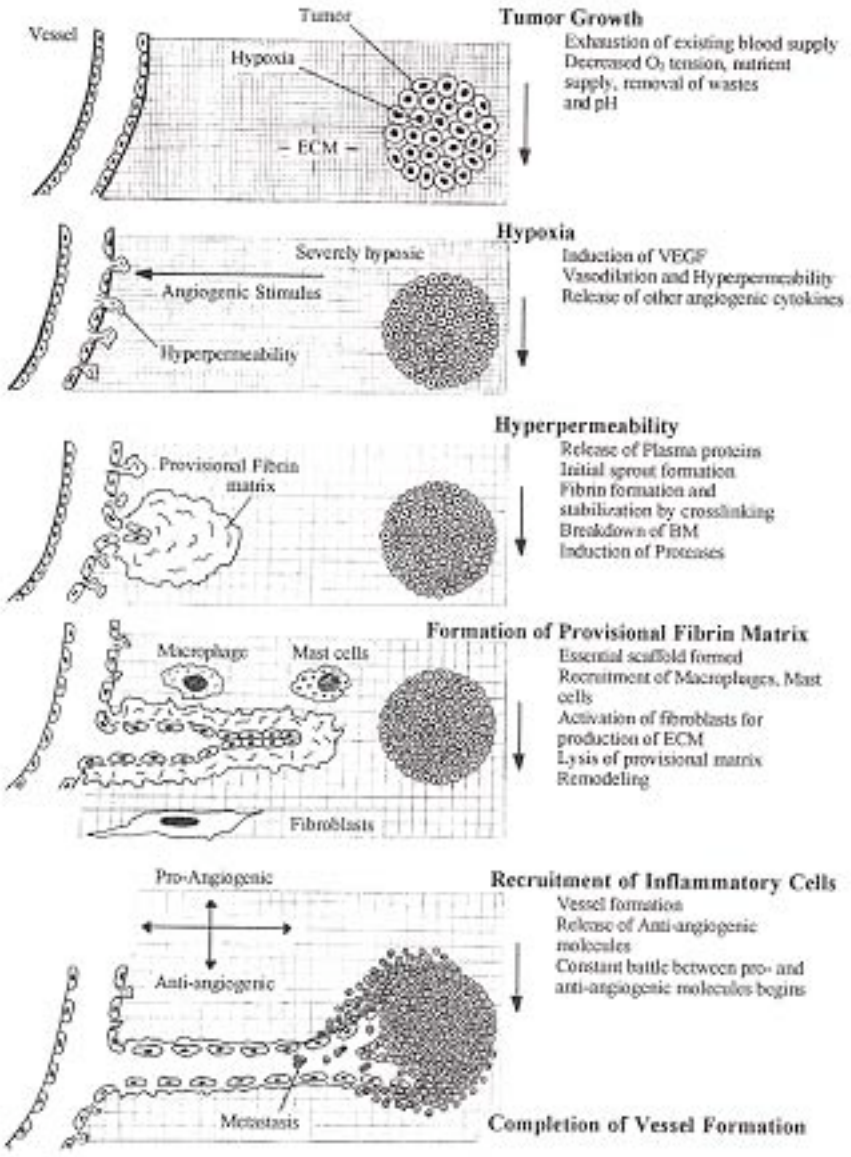

Fig. 2. A flow chart showing the current understanding of the process of tumor angiogenesis.

Furthermore, the presence of tumor hypoxia has been tied to more aggressive phenotypes in murine tumor models, as well as in human tumors. For example, in human cervix cancer and soft tissue sarcomas, presence of hypoxia prior to the start of therapy has been tied to a greater likelihood for distant metastases *(36,37)*. Additionally, the presence of tumor regions with high vascular density predicts for poorer overall survival in carcinoma of the breast, and in prostate cancer *(38)*. Taken together, these data strongly suggest that hypoxia upregulates angiogenesis, which in turn provides vascular access for metastasis.

Oncogenic transformations of tumor cells may also play a direct role in induction and propagation of angiogenesis, through production of angiogenic factors. It has been shown

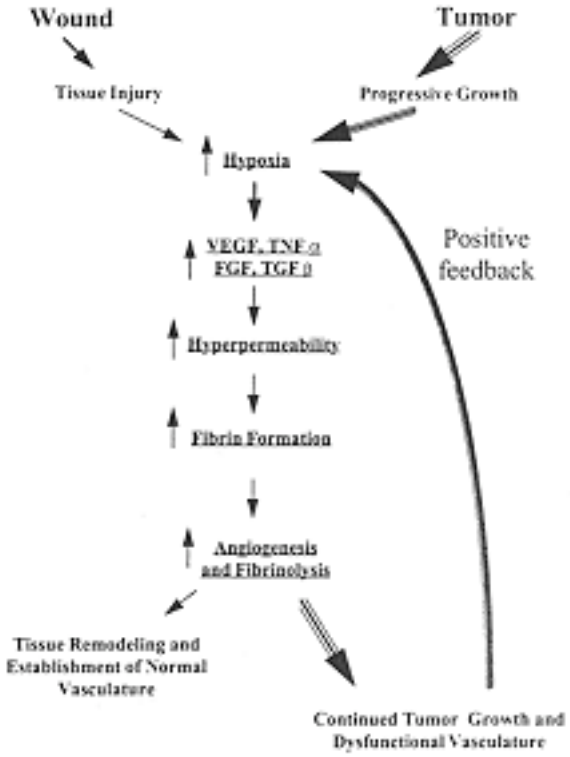

Fig. 3. Both normal tissue repair/remodeling mechanisms (wound healing) and tumors share common pathways to stimulate angiogenesis. Tissue repair, being a self-limited process, leads to regeneration of normal vasculature; tumor-induced angiogenesis produces a dysfunctional vasculature, which, coupled with progressive growth of tumor cells, results in continuing hypoxemia and angiogenesis.

Table 4
List of Proangiogenic Cytokines and Receptors Whose Expression Is Modulated by Hypoxia

Cytokine or receptor	*Hypoxia level*		*Gene expression*
	PO_2	*Time*	
Angiopoietin 1	NS	18 h	↓ *(27)*
aFGF, bFGF	2.0%	24 h	↑ *(28)*
Flk-1/KDR	2.0/2.0%	24/24 h	↓/↑ *(29[a],30[b])*
Flt-1	2.0%	24 h	↑ *(30)*
Interleukin-8	2.0%	24 h	↑ *(31)*
PDGF-A, PDGF-B	1.0%	16 h	↑ *(32)*
TGF-β	0.0%	24 h	↑ *(33)*
TNF-α	1.0%	24 h	↑ *(34)*
VEGF	1.0%	06 h	↑ *(35)*

↑, increase, ↓, decrease, NS, not specified.
*a*Reoxygenation performed in the experiments.
*b*Conflicting data has been reported in the papers.

Table 5
Short List of Molecules That Exhibit Potent Antiangiogenesis Activity

Angiostatin
Endostatin
Interferon-α
Metalloproteinase inhibitors
Platelet factor 4
Somatostatin
Thrombospondin

that VEGF expression is induced by mutant H- or K-*ras* oncogenes, as well as v-*src* and v-*raf,* in transformed fibroblasts and epithelial cells *(39)*. Other angiogenic growth factors, such as TGF-β and TGF–α, have also been shown to be upregulated by mutant ras *(39)*. These effects may be mediated through a ras-raf-MAP kinase signal transduction pathway, which results in activation of promoter regions of genes of angiogenic growth factors. Several of these proangiogenic cytokines have been shown to be produced by the same tumor *(40)*. This evidence points to a potential pitfall in approaching antiangiogenesis therapy directed against a singular angiogenic cytokine.

Aggressive neovascularization also leads to production and generation of numerous antiangiogenic compounds (Table 5). These substances are vital in regulating the process of angiogenesis, and are discussed in detail in later chapters.

4. TUMOR PATHOPHYSIOLOGY LEADING TO HYPOXIA

It is generally believed that hypoxia in tumors develops in two ways. The first form, referred to as chronic hypoxia, has classically been thought to result from long diffusion distances between tumor vessels *(41)*. The second form, known as perfusion limited, or acute, hypoxia, has been attributed to transient blockages in, or collapse of, tumor vessels *(42)*. This chapter will distinguish between these two forms of hypoxia in the following manner: Cells that are chronically hypoxic exist in an environment where the PO_2 is <10 mmHg for many hours at a time. Such cells may never experience a normoxic condition once they become hypoxic. Cells that are acutely hypoxic exist in an environment where the PO_2 is <10 mmHg for many minutes at a time. These cells probably experience many cycles of hypoxia and reoxygenation. Mechanistic understanding of the relative importance of the factors leading to both forms of hypoxia may lead to additional lines of investigation regarding microenvironmental control of gene expression in tumors.

4.1. Origins of Chronic Hypoxia

The balance between how much oxygen is delivered and how much is consumed leads to the oxygen concentration that is found in tissue. Several factors contribute to the development of chronic hypoxia, including longitudinal gradients of PO_2, irregular vascular geometry and low vascular density, altered blood viscosity, and oxygen consumption that is out of balance with supply. Of these factors, oxygen consumption has the most dynamic effect on tissue PO_2.

4.1.1. Deficiencies in Tumor Oxygen Supply Longitudinal Gradients

It is now well recognized that tumor microvessels can be hypoxic, even when there is movement of red cells through the vessel *(43,44)*. The most likely origin of intravascular hypoxia comes from longitudinal or axial gradients in oxygen tension, which are defined as declines in oxygen tension along the vascular tree. Two features of longitudinal gradients lead to intravascular hypoxia in tumors. First, tumor-feeding arterioles are more deoxygenated than comparable arterioles in normal tissues. Dewhirst et al. *(45)* reported that the PO_2 of tumor-feeding arterioles averaged 60% that of comparable arterioles of normal granulating tissues, yielding an average PO_2 of 32 mmHg, when aortic blood gas PO_2 averaged near 100 mmHg. The relative deoxygenation of these arterioles is probably caused by one or more factors: tissue pH—there is a right shift of the hemoglobin saturation curve as the arteriole enters a region of tissue acidosis (a typical condition in tumors); higher oxygen consumption—it is likely that the arterioles pass through a region of high cell proliferation, where the oxygen consumption rate may be very high. It is well established that longitudinal gradients occur in normal tissues as well, but such conditions do not normally lead to vascular hypoxia, probably because of the normal abundance of arteriolar supply *(46)*.

It has been estimated recently that the majority of oxygen transport in normal tissues actually occurs at the level of the terminal arteriole, rather than in capillaries *(46)*. There are arteriolar supply vessels to tumors, but they typically do not enter the tumor parenchyma *per se,* unless the tumor happens to grow around them *(47)*. Thus, oxygen transport in tumors cannot occur via arterioles. This means that the majority of oxygen transport is delivered via capillaries or veins, and it is likely that the blood must travel unusually long distances in postarteriolar microvessels before exiting the tumor *(48)*. There are several sets of data to support this hypothesis. Using phosphorescence quench imaging, it has been shown that longitudinal gradients exist in skin window chamber tumors, where those microvessels nearest the arterioles are much better oxygenated than vessels most distant from the arterioles *(49)*. The most distant vessels appear to be hypoxic (PO_2 averaging <10 mmHg). Studies with oxygen microelectrodes *(43)* and phosporescence quench imaging studies of Helmlinger et al. *(44)* have independently demonstrated the presence of hypoxemic tumor microvessels, in the absence of vascular stasis. Collectively, these data substantiate the notion that vascular hypoxia in tumors occurs because of the relative lack of arterioles. Vascular hypoxia, of the type described here, does not occur in normal tissues.

4.1.2. Altered Blood Viscosity

The vascular hypoxia found in tumors was speculated to be responsible for altered blood viscosity, which could contribute to sluggish flow that is often found in tumors. Using a viscometer, suspension viscosity was found to be increased by 40% when PO_2 and pH were decreased below 10 mmHg and 6.8, respectively. This effect was caused by shrinking of red cells. When the calcium channel blocker, flunarizine (5–10 mg/L-1), was co-administered with red cell suspensions, viscosity returned to control levels *(50)*. When the effect of flunarizine was examined in vivo, the drug improved blood flow and oxygenation, selectively, in the center of tumors, where microvessels are more hypoxic *(51)*. In summary, the reduced vascular oxygenation observed in tumors leads to rheologic changes in red cells that increase viscosity and decrease tumor blood flow. This leads to a viscous cycle, as the resultant reduction in blood flow further exacerbates the vascular

hypoxemia. The use of agents, such as flunarizine, could break this cycle, and at least restore viscosity to normal limits.

4.1.3. Vascular Geometry

The classic theory, which originated with Thomlinson and Grey, is that chronic hypoxia develops because intervascular distances are too long in tumors *(41)*. However, the irregular geometry of vascular networks is also important. Dewhirst et al. *(52)* have performed simulations of oxygen transport in two microvascular networks. The simulations were done using microvessel flow velocities and hematocrits that are typical for the R3230Ac tumor line growing in skin window chambers. Oxygen consumption rates were assumed to be in the midrange of what has been measured for this tumor line. Two models were compared (actual geometry shown in Fig. 4A). The Greens' function model utilizes the actual microvascular geometry; the Krogh cylinder model assumes that all of the vessels are straight and parallel. In simulations, the Krogh cylinder model predicted no regions of tissue with PO_2 <10 mmHg; the Green's function model predicted substantial regions of hypoxia (Fig. 4B). These represent scenarios in which there is adequate vascular density, but the chaotic nature of the vascular geometry creates hypoxia *(47,53)*.

The presence of highly disordered vascular networks also leads to plasma channels. These are vessels that do not contain appreciable red cells, but do carry plasma. In the skin-fold chamber tumor model, it has been estimated that 8–9% of all microvessels have this characteristic *(54)*. These channels probably develop because of the abnormal branching angles and altered rheology of red cells. Both of these conditions will tend to favor concentration of red cells in vessels with the lowest flow resistance.

4.1.4. Oxygen Demand vs Oxygen Supply

Microregional measurements of oxygen consumption have been performed by fitting the profile of oxygen tension between vessels to a diffusion equation *(52)*. For the R3230 Ac tumor, oxygen consumption rates were observed in the range of what has been measured in murine and human tumor xenografts *(48,55)*, averaging 1.5 mL O_2/100 g/min (range 0.8–2.2 mL/100 g/min). The oxygen consumption rates that have been measured in tumors are in the midrange of what has been reported for normal tissues *(48)*. Thus, one cannot make the claim that tumor hypoxia is the result of oxygen consumption rates that are abnormally elevated.

The Green's function method described above has also been used to compare the relative importance of tissue perfusion, oxygen content, and oxygen consumption in controlling tumor oxygenation (Fig. 4C; *48,56)*. The object of the simulations was to

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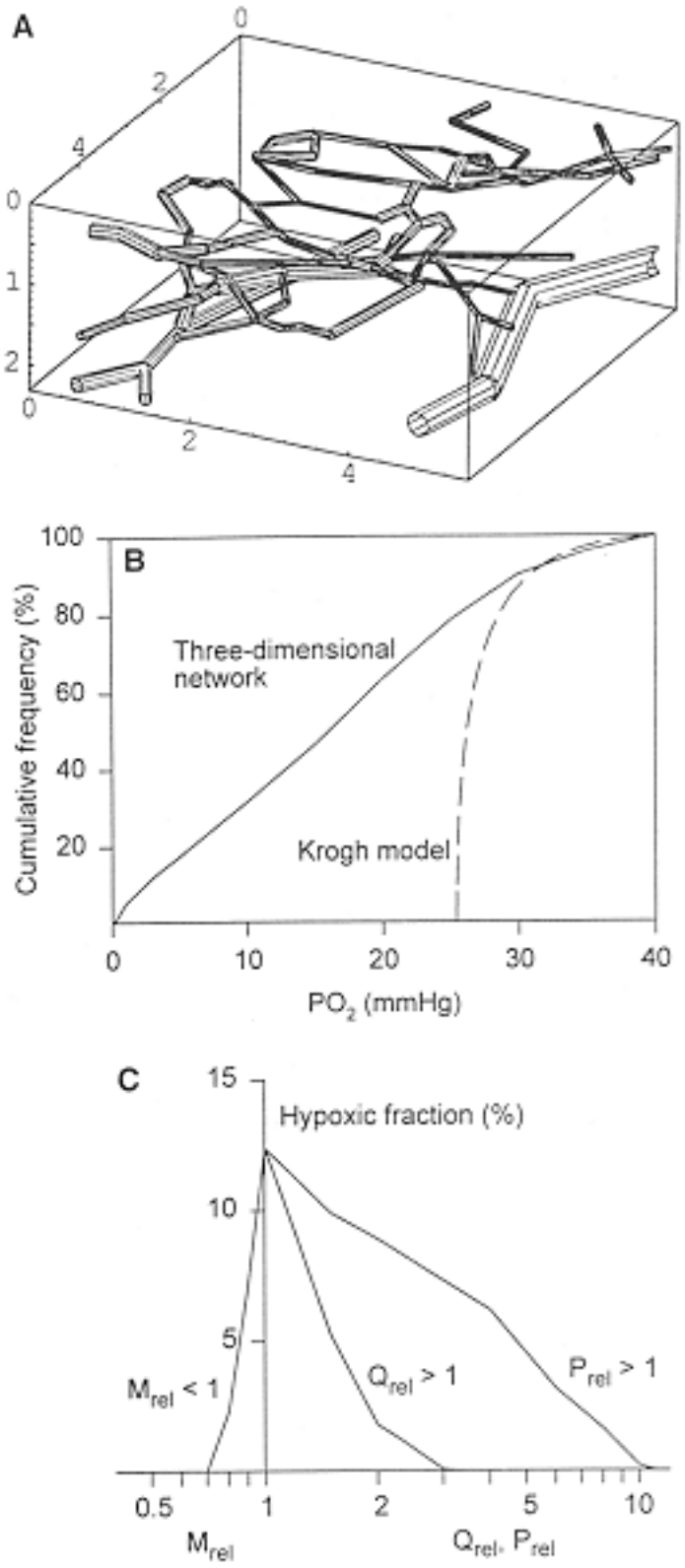
Fig. 4. Three-dimensional vascular architecture and its effects on oxygen transport. **(A)** Three-dimensional reconstruction of a microvascular network in a window-chamber tumor, as determined using confocal microscopy. **(B)** Comparison of the distribution of PO_2 values for actual geometry vs the same vascular density, but with evenly spaced, parallel vessels (Krogh cylinder simulation). The irregular geometry leads to hypoxia (PO_2 values < 10 mmHg), but no hypoxia is predicted for the Krogh cylinder simulation. **(C)** Relative effects of microvascular perfusion, oxygen content, and oxygen consumption rate on hypoxic fraction. This simulation indicates that the most sensitive parameter for reduction of hypoxia is manipulation of oxygen consumption rate. (Reproduced with permission from ref. *48*)


A
0
2
4
0
1
2
0
2
4
B
100
80
60
40
20
Cumulative frequency (%)
Three-dimensional network
Krogh model
0
10
20
30
40
PO2 (mmHg)
C
15
10
5
Hypoxic fraction (%)
Mrel < 1
Qrel > 1
Prel > 1
0.5
1
2
5
10
Mrel
Qrel, Prel

determine how much perfusion, oxygen content, or consumption rate would have to be changed, to eliminate hypoxia in the simulated region. Under two baseline conditions of oxygen consumption, the most efficient method to reduce hypoxia was via reduction in oxygen consumption rate. In one example, the relative efficiency of changing oxygen consumption vs oxygen content of blood was a factor of 30. To be specific, an 11-fold increase in oxygen content would be needed to eliminate hypoxia, compared with a 30% reduction in oxygen consumption rate. Thus, even though oxygen consumption is not greatly elevated, relative to many normal tissues, it still has the most significant impact on tissue oxygenation in tumors.

4.2. Acute Hypoxia

Until recently, the commonly held view has been that acute hypoxia results primarily from vascular stasis, and that this occurs from one of three causes: vascular collapse, leukocyte plugging, or impingement of tumor cells in the vascular lumen. Although these factors may play a role in vascular stasis, they are not the only cause for acute hypoxia. Using window chamber tumors to study the kinetics of tumor microvessel flow, total vascular stasis was observed in approx 5% of vessel segments, and the duration of stasis was <20 s *(57)*. These data are similar in magnitude to prior reports by Chaplin et al. using matched dye methods *(58)*. Based on these observations, it seems that total vascular stasis is not the most common source of transient hypoxia.

Flow instabilities are a rather common phenomenon, and there is strong evidence that instabilities in tumor perfusion, short of vascular stasis, can lead to transient hypoxia *(60)*. Trotter et al. performed a series of studies in murine tumors using, pairs of intravenously administered fluorescent dyes to monitor functional vessels *(59)*. They noted differences in staining intensity around groups of vessels, and theorized that this may be caused by fluctuations in blood flow rate within small networks of vessels. They also suggested that such behavior could lead to transient hypoxia, without a requirement of total vascular stasis. Temporal variations in perfusion have been seen in both experimental and human tumors, using laser Doppler flowmetry *(60,61)*. Similarly, the temperature of murine tumors has been shown to be temporally unstable, which must be related to variations in perfusion rate *(62)*. These data suggest that variations in blood flow rate occur in tumors, but until recently there was no direct proof that such variations in flow could lead to transient hypoxia.

This issue was also addressed by simultaneously monitoring microvessel red cell flux and PO_2 for periods up to 1 h, using window chamber tumors by Kimura et al. *(57)*. In most cases, the red cell flux in groups of microvessels was unstable, and the variations in red cell flux could be as much as several orders of magnitude, but more commonly is in the range of a factor of two. The Green's function method was used to simulate the effects of a twofold change in red cell flux on tissue PO_2 distribution. The simulation showed that as much as 25% of the tumor region could fluctuate above and below a hypoxic threshold, under these conditions. Considering the fact that the observed fluctuations were of at least this order of magnitude in nearly all vessels studied, the investigators concluded that transient hypoxia must be a common phenomenon, and that it is most frequently brought on by fluctuation in blood flow, rather than by total vascular stasis.

More recently, temporal instabilities of PO_2 and perfusion in hind limb tumors have been investigated *(63)*. To accomplish this task, 10-μm diameter recessed-tip electrodes and laser Doppler probes were introduced into tumors, and monitored continuously for

Table 6
Temporal Observations of Intermittent Hypoxia in R3230 Ac Flank Tumors *(63)*

Parameter	*PO_2 < 5 mmHg*
Fraction of experiments never hypoxic	5/13
Fraction of experiments always hypoxic	3/13
Median number of hypoxic events/h	3.9 (0.9–17.9)[a]
Median % time hypoxic	60.1 (16.2–84.7)[a]
Median duration of hypoxic episodes (min)	7.4 (0.9–44.7)[a]

[a]Numbers in parentheses indicate range of data.

periods ranging from 60 to 120 min. The observed fluctuations in PO_2 were similar in kinetics to those previously published for laser Doppler flowmetry by Chaplin and Hill *(60,61)*, and were consistent with Kimura and Braun *(57,63)* in the window chamber model (Table 6). In these studies, some measurements never dropped below a threshold value of 10 mmHg, and others remained below this threshold for periods up to 90 min of observation. The remaining experiments, however, demonstrated temporal variations above and below this threshold.

It has been reported that hypoxia might create negative selection pressure on cells with wild-type p53 suppressor gene, since activation of p53 leads to apoptosis *(64)*. Thus, cells with mutant forms of p53 would gain a survival advantage over cells with wild-type p53. The instabilities in tumor oxygenation perhaps suggest a more sinister effect of hypoxia *(57,63)*. If the process of oxygen transport is as unstable as these results would indicate, then it is possible that, once a tumor becomes vascularized, it is subjected to repeated cycles of ischemia-reperfusion injury. Since the primary bioactive product of this process is superoxide anion *(65)*, it is possible that mutagenic events leading to tumor progression could result. Recent evidence for the mutagenic effects of hypoxia reoxygenation injury comes from the work of Reynolds et al. *(66)*. In these studies, a phage shuttle vector reporter system was transfected into the tumorigenic cell line, LN12. In tissue culture under normoxic conditions the incidence of mutations averaged $<2 \times 10^{-5}$, even after more than 20 passages. However, the incidence increased by a factor of four after one episode of hypoxia/reoxygenation, and doubled again after another episode. The incidence was much higher when the tumor was grown in vivo, compared with in vitro culture, as well.

The causes of acute hypoxia are not well defined at this time. Certainly, all of the factors that contribute to chronic hypoxia will also contribute to acute hypoxia, since they create the baseline conditions that exist without blood flow fluctuations. Factors that may contribute to flow fluctuations include arteriolar vasomotion *(54)*, rapid vascular remodeling *(67)*, and other hemodynamic effects *(68,69)*.

It is clear from the above discussion that tumor hypoxia is a complex and dynamic process that is the result of several factors. Chronic hypoxia probably develops as a result of exaggerated longitudinal gradients, leading to intravascular hypoxia; irregular vascular geometry and/or long intervascular distances; irregular vascular branching patterns; and rheologic effects, leading to plasma channels and oxygen consumption rates that are out of balance with oxygen delivery. These features, combined with the dynamic nature of tumor blood flow, lead to an overall pattern of hypoxia that is both chronic and acute (Fig. 5).

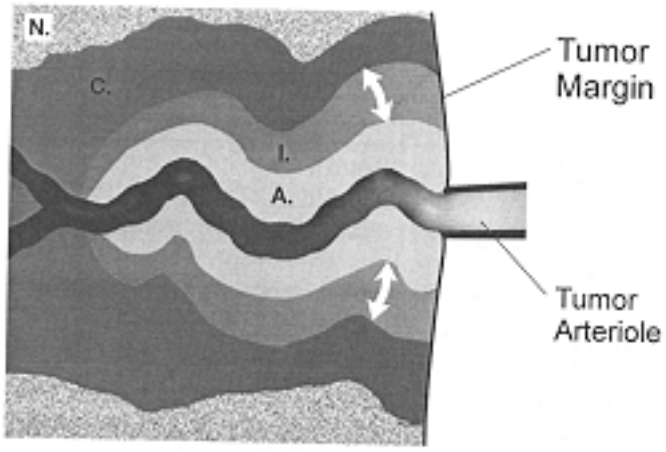


Fig. 5. Composite working model for interrelationships between chronic and acute hypoxia. Arrows indicate diffusion distance of oxygen that is influenced by instabilities in red cell flux. At the extremes, there are regions of vascular hypoxia where such fluctuations are of little consequence, since tissues immediately surrounding the vasculature would be hypoxic (C = chronically hypoxic). Conversely, there are regions near well-oxygenated vessels that are unaffected by changes in red cell flux (A = aerobic). It is likely, however, that large regions of tumor will lie between these extremes, and will be subjected to periodic fluctuations in PO_2 that lead to hypoxia reoxygenation injury. I = intermittently hypoxic; N indicates regions of necrosis.

It is of interest to note that many of the genes involved in regulation of angiogenesis are regulated by hypoxia (Table 4). However, there is very little information on whether such genes are also regulated by hypoxia reoxygenation injury, particularly with the kinetics shown in this chapter. Clearly this is an important area for future investigation.

5. NITRIC OXIDE

There is increasing evidence that tumors produce varying levels of NO *(70)*. A number of studies have been published that have investigated the effects of NO on angiogenesis. Consistently the story is emerging that NO definitely has an effect, but whether it up- or downregulates the process is controversial. It is important to note that the hyperpermeability of vascular endothelium that is stimulated by VEGF occurs via stimulation of NO synthesis *(71)*. Evidence for a proangiogenic effect of NO comes from the following observations: Exposure of glioblastoma and hepatocellular carcinoma cell lines to SNAP and NOR3 (both NO donor compounds) increased VEGF production, primarily by stabilizing mRNA levels *(72)*; use of NO donors leads to increased angiogenesis in the cornea pocket assay, when angiogenesis is stimulated by substance P *(73)*; use of NO donors stimulates proliferation of coronary postcapillary endothelial cells, in vitro *(74)*; and the human breast tumor line, DLD1, which was transfected with the NO synthase gene, grew more quickly and had better-vascularized tumors than the parent line *(75)*. There are also data that indicate that NO downregulates angiogenesis. Examples of such evidence include: VEGF production by arterial smooth muscle cells is downregulated by NO (in this case, the inhibition occurs by inhibition of AP1 binding to the VEGF promoter *[76]*); production of VEGF and its receptors are downregulated when NO is added to the perfusate of ex vivo perfused lungs, and angiogenesis is inhibited in the chick

corioallantoic membrane when exposed to exogenous NO, and is upregulated when NO synthase inhibitors are used *(77,78)*; primary tumor growth and metastasis frequency are lowered in the Lewis lung tumor model when animals are administered NO donor drugs *(79)*; proliferation and migration of endothelial cells is inhibited in vitro in the presence of NO donor drugs *(80,81)*.

The data that are available thus far suggest that NO has both positive and negative effects on angiogenesis. However, two sets of data in tumor models suggest that it plays a positive role in stimulating angiogenesis *(75,79)*. Clearly, the issue is very complicated, and additional work is needed to further elucidate the role that it plays in tumor angiogenesis.

6. SUMMARY

There is no question that there is a complex interrelationship between tumor hypoxia and tumor angiogenesis. A question that remains unanswered is whether the hypoxia in tumors is responsible for abnormal angiogenesis, or whether the abnormal angiogenesis is responsible for hypoxia. In all likelihood, dysfunction in both processes feed on each other to continue the process of tumor growth. At issue, then, is whether there is any benefit to be gained from trying to modulate tumor hypoxia as part of antiangiogenesis therapy. Presumably, if one were to improve tumor oxygenation by some means, there might be a reduction in the angiogenic stimulus, but, under such conditions, additional tumor cell growth might be favored. Alternatively, pharmacologic manipulation designed to increase tumor hypoxia may stimulate more angiogenesis, thus stimulating additional tumor growth. If either of these types of manipulations were combined with antiangiogenesis therapy, it is difficult to predict what the net result would be on tumor growth and metastasis. Alternatively, it is not clear what the physiologic consequences of antiangiogenesis therapy are on tumor oxygenation. Will the use of antiangiogenic agents exacerbate hypoxia, leading to altered cytokine expression, additional intermittent hypoxia, and tumor progression? Clearly additional studies are needed to begin to unravel these complex processes, since the answers may have important implications in how to implement these exciting new forms of therapy.

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2 Oxygenation of Solid Tumors in Animals and Patients

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CONTENTS

1. INTRODUCTION

The biological effectiveness of low energy transfer (LET) ionizing radiation is in part related to the amount of oxygen present at the time of the energy deposit. This dose modifying role of oxygen (oxygen enhancement ratio) is principally caused by the indirect effect of radiation on DNA *(1,2)*. Hypoxic cells are present in rodent and xenografted human tumors, and it has been known for a long time that the absence of oxygen in tumors is a factor of resistance against ionizing radiation *(3–5)*. More recently, it has been shown that the decrease in tumor oxygen tension could also be a factor of resistance for treatment with some cytotoxic drugs *(6–8)*, not only directly through the low-oxygen partial pressures, but indirectly through modifications in some gene expression by O_2 and other environmental factors (vascularization, pH, metabolism, angiogenic factors, and so on) *(9,10)*. In patients, tumors are known to contain hypoxic areas *(11–14)*, and the local control of human solid tumors could be improved if a clinically relevant test was able to identify tumors that would benefit from radiosensitization *(5)*. Oxygen availability is dependent on oxygen supply, which depends on many parameters: microvasculature, blood flow, tissue temperature, and pH *(15,16)*. Tissue oxygenation will result directly from O_2 availability, and from the respiration rate of the cells. For normal tissues, changes in oxygenation reflect variations in blood flow, and partial oxygen pressure (pO_2) distribution has been evaluated as a function of hemoglobin concentration, temperature, pH,

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and so on *(15)*. In tumors, tissue vascularization is qualitatively poor with shunts, vessel collapses, and high interstitial pressure. All these parameters may represent a potential therapeutic target *(16–18)*.

This chapter describes the methods used for the evaluation of tumor oxygenation, and the results obtained in animals and patients.

2. OXYGEN AVAILABILITY AND CONSUMPTION

In mammals, most of the energy for cellular life comes from oxidation/reduction reactions with ATP synthesis. This requires one oxidant (O_2) and different redox molecules, which are absorbed from ingested nutriments, and stocked as fat or in the liver *(19)*. Oxygen must be present according to the tissue requirement for energy, and any variation in its concentration will lead to modifications in organ metabolism *(15,19)*. Oxygen needs to be activated to react with substances, and is in general, bound to a metal (Fe, Mn, Cu).

Large variations in vessel densities have been demonstrated during tumor growth *(16)*, and hypoxia is present not only in large tumors with necrosis, but also in small tumors *(19)*. This is related in part to the growth site, tumor cell properties (respiration rates and cell density), and to variations in vascularization patterns *(20,21)*. Tissue oxygenation is the result of the O_2 blood supply, diffusion in the extravascular space, and respiration rate of the cells. The availability of oxygen represents the amount of oxygen carried by the blood per unit of time to a given tissue *(16)*. It is the product of the blood flow rate (perfusion rate) and the arterial O_2 concentration. It is considered that the arterial O_2 concentration is comparable for most tissues (tumor or normal tissues) *(19)*. The oxygen supply for a defined tumor at a precise time and in a specific point will depend on the tumor blood flow at that precise time through that region. However, one of the most important difference between tumor and normal tissues oxygenation could be the result of differences between oxyhemoglobin saturation, which could be significantly lower in tumors than in normal host tissues, with lower values related to an increase in tumor volume. It must be emphasized that the mechanisms of oxyhemoglobin saturation are different from animals to humans *(18)*. The diffusion flux is proportional to the difference in O_2 partial pressures between vessels and tissues, diffusion coefficient, and exchange area, and inversely proportional to the diffusion distance. The diffusion coefficient has been measured for tumor tissue, and corresponds to values of normal tissues with similar water content *(19)*.

It is considered that tumors have an impaired respiratory rate and an increased rate of aerobic glycolysis. Tumor oxygen consumption rate is close to the one observed in normal tissues with relatively low metabolism. The most important parameter is that the oxygen extraction by tumor cells is not significantly modified, compared to normal cells *(19)*. Finally, tumors are able to adapt their consumption rates as a function of their blood supply.

3. TECHNIQUES FOR OXYGEN MEASUREMENT

Methods for measuring oxygen present in tissues can be divided into invasive and noninvasive techniques *(13)*. The ideal method for measurement needs to be accurate and reproducible, and needs to have a good spatial resolution, a sensitivity in the clinically relevant range, and to be user friendly and safe. For clinical usefulness, a noninvasive

method should be preferred. However, most of the available techniques give only an indirect estimation of oxygen pressure in the tissue assessed.

3.1. Polarographic Electrodes

The first results of oxygen measurement in tissue with inserted electrodes was published in 1946 *(22)*. Later, the technique was improved by implanting an anode and a cathode in a glass needle permeable to gas, but electrically insulated *(23)*. For the polarographic technique, an electrical current is generated between a cathode (probe) and an anode. The variations in oxygen concentration in the tissue will induce oxidation of the electrode, leading to modifications in the current that are proportional to pO_2. The electrode must have a minimal oxygen consumption, should avoid tissue compression, have a fast response, be easily calibrated with minimal drift, and be specific for oxygen. The best metals to make an electrode are gold and platinum. In patients, the electrodes must be sealed in unbreakable needle microelectrodes. The exact volume of oxygen detection in tissues by modern probes is still debated, but certainly corresponds to the microenvironment of the tumor cells *(23)*.

3.2. Histology and Hypoxia Markers

Historically, Thomlinson and Gray *(3)* were the first to suggest that hypoxia could exist in human tumors. This analysis was based on the pathological examination of vascular density and tissue necrosis in bronchus carcinoma. The most important disadvantage of any technique that correlates tissue oxygenation and vascular density is that the measurements are indirectly related *(9,13,19)*. With cryospectrophotometry techniques, the oxygen present in hemoglobin in microvessels is assessed on frozen tissue sections *(13)*. Bioluminescent imaging of metabolites within tumor cells can be performed on microscopic frozen sections of biopsy and can show the distribution of metabolites in tumors, giving only indirect information about the oxygenation status.

The principle of hypoxia markers is based on the fact that the binding of hypoxic cell radiosensitizers, for example, 2-nitroimidazole, to tumor cells is dependent on oxygen concentration *(24,25)*. These techniques are limited because of the uncertainty in the biodistribution of the marker caused by variations in tumor blood flow, and by the exact correlation between the intensity of the binding and the partial oxygen pressure in the tissue *(24)*. This means that the measure will serve more as an individual monitoring of oxygen distribution in time, than as a comparison between different tumors. The binding can be detected by invasive (biopsy, immunochemical techniques) or noninvasive techniques (PET, MRI). Hypoxia is detected at a cellular level with the invasive techniques (biopsy or needle), and can be associated with the evaluation of other parameters (proliferation) *(25)*. For noninvasive techniques, the microenvironment is by definition not modified by the sampling, but the recording corresponds more to a tissular than cellular evaluation. Tritiated misonidazole has been the most commonly used marker in animal models. For SPECT and PET analysis, ^{123}I and ^{18}F-2-nitroimidazoles have been used *(26)*.

3.3. Oxyhemoglobin Saturation and Fluorescence

Oxygen is able to diffuse rapidly, and will react with many molecules. It can be detected by spectroscopic techniques because of its capacity for modifying the relaxation times of excited species. Spectroscopy (cryospectroscopy and near-infrared spectroscopy) is based on the spectral differences between oxygenated and deoxygenated hemo-

globin. The oxyhemoglobin saturation (HbO_2) is related to the tissue respiration and is an indirect reflect of tissue oxygenation. The mean HbO_2 saturation values observed in the tumors are in general lower than those found in normal tissue. The HbO_2 frequency distribution decreases from normal tissues to tumors (80–49 sat.%), and is sometimes correlated with vascular density. Near-infrared spectroscopy could be interesting for superficial tumors; it has been used in normal muscles *(27)*. Tissue oxygenation can be indirectly evaluated with oxygen sensors, based on the recording of oxygen quenching by a fiberoptic fluorosensor *(28)*.

3.4. Comet Assay and DNA Strand Breaks

It is in general considered that the formation of DNA strand breaks is dependent on oxygen. The comet assay is based on images of damaged DNA moving from the nucleus into a tail when electrophoresis is applied. This tail is caused by the difference in migration between small fragments of damaged DNA and intact DNA. A recording is obtained by a camera with an image-analysis system. The fraction of hypoxic cells is estimated, assuming that DNA damage differs by a factor of 3 between aerobic and hypoxic cells. The method could compare hypoxia and other biological parameters, and requires doses of irradiation >3 Gy, and a rapid handling of the sample *(29)*.

3.5. Magnetic Resonance

The effect of oxygen on relaxation time is a function of the diffusion coefficient and mostly of its concentration. For magnetic resonance, the measurement will depend on the field strength of the magnet and the probe relaxation time. This is a noninvasive procedure that can give an indirect assessment of tissue oxygenation through the evaluation of tumor metabolism *(30,31)*. The most widely used techniques are ^{31}P-MRS and ^{1}H-MRS (lactate). The latter techniques is promising because lactate production is markedly increased in tumor cells, compared to normal tissues *(31)*.

3.6. Comparison Between Techniques

In a National Institutes of Health consensus workshop held in November 1992, the various advantages and disadvantages of techniques previously presented were reviewed. For measuring tissue oxygenation, it was concluded that a comparison of techniques would be advisable, with oxygen electrodes serving as the gold standard *(32)*, because those techniques were the only ones able to predict treatment outcome, based on tumor oxygenation assessment. The different techniques used for measuring oxygen tension in tumors might give different information than information on biologically significant tumor hypoxia.

4. MEASUREMENTS OF OXYGEN TENSION IN TISSUES

4.1. Background and Technique

To assess the oxygenation status of human tumors, needle probes were first used in the 1950s, and changes in oxygen tension were monitored in patients breathing air or oxygen *(33–39)*. Many data have been published on the subject, some of them demonstrating a clear correlation between pO_2 and treatment outcome *(34,37–39)*. However, most of these results have been criticized, because of the lack of reproducibility with the various systems used, and because of some technical limitations *(37,38)*. Improvements were

made in the late 1980s with new equipment, the KIMOC-6650 histograph (Eppendorf, Hamburg, Germany). Most recently published results on direct oxygen tension measurements in humans have been obtained with this computerized polarographic system. This subheading will focus on the results obtained with that equipment, which can now be considered as giving base line information on normal tissues and tumors oxygenation.

This histograph uses fast responding electrodes programmed to minimize the effects of tissue compression and massive oxygen consumption by the electrode. The technical data on this equipment have been published *(40–45)*. Briefly, a gold microcathode (12 μm in diameter) is placed within an unbreakable stainless steel needle probe (300 μm in diameter). The cathode is polarized against an Ag/AgCl anode placed on the skin of the patient. The resulting current is proportional to the partial oxygen pressure in the tissue. All electrical connections to the patient are insulated at a level of 8 kV. Values are corrected for the barometric pressure and temperature of the tissues (recorded with a thermocouple). The electrodes are cleaned, dried, and sterilized. For most patients, one track is recorded in normal tissues surrounding tumors, followed by independent tracks in the tumor. The probe is automatically moved through the tissues as defined at the beginning of each experiment, with each forward movement followed by a backward step, and, at the end of the measurement, the needle probe is automatically removed. The results appear as a histogram, together with the mean, median, and 10 and 90 percentiles of recorded values along tracks. Tissue architecture is not significantly modified by oxygen-tension measurement, as assessed by histology *(40)*. However, it has been shown that some systematic overestimation of partial oxygen pressure was found with this equipment (which could be caused by bleeding along the probe track) when compared to measurements made with microelectrodes.

4.2. Distribution in Normal Tissues

Distributions of pO_2 values in various normal human tissues have been described in detail *(49–53)*. The normal tissues were subcutaneous tissues surrounding tumors for ear, nose, and throat (ENT), sarcoma, and melanoma patients, normal brain for gliomas, and vaginal mucosa for cervix tumors *(49–55)*. As expected, there is a scattering of the individual pO_2 values in normal tissues between 1 mmHg and values typical for arterial blood (80–100 mmHg). Whatever the normal tissue, median pO_2 ranged from 15 to 70 mmHg. For example, in patients with head and neck tumors, median pO_2 was 52 mmHg in the Institut Gustave-Roussy (IGR) experience (mean 54 mmHg) *(52)* and mean pO_2 was 57 mmHg at Stanford *(55)*.

The results obtained with the KIMOC 6650 have demonstrated that normal tissues are in general better oxygenated than solid tumors. This finding probably reflects the better vascularization of normal tissues than tumors, and the higher interstitial pressure present in tumors. In most studies, more than 90% of the values recorded in normal tissues were above 10 mmHg. At this oxygen tension, the relative radiosensitivity is close to a maximum, and only a small increase in sensitivity can be expected with an increase in oxygen delivery.

5. OXYGENATION OF SOLID TUMORS IN ANIMALS

Many oxygen partial pressure distributions for murine and xenografted tumors have been published *(41–45)*. Most of the tumors assessed reveal hypoxic areas, which are heterogeneously distributed within the tumor. The effect of different factors on tumor

oxygenation has been assessed. In one study, tumor type, site of implantation, and anesthesia were studied *(20,21)*. Oxygenation of two sarcomas (Sa-NH and Fsa-II), one mammary carcinoma (Mca-4), and one ovarian carcinoma (Oca-1) was compared to normal tissue (muscle). Large variations were found in hypoxic fraction (%<2.5 mmHg) and median pO_2 between the different tumors. Three of the four tumors growing subcutaneously were less well oxygenated than their intramuscular counterparts (significantly, for Mca-4). Furthermore, anesthesia reduced pO_2, leading to the conclusion that the need for standardization was very important, to have pO_2 measurements as a real predictive assay.

Experiments in animals have demonstrated that pO_2 could be measured without any adverse effects on tumor growth or pulmonary metastasis rate in two human xenografted tumors (HRT18, $Na11^+$) and one rodent tumor (Lewis lung carcinoma 3LL) *(41)*. Mean pO_2 was 6.8 mmHg in 3LL, 11.4 in HRT18, and 9.4 in $Na11^+$. In normal tissues, mean pO_2 was 35.2 mmHg in kidney and 45 mmHg in subcutaneous tissues. Such results have been found in most of the animal studies *(42–44)*, with, for example, median pO_2 of 4 mmHg in C3H tumors *(43)*. Different ways of increasing tumor oxygenation have been tested in experimental models (oxygen, carbogen, nicotinamide, hyperthermia, and so on) *(45–47)*, some of them combined with the recording of pO_2 variations. Thus, it has been possible to demonstrate a clear relationship between pO_2 and radiosensitivity in xenografted cell lines after carbogen and nicotinamide administration *(47)*. However, it has been impossible to demonstrate a clear correlation between pO_2 and the percentage of radiobiologically hypoxic cells across different cell lines *(45)*, even if this was possible throughout a single cell line. Furthermore, comparisons of interlaboratory variations in oxygen tension measurements by Eppendorf Histograph have been performed. Six laboratories have measured hypoxia in a *Fsa*II tumor, and 77% of values were below 10 mmHg. Some variations were found in the measured median tumor pO_2, from 1.5 to 5.6 mmHg, as well as in the hypoxic fraction. Some caution was advocated by the authors, when comparing absolute pO_2 values by such technique, even if the measurements were reproducible for a given laboratory *(48)*.

6. OXYGENATION OF SOLID TUMORS IN PATIENTS

6.1. Distribution in Tumors

6.1.1. Head and Neck Tumors

Historically, neck nodes were the first tumors to be investigated with the KIMOC 6650. In 1990, Fleckenstein published results in seven patients *(53)*. Most tumors were hypoxic, with recorded pO_2 values below 10 mmHg. In 1993, results were published on patients treated at the IGR *(51,52)*. Oxygen tension was measured in tumors of 35 patients (mean age 56 yr, range 36–79 yr) with a metastatic neck node from a primary squamous carcinoma of the head and neck (ENT). The site of the primary disease was the oropharynx in 31 of 35 patients. Oxygen tension was measured on conscious patients, with the approval of the local ethical committee, and after obtaining informed consent. None of the patients had previously been treated for their malignancies. The mean dimension of the tumors in which pO_2 was measured was 5.3 cm (range 2.0–11.0). All pO_2 measurements were performed immediately before treatment (surgery or radiotherapy). Median pO_2 was 10 mmHg, with mean pO_2 of 21 mmHg, 10 percentile <1 mmHg,

and 90 percentile of 61 mmHg. Low pO_2 values (values <10 mmHg) were found in 83% of the tumors. The tumor-to-tumor proportion of very low values (<2 mmHg) varied from 2.5 to 100%. These results are comparable to those obtained at Stanford University in 16 patients (mean pO_2: 22.7 mmHg) *(55)*, and at Aarhus University in 35 tumors with a median pO_2 between 10 and 14 mmHg *(54)*.

Tumor pO_2 values generally varied along the electrode tracks, but, in general, there appeared to be no systematic variation in pO_2 as a function of the electrode position in the tumor (i.e., no decrease in pO_2 from the superficial to the deep part of the tumor). The pO_2 distribution did not differ according to the age of the patient or the histological grading of the tumor. The distribution of oxygen tension varied greatly from one tumor to another, indicating differences in tumor oxygenation between patients with the same histological tumors (intertumor variability). The pO_2 also varied along the electrode tracks within a given tumor (intratumor variability); this intratumor heterogeneity makes it necessary to record the greatest number of individual values per tumor, with at least two tracks (same or different entrance points), to obtain a geographical representation of the pO_2 distribution.

6.1.2. Breast Carcinoma

In the Mainz experience, oxygen tensions measured in the normal breast of 16 patients revealed a mean (median) pO_2 value of 65 mmHg; in 18 cancers of the breast (stages pT 1–4), the median pO_2 was 28 mmHg. Six of 18 breast cancers exhibited pO_2 values between 0 and 2.5 mmHg. Thirty-three percent of the tumors investigated contained hypoxic areas, and the proportion of pO_2 readings between 0 and 2.5 mmHg ranged from 4% (in a T4 breast cancer) to 64% (in a T3 tumor). Furthermore, the oxygenation patterns did not correlate with the histological grades, menopausal status, tumor histology (ductal vs lobular), or extent of necrosis or fibrosis *(49,56)*.

6.1.3. Cervical Carcinoma

In 37 cancers of the cervix (stages FIGO I–IV), the median pO_2 was 12 mmHg. The frequency of pO_2 readings between 0 and 2.5 mmHg ranged from 1 to 82%. At IGR, median pO_2 measured in the lateral vaginal wall (normal tissues) was 49 mmHg, and 21 mmHg in six tumors *(57,58)*. The heterogeneity of cervical cancer oxygenation has been studied. The optimal sampling is obtained with five independent tracks, with 20–30 measurements per track. No significant difference in pO_2 was found as a function of the location of the tracks (circumference or depth along the tracks) *(59)*.

6.1.4. Melanoma

In 20 patients, oxygen tension was measured the day before the surgical removal of a suspected metastatic lesion from a primarily treated melanoma *(52)*. An histological confirmation of the malignant origin of the removed lesion was obtained in 18 cases. In 2 cases, the removed nodes were histologically noninvaded by the known melanoma. The median pO_2 for normal tissues was 40.5 mmHg. For tumors, median pO_2 was 11.6 mmHg, 17.1 mmHg in nodes, and 6.7 mmHg in skin metastases. Very low values (<2 mmHg) represented 20% of the recorded values in nodes and 15% in skin metastases. Median pO_2 was 10.4 mmHg in nodes larger than 3 cm (6 patients), and 53.3 mmHg in nodes smaller than 3 cm (6 patients). For nonmetastatic nodes, median pO_2 was 20.9 and 25.1 mmHg, without any value below 10 mmHg. A decrease in pO_2 values, probably corresponding

to tumor hypoxia, was found in most of the largest metastatic tumors, compared to normal tissues.

6.1.5. Brain Tumors

Mean and median pO_2 recorded in brain tumors have been lower than values recorded in normal brain *(60)*. For 10 glioblastomas, pooled median pO_2 was 7.4 mmHg, and 28% of the values were below 2.5 mmHg. Hypoxic regions were found in anaplastic (WHO G3) astrocytomas, with up to 41% values below 2.5 mmHg in one tumor. There was no correlation between tumor size and either median pO_2 or hypoxic fraction in the glioblastomas.

6.1.6. Sarcomas

Oxygen tension has been evaluated in patients with nonpreviously treated sarcomas *(61)*. The average hypoxic fraction defined by the authors (<5 mmHg) was 29%. Arterial pO_2 was related to individual mean and median pO_2 but not to the hypoxic fraction. Median pO_2 values were lower in malignancies than in the surrounding normal tissues, and there was an accumulation of pO_2 values in the lower pO_2 classes, indicating tissue hypoxia in tumors.

6.2. Factors of pO_2 Variation

6.2.1. Tumor Size

In head and neck tumors, a trend to have a decrease in the median pO_2 (increase in the frequency of very low values <2 mmHg) with an increase in tumor size (N2 vs N3, $p = 0.012$) has been found in the IGR experience *(51)*. This was confirmed in the Stanford data *(55)*, with a mean pO_2 of 44 mmHg for tumors <4 cm in diameter and 18 mmHg for tumors >4 cm in diameter. The same trend was found in sarcomas *(61)*.

In contrast, no statistically significant differences were found between small and large breast tumors (median pO_2 in T1–2 tumors: 26 mmHg ; median pO_2 in T3–4 tumors: 30 mmHg). This could imply that the oxygenation pattern in breast cancers and the occurrence of hypoxia and/or anoxia do not correlate with the pathological stage. This is in agreement with blood flow data of breast cancers, because there was no association between tumor size, as determined by TNM staging, and blood flow.

6.2.2. Hemoglobin Concentration

In 16 patients with head and neck tumors, no correlation was found between hematocrit level and tumor oxygenation *(55)*. In breast carcinoma, no association was observed between median tissue pO_2 values and hemoglobin concentration *(49)*. Evaluation of the oxygenation status of 65 cervix cancers revealed a significant inverse correlation between the fraction of low tissue pO_2 values (0–2.5 mmHg) and hemoglobin concentration, and the median pO_2 values tended to rise with increasing hemoglobin concentrations ($p = 0.05$) *(57,58)*.

6.2.3. Tumor Treatment

The variations in tumor oxygenation during a very accelerated radiotherapy for advanced head and neck carcinoma have been studied in 14 patients at IGR. All the patients (12 men and 2 women) had T3–T4, N0–N2c, M0 tumors of the oropharynx and/or oral cavity, and were previous smokers. The accelerated radiotherapy delivered 70 gy in 3.5 wk on primary tumor and clinically involved nodes *(62)*. A median number of

70 pO_2 values per tumor (range 51–100) was recorded. One set of pO_2 measurements was performed before the start of the radiotherapy, and a second one at the end of the second week (dose delivered: 34.3 gy). For normal tissues outside the radiotherapy field, oxygen distribution was not significantly increased during treatment, with pooled median pO_2 of 38 mmHg before treatment, and 46 mmHg after 2 wk of treatment. The pooled 10 percentiles were, respectively, 11 and 15 mmHg before and during treatment. For tumors, pooled median pO_2 was 13 mmHg before treatment, and 33 mmHg after 2 wk of treatment. This difference was significant ($p = 0.05$). Very low values (<2 mmHg) represented 20% of the recorded values before treatment and 10% after 2 wk, and low pO_2 values (<10 mmHg) accounted for, respectively, 45% and 25% of the recorded values. An increase in median pO_2 was shown in nine tumors on 14, a decrease in 4, and no changes in one. For the nine tumors with values recorded below 2 mmHg, a decrease in the percentage of very low values (increased oxygenation) was found in seven cases after 2 wk of treatment.

6.2.4. Carbogen Breathing

One efficient way to decrease the percentage of low pO_2 values in tumors is to breathe carbogen, or pure oxygen, throughout every radiotherapy fraction *(63–65)*. Oxygen tension has been recorded in tumors before, and at the time of, carbogen breathing *(66,67)*. In IGR studies, carbogen was started 5 min before the beginning of each radiotherapy fraction, and was stopped immediately at the end (1 atm, flow rate 15 L/mn). It was breathed via a rubber mouthpiece connected to a plastic two-way valve; this system was connected to a 3-L. breathing bag. A nose-clip prevented room-air breathing *(66)*. Pooled median pO_2 was 20 mmHg before carbogen (20 patients), and 61 mmHg at the time of carbogen breathing (13 patients). A decrease in the percentage of very low values (below 2 mmHg) was obtained in 9 of 13 patients. Comparable results were found by another group working with a different methodology *(67)*.

These data could be useful for monitoring new protocols using carbogen breathing during radiotherapy (i.e., ARCON protocol), which has not been possible in the past. Based on these results, it could be worthwhile to select patients who should benefit from carbogen breathing, on the basis of preirradiation pO_2 measurements. This finding, observed for both air- and carbogen-breathing patients, means that the impact of carbogen breathing on tumor response to treatment could be less than predicted by preirradiation pO_2 investigations.

6.3. Predictive Value of pO_2 Measurements

Techniques using pO_2 electrodes do not allow for estimate of proportion of clonogenic hypoxic cells and for discrimination between chronic and intermittent hypoxia. In 1968 and 1988, two studies presented results on the correlation between pO_2 and radiotherapy outcome *(34,38)*. Some comments on these data have already been made. Two recently published studies in uterine cervix and head and neck carcinomas have confirmed the predictive value of pO_2 measurements with convincing data, demonstrating that pretreatment pO_2 measurements by needle probe were of predictive value for the tumor response to radiotherapy *(54,57,58)*.

In locally advanced cervical cancers (56 patients), it has been shown that tumor oxygenation was a significant and independent parameter for prediction of patient survival and local tumor control. In a Cox regression multivariate analysis, the fraction of pO_2

values between 0 and 5 mmHg (hypoxic fraction) was the most powerful single predictor of overall survival ($p = 0.0017$) and recurrence-free survival ($p = 0.014$) *(57,58)*.

In locally advanced head and neck tumors (35 patients), Nordsmark *(54)* has demonstrated that pO_2 was a significant and independent parameter for prediction of local tumor control after exclusive radiotherapy. Actuarial tumor control probability at 2 yr was 70% for well-oxygenated tumors, vs 36% for poorly oxygenated ones ($p = 0.01$). The parameter used was the fraction of pO_2 values <2.5 mmHg.

Even if tumor hypoxia is a highly relevant parameter for treatment outcome, the best parameter to demonstrate predictive tumor hypoxia is still not clearly defined. Two distinct parameters have been described: the hypoxic fraction based on median pO_2, and the percentage of oxygen tension values below 2.5 mmHg. Determination of the oxygenation status may thus enable pretherapeutic selection of hypoxic cervical cancers (and probably other solid tumors as well) as candidates for modified treatment approaches. However, the results obtained do not definitely answer the question of whether the poor prognosis of poorly oxygenated cancers is related to the limitation in the treatment (oxygen-enhancement ratio) or represents the malignant potential of the tumor itself (gene modulation). Whatever the reason, since the majority of the patients have been treated with radiation, the results demonstrate that hypoxic carcinomas are less sensitive than well-oxygenated ones.

7. CONCLUSION

Finally, it can be said that most of the solid tumors in man contain areas of hypoxia. The measurement of tumor pO_2 can be considered as a predictive indicator of treatment response in cervix and head and neck tumors; oxygen tension can be modulated by carbogen breathing, and can vary among radiotherapies. Taking this into account, the remaining question is to determine the best time to sensitize tumors, i.e., at the start or during a fractionated radiotherapy *(68)*. One of the more logical ways could be to have the maximum increase in tumor oxygenation at the start when the maximum hypoxia is present, with one of the following methods: erythropoietin injections, perfluorochemical emulsions, hypoxic cell sensitization, and/or carbogen breathing *(69,70)*. Furthermore, this approach could be combined with the use of bioreductive drugs *(71–73)*, or hyperthermia *(74)*, over the course of the treatment, in order to be active on the nonsensitized clonogenic hypoxic cells remaining in the tumor. The clinical impact of such oxygen-based radiotherapy remains to be demonstrated.

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3 Role of Inflammatory Mediators in Angiogenesis

Federico Bussolino and Alberto Mantovani

CONTENTS

1. INTRODUCTION

Infiltrating leukocytes and angiogenesis are commonly observed in solid tumors and in chronic and acute inflammatory diseases. The endothelium of the vascular bed near the injured tissues actively participates to create this picture, through the formation of an interlaced network with circulating blood and/or neighboring cells and tissues. An impressive repertoire of molecules (cytokines, autacoids, growth stimulators and inhibitors, vasoactive peptides, pro- and anticoagulants, and fibrinolytic factors), with activities often partially overlapping, represent the "area codes" used to control this network, and can address endothelial cells to assume an inflammatory and/or an angiogenic phenotype *(1–4)*.

The key event characterizing the inflammatory phenotype is the expression on the endothelial surface of adhesion molecules, which permit the adhesion and then the transmigration of leukocytes. Among these, monocytes/macrophages represent a constant feature of solid tumor, and, in addition to cancer cells, produce molecules that induce an angiogenic program in endothelial cells *(1,2,4)*.

This review will provide a perspective on the role of humoral stimuli produced by, and active on, inflammatory cells, which regulate the angiogenic process.

2. CHEMOKINES

2.1. Action

By and large, the spectrum of action of chemokines is restricted to leukocytes, but recent evidence suggests that some members of this superfamily of inflammatory media-

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tors may affect endothelial cell function. Interleukin (IL)-8, groα and other C-X-C chemokines were reported to induce endothelial cell migration and proliferation in vitro, and to be angiogenic in vivo *(4–7)*. The expression of high-affinity receptors and responsiveness to IL-8 of endothelial cells has, however, been the object of conflicting results *(8,9)*.

Platelets factor 4, a C-X-C chemokine contained in the α-granules of platelets, inhibits growth-factor-induced proliferation of endothelial cells and angiogenesis *(10)*. Also, interferon (IFN)-γ-inducible protein-10 (IP-10) was shown to have angiostatic properties in vivo, though conflicting results have been obtained about its capacity to inhibit basic fibroblast growth-factor-induced proliferation of endothelial cells in vitro *(11,12)*. IP-10 induced via IFN-γ, may represent the ultimate mediator of the antiangiogenic activity of IL-12 *(12)*.

A 3-aminoacidic motif (Glu-Leu-Arg) (ELR), is highly conserved in all members of the C-X-C family that activate neutrophils. Recent results, including the action of ELR+ vs ELR– molecules and the activity of IL-8 muteins, suggest that the presence or absence of an ELR motif dictate whether C-X-C chemokines induce or inhibit angiogenesis *(13)*. In idiopathic pulmonary fibrosis, a chronic disease characterized by an exaggerated angiogenesis and deposition of extracellular matrix, a significant imbalance in the presence of C-X-C chemokines favoring the expression of IL-8, compared with IP-10, has been demonstrated *(14)*. This imbalance dictates the prominent neovascularization. However, the observation that groβ inhibits angiogenesis is not consistent with this model of function *(15)*. The C-C chemokines, monocyte chemotactic protein-1 (MCP-1), -2, and -3, do not affect endothelial migration and proliferation.

Three types of chemokine binding sites have been identified *(16)*. Chemokines activate leukocytes via 7-transmembrane-domain, G-protein-coupled receptors. A promiscuous chemokine receptor, identical to the Duffy blood group antigen, has been identified on erythrocytes, where it may act as a sink for chemokines leaking from inflamed tissues. This promiscuous receptor is expressed by endothelial cells at postcapillary venules in vivo, but not by endothelial cells in vitro *(16,17)*. The structure of the promiscuous chemokine receptor is that of a 7-transmembrane-type molecule, but there is no clear evidence of signaling activity. Finally, chemokines bind heparin and heparin-like proteoglycans, and these molecules on endothelial cells present at least certain chemokines to leukocytes in the multistop process of recruitment. The molecular basis underlying the pro- and antiangiogenic has not been clearly established. The expression of classic chemokine serpentine receptors in endothelial cells is controversial *(9,10)*. The antiangiogenic chemokine IP-10 was suggested in one study to inhibit basic FGF-induced endothelial proliferation via interaction with proteoglican structures *(12)*, but did not affect endothelial proliferation in another *(11)*. In the same vein, groα affected endothelial cell proliferation in one study *(7)*, but others failed to observe binding and activity of this chemokine on endothelial cells *(18)*. The mouse C-C chemokine FIC has been shown to bind endothelial cells *(19)*.

2.2. Production

Endothelial cells produce various chemokines in response to signals representative of inflammatory reactions, immunity, and thrombosis *(1,2)*. IL-1, tumor necrosis factor-α (TNF)-α, and bacterial lipopolysaccharide induce expression and release of IL-8, MCP-1, and groα *(20–25)*. Monocyte-associated IL-1 induces IL-8 expression in endothelial cells via a juxatacrine mechanism *(26)*. IL-4, IL-3, and IL-13 are weak inducers of IL-8 expression, and amplify induction by inflammatory cytokines *(27–29)*. Histamine induces

IL-8 production in endothelial cells *(30)*. Hypoxia has recently been shown to induce IL-8 and MCP-1 expression in endothelial cells, a finding potentially relevant for pathological conditions in which activation and recruitment of leukocytes may amplify tissue damage *(31,32)*. Platelets contain IL-1 and, when they interact with vascular endothelial cells, induce IL-8 gene expression *(31)*. Fibrin induces IL-8 in endothelial cells *(33)*.

As a result of proteolytic cleavage, IL-8 versions with a different NH_2 terminus and length can be produced *(34)*. It has been suggested that endothelial cells predominantly release a 77aa version of IL-8, which is a less active species at activating leukocytes than the most common 73 residue form *(35)*.

The influence of IL-8 on the interaction of neutrophils with vascular endothelium has been the object of seemingly conflicting observations, which seem now to reflect different experimental protocols and different functions exerted by this cytokine under different pathophysiological conditions. IL-8 increased the adhesiveness of normal neutrophils for normal endothelial cells *(36)*. In apparent contrast with these findings, endothelial-derived IL-8 was reported to inhibit binding of the neutrophils to activated endothelial cells *(37)*. Although it elicits neutrophil extravasation when given locally, IL-8 inhibits recruitment if administered systemically by the iv route *(38)*. The seemingly paradoxical anti-inflammatory effects of high levels of systemic IL-8, possibly dependent upon the action of a reverse chemotactic gradient and leukocyte deactivation, may represent a feedback mechanism to control tissue damage.

Endothelial cells activated in vitro by inflammatory cytokines express groα, which, according to one report, could in turn act on endothelium *(7)*. It has been suggested that endothelial-bound groα may promote monocyte adhesion *(39)*.

IP-10, a member of the C-X-C family, but unique in that it is inactive on neutrophils, is expressed in certain endothelia of mice exposed in vivo to IFN-γ, or to lipopolysaccharide *(40,41)*.

Endothelial cells produce substantial amounts of the C-C chemokine MCP-1 *(24,25)*. The proinflammatory signals IL-1, TNFs are potent stimuli for MCP-1 production *(24,25)*. Under the same conditions, MCP-2 was undetectable in endothelial cell supernatants (J. Van Damme, personal communication). IL-4 and IL-13 are active, though less-potent, inducers of MCP-1 expression *(28,29)*. IFN-γ was recently shown to induce MCP-1 in human microvascular endothelium *(42)*.

Given the role that lipids and monocytes play in the natural history of atherosclerosis, it is of interest that minimally modified low density lipoproteins induce MCP-1 production in endothelial cells and smooth muscle cells *(43)*. Thrombin was recently found to induce expression of MCP-1 in monocytes and, less prominently, in endothelial cells *(44)*. The C-C chemokine RANTES was produced by endothelial cells exposed to TNF and IFN-γ *(45)*.

Recently, Bazan et al. *(46)* reported the identification of a unique chemokine, fractalkine, representative of a new class: It is a transmembrane molecule consisting of a mucin and a chemokine domain. It is induced by IL-1 in endothelial cells, and is recognized by mononuclear cells.

3. CYTOKINES

3.1. Tumor Necrosis Factor

TNF is a primary inflammatory cytokine with profound effects on endothelial cells. TNF activates a proinflammatory, prothrombotic, and proangiogenic program in endo-

thelial cells *(1–3)*. Endothelial cells express both the p55 and the p75 TNF receptor. The p75 receptor is most abundant on the cell; p55 is mostly localized in the Golgi apparatus and in cytoplasmic vacuoles *(47,48)*. Endothelial cells respond to TNF via the p55 receptor, and p75 amplifies the response to low concentrations of the agonist by a process referred to as ligand passing *(49–51)*. TNF regulates the life-span of endothelial cells and angiogenesis with seemingly contradictory actions. TNF is not growth factor for endothelial cells, but it induces endothelial cell migration in vitro and angiogenesis in vitro and in vivo (reviewed in ref. *3*). These functions may be indirect. TNF induces production of the secreted protein B.61, which functions as an endothelial chemoattractant and angiogenic factor by acting as the ligand for the eck tyrosine kinase receptor *(52,53)*. Platelet-activating factor (PAF; *see* Section 4.1.) and nitric oxide (NO; *see* Section 4.2.) are two other molecules produced by endothelium after TNF challenge, which contribute to the angiogenic effect of the cytokine *(54,55)*. Finally, TNF upregulates the expression of urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR), thus favoring the degradation of proteins of extracellular matrix, a key step in angiogenesis (reviewed in refs. *1,2*, *56*). uPAR binds to the inactive form of uPA, and converts it into the active form, which in turns is able to degrade extracellular matrix, and to activate matrix metalloproteinases. The upregulation of uPAR is required for the in vitro angiogenesis of human microvascular endothelial cells stimulated by vascular endothelial growth factor (VEGF)-A or bFGF *(56)*.

Furthermore, other molecules cloned as TNF-inducible genes in endothelial cells may be relevant to vasculogenesis or angiogenesis *(57)*. A20 is a zinc-finger transcription factor that may be important in protection of endothelial cells and other cell types against TNF toxicity. B94 is a gene of unknown function, expressed in a complex way during vasculogenesis.

3.2. Granulocyte–Macrophage Colony-Stimulating Factor

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that regulates proliferation and differentiation of myeloid progenitor cells, and functional activation in host defense circuits of mature monocytes and granulocytes. It is produced by immune-competent cells, and by mesenchymal cells present in bone marrow, including endothelium and fibroblasts (*see* ref. *58*). GM-CSF heterodimeric receptor is composed by two transmembrane glycoprotidic subunits, termed α and β. The 60–90 kDa α-chain specifically binds the ligand with low affinity and dimerizes with the 120–140 kDa β-chain, which is also shared by IL-3 and IL-5 receptors (*see* ref. *59*).

Several studies have suggested that GM-CSF responsiveness may not be limited to hematopoietic lineages *(59)*, and endothelial cells is another target for this cytokine *(60–64)*. GM-CSFR-mediated endothelium activation includes functions related to angiogenesis and inflammation (i.e., proliferation, migration, expression of adhesion molecules). Although GM-CSF is less potent that other more classic angiogenic molecules (i.e., fibroblast growth factor) in promoting endothelial proliferation, it activates a fully migratory phenotype *(65)*. In addition, it has also been shown that GM-CSF interaction with specific binding sites on endothelial cell surface induces the expression of c-fos mRNA *(60)*, rapid activation of Na+/H+ exchanger, and JAK-2 tyrosine kinase *(66,67)*. It is emerging that other cytokines, which act primarily on hematopoietic cells (IL-3, granulocyte-CSF), affect endothelial cell function related to angiogenesis and inflammation *(27,60,61,65,68,69)*, and many angiogenic molecules have positive or negative effect

on hematopoiesis (reviewed in ref. *70*). Therefore, the ability of GM-CSF and other CSF-like molecules to induce migration and proliferation of endothelial cells, including that of bone marrow *(64)*, could be essential for the survival and renewal of bone marrow microenvironment in physiologic and pathologic conditions.

4. AUTACOIDS

4.1. PAF

Alkyl phosphoglycerides in membrane of mammalian cells are sources of autacoids with angiogenic activities. Phospholipase A_2 activation in inflammatory cells results in the hydrolysis of membrane alkyl-arachidoyl-phosphoglycerides to generate free arachidonic acid and alkyl-2-lyso-glycerophosphoglyceride. Class E prostaglandins generated by the conversion of arachidonic acid are in vivo angiogenic (reviewed in ref. *3*). The lyso-compound is the substrate of a specific acetyltransferase that catalyzes the transfer of the acetyl moiety from acetyl-CoA to the free hydroxyl at *sn*-2 of the lysophospholipids. The generated acetyl phosphoglyceride is named PAF, and is mediator with a wide spectrum of biological activities relevant for the development of inflammatory reaction embryogenesis and cell differentiation *(71)*. Many cell types that synthesized PAF, including endothelial cells, express PAF-binding sites on their surface *(72)*, and are target for PAF action *(73,74)*. PAF directly enhances the permeability of endothelial cell monolayer, and induces changes of the cell cytoskeleton characteristic of cells undergoing translational movements *(73,74)*. Subsequently, it has been reported that PAF is chemotactic in the Boyden's chamber *(75)*. PAF-induced migration of endothelial cells was a receptor-dependent phenomenon, because pretreatment with PAF receptor antagonists inhibited the migration, and the D-stereoisomer of PAF was ineffective *(75)* PAF receptor, the cDNA of which has been recently cloned, belongs to the family of serpentine receptors containing seven α-helical domains that weave in and out of the plasma membrane (reviewed in ref. *71*) and expressed in endothelial cells *(75)*. By using the Matrigel model in mouse, the author observed that PAF induced in vivo the formation of canalized vessels *(75)*, already evident after 16–24 h. Similar results have been obtained in rabbit cornea (M. Ziche, unpublished results). The minimal concentration of PAF able to induce angiogenesis was 10 n*M*, an amount compatible with levels of bioactive PAF produced in vivo within inflamed tissues. PAF was found to induce an in vivo angiogenic response either in an heparin-independent or an heparin-dependent manner, depending on the dose used *(75)*. At pharmacological concentrations (1–5 μM), PAF induced a heparin-independent angiogenesis. In contrast at physiological concentrations of PAF (10–50 n*M*), heparin was required, suggesting that the angiogenic effect of PAF may depend on the secondary involvement of heparin-binding endothelial cell growth factors with angiogenic properties. Indeed, several studies have suggested that PAF may promote transcription of genes coding for cytokines and growth factors, including that of VEGF-A *(76)*. PAF has been implicated as mediator of angiogenesis induced by tumor necrosis factor *(77)* since it is actively synthesized in vivo during the angiogenic process, and PAF-receptor antagonists significantly reduce the angiogenic response to this cytokine that sustains in vivo angiogenesis. PAF is also involved in the angiogenic effect triggered by hepatocyte growth factor (HGF). This growth factor activates a direct angiogenic program in endothelial cells by activating the tyrosine kinase activity of its cognate receptor *(78)*. Furthermore, it promotes the migration of monocytes and the synthesis of PAF by

this cell type, thus amplifying the angiogenic effect. Therefore, PAF receptor antagonists partially block the angiogenesis induced by HGF *(79)*. Finally, it has been shown that PAF participates in vivo in the permeability activity induced by VEGF-A *(80)*. Evidence of a role of PAF in angiogenesis associated with cancer is supported by the inhibitory role of PAF antagonist in lung metastatization of B16 murine melanoma cells *(81)*, and by the positive correlation between the presence of PAF and the microvessel count in hot spots in breast cancer *(81a)*.

4.2. Nitric Oxide

Vasodilation and hyperemia of the pre-existing capillaries, and the persistence of a dilation state of the newly formed vessels, are typical findings in angiogenesis and in vasculature of tumors. Furthermore, several angiogenesis factors/mediators promote relaxation in vascular preparations, and peptides known to induced endothelium-mediated vasorelaxation are angiogenic *(3)*. Nitric oxide (NO) is a an endothelial derived relaxing factor that is a good candidate to mediate the vasodilation of capillaries that precedes the vascular sprouting *(3)*. NO is an inorganic free radical gas synthesized from L-arginine by a family of enzymes called NO synthases. Two of these are constitutively expressed, and a third is inducible by immunological stimuli. The NO released by the constitutive enzyme acts as an important signaling molecule; NO released by the inducible NO synthase is generated for long periods, and has been shown to be cytostatic/cytotoxic for tumor cells *(82)*. The release by endothelial cells of NO can be blocked by L-arginine analogs like N^G-mono-methyl-L-arginine and L^G-nitro-L-arginine methyl ester, but the D-enantiomers are ineffective. NO acts as an angiogenesis effector and as a modulator of the activity and/or the production of angiogenesis factors. The role of NO in angiogenesis was assessed by testing the activity of molecules regulating NO production on proliferation and migration of capillary endothelium. By using the vasodilator, sodium nitroprusside, which provides an exogenous source of NO, and the neuropeptide, substance P, which induces NO-production in endothelial cells, it has been shown that NO generating compounds promote endothelial cell proliferation and migration *(83)*. At least in coronary microvascular endothelial cells, NO promotes cell proliferation by inducing endogenous FGF *(84)*. The positive role of NO in cell proliferation seems to be restricted to some endothelial subtypes. Therefore, NO inhibits the proliferation of bovine pulmonary arterial endothelial cells *(85)*, and is ineffective in spontaneous rat microvascular endothelial cell growth *(86)*.

The relevance of NO in angiogenesis in vivo was assessed in animal models receiving angiogenesis effector during systemic treatment with NO synthase inhibitors, which suppress angiogenesis induced by vasodilating effectors like substance P, prostaglandin E1, and VEGF *(83,87)*. VEGF in human endothelium activates the synthesis of NO *(88)*, which is upstream from the activation of guanylate cyclase activity. The growth-promoting effect of VEGF appears to be linked to cGMP generation, as inferred by the specific inhibition of the cyclase by LY 83583 *(55,87)*. Conversely, exogenous bFGF can elicit angiogenesis in vivo and endothelial cell proliferation in vitro, despite the block of NO production *(55,87)*. However, NO downregulates VEGF promoter activity, suggesting that this radical has an opposite effect on the synthesis and on the action of VEGF *(89)*.

Other reports have implicated a role for NO synthase in angiogenesis. For example, it was shown that in vitro angiogenesis triggered by transforming growth factor-β_1 (TGF-β_1) requires NO *(86)*. The angiogenic activity was only released from bacterial endotoxin

treated human monocytes in the presence of L-arginine *(90)*. Although the source of angiogenic activity was not identified in the above study, it was nonetheless blocked by the NO synthase inhibitors. Similarly L-arginine was shown to favor healing and angiogenesis in gastric ulcerations, but NO synthase inhibitors delayed it *(91)*. The in vivo progression of murine hemangiomas induced by transplantation of endothelial cells immortalized by middle T-antigen of polyomavirus is reduced by canavanine, a NO synthase inhibitor *(92)*. Other observations have indicated a cytotoxic/cytostatic effect of NO on the vascular development of the chorioallantoid membrane, suggesting a diversity of effects in embryonic vs adult tissue *(93)*.

Most of the cellular components of the tumor mass (the tumor cells themselves and the immune cells infiltrate) have been shown to generate NO in vitro. However, the role of NO in tumor biology is still poorly understood. Malignant tumors exert a powerful influence on neighboring blood vessels. Hemodynamic studies have shown that vasculature associated with tumors is insensitive to vasoactive agents, and appears to be in an almost maximal state of vasodilation *(94)*. When compared to normal arteries in vitro, tumor-associated vessels were unresponsive to vasoconstrictor agents like phenylelphrine, an effect evidenced to be linked to increased NO synthase activity *(95)*. Transfection of the inducible NO synthase into a colon adenocarcinoma line gave a cell line, which, despite growing more slowly in vitro, promoted tumors that grew more rapidly, and were more vascularized than wild-type cells *(96)*. Other observations in agreement with NO being a specific signal for tumor vascularization show that blocking NO-synthase activity retards the growth of xenografted tumors *(96)*. Thus, several data exist in support of NO as a signaling molecule in tumor angiogenesis. Based on these considerations, the authors hypothesize that NO released by capillaries in proximity of a tumor under the control of a local growth factor can be instrumental for tumor angiogenesis.

5. ANGIOGENESIS IN KAPOSI'S SARCOMA: AN EXAMPLE OF COMBINED ACTION BETWEEN ANGIOGENIC AND INFLAMMATORY INDUCERS

Kaposi's sarcoma (KS) is a multicentric, highly vascularized neoplasm, probably arising from a monoclonal population of circulating progenitor cells that home to multiple visceral and cutaneous sites *(97,98)*. KS incidence is greatly enhanced in AIDS patients, being detected in about 20% of the affected individuals, who develop a particularly aggressive form *(99)*. Four principal features of KS include the presence of spindle cells, which represent the core of the lesion; an aberrant proliferation of endothelial cells with prominent angiogenesis; the presence of infiltrating mononuclear cells; and increased vascular permeability. The histogenesis of spindle cells is still debated, but recent data, showing the co-expression of endothelial and macrophage antigens, permit speculation that the origin of spindle cells is in a precursor that differentiates in normal tissue in the sinus-lining cells of spleen and lymph nodes *(100)*. The molecular mechanisms regulating the differentiation of a putative precursor into spindle cells are unknown. However, an unbalance of soluble mediator network seems to be pivotal in differentiation and growth of spindle cells, as well as in the recruitment of vascular and lymphomononuclear cells. A new herpesvirus, HHV8, has been associated with KS of all origins *(101)*, and some of its genes are homologs to human genes. Among these, there are the homologous of IL-8 receptor *(102)*, IL-6, and macrophage inflammatory protein-1 *(103)*. Human

IL-6 regulates KS proliferation *(104)*, and viral IL-6 can be integrated in an autocrine pathway. HIV-1-Tat is also thought to be the link between increased incidence and aggressiveness of KS in infected patients. Tat is officially the HIV-1 transactivating protein, responsible for viral transcription, but it has a number of extracellular activities relevant for KS pathogenesis. Through the activation of VEGF receptor-2 and -1, Tat promotes, respectively, angiogenesis and monocytes recruitment *(105,106)*. Furthermore, Tat promotes the growth of KS cells *(107)* and the upregulation of adhesion molecules in endothelium, thus favoring leukocyte infiltration *(108)*.

Besides these viral proteins, the production of autocrine and paracrine human soluble mediators has been demonstrated as relevant in the features of KS lesions. FGF *(109)*, HGF *(110)*, PAF *(111)*, and VEGF-A *(112)* are instrumental for vascularization; platelet-derived growth factor (PDGF; *113*) and TGF-β *(114)* are instrumental for the maturation of nascent vessels, through the recruitment of pericytes and smooth-muscle cells and the matrix remodeling. KS cells also produce chemokines *(115)*, which, besides VEGF *(3)* and PAF *(71)*, provide signals for monocytes. Inside the lesion, proteolytic enzymes released by macrophages, PAF, and VEGF participate in the increasing vascular permeability. Furthermore, FGF *(109)*, HGF *(110)*, VEGF *(112)*, PAF *(111)*, IL-1 *(116)*, PDGF *(113)*, TGF-β *(114)*, oncostatin *M (117)*, IL-6 *(104)*, and GM-CSF *(118)* are also autocrine/paracrine activators of KS spindle cells.

Indeed, KS is an example of co-operation between growth and inflammatory factors supporting the expansion of neoplastic cells, and can represent an appropriate pathological condition to test antiangiogenic compounds in adjuvant therapy of cancer.

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4 Intra- and Extracellular pH in Solid Tumors

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Contents

1. INTRODUCTION

It has long been known that the interstitial environment in malignant tumors is acidic as a result of accumulation of lactic acid and other acidic metabolites. Neoplastic cells metabolize glucose preferentially through glycolysis, even in an aerobic environment resulting in the production of lactic acid *(1)*. In solid tumors, the supply of oxygen is limited because of sluggish blood perfusion through a heterogeneously distributed vascular network, resulting in diffusion-limited hypoxia *(2–5)*. Furthermore, it has been observed that blood perfusion through capillary-like tumor vessels often slows down, stops, or even completely reverses its direction, creating perfusion-limited hypoxia *(5,6)*. Under such hypoxic conditions, cells metabolize glucose through anaerobic glycolysis and produce lactic acid *(1,7–9)*. Furthermore, a sudden induction of hypoxic condition caused by sudden cessation of blood perfusion may cause hydrolysis of ATP, resulting in the generation of H^+ ions *(10)*. For reasons unknown, small amounts of β-hydrobutyric acid were detected in experimental tumors *(9)*. Such overproduction of acidic metabolites, notably lactic acid combined with slow discharge of the acidic metabolites via sluggish tumor blood flow, results in accumulation of acidic metabolites in the tumors. The acidic interstitial or extracellular environment in tumors inevitably influences the intracellular acidity, which in turn would exert a marked influence on the proliferation of parenchyma and endothelial cells. This chapter discusses the relationship between the

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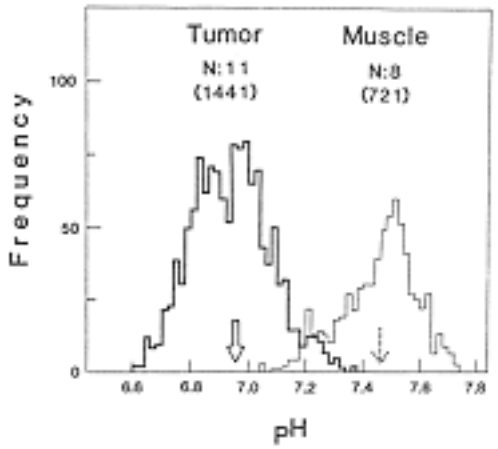

Fig. 1. Histograms of interstitial (extracellular) pH in the SCK tumors grown sc in the leg of A/J mice, and that in the leg muscle of A/J mice. The pH was measured with glass electrodes 50–80 μm in diameter (adapted with permission from ref. *12*).

extracellular and intracellular acidity in tumors, the influence of the intracellular acidity on angiogenesis, and of that on the response of tumor cells to various antineoplastic regimens.

2. EXTRACELLULAR pH IN TUMORS

A brief discussion about the definition of pH and how pH is determined would be helpful to clearly understand tumor pH. The degree of acidity in nonideal solution, such as interstitial fluid in tumors, is determined by the activity of H^+ ions, $^aH^+$, and not by the concentration of H^+ ions, $[H^+]$ *(11)*. The logarithmic units for the H^+ activity is used to express the H^+, which is defined as $pH = -\log {}^aH^+$. As the equation indicates, an increase in $^aH^+$ means a decrease in pH value. A decrease in 1.0 pH unit is equal to an increase in $[^aH^+]$ by a factor of 10. An increase in $[^aH^+]$ by a factor of 2 would result in a decrease in 0.3 pH units.

For nearly a century, pH was measured with pH electrodes constructed with H^+-permeable soft soda-lime glass. The principle of determining the pH with such glass pH electrodes is that H^+ ions diffuse across the pH-sensitive glass membrane until an opposing electrical potential is developed. The pH value and the potential (E), as determined with the pH electrode, together with the reference electrode incorporated in the system, are related by the following equation: $E = E_0 - 58.2$ (pH), where E is expressed in millivolts and E_0 is a constant *(11)*.

In the determination of pH in tumors or normal tissues, it is highly desirable to use small-diameter pH electrodes to minimize disturbances in the intratumor environment, and to avoid destruction of blood vessels. However, the electrode should be rigid enough to be advanced through the tumors for the determination of the distribution of pH values in the tumors. The size of electrodes used in the past, to determine the pH in various animal and human tumors, varied from 50 μm to as large as 2.0 mm in diameter. To determine the pH in several rodent tumors in this laboratory, 50–80 μm od glass capillary electrodes were constructed *(12)*. The sensitivity of these pH electrodes was 58–60 mV per 1.0 pH unit at 34°C.

As shown in Fig. 1, the mean pH value in SCK tumors, determined with the 50–80-μm diameter electrodes, was 6.96. A previous study used a commercially available electrode

with 800-μm tip diameter *(13)*. The pH of the SCK tumors, determined with such relatively large electrodes, was about 7.05, which was slightly higher than the pH value obtained with the smaller electrodes mentioned above, indicating that the tumor pH value determined with electrodes is almost independent of the diameter of electrodes within the range of 50–800 μm. It is believed that the tumor pH values obtained using the microelectrodes or miniaturized electrodes primarily represent pH of the extracellular fluids, i.e., extracellular pH (pH_e), in tumors.

It has been more than half a century since the pH in rodent tumors was found to be acidic *(14)*. These pH values probably represented the extracellular pH values, i.e., pH_e, because they were obtained with relatively large capillary electrodes. Since then, a number of investigators determined pH_e in a variety of tumors and normal tissues of rodents *(15–19)*. Wike-Hooley et al. *(17)* reviewed a number of reports on the pH values in tumors and normal tissues of animals, and reported that the tumor pH_e ranged from 5.80 to 7.68, with an average of 7.09; the pH_e values in normal tissues, such as the muscle and liver, was about 0.5 pH units higher than that in the tumors. The authors' results, shown in Fig. 1, demonstrate that the average pH_e values of the SCK tumors were 6.6–7.4, with a mean pH value of 6.9; the mean pH value in the leg muscle was 7.4 *(12)*. Jähde et al. *(18)* reported that the pH_e values in 1.0–2.5 g of neuroectodermal TV1A tumors, grown subcutaneously in the flank of BDIX rats, ranged from 6.8 to 7.1, with a mean of 7.0. The pH_e values in the brain and kidney of BDIX rats were acidic, as in the tumors. Vaupel et al. *(16)* found that the pH_e values in a C3H mouse mammary carcinoma ranged from 6.4 to 7.1, with a mean and median value of 6.73 and 6.75, respectively. In large ulcerated tumor, there were microareas with very low pH_e values (5.8–6.3); in extensively necrotic areas, pH_e values were even higher than the arterial pH. Such high pH_e values in the necrotic area appeared to result from a lack of lactic acid formation because of cell death. Kallinowski and Vaupel *(19)* reported that the mean pH_e values in malignant spontaneous mammary tumors of rat were 6.94; those in benign tumors were 6.95, indicating that the pH_e value in tumors are independent of the degree of malignancy. On the other hand, tumors with the same degree of malignancy, but with different histological makeups, had different pH_e distribution.

The pH_e distribution in human tumors was reported as early as 1948 by Meyer et al. *(20)*. As in animal tumors, it was observed that the pH_e values in human tumors were significantly lower than those in normal tissues. Essentially similar results have been observed in numerous studies conducted during the past half century *(17,21–31)*. Wike-Hooley et al. *(24)* reported that mean pH_e in 77 different human tumors was 7.25 ± 0.99; that in normal subcutaneous tissue was 7.54 ± 0.09. The tumor pH_e values were not related to the tumor histology, degree of differentiation, tumor size, patient age, or treatment history. However, the pH_e in primary tumors was significantly lower than that in their metastases. Vaupel et al. *(29)* reviewed the reports on the pH_e values in various human tumors, as measured with microelectrodes, and concluded that most human tumors exhibit pH_e values between 6.15 and 7.40; normal tissues usually have pH_e values between 7.0 and 7.4. Engin et al. *(31)* investigated the relationship among pH_e, tumor histology, and tumor volume. The range of pH_e in 67 tumor nodules in 58 patients ranged from 5.66 to 7.78, with a mean of 7.06 ± 0.05. The pH_e of adenocarcinoma and soft-tissue sarcoma was lower than that in the squamous cell carcinoma and malignant melanoma by 0.26 pH units, indicating that a relationship exists between the pH_e and tumor type in human tumors. This conclusion was at variance with the

conclusion by Wike-Hooley et al. *(24)* that the human tumor pH value is not related to the tumor histology, as mentioned above.

3. INTRACELLULAR pH IN TUMORS

There have been attempts to determined the intracellular pH (pH_i) with glass microelectrodes with a diameter as small as 1 μm, but the results of these studies were inconclusive. In the late 1970s, magnetic resonance spectroscopy (MRS) was introduced as a means to determine pH_i in tumors *in situ,* or in normal tissues in animals and humans. Since then, there has been a marked improvement in the MRS technique, and it is now widely used to determine the pH_i, as well as the metabolic state of tumors. The use of MRS for assessing tumor pH is attractive, because it is noninvasive, can be used for deep-seated tumors, and it may provide information about spatial pH distribution.

The measurement of intracellular pH (pH_i) with MRS relies on the chemical-shift difference between P_i and an endogenous reference, such as PCr or a-ATP *(32)*. The relative concentrations of the major phosphate compounds comprising the ^{31}P MR-observable signal (e.g., $H_2PO_4^-$ and HPO_4^{2-}) are dependent on pH, since there is a rapid exchange between these two species ($H_2PO_4^- \hat{U} HPO_4^{2-} + H^+$). Thus, the observed signal consists of a single peak in which the chemical shift is pH-dependent. Since the P_i peak arises from phosphates, which are primarily intracellular *(33,34)*, the use of ^{31}P MRS has been widely used to estimate pH_i of cells and intact tissues, such as tumors *(33,35)*. It has also been shown that necrotic regions within tumors do not contribute to the overall pH_i value, since P_i does not accumulate in these regions of the tumor *(36)*. Therefore, ^{31}P MRS has been accepted as a very accurate method for measuring the pH_i of living cells *(37)*. Early pH_i measurements of solid tumors using ^{31}P MRS demonstrated average values for the whole tumor; thus, no spatial discrimination was achieved. More recently, with advances in spectroscopic localization techniques, single- or multivoxel ^{31}P localized spectra could be obtained. This capability provides for the ability to monitor spatial differences of pH_i throughout the tumor mass, but this application is limited to relatively large voxels, and thus can be only applied to larger tumors such as can occur in humans.

Recently, Gillies et al. *(38,39)* reported that both pH_i and pH_e can be measured simultaneously with ^{31}P MRS, using 3-aminopropylphosphonate (3-APP) as the pH_e probe. 3-APP was chosen for this purpose because it is not significantly internalized nor metabolized by cells. Furthermore, 3-APP is relatively nontoxic, it has a pKa in the physiological range, and its resonant frequency is not significantly affected by such variables as ionic strength and temperature. Figure 2 shows an in vivo ^{31}P MR spectrum of a MCF-7/mitox tumor grown in the mammary fat pad of a female SCID mouse, which had received an ip injection of 3-APP prior to obtaining the spectrum. The pH_e from the chemical shift of 3-APP was determined to be 6.62; the pH_i from the chemical shift of the P_i peak was found to be 7.06. This result shows that it is possible to measure both pH_i and pH_e, noninvasively, in the same tumor using ^{31}P MRS.

The spatial resolution of pH_e using 3-APP is limited by the sensitivity of the ^{31}P nuclei, and ^{31}P spectra can typically only provide a pH range averaged over the whole tumor volume. Having a compound with similar properties to 3-APP, but with a MR-visible ^1H nuclei, would allow for a greatly improved spatial discrimination of pH within tumors. A series of imidazole derivatives has been recently developed and tested for this purpose *(39)*. Using such compounds, localized pH values in experimental mouse tumors could

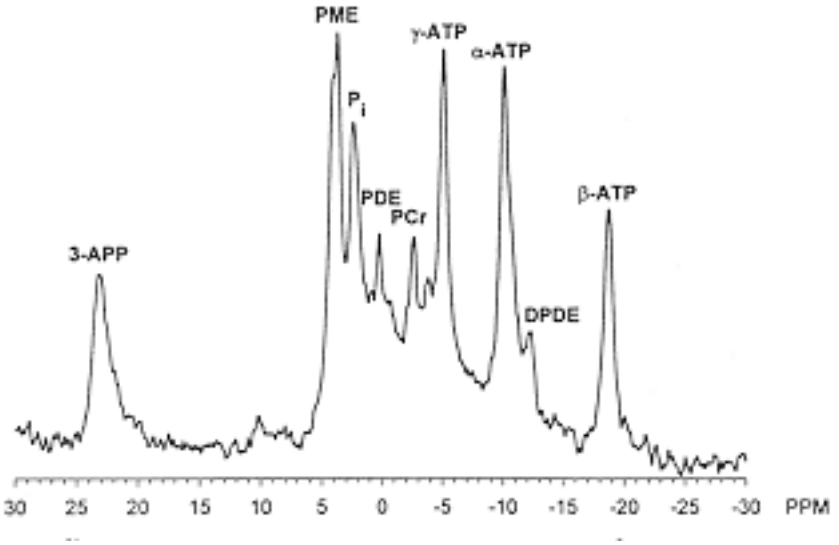

Fig. 2. In vivo ^{31}P MR spectrum of MCF-7/mitox tumor (1200 mm^3) grown in the mammary fat pad of a female SCID mouse. The pH_e from the chemical shift of 3-APP is 6.62, and the pH_i from the chemical shift of the P_i peak is 7.06. Abbreviations: 3-APP, 3-aminopropylphosphonate; PME, phosphomonoesters; Pi, inorganic phosphate; PCr, phosphocratine; ATP, adenosine triphosphate; and DPDE, diphosphodiesters. (Spectrum kindly provided by Dr. R. J. Gillies).

be obtained with ^{1}H MRS. It has recently been demonstrated that 6-fluoropyridoxol (6-FPOL), a vitamin B^6 analog, may be used to simultaneously determine pH_i and pH_e in tumors *(40)*. The ^{19}F NMR from 6-FPOL showed two well-resolved peaks, corresponding to pH_i and pH_e. The usefulness of this method to determine tumor pH_i and pH_e, however, has not been demonstrated.

The pH_i of cells in tumors determined with ^{31}P MRS turned out to be considerably higher than the pH_e determined with electrodes *(29,30,41,42)*. According to the data pooled by Vaupel et al. *(29)*, the pH_i of various sarcomas, squamous cell carcinomas, breast cancer, non-Hodgkin lymphomas, and other miscellaneous malignancies of humans ranged from 6.9 to 7.4 (Fig. 3). The mean pH_i of various human tumors was conspicuously consistent at 7.17–7.19, except for the mean pH_i of brain tumors, which was about 7.10. The ^{31}P MRS studies revealed that the normal-tissue pH_i is either similar to or slightly lower than the tumor pH_i. The pH_i of the normal brain was about 7.01, which was significantly lower than the approx 7.10 pH_i of brain tumors, as mentioned above. Unequivocal conclusions may be drawn from these results: In tumors, the intracellular environment is either neutral or only mildly acidic; the extracellular environment is acidic because of the overproduction of acidic metabolites. These facts point to the presence of cellular mechanisms that effectively regulate pH_i.

4. REGULATION OF pH_i

Numerous studies, using pH-sensitive fluorescent probes and a variety of cell lines in vitro, demonstrated that most mammalian cells, including neoplastic cells, possess effective pH_i-regulatory mechanisms through which the pH_i is maintained at neutral pH range *(43,44)*. Sudden acid loading inside the cells is controlled temporarily by the intracellular

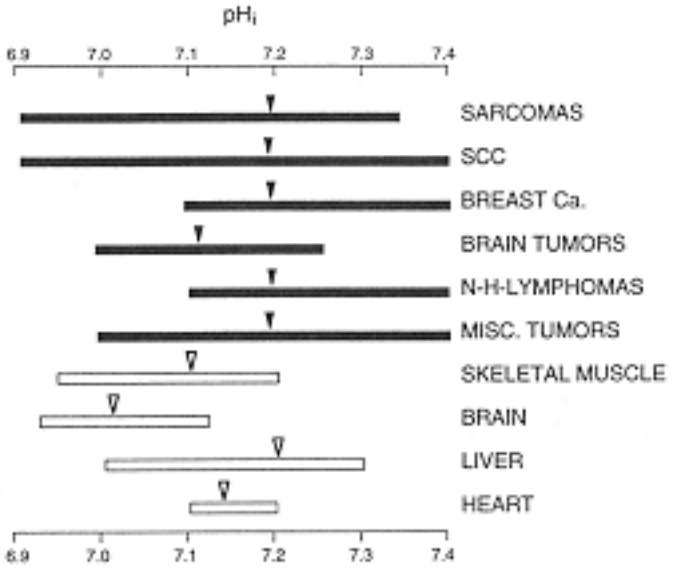

Fig. 3. Range of intracellular pH (pH$_i$) in solid human tumors and normal tissues measured with ^{31}P NMR. Arrowheads indicate the mean pH. Modified from Vaupel et al. *(29)*.

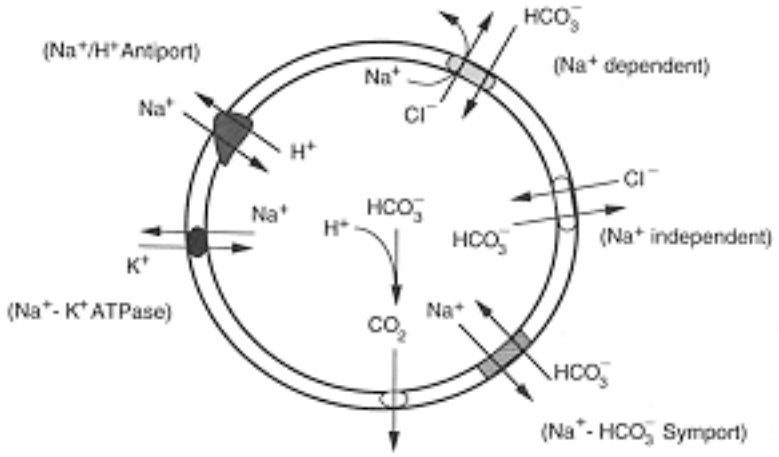

Fig. 4. Schematic illustration of the most common membrane-based pH$_i$ regulatory mechanisms in mammalian cells.

physicochemical buffering, metabolic consumption of nonvolatile acids, and transfer of acids from the cytosol to the organelles *(43,44)*. However, these mechanisms are only for a temporary prevention of acute acidification of the intracellular environment, and the prolonged regulation of pH$_i$ relies on cell-membrane-based ion exchange. Among a number of different membrane-bound ion exchangers identified, the Na^+/H^+ antiporter, Na^+-dependent and Na^+-independent HCO_3^-/Cl^- exchangers, lactate/proton symporter, and ATPase-driven H^+ pump play the major roles in regulating pH$_i$ (Fig. 4). It may be inferred that the relative importance of various pH$_i$ regulatory mechanisms in different tumor types varies considerably, depending on the tissue origin of the tumors.

The Na^+/H^+ antiport exists in all mammalian cells *(43–47)*. There are at least three different subtypes of Na^+/H^+ antiporters, and their distribution varies, depending on cell and tissue types *(48)*. The Na^+/H^+ antiporter facilitates the transport of intracellular H^+ to the outside of cells across the cell membrane, in exchange for inward transport of Na^+. The antiport is activated when the intracellular H^+ ions bind to the allosteric H^+ binding site in the inner site of the cell membrane, and Na^+ binds to the cell surface *(45–47)*. The gradient of Na^+ across the cell membrane is the driving force of the Na^+/H^+ antiport, although the antiport system is secondarily dependent on the Na^+/K^+-ATPase *(49)*. The Na^+/H^+ antiport can be inhibited by the diuretic drug amiloride and its analogs *(43,46,50–53)*. Recently, several new drugs have been developed that can selectively inhibit the three subtypes of Na^+/H^+ exchange (NHE). HOE642 (Hoechst Chemical Research, Frankfurt, Germany) has been demonstrated to selectively inhibit the NHE subtype 1 *(48)*.

Through the Na^+-dependent $HCO3^-/Cl^-$ exchange mechanism, the intracellular Cl^- is expelled in exchange, with the influx of extracellular HCO_3^-, which subsequently reacts with the intracellular H^+ and forms H_2O and CO_2 *(43–46,54,55)*. The activity of this exchanger is driven by the inward Na^+ gradient. The Na^+-dependent HCO_3^-/Cl^- exchange can be inhibited by stilbene derivative 4,4'-diisothiocyanostilnene 2'2-disulfonic acid (DIDS) *(43,46,51–58)*. The Na^+-independent HCO_3^-/Cl^- exchanger is activated on the rare occasion when the intracellular environment becomes alkaline and it exchanges the intracellular HCO_3^- for the extracellular Cl^- *(54,55,59,60)*.

Lactate is expelled from the cells by the lactate/proton symporter, and this process can be inhibited by quercetin *(60,61)*. Another specialized pH_i regulatory system in mammalian cells is ATPase-driven H^+ pump *(46)*, which also appears to regulate the pH in a number of intracellular organelles. The presence and relative importance of these membrane-bound pH_i-regulatory mechanisms are dependent on cell type. For example, although the Na^+/H^+ antiporter plays the major role in gastric glands, HCO_3^-/Cl^- exchanger plays the major role in the neighboring oxyntic cells *(46)*.

When tumor cells cultured in neutral pH medium are exposed to low pH medium, the pH_i immediately drops, but is stabilized at a pH significantly higher than the medium pH, because of the presence of the pH_i-regulatory mechanisms, as discussed above. The authors have investigated the relationship between the pH_e and pH_i by measuring pH_i with the pH-dependent fluorescent probe acetyoxymethyl ester of 2'7'-bis-(2-carboxyethyl)-5-(and 6-)carboxy-fluorescein (BCECF) *(51–53)*. It was observed that the pH_i of tumor cells is substantially higher than the medium pH (pH_e) at pH_e lower than 7.0, and the pH_i and pH_e become nearly equal as the pH_e is raised to 7.0–7.5 *(62)*. It should be remembered that tumor cells in vivo are chronically exposed to, and thus adapt to, a low-pH_e environment. The authors have observed that the pH_i regulatory mechanisms are upgraded when cells are chronically exposed to an acidic environment (unpublished data). It may be concluded that the large gradient between the pH_i and pH_e in tumors results from the presence of upgraded pH_i-regulatory mechanisms in the tumors.

5. THERAPEUTIC IMPLICATION OF pH_i AND pH_e

It is not surprising that acidification of the intracellular environment is cytotoxic. The therapeutic potential of intracellular acidification alone, or in combination with other modalities, such as hyperthermia or chemotherapy, has been investigated *(51–53,62–66)*.

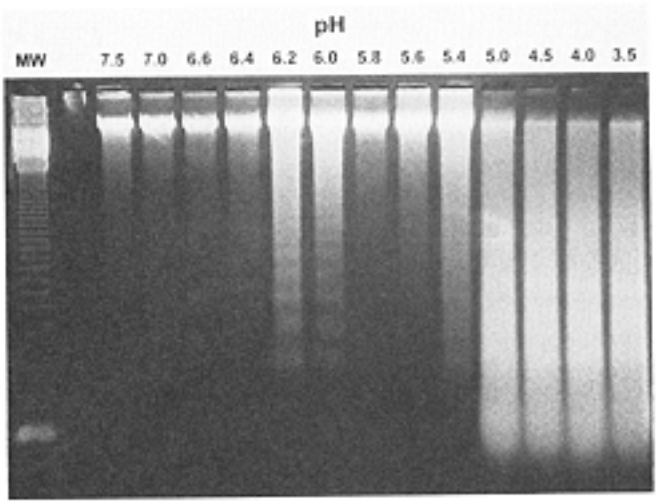

Fig. 5. Agarose gel electrophoresis of DNA from Ramos cells incubated for 7 h in different pH media.

The authors have observed that an acidic environment kills cancer cells by apoptosis or necrosis, depending on the degree of acidity, and also on the cell type. Figure 5 shows that incubation of Ramos human leukemic cells in low pH media caused apoptosis, resulting in DNA fragmentation, a hallmark of apoptosis. The DNA fragmentation occurred when the medium pH was lowered to 6.4–6.0. The optimal medium pH for the induction of apoptosis in Ramos cells was 6.2, at which the pH_i was about 6.6. An incubation for 7 h in pH 4.0–5.4 medium caused massive DNA degradation in Ramos cells, suggesting that an extremely acidic environment induces necrosis. In HL-60 cells, marked apoptosis occurred when the cells were incubated for 7 h in pH 6.4 medium, at which the pH_i was about 6.8 *(64)*. In nonlymphoid cells, such as SCK tumor cells of A/J mice, apoptosis, accompanied by DNA fragmentation, occurred when the cells were incubated for 24–48 h in medium with a pH below 6.2 *(65)*. In HL-60 cells, the inhibition of Na^+/H^+ antiport with amiloride or its analogs, such as HMA, together with inhibition of HCO_3^-/Cl^- exchange with DIDS, caused apoptosis, even in pH 7.5 medium *(64)*. Similar results were obtained in Ramos cells (unpublished data). Nigericin is an ionophore of K^+, and it lowers pH_i by transporting extracellular H^+ ions into the cells *(51)*. A combination of HMA, DIDS, and nigericin lowered pH_i, and caused apoptosis in HL-60 cells, even in pH 7.5 medium *(64)*.

It is well known that an acidic environment enhances hyperthermic cell killing, inhibits expression of heat-shock proteins, and inhibits development of thermotolerance *(66)*. Lowering the pH_i with amiloride or its analogs, DIDS and nigericin, potentiates the hyperthermic antitumor effect both in vitro and in vivo *(51–53,62,63)*. The authors have recently reported that an acidic environment enhances the expression of certain genes, such as *p*53 and *p*21 *(67)*, which are known to play cardinal roles in apoptosis and cell-cycle progression after exposing cells to various stresses, such as ionizing radiation. The authors have also observed that an acidic environment suppresses radiation-induced apoptosis, and prolongs the radiation-induced G_2/M block *(65)*. It appeared that the acidic environment suppressed the activity of cyclins and kinases responsible for the progression of cells through G_2 phase, thereby suppressing the postmitotic apoptosis. In agree-

ment with these results, it has been reported that radiation-induced clonogenic cell death could be protected when the cells were preconditioned in an acidic environment prior to radiation exposure, and when maintained in the same acidic medium after the irradiation *(68)*.

Most chemotherapeutic drugs would not be able to kill the target cells, unless they are internalized in the cells. The internalization of drugs requires dissociation of the drugs, which depends on their pKa and the acidity of extracellular fluid *(62,69)*. Once the drugs are internalized in the cells, the retention of the drugs inside the cells, and the interaction between the drugs and target molecules, are markedly affected by pH_i *(69)*. For example, low pH_i enhanced the effect of mitomycin C in causing DNA crosslinking *(70)*. The structure of topoisomerase I inhibitors, camptothecin and its analogs, were found to be strongly pH-dependent *(71)*. Furthermore, prolonged exposure in a clinically relevant low-pH environment, e.g., pH 6.2–6.8, markedly enhanced the cytotoxicity of the drugs *(71)*. Such an increase in the effect of these drugs in vitro and in vivo was caused by an increase in cellular uptake and retention in an acidic environment. It is known that an acidic environment increased the effect of cisplatin, melphalan, and cyclophosphamide, but it decreased the effect of cyclohexyl-chloroethyl-nitrosourea (CCNU), doxorubicin, and vinblastine, as mentioned above *(62)*.

The aforementioned results strongly suggest that acidification of the intratumor and intracellular environments may enhance the effect of hyperthermia and certain chemotherapeutic drugs. Indeed, the feasibility of exploiting the high glycolytic acidity in tumors for selectively acidifying the tumors has been investigated. It was demonstrated that administration of relatively large doses of glucose preferentially acidifies the tumors *(72–76)*.

6. EFFECT OF pH ON ANGIOGENESIS

The vascular and perfusion insufficiencies in tumors may be incriminated, at least in part, for the development of acidic and hypoxic environment in the tumors. Conversely, such an adverse environment may greatly affect the vascularization, i.e., angiogenesis, and also affect the functional integrity of the tumor vasculature. In the early stage of tumor growth, the tumor cells evoke various pathological changes in the nearby host cells and tissues. These affected normal cells, as well as the tumor cells themselves, secrete stimuli, which in turn induces focal fragmentation in the basement membranes of nearby venules. Subsequently, the endothelium of the affected vessels migrate and form sprouts through the fragmented basement membrane. As the endothelial cells are stimulated to proliferate by various growth factors secreted from the tumor cells, the sprouts elongate and interlink, forming a vascular network *(2–6)*. It is not difficult to envision that microenvironmental factors, such as pH and pO_2, greatly affect the extremely complex biochemical and molecular process involved in the proliferation and differentiation of endothelial cells. Likewise, the local microenvironment may significantly influence the interaction among endothelial cells and the interaction of endothelial cells with tumor cells.

It has been reported that hypoxia increases expression of vascular endothelial growth factor (VEGF), thereby amplifying angiogenesis *(77)*. Kim et al. *(78)* recently reported that insulin-like growth factor II (IGF-II) enhances the expression of VEGF in human hepatocellular carcinoma cells, and that this process is enhanced by hypoxia. The binding of insulin to endothelial cells is pH-dependent, with a sharp optimum at pH 7.8, so that the binding of insulin to endothelial cells at pH 7.8 was about 5 times greater than that

at pH 6.8 *(79)*. These results strongly suggest that the involvement of IGF-II and VEGF in angiogenesis may be greatly influenced by the intratumor pO_2 and pH. Griffiths et al. *(80)* recently reported that the expression of angiogenic enzyme platelet-derived endothelial cell growth factor (PDECGF) in a breast cancer cell line markedly increased when the medium pH was lowered to 6.3–6.7, and also when the oxygen concentration was lowered to 0.3%. It would be of interest to know how the expression of endothelium growth factors changes when the environment is both acidic and hypoxic, as inside the tumors. The acidic and basic fibroblast growth factors (aFGF and bFGF) also play major roles in proliferation and migration of endothelial cells *(81)*. The pI of aFGF is 5.0, and that of bFGF is 9.6, indicating that their activity may vary, depending on the environmental acidity.

It is known that the mitogenic response of a variety of cells to growth-stimulating agents are accompanied by an transient increase in Na^+/H^+ exchange, with a resultant increase in pH_i, which suggests that alkalization of the intracellular environment may be an initial signal for cell proliferation, as well as possible cell differentiation. In this connection, Grass et al. *(82)* reported that cellular differentiation was stimulated by agents that increase pH_i and was inhibited by agents that block the ATP-dependent H^+ pump (Na^+/H^+ ATPase). The pH_i in mammalian cells has been demonstrated to vary, depending on the stages of the cell cycle. In tumor cells, the pH_i of S-phase and G_2/M cells was slightly higher than that of G_0/G_1 cells *(83)*. Taylor and Hodson *(84)* observed that the growth rate of PMC-22 human melanoma cells in culture was normal at pH_e 7.2–6.8; the growth rate was reduced as the medium pH was lowered to below 6.8. When the medium pH was lowered to 6.7–6.4, the cells accumulated in G_1 phase, and the cell-cycle progression was completely halted as the medium pH was lowered to below 6.3. Note that the above-mentioned studies on the effect of pH on cell proliferation and cell-cycle progression pertain to tumor cells, and there have been no comparative studies for endothelial cells. Nevertheless, assuming the effect of pH_e on proliferation of endothelial cells is similar to that on tumor cells, one may surmise that moderately hypoxic and acidic intratumor environments may enhance the activity of various endothelial cell growth factors and promote proliferation of endothelial cells and angiogenesis. On the other hand, extremely hypoxic and acidic environments inhibit the proliferation of endothelial cells.

7. CONCLUSION

The extracellular environment in malignant tumors is acidic, mostly as a result of the inherent nature of malignant cells to metabolize glucose by glycolysis, and also to hypoxia caused by poor blood circulation, resulting in anaerobic glycolysis. The newly developed ^{31}P MRS method clearly demonstrates that the intracellular pH (pH_i) is considerably higher than the extracellular pH (pH_e). Such a large gradient between pH_i and pH_e is caused by effective pH_i regulatory mechanisms. The pH_i can be lowered by drastically lowering the pH_e or by interfering with the pH_i-regulatory mechanisms. An acidic environment induces cell death through apoptosis and necrosis. The effects of environmental acidity on the proliferation of endothelial cells and angiogenesis have not been elucidated, but hypoxia has been demonstrated to induce or enhance angiogenesis. In tissues, hypoxia usually exists with acidosis. These facts, along with other evidence, suggest that a moderately acidic environment may enhance the effect of various molecular factors involved in angiogenesis, and that a severely acidic environment may inhibit proliferation of endothelial cells and angiogenesis.

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5 Contribution of the Extracellular Matrix and Macrophages in Angiogenesis

Peter J. Polverini

CONTENTS

1. INTRODUCTION

The formation of new capillary blood vessels, or angiogenesis, is one of the most fundamental processes encountered in mammalian organisms *(1–4)*. Angiogenesis is driven by a diverse array of soluble mediators, matrix molecules, and accessory cells that function to coordinate the growth, differentiation, and maturation of new capillaries in a strictly defined temporal and spatial pattern. In recent years, the role of the extracellular matrix (ECM) in angiogenesis and the enzyme systems responsible for its continuous remodeling have received considerable attention *(5–9)*. In addition to providing a scaffold for the transmission of essential morphogenetic signals during capillary development, the ECM, by virtue of its ability to transmit biomechanical forces to cells, has been shown to exert complex local controls on the functions of endothelial cells. Depending on the composition and the local activity of proteolytic enzymes, the ECM is able to regulate the availability of soluble angiogenic mediators to endothelial cells and specify the nature and type of interactions that endothelial cells have with integrins and cellular adhesion molecules *(10–16)*. By exerting mechanical forces along the course of developing blood vessels, the ECM is also able to alter signaling patterns of growth and

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differentiation factors and promote coordinated changes in the endothelial cytoskeleton and nuclear architecture and function *(17–19)*.

Early studies of the ECM investing new capillaries revealed that angiogenesis was dependent on the synthesis of key extracellular matrix molecules and the precise degradation of specific components in the basement membrane *(5,19,20)*. In vitro studies of the organization of new capillaries derived from primary endothelial cell cultures or endothelial cell lines has revealed the importance of the ECM in the morphology, proliferation, cytoskeletal organization, and shape of endothelial cells *(21–23)*. The ECM transmits a number of important morphogenetic signals to endothelial cells and growing capillaries. Alterations in the secretion of collagen or the deposition and assembly of other ECM proteins has been shown to promote the regression of growing capillaries in several model systems *(24–28)*. The ECM has been shown to induce changes in gene expression and the secretory phenotype of endothelial cells during capillary morphogenesis. These include, among other changes, alterations in fibronectin, laminin, and collagen during tubular reorganization, initiation of the expression of type 1 collagen, and the upregulation of a secreted protein, acid and rich in cysteine (SPARC) *(24,29,30)*. It has been shown that in cultures of endothelial cells that spontaneously give rise to capillary-like structures, type I collagen is a necessary substrate for the attachment and spreading of endothelial cells during the formation of endothelial tubes. SPARC is thought to play an important role in inducing changes in the cytoskeleton that promote cell rounding and migration and initiate the reorganization of endothelial cells *(24,29,30)*.

The significance of these data is further supported by in vivo studies in which the systemic or local administration of pharmacological agents that interfere with ECM structure and function were shown to disrupt capillary morphogenesis. Agents that suppress collagen accumulation, crosslinking, or alter the metabolism of the ECM have been shown to interfere with the induction and maintenance of a stable endothelial tubular network *(8,28)*. In vitro models designed to investigate early events in angiogenesis have demonstrated the importance of the extracellular matrix remodeling in angiogenesis. These studies have revealed that the balanced production of degredative proteinases and their inhibitors is required for normal vascular morphogenesis and invasion. Migrating endothelial cells produce type VI collagenase and other members of the matrix metalloproteinase family *(31,32)*. Specific inhibitors of type VI collagenase, general metalloproteinase inhibitors, and serine proteinase inhibitors block endothelial invasion of the extracellular matrix *(31–35)*. Conditions that tip this balance and lead to suppression of proteolysis by decreasing protease synthesis and secretion can block angiogenesis. For example, strategies used to block TIMP transcription and translation have been shown to markedly attenuate angiogenesis in vivo *(35)*. Agents that induce angiogenesis, such as bFGF, also work through the ECM by inducing urokinase-type plasminogen activator (u-PA) as well as plasminogen activator inhibitor (PAI-1) *(34)*.

The ECM plays an important role in modulating the responsiveness of endothelial cells to endogenous growth factors. Matrix proteins, such as laminin, collagen, and fibronectin, often contain bFGF sequestered in the ECM complexed to heparin sulfate proteoglycans *(7,11)*. It has been proposed that during blood vessel injury, bFGF is released as part of a "self repair" process following injury to blood vessels. This may represent an efficient mechanism for initiating repair and angiogenesis *(7,11)*. At another level, the ECM can also serve as an effective [barrier to angiogenesis *(8)*. Degradation of the ECM by endothelial cell proteinases is thought to facilitate capillary migration; hence, there is consider-

able interest in collagenase and proteinase inhibitors as antiangiogenic therapeutic agents *(8,36)*. The angiogenic stimulator bFGF induces plasminogen activator (PA) and collagenase production by endothelial cells, suggesting that one of its major functions in angiogenesis is to stimulate ECM remodeling. On the other hand, the inhibitory effects of TGFβ on endothelial growth have been attributed to enhanced ECM production, which facilitates quantitative changes in the ECM and inhibits integrin expression *(16)*.

2. TUMOR CELLS SUBVERT HOST DEFENSES DESIGNED TO SUPPRESS ANGIOGENESIS

Tumors cells can induce angiogenesis by several different mechanisms; however, none of them are mutually exclusive. First, tumors are able to assure themselves a growth advantage by inducing angiogenesis. Expression of angiogenic activity has been shown to be an early and predictable property of many preneoplastic cells and may represent one of the earliest indications that a cell population has become committed to malignancy *(1–3)*. One of the earliest observations demonstrating the angiogenic potential of preneoplastic cells was a series of studies from Pietro Gullino's Laboratory at the National Cancer Institute. Using a series of murine breast cell lines derived from mouse strains that exhibited either a high or low incidence of spontaneous mammary tumors, these workers showed that mammary cells derived from mouse strains at risk for mammary tumors consistently were able to stimulate angiogenesis when implanted into the rabbit cornea, whereas cells derived from mice with a low incidence of spontaneous tumors exhibited little or no angiogenic activity *(37–39)*. Similarly, Polverini et al. *(40)* showed that cloned populations of preneoplastic keratinocytes derived from carcinogen-treated, nontumor-bearing hamster buccal pouches were potently angiogenic yet were not tumorigenic when injected into nude mice or neonatal hamsters. The same situation appears to hold true for some human tumors and their preneoplastic progenitors. Brem and colleagues *(37)* and Jensen et al. *(41)* examined a series of human breast lesions of varying malignant potential. They showed that there was a strong correlation between the angiogenic activity of a particular breast lesion and its malignant potential that preceded morphological and functional changes, indicative of neoplastic transformation. Chodak, in examining the urine of patients with neoplastic and nonneoplastic lesions of the bladder for the presence of endothelial-cell-migration-stimulating activity, was able to distinguish between benign and premalignant conditions as well as to detect the emergence of recurrent tumors *(42)*. Thus, it would appear that expression of angiogenic activity may be predictive of malignant potential that is acquired early in the carcinogenic process.

Second, tumors often lose the ability produce natural inhibitors of angiogenesis with or without the enhanced production of proangiogenic factors. There is now substantial evidence that one of the earliest changes that occurs in cells undergoing neoplastic transformation is loss of production of endogenous angiogenesis inhibitors *(53,54)*. In at least two instances, this loss in inhibitor activity has been directly linked to the loss or inactivation of tumor suppressor genes *(54,64)*.

Third, tumors can recruit host cells, such as macrophages, which contribute a rich array of proangiogenic cytokines to an environment already saturated with angiogenic factors. Mononuclear phagocytes are a frequent component of the stroma of neoplastic tissues *(71)*. Interest in cells of the mononuclear phagocyte system in relation to the growth of neoplasms stem largely from the observation that these effector cells, when appropri-

ately activated, are able to arrest the growth or kill neoplastic and transformed target cells. Although there is substantial in vitro evidence supporting the antitumor activity of activated Mø, the in vivo relevance of these observations has not been unequivocally demonstrated even under conditions in which Mø are likely to have antitumor activity. Mø express diverse functions that are essential for tissue remodeling, inflammation, and immunity. Analyses of tumor-associated Mø (TAM) functions suggest these multifunctional cells have the capacity to affect diverse aspects of neoplastic development, including vascularization, growth rate and metastasis, stroma development, and remodeling. There is evidence that in some neoplasms, including human cancers, the protumor functions of Mø prevail. The importance of a sustained influx of activated TAM to tumor neovascularization has been recently investigated by Lingen, Bouck, and Polverini (unpublished observations). They recently found that human squamous carcinoma treated with the chemopreventive agent retinoic acid failed to activate Mø to express angiogenic activity and exhibited a diminished capacity to stimulate chemotaxis of Mø. These results suggest that the ability of retinoic acid to reduce the incidence of secondary tumor growths may, in addition to inducing the production of an inhibitor of angiogenesis by tumor cells, block the sustained infiltration of Mø into tumors, and their subsequent activation for expression of angiogenic activity. These observations emphasize the dual potential of TAM to influence neoplastic growth and progression in opposite directions, with protumor activity often prevailing in the absence of an efficient antitumor defense mechanism.

A fourth mechanism by which tumors can elicit a sustained angiogenic response is by subverting host defenses that normally guard against aberrant angiogenesis. A good example of this mechanism is what occurs when normal endothelial cells undergo neoplastic conversion and progress toward the development of Kaposi's sarcoma (KS). KS is a complex mesenchymal neoplasm of suspected vascular endothelial cell origin *(37–40)*. It presents in several distinct pathological settings, with AIDS-associated KS being the most severe and life-threatening form of the disease. During the course of our early studies of KS carcinogenesis, we found that when HTLV-II-conditioned media was added to cultures of human umbilical vein or human dermal microvascular endothelial cells (HDMEC), within 24 h the normal epitheloid cobblestone morphology of endothelial cells changed to a "spindle"-shaped appearance with some cells demonstrating prominent dendritic processes. This "phenotypic conversion" of endothelial cells to a KS tumor phenotype not only involved a change in the morphology of endothelial cells but also a dramatic upregulation in the expression of cell surface antigens, including factor XIIIa, ICAM-1, and several cytokines, including some unique to KS tumors *(37,41)*. Upon removal of KS-conditioned media, endothelial cells rapidly reverted to their normal unstimulated phenotype. As normal endothelial cells proceed through the multistep carcinogenic process to KS they frequently begin producing elevated levels of scatter factor (SF) and express the SF receptor the c-*met* protooncogene. SF is a mesenchymal cell-derived pleotrophic mediator that may be involved in driving endothelial cells toward malignancy. We later found that the active mediator in KS-conditioned media responsible for inducing the transient "phenotypic conversion" of HDMEC to KS-like tumor cells was SF. However, the functional significance of the "phenotypic conversions" was unclear. To further define the biological implications of the KS phenotype, we examined the functional consequence of this transient conversion phenomenon *(72)*.

When normal HDMEC are exposed for 24–48 h to KS–tumor-conditioned media, they rapidly acquire the ability to potently stimulate the migration of other normal HDMEC and stimulate neovascularization in the rat cornea model of angiogenesis. We also found that pure human recombinant SF was as potent as KS-conditioned media, if not more so, in inducing HOMEC to express angiogenic activity. The role of SF in the induction of this phenotypic conversion phenomenon was also confirmed using neutralizing antibodies to SF, which abrogated most of the angiogenic activity induced by SF- or KS-conditioned media. Since expression of angiogenic activity is one of the earliest detectable changes that occurs in cells destined to become fully malignant, we speculated that the transient expression of angiogenic activity mimicked this early step, albeit transient, in KS carcinogenesis. In other experiments endothelial cells transduced to overexpress SF, and induced a vigorous angiogenic response in the rat cornea and dermal angiogenesis following transplantation into SCID mice but did not grow as a solid tumor (Liu et al., unpublished).

When "phenotypically converted" HDMEC were examined for expression of a selected panel of positive and negative regulators of angiogenesis, there was marked overexpression of a number of proangiogenic cytokines, including, among others, IL-8, SF, and VEGF (Liu et al., unpublished). Interestingly and perhaps more importantly, the level of the endogenous angiogenesis inhibitor TSP1 was markedly reduced. These results suggested that one consequence of the early conversion of normal endothelial cells to KS tumors was the loss of endogenous angiogenesis inhibitory activity. Thus, when inhibitory constraints afforded by TSP1 are removed, endothelial cells are able to express angiogenic activity without producing significantly elevated levels of proangiogenic mediators. These studies also imply that as KS tumors grow they are able to suppress TSP1 production in adjacent normal endothelial cells and effectively recruit them into the pool of stromal cells capable of augmenting KS angiogenesis.

3. MØ THAT FAIL TO SWITCH FROM A PROANGIOGENIC TO AN ANGIOINHIBITORY PHENOTYPE CONTRIBUTE TO PATHOLOGICAL ANGIOGENESIS

The angiogenic switch during tumor development, in which tumors lose their ability to produce inhibitors of angiogenesis, and thus gain the ability to stimulate angiogenesis is an emerging paradigm that is just now being validated in other angiogenesis-dependent diseases. This concept also provides a clear explanation of how angiogenesis is most likely regulated in physiological settings. There is mounting evidence that Mø, key angiogenesis accessory cells, must also undergo a similar switch if they are to effectively participate in the timely ingrowth and regression of capillaries that characterize physiological neovascularization. This phenomenon has also been reported in chronic inflammation and wound repair, where it is responsible the transient nature of granulation tissue In these cases, Mø switch from a proangiogenic to angioinhibitory phenotype, a situation that is the reverse of tumors. Therefore, one might predict that if Mø fail to undergo this conversion from a proangiogenic to an angioinhibitory phenotype, they could potentially contribute to the unwarranted angiogenesis associated with disease processes where they are a frequent participant. In the remainder of this chapter, I will provide indirect and direct evidence suggesting that when Mø fail to produce appropriate levels of the angiogenesis inhibitory ECM thrombospondin-1, they can contribute to the persistent angiogenic activity encountered in solid tumors and in the skin disease psoriasis.

Macrophages have been recognized as important angiogenesis effector cells for a number of years *(48–50)*. They have been shown to actively participate in the initiation and maintenance of wound neovascularization, where they produce a spectrum of soluble mediators capable of stimulating the growth and migration of microvascular endothelial cells. They have been shown to function during the process of tumor angiogenesis to augment both tumor growth and neovascularization *(51,52)*. Also, the persistent and unrelenting formation of granulation tissue that is a feature common to chronic inflammatory diseases, such as rheumatoid arthritis and psoriasis, are examples in which the destructive effects of persistent granulation tissue and aberrant angiogenesis are caused in large part by the promiscuous angiogenic activity of Mø *(52)*.

The macrophage may influence new capillary growth by several different mechanisms. First, Mø produce factors that act directly to stimulate angiogenesis. Macrophages are known to produce numerous growth factors, cytokines, proteolytic enzymes, and matrix molecules that stimulate endothelial cell proliferation, migration, and differentiation in vitro *(48)* and angiogenesis in vivo. A second mechanism by which macrophages might modulate angiogenesis is by modifying the ECM. The composition of the ECM has been shown to dramatically influence endothelial cell shape and morphology, and may profoundly influence new capillary growth *(9,20)*. Macrophages can influence the composition of the ECM either through the direct production of ECM components or through the production of proteases that effectively alter the structure and composition of the ECM *(51)*. A third mechanism is by producing substances that suppress angiogenesis. One of these Mø-derived inhibitors of angiogenesis that has received considerable attention in recent years in the ECM is thrombospondin-1 (TSP1).

TSP1 is one member of a family of five homologous proteins. It is a 450-kDa disulfide-linked trimer that is composed of three identical chains with a monomeric mass of about 140 kDa. Its modular structure in part enables it to interact with a variety of extracellular matrix proteins, cell surface and serum proteins, and cations. TSP1 is present in great abundance in the platelet alpha granules and is secreted by a wide variety of epithelial and mesenchymal cells *(53–57)*. It has been shown to govern the interaction of cells with their substrate and where cells have been shown to attach, spread, and migrate on insoluble TSP1 *(54,58)*. TSP1 was first implicated as an inhibitor of neovascularization when an antiangiogenic hamster protein whose secretion was controlled by a tumor suppressor gene was found to have an amino acid sequence similar to human platelet TSP1 *(60)*. Authentic TSP1 was then purified from platelets and shown to block neovascularization in vivo *(58)*. A role for TSP1 in the inhibition of angiogenesis is supported by several observations. It is present adjacent to mature quiescent vessels and is absent from actively growing sprouts both in vivo *(60)* and in vitro *(25)*. Hemangiomas, which consists of rapidly proliferating endothelial cells, fail to produce detectable TSP1 *(60)*. Antibodies to TSPI added to endothelial cell cultures enhance sprouting in vitro *(25)* and endothelial cells in which TSP1 production has been downregulated by antisense TSP1 exhibit an accelerated rate of growth, enhanced chemotactic activity, and an increase in the number of capillary-like cords *(61)*. More recently, DiPietro et al. *(64)* have shown that the addition of antisense TSP1 oligomers to wounds in the skin of mice results in delayed healing, suggesting that TSP1 is just as important in the initiation of the wound response as it is in the organization phase of wound repair. Also, Polverini et al. *(65)* has reported that mice with a targeted disruption in TSP1 show delayed wound organization, prolonged wound neovascularization, and heightened infiltration by Mø. Previous inves-

tigations have shown that both resting and activated Mø produce TSP1. DiPietro et al. *(68)* has reported an approximately sixfold increase in the steady-state levels of TSP1 mRNA expression in the murine monocyte line WEHI-3 when the cells were treated for 24 h with the potent activating agent lipopolysaccharide (LPS), with peak secretion of TSP1 protein occurring by 8 h. An examination of TSP1 knockout mice clearly demonstrated that a deficiency in TSP1 can have a subtle yet detectable effect on a physiological function, such as wound neovascularization. Also, the introduction of pure TSP1 or TSP1-expressing Mø from wild-type mice partially corrects the defect in neovascularization (Nõr et al., unpublished).

4. CHANGES IN MØ TSP1 EXPRESSION LEVELS INFLUENCE TUMOR NEOVASCULARIZATION

The importance of how alterations in the expression of TSP1 by TAM can affect tumor angiogenesis is best exemplified by recent studies by Lingen et al. *(68,69)*. These workers have demonstrated that retinoic acid, a chemopreventive agent currently used to reduce the incidence of secondary tumor growth in patients with head and neck squamous cells carcinoma, does so in part by inducing tumor cell to produce inhibitors of angiogenesis and by rendering endothelial cells refractory to proangiogenic mediators. In addition, Lingen, Bouck, and Polverini (unpublished observations) have found that human squamous carcinoma treated with the chemopreventive agent retinoic acid failed to activate Mø, resulting in a diminished capacity to express angiogenic activity in vitro and in vivo. Moreover, when these cells were phenotyped for expression of proangiogenic and angioinhibitory molecules, the most dramatic change observed was marked increase in the level of TSP1 production. These results suggest that the ability of retinoic acid to reduce the incidence of secondary tumor growths may, in addition to affecting tumor cells and endothelium, interfere with the sustained infiltration and activation of Mø into tumors, and drive TAM toward an angioinhibitory phenotype.

Another piece of evidence linking a defect in the acquisition of the angioinhibitory activity by Mø to pathological angiogenesis in the skin disease psoriasis. Psoriasis is a chronic skin disease linked to both genetic and environmental triggering factors *(72)*. It is characterized pathologically by excessive growth of epidermal keratinocytes, inflammation, and microvascular proliferation, which is believed to result from a disruption in the complex and reciprocal molecular crosstalk between activated keratinocytes and dermal cells *(73)*. Several lines of evidence have implicated psoriatic keratinocytes, inflammatory Mø, and dermal dendritic cells in the persistent vascular proliferation that accompanies this disease. Using fresh human psoriatic lesional tissue that was separated into epidermal and dermal components, the angiogenic potential of the lesion was found by two different groups to reside in both the dermal and the epidermal compartment *(74–76)*. Psoriatic keratinocytes are known to produce a variety of pro-angiogenic cytokines, such as basic fibroblast growth factor, interleukin-1 (IL-1), transforming growth factor-alpha (TGF-α), and IL-8 *(73)*. In addition to expressing several candidate mediators of angiogenesis, keratinocytes are also known to be a source of the angiogenesis inhibitor TSP1 *(78)*. In an attempt to further define the molecular mechanism underlying this chronic inflammatory skin disease, Nickoloff et al. *(77)* examined the mechanisms responsible for the disregulated vasoproliferation that characterizes psoriasis. These workers showed that psoriatic keratinocytes appeared to have a combined defect in both the overproduc-

tion of the pro-angiogenic cytokine IL8 and a deficiency in the production of the angiogenesis inhibitor TSP1. Previous studies have described differences between normal and psoriatic keratinocytes with respect to their growth response *(78)* and immunomodulating capacity *(73)*. It would appear that psoriatic keratinocytes also have a defect in which there is an imbalance in the production of positive and negative angiogenic mediators that governs the orderly growth of new capillary endothelial cells.

Direct evidence implicating inflammatory Mø and/or dermal dendritic cells in psoriatic angiogenesis are not as clear. However, Polverini and Nickoloff (unpublished data) have found that dermal dendritic cells isolated from psoriatic and symptomless skin as well as human monocyte-derived Mø and dendritic cells from normal skin when exposed to conditioned media from psoriatic keratinocytes potently express angiogenic activity, as one might predict. Interestingly, when TSP1 levels in these Mø or dendritic cells were examined and compared to dendritic cells derived from symptomless or normal skin, TSP1 was virtually undetected. These observations therefore suggest that inflammatory Mø and perhaps dermal dendritic cells can be activated by psoriatic keratinocytes to express angiogenic activity and interfere with their ability to express sufficient levels of TSP1 that would enable them to counterbalance their heightened vasoproliferative activity.

In summary, capillary growth and differentiation may be controlled in a tissue environment that contains high levels of soluble angiogenic mediators by altering either the composition or function of the ECM. Changes in ECM composition can be induced by soluble factors produced by tumor cells or host cells, such as macrophages, that alter ECM gene expression, protein synthesis, or protein secretion. ECM molecules can have different effects on capillary morphogenesis depending on the type and number of ligands that ECM binds to. Angiogenesis can be modulated by altering the ECM-cell surface interactions on focal contacts, or by reprogramming cells and driving them toward a proangiogenic phenotype. It is eminently clear that Mø can influence angiogenesis in several physiological and pathological settings. Mø have been shown to produce both stimulators and inhibitors of angiogenesis and thus have the ability to modulate angiogensis in either a positive or negative fashion. In addition, tumor cells are able to recruit and modify the angiogenic activity of Mø in a manner that will insure expression of their protumor activities. Regardless of the diverse settings in which angiogenesis is encountered and the great redundancy of mediator and ECM that participate in this process, the sorting out of the mechanisms that control the balanced production of positive and negative regulators of angiogenesis by accessory cell populations, such as Mø, is essential in order to develop novel approaches for the treatment of angiogenesis-dependent diseases, such as neoplasia and chronic inflammatory diseases.

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6 Regulation of Angiogenesis by the Organ Microenvironment

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CONTENTS

1. INTRODUCTION

Most deaths from cancer are caused by metastases that are resistant to conventional therapies *(1–3)*. The major barrier to the treatment of metastases is the biological heterogeneity of cancer cells in primary and secondary neoplasms. This heterogeneity is exhibited in a wide range of biologic characteristics, such as cell-surface receptors, enzymes, karyotypes, cell morphologies, growth properties, sensitivities to various therapeutic agents, and in the ability to induce angiogenesis, and to invade and produce metastasis *(3–6)*.

The process of cancer metastasis consists of a series of sequential steps, each of which can be rate-limiting *(3)*. After the initial transforming event, growth of neoplastic cells must be progressive. Extensive vascularization must occur if a tumor mass is to exceed 2 mm in diameter *(7,8)*. Local invasion of the host stroma by some tumor cells could occur by several mechanisms that are not mutually exclusive *(9)*. Detachment and embolization of small tumor cell aggregates occur next, and tumor cells that survive the circulation can arrest in the capillary beds of organs. Extravasation occurs next, probably by the same

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mechanisms that influence initial invasion. Proliferation within the organ parenchyma completes the metastatic process. To produce detectable lesions, the metastases must develop a vascular network, evade the host immune system *(10)*, and respond to organ-specific factors that influence their growth *(11,12)*. Once they do so, the cells can invade host stroma, penetrate blood vessels, and enter the circulation to produce secondary metastases: the so-called metastasis of metastases *(1–3)*.

2. ANGIOGENESIS IN PATHOGENESIS OF METASTASIS

The progressive growth of primary neoplasms and metastases is dependent on adequate blood supply. The process of neovascularization, i.e., angiogenesis, allows tumors to expand beyond 1–2 mm^3 *(7,8,13–15)*. With few exceptions, benign tumors are sparsely vascularized and tend to grow slowly, but malignant neoplasms are highly vascular and fast-growing *(13–15)*. The increase in vasculature also increases the probability that motile-invasive tumor cells will enter the circulation to disseminate to distant organs *(16)*. The extent of vascularization in different malignancies has been shown to correlate directly with their metastatic potential *(17–19)*.

The process of angiogenesis consists of sequential and interdependent steps. The process begins with local degradation of the basement membrane surrounding capillaries, followed by invasion of the surrounding stroma by the underlying endothelial cells in the direction of the angiogenic signal. Endothelial cell migration is accompanied by cell proliferation at the leading edge of the migrating column. The endothelial cells begin to organize into three-dimensional structures to form new capillary tubes *(7,8,13,20)*. The vascularization of many neoplasms may differ from that of normal tissues. There are differences in cellular composition, vascular permeability, blood vessel stability, and growth regulation *(8,21,22)*.

The induction of angiogenesis is mediated by positive and negative regulatory molecules released by both tumor and host cells, including endothelial cells, epithelial cells, mesothelial cells, and leukocytes *(8,13,15,20)*. The balance between regulating molecules determines the extent of vascularization *(20,21)*. Molecules that stimulate angiogenesis include members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), interleukin-8 (IL-8), angiogenin, angiotropin, epidermal growth factor (EGF), fibrin, nicotinamide, platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF), transforming growth factor-alpha (TGF-α), and members of the matrix metalloproteinase family (MMP-2, MMP-9) *(8,20)*. Negative regulatory molecules include thrombospondin, platelet factor IV, interferons, angiostatin, and endostatin *(20,23–27)*.

In quiescent normal tissues, factors that inhibit angiogenesis predominate, but in rapidly dividing tissues, the balance of angiogenic molecules favors stimulation of the process *(15,28,29)*. For example, in cultured fibroblasts, the loss of wild-type allele of the TP53 tumor-suppressor gene coincides with the acquisition of the angiogenic phenotype, and is the result of reduced production of thrombospondin-1 (TSP-1) *(30,31)*. Moreover, experiments with different transgenic mouse models have shown that angiogenesis is activated via different pathways during the preneoplastic stages that precede the appearance of solid tumors and that the mechanisms regulating the angiogenic switch may be tissue-specific *(32)*.

3. ROLE OF ORGAN ENVIRONMENT IN PATHOGENESIS OF METASTASIS

Clinical observations of cancer patients, and studies with experimental rodent tumors, have revealed that certain tumors produce metastasis to specific organs, independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ. The distribution and fate of hematogenously disseminated, radiolabeled melanoma cells in experimental rodent systems amply demonstrate that tumor cells reach the microvasculature of many organs *(33–36)*. Extravasation into the organ parenchyma and proliferation of tumor cells occur in only some organs; therefore, the mere presence of viable tumor cells in a particular organ does not always predict that the cells will proliferate to produce metastases *(33,34,37)*.

The search for the mechanisms that regulate the organ distribution of metastasis began a century ago, when, in 1889, Stephen Paget *(38)* questioned whether the pattern of metastasis was the result of chance. Paget analyzed a large number of autopsy records of women with breast cancer, and found that the pattern of visceral metastases was not random. Paget concluded that the process was not because of chance, but, rather, that certain tumor cells (the seed) had a specific affinity for the milieu of certain organs (the soil). Metastases resulted only when the seed and soil were matched *(38)*.

Experimental data supporting the "seed and soil" hypothesis were derived from studies on the preferential invasion and growth of B16 melanoma metastases in specific organs of syngeneic mice *(39)*. The B16 melanoma cells injected intravenously produced lesions in the lungs and in fragments of pulmonary or ovarian tissue implanted intramuscularly. In contrast, metastatic lesions did not develop in renal tissue implanted intramuscularly as a control, or at the site of surgical trauma *(39)*. This study confirmed that sites of metastasis are determined not solely by hemodynamic considerations, or by the characteristics of the neoplastic cells, but also by the microenvironment of the host tissue. In vitro experiments demonstrating organ-selective adhesion, invasion, and growth also support Paget's hypothesis. With the B16 melanoma system, cells with increased capacity for organ adhesion, invasion, and growth have been isolated *(40,42)*. Moreover, experiments with organ tissue-derived soluble growth factors indicate that soil factors can have profound effects on certain tumor cell subpopulations *(11)*.

A current definition of the seed-and-soil hypothesis consists of three parts. First, neoplasms are biologically heterogeneous and contain subpopulations of cells with different biologic properties *(3,43)*. Second, the process of metastasis is highly selective, favoring the survival and growth of cells that pre-exist in the parent neoplasm *(44)*. Third, the outcome of metastasis depends on multiple interactions of metastatic cells with homeostatic mechanisms, which can vary among tumors arising from different tissues, and in tumors of similar histologic origin in different patients *(45,46)*.

There is no question that the circulatory anatomy influences the dissemination of many malignant cells; however, it cannot, as Ewing proposed *(47)*, fully explain the patterns of distribution of numerous tumors. Ethical considerations rule out the experimental analysis of cancer metastasis in patients in the same way as they are studied in laboratory animals. The introduction of peritoneovenous shunts for palliation of malignant ascites has, however, provided an opportunity to study some of the factors affecting metastatic spread in humans. Good palliation with minimal complications was reported for 29 patients with different neoplasms. The autopsy findings in 15 patients substantiated the

clinical observations that the shunts do not significantly increase the risk of visceral organ metastasis. In fact, despite continuous entry of hundreds of millions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare *(48)*. These results provide compelling verification of the seed-and-soil hypothesis.

4. HOST MICROENVIRONMENT-DEPENDENT EXPRESSION OF ANGIOGENESIS

Previous studies from this laboratory and others have demonstrated that the orthotopic implantation of human renal cell cancer (HRCC) cells *(49–51)* and human colon cancer (HCC) *(52,53)* cells is associated with intense angiogenesis, invasion, and metastasis, but the ectopic implantation (subcutaneous) is not *(54,55)*.

The production of degradative enzymes is differentially regulated by the organ microenvironment *(56–58)*. HCC cells (KM12), implanted in the cecum of nude mice, produced high levels of type IV collagenase (gelatinase) and heparinase (heparan sulfate-specific endo-β-D-glucuronidase), but the same cells implanted subcutaneously did not *(56)*. Ectopic tumors did not produce visceral metastases; orthotopic tumors metastasized to mesenteric lymph nodes and to the liver. A similar increase in gelatinase activity was found, using an HRCC line (KG-2) injected into the kidney of nude mice, compared to the subcutis *(58)*. When HCC or HRCC cells were co-cultured with skin fibroblasts, their production of type IV collagenase was inhibited. Collagenase levels were not inhibited when HCC cells were co-cultured with colon or lung fibroblasts, and collagenase levels in HRCC cells were not inhibited by kidney fibroblasts *(57,58)*. Interferon-β (IFN-β) is produced by fibroblasts and differentiated epithelial cells in the skin. When HCC (KM12SM) cells were incubated in serum-free medium containing IFN-β, gelatinase activity was significantly reduced *(57)*. Similar results were obtained with HRCC cells and choriocarcinoma cells *(59,60)*. These results indicate that organ-specific fibroblasts can regulate an important step in angiogenesis and metastasis, namely, invasion.

An example of host factors regulating angiogenesis can be seen in psoriasis, a common inherited skin disease characterized by hyperproliferation of keratinocytes and excessive dermal angiogenesis. Keratinocyte conditioned media from symptomatic and psoriatic plaques induced a vigorous angiogenic response, but media conditioned from normal keratinocytes did not *(61)*. Furthermore, keratinocytes from psoriatic skin expressed a 10- to 20-fold increased level of IL-8 and a sevenfold reduction of TSP-1. These data suggest that aberrant angiogenesis in psoriatic skin might be caused by the overproduction of the positive angiogenic molecule IL-8, and by the concomitant deficiency in the negative angiogenic molecule, TSP-1.

5. ORGAN-DEPENDENT EXPRESSION OF BASIC FIBROBLAST GROWTH FACTOR BY TUMOR CELLS

This laboratory has shown that expression of basic FGF (bFGF) by tumor cells is dependent on the site of implantation. When HRCC (SN12) cells were implanted in different organ microenvironments in nude mice, the expression of bFGF was 10–20 times higher in those tumors implanted in the kidney than those implanted in the subcutaneous tissues *(51)*. The kidney tumors were more highly vascularized than tumors implanted in the subcutis. In sharp contrast, the expression of IFN-β was high in epithelial cells and fibroblasts surrounding the subcutaneous tumors, but no IFN-β was found in or

around HRCC tumors growing in the kidney. The parental cell line (SN12) and metastatic clone also differed in bFGF expression. The alteration in bFGF level by the site of implantation was caused by adaptation to the organ microenvironment, as was demonstrated when the cells were reestablished in culture, and the levels of bFGF returned to the previously in vitro concentration after 4 wk *(51)*.

Expression of bFGF in HRCC (SN12) is cell-density-dependent. By in situ mRNA hybridization (ISH) and Northern blot analysis, an inverse correlation was found between increasing cell density and bFGF expression *(62)*. Fluorescence-activated cell sorting (FACS), immunohistochemistry, and ELISA confirmed this finding at the protein level. Tumor cells harvested from dense cultures (low bFGF expression), and then plated under sparse conditions, expressed high levels of bFGF. Similar data was obtained using endothelial cells. The effect was not mediated by soluble factors released into the culture medium.

The in vitro conditions of sparse vs confluent can be argued to be irrelevant under in vivo conditions, in which almost all cells are in contact with neighboring cells. The in vivo manifestation of cell-density-dependent regulation may be interpreted as the difference in gene expression in the center of a tumor vs the periphery or leading edge. This laboratory investigated the expression of multiple metastasis- and angiogenesis-related genes, including bFGF, in patients with various stages of HCC. By ISH, the level of bFGF was significantly higher in patients with Dukes' stage C (node-positive tumors without further evidence of metastasis) and Dukes' stage D (distant metastases), than in those with Dukes' stage B disease (node-negative tumors without evidence of metastasis) *(63)*. Of particular interest was the fact that Northern blot hybridization did not detect mRNA transcripts for bFGF. Careful analysis by ISH revealed that bFGF was expressed at the highest levels in subpopulations of cells at the periphery of the tumor (invasive edge), which probably represents the most active portion of the invading tumor. A follow-up study utilized the same ISH technique to predict the disease recurrence in patients with colon cancer. The authors were able to identify patients who appeared to be free of metastasis at the time of initial surgery (Dukes' stage B), yet developed distant metastases at a later date. These patients had relatively high bFGF expression (along with increased expression of other metastasis-related genes) *(64)*. These studies demonstrate that host cells surrounding the tumor may affect angiogenesis-related gene expression, possibly by direct cell-to-cell contact or by secretion of paracrine factors.

Recent clinical observations have noted antiangiogenic effects in vascular tumors, including hemangioma *(65–70)*, Kaposi's sarcoma *(71–74)*, melanoma *(75)*, basal cell and squamous cell carcinoma *(76)*, and bladder carcinoma *(77)*, using recombinant interferons. These tumors have also been documented as producing high levels of bFGF, often detectable in the urine or serum of these patients *(78,79)*. These findings, along with in vivo observations, prompted investigation of whether IFNs could modulate the expression of the angiogenic molecule, bFGF. The authors found that IFN-α and IFN-β, but not IFN-γ, downregulated the expression of bFGF mRNA and protein in HRCC, as well as in human bladder, prostate, colon, and breast carcinoma cells *(80)*. The inhibitory effect of IFN-α and -β on bFGF expression was cell-density dependent and independent of the antiproliferative effects of IFNs *(80,81)*. The authors recently confirmed that IFN can inhibit bFGF production in an in vivo model system. Systemic administration of human IFN-α decreased the in vivo expression of bFGF, decreased blood vessel density, and inhibited tumor growth of a human bladder carcinoma implanted orthotopically in nude

mice (Dinney et al., submitted for publication). Both in vitro and in vivo, the downregulation of bFGF required a long exposure of cells to low concentrations of IFNs. In addition, when IFN was withdrawn, cells resumed production of bFGF. These observations are consistent with the findings from Ezekowitz, Mulliken, and Folkman *(65)*, in which complete regression of fatal hemangioma required daily subcutaneous injections of low level IFN-α-2a over a course of 7–8 mo.

6. ORGAN-SPECIFIC MODULATION OF IL-8 EXPRESSION IN MELANOMA CELLS

Human melanoma is an excellent example of a hematogenous malignancy. Melanoma cells secrete a variety of angiogenic molecules, e.g., VEGF, bFGF, and IL-8, and are regulated by complex interactions with keratinocytes in the skin *(82)*. Recent reports from this laboratory show that IL-8 is an important molecule in melanoma growth and progression. Constitutive expression of IL-8 directly correlated with the metastatic potential of the human melanoma cell lines tested. Further, IL-8 induced proliferation, migration, and invasion of endothelial cells, and, hence, neovascularization *(83)*. Several organ-derived cytokines (produced by inflammatory cells) are known to induce expression of IL-8 in normal and transformed cells *(83)*. Since IL-8 expression in melanocytes and melanoma cells can be induced by inflammatory signals, the question of whether specific organ microenvironments could influence the expression of IL-8 was analyzed. Melanoma cells were implanted into the subcutis, the spleen (to produce liver metastasis), and the lateral tail vein (to produce lung metastasis) of athymic nude mice. Northern blot and immunohistochemistry analyses determined that subcutaneous tumors, lung lesions, and liver lesions expressed high, intermediate, and no IL-8 mRNA and protein, respectively *(84)*. Melanoma cells established from the tumors growing in vivo exhibited similar levels of IL-8 mRNA transcripts as continuously cultured cells, thus demonstrating that the differential expression of IL-8 was not caused by the selection of a subpopulation of cells. Crossover experiments suggested that IL-8 mRNA levels were always higher in the skin and lower in the liver tumors, regardless of whether the melanoma cells had first been harvested from subcutaneous or liver tumors *(84)*.

IL-8 expression can be upregulated by co-culturing melanoma cells with keratinocytes (skin), and inhibited by co-culturing melanoma cells with hepatocytes (liver). Similar modulation of IL-8 can be seen with conditioned media from keratinocytes or hepatocytes, suggesting that soluble organ-derived factors can influence IL-8 expression *(84)*. To explore the complex situation of multiple cytokine interactions, the authors investigated the effects of two cytokines produced by keratinocytes (IL-1, IFN-β) and two cytokines produced by hepatocytes (TGF-α and TGF-β) on the regulation of IL-8 in human melanoma cells. IL-1 upregulated the expression of IL-8 in human melanoma cells at both the mRNA and protein levels in a dose- and time-dependent manner in the presence of *de novo* protein synthesis *(86,86)*. IFN-β did not affect constitutive IL-8 mRNA and protein production in human melanoma cells, but it did block the induction of IL-8 by IL-1 *(85–87)*. The authors found that TGF-β inhibited the expression of IL-8, but that TGF-α had no effect on IL-8 expression. Taken together, these data suggest that the organ microenvironment modulates the expression of IL-8 in human melanoma cells.

7. MOLECULAR DETERMINANTS OF ANGIOGENESIS IN CUTANEOUS HEMANGIOMAS

A specialized case of tumor dependence on angiogenesis can be seen in hemangiomas. In these benign tumors of capillary origin, tumor growth is absolutely dependent on capillary growth. Infantile cutaneous hemangiomas represent a unique form of pathologic angiogenesis, in which tumors grow rapidly in the first year of life (proliferative phase), followed by a slow regression during the next 5 yr (involuting phase), and eventual involution or complete regression (involuted phase) by the age of 10–15 yr. Treatment with systemic IFN-α has been shown to accelerate the involution of fatal hemangiomas *(65–70)*. This laboratory investigated whether the progression and involution of infantile cutaneous hemangiomas was associated with an imbalance between positive and negative regulators of angiogenesis, and found that proliferating hemangiomas expressed high levels of bFGF and VEGF/VGF, but not IFN-β (mRNA and protein) (Bielenberg et al., in press). The epidermis from normal individuals and the epidermis overlying involuted lesions, or at sites distant to the proliferating hemangiomas, was not hyperplastic and expressed bFGF, VEGF/VPF, and IFN-β, suggesting that the proliferating hemangiomas may induce hyperplasia in the surrounding normal tissues, which also produce bFGF and VEGF/VPF, but not IFN-β (Bielenberg et al., in press). This represents another example of the concept that neoplastic cells subvert and usurp host homeostatic mechanisms for their growth advantage.

To study the relationship between hemangiomas and the microenvironment, the authors developed an in vivo model to study transitive epidermal hyperplasia and angiogenesis using UV-B irradiation of mice. Mice exposed to 10 kJ/m^2 UV-B developed increasing epidermal hyperplasia and dermal angiogenesis and telangiectasia during the first week following irradiation, but these slowly subsided over the following 7 wk. The authors investigated the production of positive and negative regulators of angiogenesis in this model, and found that the first striking event following UV-B irradiation was the increase in production of bFGF in the keratinocytes of the epidermis (Bielenberg et al., in press). The increase in bFGF proceeded, or at least coincided with, the division of epidermal cells, recognized by immunohistochemical staining with antibodies to proliferating-cell nuclear antigen (PCNA). Days 3–7 following UV-B irradiation showed marked hyperplasia and angiogenesis. The expression of VEGF/VPF was slightly increased by d 5. The expression of IFN-β in the epithelium decreased as the epidermis became increasingly hyperplastic, but was again expressed as the hyperplasia and angiogenesis subsided (Bielenberg et al., in press). Whether expression of IFN-β caused the eventual decrease in epidermal and endothelial cell proliferation remains unclear.

8. MODULATION OF ANGIOGENESIS BY HOST LYMPHOID CELLS

The regulation of physiologic angiogenesis by lymphoid cells is well established. Angiogenesis is influenced by cytokines released from mast cells, T-lymphocytes, and macrophages in the tumor microenvironment *(88–95)*. Mast cells have been associated with disease progression in infantile cutaneous hemangiomas *(96)*. In fact, the presence of elevated numbers of mast cells has been used to distinguish hemangiomas from vascular malformations *(97)*. Lymphoid-mediated angiogenesis has been recognized in cutaneous melanoma. Increased vascularity at the vertical base of human melanoma is associated with poor prognosis *(98)*. A local inflammatory reaction is often associated

with invasive cutaneous melanoma. An intense inflammatory reaction is often associated with increased risk of metastasis, suggesting that inflammatory-associated angiogenesis may contribute to melanoma dissemination *(99,100)*. This laboratory has shown an important role for host-infiltrating leukocytes and their products in the induction of tumor neovascularization. The authors investigated angiogenesis around melanomas growing subcutaneously and their correlation with tumor size in normal and myelosuppressed mice. The subcutaneous growth of weakly immunogenic B16 melanoma cells was slow in myelosuppressed mice, and neovascularization was likewise low. The reconstitution of myelosuppressed mice with normal splenocytes resulted in rapid vascularization around the tumor implants, and then rapid growth of the tumors *(101)*. Whether this lymphoid-mediated angiogenesis was caused by the production of angiogenic molecules is still unclear.

A more recent study of HCC specimens examined the role of infiltrating cells in angiogenesis. VEGF expression, vessel counts, and metastasis were found to correlate with stage of disease in HCC *(102)*. HCC patients with low expression of VEGF, but high vessel counts, were found to contain infiltrating macrophages and lymphocytes expressing PD-ECGF *(103)*. The intensity of staining for PD-ECGF in infiltrating cells may contribute to angiogenesis in HCC and may provide a redundant mechanism for tumor neovascularization.

8. ANTIANGIOGENIC ACTIVITY OF INTERFERON-β

The IFN family consists of three major glycoproteins that exhibit species specificity: leukocyte-derived IFN-α, fibroblast-derived IFN-β, and immune-cell-produced IFN-γ. Although IFN-α and IFN-β share a common receptor (the type I IFN receptor) and induce a similar pattern of cellular responses, certain cellular reactions can be stimulated only by IFN-β, probably by the phosphorylation of a receptor-associated protein that is uniquely responsive to IFN-β *(104)*. Although IFNs originally captured interest as antiviral agents, it has become increasingly clear that IFNs regulate multiple biological activities, such as cell growth *(105,106)*, differentiation *(107)*, oncogene expression *(108,109)*, host immunity *(110–112)*, and tumorigenicity *(113–118)*. A growing body of evidence has implicated IFNs as inhibiting a number of steps in the angiogenic process. IFN has antiproliferative properties, especially on tumor cells *(119–121)*, and this effect has also been demonstrated on endothelial cells in vitro *(122–125)*. IFN-α inhibited FGF-induced endothelial proliferation *(122)*, and IFN-γ inhibited ECGF-induced endothelial proliferation *(123)*. IFN-α and IFN-γ were cytostatic to human dermal microvascular endothelial cells *(124)* and human capillary endothelial cells *(125)*. The mechanisms by which IFNs exert their direct antiproliferative effects are not clearly understood, but they appear to lengthen all phases of the cell cycle, and deplete essential metabolites, such as ornithine decarboxylase *(118)*.

The antiangiogenic effect of IFNs cannot be explained solely on the basis of antiproliferation of endothelial cells. IFN-α/β inhibited endothelial cell and tumor cell migration *(126,127)*. Subcutaneous injection of IFN-α/β adjacent to a wound delayed the healing process by inhibiting the proliferation, migration, and invasion of many cell types, including capillary buds, fibroblasts, and epithelium *(128,129)*. IFN-α/β injected intratumorally or peritumorally into tumor cells resistant to the antiproliferative effects of IFN resulted in damaged tumor blood vessels, which disrupted blood flow and led to

ischemia and necrosis *(129)*. Sidky and Borden *(25)* demonstrated that IFN-α/β can inhibit tumor-induced and lymphocyte-induced angiogenesis. Specifically, murine IFN-α/β inhibited vascularization of murine tumor cells, and human IFN-α/β suppressed vascularization of human tumor cells in nude mice, thus demonstrating that IFN-α/β can inhibit angiogenesis without a direct cytostatic effect on murine endothelial cells, possibly by suppressing tumor cell-produced angiogenic factors *(25)*. As mentioned previously, this laboratory has gone on to show that IFN-α/β can directly or indirectly affect the expression of several angiogenic factors, including bFGF *(80,81)*, IL-8 *(85)*, and collagenase type IV *(57,58)*, but a direct effect of IFN-α/β on the expression of VEGF has not been seen.

This laboratory recently demonstrated that IFN-β gene therapy can eradicate tumor cells of various histological origin, and found that the sustained local production of murine IFN-β could inhibit the tumorigenicity and metastasis of human and murine tumor cells implanted into nude mice *(130)*. All human tumor cell lines transfected with the murine IFN-β gene grew well in vitro, but none grew in vivo. IFN-β-transfected cells could prevent the outgrowth of parental or control-transfected cells when injected at the same site, but not when injected at distant sites, suggesting that IFN-β promoted a local lysis of the bystander cells *(130)*. Similar results were found when murine UV-2237m fibrosarcoma cells were infected with the murine IFN-β gene, using a retroviral vector. The transduced UV-2237m IFN-β cells did not grow in syngeneic mice when injected either subcutaneously or intravenously, but did produce small tumors in nude mice and in SCID/Beige mice (Dong et al., in press). Further analysis revealed that IFN-β-transduced cells were sensitive to natural killer (NK)-cell-mediated lysis, and that NK cell selective antiserum (antiasialo GM1) could partially abrogate the cytotoxic activity of these cells in syngeneic C3H/HeN mice. All transfected and transduced cells were able to stimulate a high level of nitric oxide in murine macrophages, which correlated with the vigorous antitumor activities. Therefore, the local production of IFN-β can suppress tumorigenicity and metastasis, in part, because of the activation of host-effector mechanisms.

In order to evaluate the effect of IFN on angiogenesis specifically, the authors transfected a human metastatic bladder carcinoma (253J-BV^R, resistant to the antiproliferative effects of IFN) with the human IFN-β gene, and injected the cells orthotopically into nude mice. Although parental cells and control-transfected cells were able to grow rapidly and produce highly vascular tumors that metastasized to regional lymph nodes, the IFN-β-transfected cells were weakly tumorigenic (Bielenberg et al., unpublished data). In vitro, IFN-β-transfected cells constitutively produced less bFGF and collagenase type IV than parental or control-transfected cells. These experiments suggest a causal role for IFN-β in suppressing tumor-derived angiogenic factors and thus inhibiting angiogenesis and metastasis.

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7 Fibroblast Growth Factors in Tumor Progression and Angiogenesis

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CONTENTS

1. INTRODUCTION

Based on their wide spectrum of target cells, FGF family members have been suspected of playing important roles in many biological events. Some members of the FGF family are potent mitogens for a large number of cell types of the mesodermal and neuroectodermal lineage, and thus could be responsible for the proliferative stimulus in tumors derived from these cells. At the same time, some of the FGFs are mitogenic for endothelial cells, and are capable of promoting tumor angiogenesis. It is therefore difficult to separate the functional role of FGFs in tumorigenesis from their role in tumor angiogenesis.

This chapter reviews the current knowledge about the FGF family members and their receptors, discusses the role of FGFs in the autocrine and paracrine stimulation of tumor cell proliferation and in tumor angiogenesis, and describes potential pathways that may inhibit FGF activity.

2. FGF FAMILY MEMBERS AND INVOLVEMENT IN TUMOR PROGRESSION

Members of the FGF family are structurally related, with up to ~55% sequence identity at the amino acid level. They are also highly conserved between species. All FGFs are 18–30-kDa proteins with high heparin-binding affinity, which has given them their early family name of heparin-binding growth factors (HBGF) *(1)*. The genes for FGFs are all structured in three exons separated by two introns exhibiting dramatically different length

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within the family members; the total length of the introns is 1.1 kb in the FGF-4 gene, 19 kb in the FGF-5 gene, and more than 30 kb in the FGF-2 gene. The coding regions are highly conserved within a central core region, but the more N-terminal and C-terminal regions have diverged. FGFs are mitogens for a wide variety of cells of mesodermal and neuroectodermal origins, and the various FGF family members exhibit diverse biological activities by targeting a large number of different cell types. They have been implicated in many physiological and pathological processes, including embryonic development, wound healing, angiogenesis, differentiation, neuronal outgrowth, cell survival, migration, invasion, and transformation *(1,2).* Some of the features of members of the FGF family are summarized below.

2.1. FGF-1 and FGF-2

FGF-1 (acidic FGF) is a 18-kDa, 154-amino acid protein, first identified as a mitogen in neural tissue extracts and in bovine brain. It was named acidic FGF because of its acidic pI value of 5 *(1)*. Most curiously, FGF-1 does not have a signal peptide for secretion. It is found in the nucleus of many cells, and a putative nuclear localization signal has been located at amino acids 21–27 *(3)*. Heparin dramatically stimulates the mitogenic activity of FGF-1 on target cells by stabilizing the relatively labile FGF-1 in an active conformation, and protecting it against denaturation by heat, extreme pH, and proteolysis *(4)*. The resolution of the crystal structure of FGF-1, and peptide competition experiments, revealed that heparin binding and receptor binding are mediated by different domains of the molecule *(5)*.

FGF-1 is a potent inducer of DNA synthesis, proliferation, cell survival, differentiation, and cell migration in a large number of normal diploid cell types and established cell lines of mesodermal origin *(2)*. It may play an important role in the autocrine and paracrine growth regulation of tumor cells, and in angiogenesis in vitro and in vivo.

FGF-2 (basic FGF) was initially purified from bovine pituitary, and, based on the pI of 9.6, it was named basic FGF. Subsequently it was also purified from many other tissues. FGF-2 is a 18-kDa, 155-amino acid protein. FGF-2, like FGF-1, does not have a signal sequence for secretion (reviewed in refs. *1* and *2*). However, N-terminal extended isoforms of approx 22, 23, and 25 kDa mol wt have been described. The 18 kDa form of FGF-2 is localized to the cytosol, but these high-mol wt forms are predominantly associated with the nucleus because of a nuclear localization signal in the alternatively expressed N-terminal region *(6)*. FGF-2 has a higher affinity for heparin than FGF-1; however, unlike FGF-1, heparin does not exhibit a stimulatory effect on the activity of FGF-2. Resolution of the crystal structure of FGF-2, and peptide competition experiments, localized the receptor binding site in two regions distinct from the heparin binding sites *(5,7)*.

Many tumors and tumor cell lines express FGF-2 as an autocrine growth regulatory factor. FGF-2 has essentially the same target cell types as FGF-1 *(1,2)*. The only exception may be melanocytes, which respond to FGF-2, but not to FGF-1 *(8)*. Based on its pleiotropic activities, FGF-2 has been proposed to be involved in many different physiological and pathological processes, including embryonic development, cell differentiation, cell survival, angiogenesis, and tumorigenesis.

2.2. Transforming Potential of FGF-1 and FGF-2

Many experiments investigating the transforming potential of FGF-1 and FGF-2 have been performed; however, a clear correlation between export of FGF-1 and FGF-2 and

transformation was not apparent *(2,9)*. For example, expression of the original FGF-2 coding sequence lacking a signal peptide in Balb/c 3T3 fibroblasts or BHK cells, resulted in transformation of the cells, and the detection of FGF-2 in the culture supernatant. However, transformation was not affected by neutralizing antibodies to FGF-2, indicating that the FGF-2 activation occurred within the cells *(10)*. Forced secretion of FGF-2, by fusing a signal peptide onto the N-terminus of FGF-2, resulted in complete autocrine transformation of NIH 3T3 fibroblasts, as assessed by growth in soft agar, focus formation, and tumorigenicity and metastasis in athymic mice *(9,11,12)*. Transformation could not be inhibited by neutralizing antibodies, but was reversed by suramin, a polyanionic drug known to inhibit growth factor–receptor interaction *(9)*. Similar results were obtained with FGF-1 *(13)*. FGF-1, fused to a signal peptide, was readily secreted by the transfected NIH 3T3 cells, resulting in transformation of the cells, and the formation of highly vascularized tumors when injected into athymic mice. It appears that wild-type FGF-1 and FGF-2 by themselves transform cells only when expressed at high levels. In contrast, acquisition of a signal peptide converts FGF-1 and FGF-2 into highly transforming proteins analogous to other members of the FGF family. Consistent with these findings, the deletion of the signal sequence from FGF-4 significantly diminished its transforming activity *(14)*.

2.3. FGF-1 and FGF-2 in Tumor Development

Extensive studies on the expression of FGF-1 and FGF-2 in tumor biopsies in vivo together with functional studies on tumor cell proliferation in vitro, provided a plethora of evidence for the involvement of FGF-1 and FGF-2 in tumor development. In some cases, expression of FGF-1 or FGF-2 correlated with malignancy, and interference with the activities of FGF-1 or FGF-2 diminished tumor growth and cell transformation *(15)*. FGF-1 and FGF-2 were significantly upregulated in human gliomas, proportional with the degree of malignancy *(15)*. Furthermore, the malignant phenotype of glioblastoma cell lines could be inhibited by FGF-2-specific antibodies, indicating that FGF-2 utilized an autocrine pathway to promote tumor progression *(16)*. FGF-2 and sometimes FGF-1 are constitutively expressed at high levels in melanomas, but not in normal melanocytes *(8)*. Proliferation and growth in soft agar was inhibited by antisense oligonucleotides to FGF-2 or FGFR-1 in melanoma cell line, indicating that FGF-2 might be a major autocrine growth factor for melanoma cells *(17)*. In Kaposi's sarcoma (KS), FGF-2 and sometimes FGF-1 were detectable in tissue specimen and KS-derived cell lines. In fact, proliferation, angiogenesis, and lesion formation by KS-derived cell lines in athymic mice could be prevented by treatment with antisense oligonucleotides to FGF-2 *(18)*.

Elevated expression of either FGF-1 or FGF-2 has been demonstrated in a variety of other tumor types in vivo, and in tumor cell lines in vitro. In some cases, FGF expression correlated significantly with advanced stages of tumor development and malignancy (reviewed in refs. *1* and *2*). Examples include the expression of FGF-2 and FGF-1 in squamous cell carcinoma, human leukemic cells, human bladder carcinoma, human pancreatic carcinoma, human ovarian cancer, and gastric carcinoma; and the expression of FGF-2 in pituitary tumors, renal tumors, mammary carcinoma cell lines, prostate carcinoma cell lines, colon carcinoma cell lines, hepatoma cell lines, chondrosarcoma cell lines, osteosarcoma cell lines, and the expression of FGF-1 in rat Morris hepatoma cell lines *(19)*.

2.4. Other Species of FGF

FGF-3 was originally identified as a result of the insertion of mouse mammary tumor virus next to the FGF-3 gene, causing transcriptional activation of FGF-3 and tumor induction *(20)*. Because by the insertion event, the gene was named int-2. It was later discovered that int-2 had 44% identity to FGF-2, and it was incorporated as FGF-3 into the FGF family *(20)*. Secretion of FGF-3 is rather inefficient, and it has been shown that immature forms of FGF-3 accumulated in the Golgi, from where they were only slowly released into the extracellular matrix. FGF-3 was shown in transgenic mouse models to induce the development of mammary hyperplasia and neoplasia and prostate hyperplasia *(21)*. However, in most cases, FGF-3 by itself caused only the development of hyperplastic lesions, which spontaneously regressed, indicating that additional events are required for complete tumor progression. Such a synergistic cooperation in the progression to full malignancy has been demonstrated between FGF-3 and FGF-4 in transgenic mice *(22)*. Consistent with its function in transgenic mice, amplification of the gene for FGF-3 was found in a proportion of human breast cancer biopsies and squamous cell carcinoma of head and neck *(23)*. In addition, FGF-3 was expressed at elevated levels in a significant number of KSs *(24)*.

FGF-4 was discovered by its transformation capability as hst-1 in DNA derived from human stomach cancer, colon carcinoma, and hepatoma *(25)*, and as KFGF in DNA extracted from KS lesions *(26)*. The N-terminal region (30–31 amino acids) contains the signal peptide that is cleaved, resulting in an efficiently secreted protein of 175 or 176 amino acids. Together with N-linked glycosylation, the mol wt is approx 22–23 kDa. FGF-4 has been demonstrated to play a pivotal role in limb development, myogenesis, and tooth development. Transfection and high expression of FGF-4 leads to autocrine transformation of cells via FGF receptors on the cell surface. Furthermore, gene amplification and/or upregulated expression of FGF-4 has been found to correlate with the malignant phenotype in many types of cancer cells, suggesting that FGF-4 plays a major role in oncogenic transformation and tumor progression. Expression of FGF-4 in NIH 3T3 fibroblasts completely transforms the cells, as assayed by increased motility, invasiveness, and malignancy in vitro and in vivo *(27)*. Similarly, upregulated expression of FGF-4 induced spontaneous metastasis in MCF-7 breast carcinoma cell lines *(28)*. Retroviral transfer of FGF-4 into mice induced the development of soft tissue sarcomas and meningeal tumors *(29)*. In cancer patients, FGF-4 is expressed in a number of tumors, including teratocarcinomas and germ cell tumors *(30)*. Amplification of both the genes for FGF-3 and FGF-4 was found in mammary tumors and squamous cell carcinomas, with a frequency of 20% *(2)*. FGF-4 is mitogenic for vascular endothelial cells, and is angiogenic in vitro and in vivo. Furthermore, NIH 3T3 fibroblasts transfected with FGF-4 appeared to develop highly vascularized tumors in immunodeficient mice *(31)*. FGF-4 might thus be involved in the regulation of tumor angiogenesis *(31)*.

FGF-5 was identified by transformation of NIH 3T3 cells with DNA extracted from human bladder tumors *(32)*. The core region of FGF-5 exhibits 50% identity to FGF-2. FGF-5 is a protein of 267 amino acids, and N-linked, and possibly O-linked glycosylation result in a secreted protein of 32.5–38.5 kDa mol wt *(32)*. High levels of FGF-5 expression have been found in patients with human bladder carcinoma, hepatoma, endometrial carcinoma, and KS *(33)*.

FGF-6 is structurally very similar to FGF-4, with 70% identity at the amino acid level. FGF-6 is secreted as a glycosylated protein of 25 kDa *(34)*. Little is known about the

expression of FGF-6, or about its biological role. The human gene for FGF-6 was cloned by virtue of its transformation activity as hst-2 *(34)*. FGF-6 has the ability to transform NIH 3T3 fibroblasts, as determined in focus-forming assays in vitro, and in athymic mice in vivo. FGF-6 is expressed in some KS lesions *(33)*. However, not much more is known about its possible involvement in carcinogenesis.

FGF-7 (keratinocyte growth factor) was isolated as a mitogen for cultured keratinocytes *(35)*. It consists of 194 amino acids: The 64 amino acids at the N-terminus are unique, and the remainder exhibits 30% identity to FGF-3 *(35)*. It has a hydrophobic signal sequence, and is efficiently secreted as a 28 kDa, *N*-glycosylated protein. FGF-7 is mitogenic for many epithelial and stromal cells, but exhibits only little activity on mesenchyme-derived cell lines. The highest mitogenic activity is found with keratinocytes. FGF-7 is highly upregulated upon wounding of the skin, suggesting that it is a major component of the wound healing process *(36)*. Its expression and role in human cancers have not been reported yet.

FGF-8 (androgen-induced growth factor) was purified and cloned from the androgen-dependent mouse mammary carcinoma cell line SC3 *(37)*. It is a polypeptide chain of 215 amino acids in length, 30–40% identical to the other FGF family members. FGF-8 carries a signal peptide, and is efficiently secreted as 28 and 32 kDa proteins. FGF-8 is an autocrine growth factor for androgen dependent mammary carcinoma cells *(37)*.

FGF-9 (glia-activating factor) has been purified and cloned from human glioma cell lines and rat brain *(38)*. Its transforming potential has not been reported. The amino acid sequence of FGF-9 in the conserved core region is approx 30% identical to the other family members, and is conserved to 94% between human and rat. FGF-9 lacks a consensus signal peptide, yet is found to be secreted by transfected COS and CHO cells. Proteins with mol wt of 30, 29, and 25 kDa have been purified from the culture medium.

3. FGF RECEPTORS

Two types of FGF binding sites have been identified *(39,40)*. High affinity receptors for FGF represent low-capacity (10^3–10^5 receptors per cell), but high-affinity binding sites (K_d of ~20–600 p*M*). They are typical members of the transmembrane tyrosine kinase family of receptors. Thus far, at least four members have been identified and cloned; however, an enormous diversity of alternative mRNA processing possibilities results in different extracellular domains with variable ligand specificity and affinity. Alternative splicing also produces soluble forms of some of the high-affinity FGF receptors.

Low-affinity binding sites have been characterized that exhibit high capacity ($1–2 \times 10^6$ sites per cell), but low affinity for ligand binding (K_d of ~2–200 n*M*). Low-affinity receptors for FGF are predominantly proteoglycans carrying heparan sulfate side chains that bind FGFs. They sequester and present FGFs to their high-affinity receptors, but are unable to activate a signal-transduction cascade on their own *(4)*.

3.1. High-Affinity FGF Receptors

The first receptor for FGF was purified and cloned as a high-affinity receptor for FGF-2 from chicken *(41)*. This receptor, FGF receptor-1 (FGFR-1), was highly homologous to the flg tyrosine kinase previously cloned from human endothelial cells. Subsequently, FGFR-1 was shown to bind FGF-1, FGF-2, and FGF-4 *(42)*. FGFR-2 was first identified by screening of a mouse liver expression library with antiphosphotyrosine antibodies,

and was called bek *(43)*. Subsequent molecular cloning of human, murine, and chicken homologs to bek revealed that it is a receptor for FGF-1, FGF-2, and FGF-4 *(40)*. FGFR-3 was cloned from human leukemia cell lines *(40,44)*, and turned out to be highly homologous to the previously cloned orphan tyrosine kinase receptor cek-2. FGFR-4 was also cloned from human leukemia cell lines *(44)*, and was shown to bind FGF-1 and FGF-6 with high affinity, FGF-4 with lower affinity, and FGF-2 with an even lower affinity *(40,45)*.

The overall structure of the four members of the FGFR family is identical, and can be summarized from N- to C-terminus as follows: signal peptide, two or three extracellular immunoglobulin-like loops (Ig domains), characteristic acidic region between first and second Ig domain, transmembrane domain, cytoplasmic domain with the catalytic tyrosine kinase domain split by a 14-amino acid kinase insert, and a carboxy-terminal tail *(40)*. All four receptors are highly homologous to each other, with 70–80% amino acid identity in the ligand-binding domains (Ig loops II and III), and in the tyrosine kinase domain. Other less conserved regions still exhibit 50–60% homology *(44)*. Complex alternative splicing, combined with alternative polyadenylation, creates a high diversity in receptor isoforms for FGFR-1 to -3, but not for FGFR-4, resulting both in receptors with distinct, and others with redundant, functions, ligand specificities, and signal transduction pathways *(39,40)*.

One of the more general splicing variations found in FGFR-1, FGFR-2, and FGFR-3 affects the FGF-binding domain and, thus, the ligand specificity of the receptors. Alternative usage of exons IIIb and IIIc, encoding the alternative second halves of the third Ig domain, creates different membrane spanning forms. In comparison to FGFR-1 containing the Ig loop IIIc, use of Ig loop IIIb reduces the affinity to FGF-2 about 50-fold; the affinity to FGF-1 is not affected *(42)*. Use of a polyadenylation site preceding these alternative exons produces a receptor form that lacks transmembrane and cytoplasmic domains, resulting in a soluble receptor (IIIa; *40*). This soluble form of FGFR-1 is functional in that it can bind FGF-1 and FGF-2 *(40)*. With FGFR-2, this alternative splicing event creates a dramatic difference in ligand-binding specificity: Use of Ig loop IIIc results in a receptor that binds FGF-1 and FGF-2 with high affinity, but not FGF-7 (KGF). FGFR-2 containing Ig loop IIIb binds FGF-7 (KGF) with very a high affinity and FGF-1 and FGF-2 with 50-fold lower affinity. Thus, two growth factor receptors with different ligand specificities are encoded by alternate transcripts from the same gene *(40,46)*.

Many other alternative products have been described. For example, three truncated extracellular domain forms of FGFR-1 have been identified as soluble FGF-binding proteins in blood, and in the extracellular matrix and basement membrane of vascular endothelial cells. Their biological function, however, remains unclear *(47)*. Specific cleavage of FGFR-1 by gelatinase A (MMP-2) yields a soluble ectodomain containing the three Ig domains, and is capable of binding FGF-1 and FGF-2 (*48*; Fig. 1).

Although the four members of the FGFR family are very similar to each other in activity, and are expressed in partially overlapping patterns during embryonic development, inactivation of one receptor results in a very severe phenotype, indicating that FGFRs function in a nonredundant manner, and exert very specific activities. Recently, unique mutations in FGFRs have been shown to be associated with human skeletal disorders, suggesting an important role for FGFRs in bone development. Data from many experiments suggest that the high-affinity receptor complex is an intimate ternary complex of the transmembrane tyrosine kinase receptor, heparan sulfate glycosaminogly-

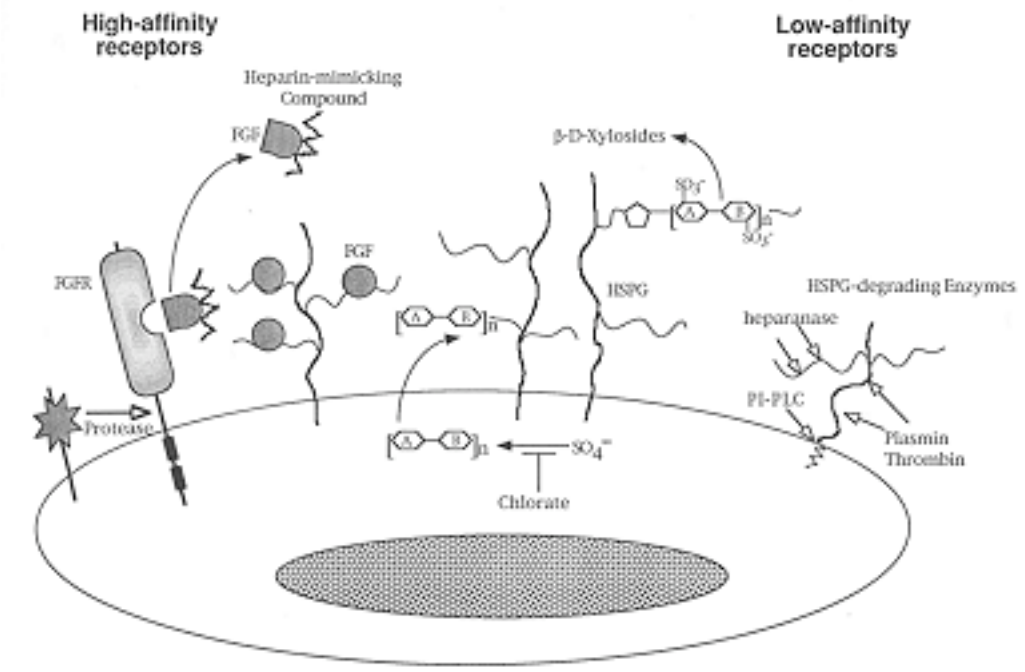

Fig. 1. Modulation of FGF-2-receptor binding and activation by metabolic inhibitors of HS synthesis and sulfation, HS-degrading enzymes, and heparin-mimicking compounds. Modulation of FGF-2 binding to low-affinity cell-surface receptor sites can be brought about by enzymes that degrade the HS side chains (e.g., heparanase) or core protein (e.g., plasmin, thrombin, PI-PLC) of HSPG (right), and by soluble primers (β-D-xylosides) of HS synthesis, and metabolic inhibitors (e.g., chlorate) of sulfation (center). Binding of FGF-2 to high-affinity cell-surface receptor sites can be modulated by heparin-mimicking compounds (i.e., compound RG-13577) that compete with HS, bind the growth factor, and prevent receptor binding and/or dimerization, and by proteolytic enzymes (e.g., MMP2) that cleave the ectodomain of the receptor (left).

cans, and FGF ligands. Together, these components define ligand specificity and binding affinity *(40,42,49)*.

3.2. FGF Receptors and Tumorigenesis

Similar to the FGF ligands, expression of FGFRs is significantly upregulated in a variety of cancer types. For example, the genes for FGFR-1 and -2 were found to be amplified and overexpressed in a subset of breast cancers. In these cases, amplification of FGFR-2 significantly correlated with amplification of the gene for c-myc; amplification of FGFR-1 correlated with the amplification of the genes for FGF-3 and FGF-4 *(50)*. The gene for FGFR-2 was found to be amplified in stomach cancer, in which it was isolated as K-sam *(40)*. FGFR-1 expression was found to be upregulated in glioblastoma; FGFR-2 was present in white matter and low-grade astrocytomas, but absent in glioblastoma. Indeed, during progression to the most malignant phenotype of glioblastoma, a gradual shift from FGFR-2 to FGFR-1, with two or three Ig domains, was observed *(51)*. Expression of both FGFR-1 and FGFR-2 was found to be upregulated in gliomas and meningiomas *(19)*. Inhibition of FGFR-1 expression by antisense oligonucleotides resulted in the inhibition of melanoma proliferation *(17)*.

Elevated expression of FGFR-2 IIIb and IIIc were reported in endometrial adenocarcinoma and FGFR-1 and FGFR-2 in KS *(19,33)*. The gene for FGFR-4 appeared to be amplified in human breast and gynecological cancers *(52)*. All four members of the FGFR family are expressed by the MCF 7 breast carcinoma cell line, and in a number of leukemia cell lines *(39)*. However, stimulation of the receptor with FGF-1 or FGF-2 resulted in growth inhibition, rather than stimulation of proliferation. In conclusion, members of the family of FGFR are expressed at elevated levels on the surface of tumor cells in vivo, and on tumor cell lines in vitro. Upregulated expression of FGF receptors is frequently found in tumor cells concomitant with the expression of FGF ligands, indicating that many tumor cells utilize FGF ligands and FGFRs in an autocrine pathway of transformation and proliferation.

3.3. Low-Affinity FGFR

FGF-2 has been localized to the extracellular matrix, basement membrane, and surface of endothelial cells, and other cell types (reviewed in refs. *4* and *53*). Low-affinity binding sites for FGF have been biochemically characterized as heparan sulfate proteoglycans. The core proteins of some of these receptors (i.e., syndecan, perlecan, glypican) for FGF have been isolated and cloned *(54)*. By binding to the heparan sulfate moiety, FGFs are sequestered on the cell surface and extracellular matrix (ECM) *(53)*. Heparan sulfate side chains are specific for this binding; other glycosaminoglycans, such as dermatan sulfate, keratan sulfate, or chondroitin sulfate, do not bind FGFs *(54)*. Binding of FGFs to heparan sulfate results in protection and storage of FGF in the extracellular matrix, and in basement membranes *(53)*. Heparin, heparan sulfates, and heparitinase are able to release stored FGF in vitro and in vivo from extracellular matrix, basement membranes, and cell surfaces *(4,53)*.

3.4. High- and Low-Affinity FGFR Cooperativity

Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues *(55)*. Besides serving as a scaffold for the attachment of various ECM components (e.g., collagen, laminin, fibronectin), the binding of HS to certain proteins induces a conformational change that may lead to the exposure of novel reactive determinants, or, conversely, may stabilize an inert protein configuration. Of particular significance is the interaction of HS with FGFs, mediating their sequestration, stabilization, and high-affinity-receptor binding and signaling *(56–60)*. Cellular responses to FGFs may hence be modulated by metabolic inhibitors of HS synthesis and sulfation, HS-degrading enzymes, and synthetic mimetics of heparin/HS that compete with HS on FGF-2 binding, but fail to present the bound FGF-2 to its high-affinity signaling receptor *(61–63)* (Fig. 1).

Examining the role that cell- or ECM-associated HSPG plays in mediating the biological activities of FGFs is difficult, because all cells that express FGFR also display HSPG on their surface or ECM. Therefore, to better assess the involvement of HS, in FGF signaling, it was necessary to remove the HS or to impair its ability to interact with FGFs. This was brought about by HS-degrading enzymes or metabolic inhibitors of HSPG synthesis and sulfation (*57,61,63*; Fig. 1). Under these conditions, FGF-2 exhibited little or no binding to its high-affinity receptors, and the biological activity of FGF-2 was impaired. FGF-2 receptor binding and response were rescued by the addition of exogenous soluble heparin or heparin sulfate *(57–60)*. Also, HS-deficient lymphoid cells were

transfected with individual FGFRs, and the ability of these receptors to bind FGFs, and to mediate their biological activities, was examined in the absence and presence of exogenously added heparin or HS *(60)*. Thus, transfection of FGFR-1 or FGFR-2 resulted in cells that are growth responsive to FGF-1 and FGF-4, but only in the presence of heparin *(60)*. These experiments could also be reproduced in cell-free systems using soluble extracellular domains of the FGFR, HSs, and FGF ligands *(49,57,58,60,62)*. Other studies showed that heparin/HS increases the affinity of FGF-2 for FGFR-1 by a factor of 2–3, but is not essential for the interaction itself *(64)*. This contention is in contrast with the prevailing opinion, but in agreement with previous studies that show FGF-2-receptor binding in the absence of heparin *(65)*. Heparin was also not required for FGF-2-mediated c-fos induction, but was necessary for stimulation of DNA synthesis and cell proliferation *(64)*, suggesting that heparin/HS principally stabilizes the FGF molecule, and hence affects primarily long-term effects of the growth factor. It is also possible that the reduced ability of FGF-2 to bind to its high-affinity receptors is a result of damage occurring during purification of the growth factor and/or its radiolabeling *(64)*, and that part of the effect of heparin is apparently because of restoration of the biological activity of the damaged growth factor *(65)*. Among the various species of HSPGs, glypican exhibits a novel type of modulatory activity that is manifested by its ability to exert a dual mode of action. It stimulates receptor binding of FGF-1 and FGF-2, but inhibits the biological activity of FGF-7 *(66)*.

Collectively, these results have been put forward in a dual-receptor model *(56,59)*. According to this model, FGF is sequestered in the extracellular matrix, basement membrane, or on the cell surface through binding to HS side chains. The large number of low-affinity, but high-capacity, receptors leads to an accumulation of FGF ligands on the cell surface, resulting in the delivery of FGFs to the high-affinity, but low-capacity, FGFRs. It was proposed that the primary function of the low-affinity receptors is to reduce the dimensionality of ligand diffusion from three to two dimensions *(67)*. In other words, binding of fluid-phase ligands, such as FGF-2, to abundant low-affinity cell-surface receptors, which are restricted to lateral mobility in two dimensions, increases the local concentration of the bound ligands, thereby enhancing the probability of their interaction with the less-abundant high-affinity signaling receptors *(67)*. Because the binding sites on FGF for HS, and for the signaling receptor, are distinct, dissociation from the low-affinity HS receptors may not be necessary for ligand binding to the high-affinity receptor, resulting in the formation of a ternary complex. Formation of such a complex may be needed for receptor dimerization and activation *(57)*. Two potential mechanisms have been proposed that may mediate the cooperativity among HSs, FGF ligands, and FGFRs. First, heparin could induce a conformational change in the ligand and/or in the receptor to bring about ligand–receptor binding (induced-fit model). In a second model, heparin would induce oligomerization of FGF, which might be important for receptor dimerization and activation *(60)*. The latter model is supported by the binding of several FGF-1 molecules to heparin and heparin sulfate (one molecule every 4–5 saccharide units). The resulting oligomerized FGF-1 complexes bind to several receptor molecules, leading to receptor dimerization, transphosphorylation, and signal transduction *(62,68)*.

On the basis of these considerations, and regardless of the exact dual-receptor mechanism of action, inhibition of FGF-2 interaction with low-affinity HS accessory receptors is likely to suppress FGF-2 mediated cell proliferation and neovascularization. In fact, heparin/HS degrading enzymes (bacterial heparinase I and III), or polyanionic com-

pounds that compete with heparin/HS, were shown to inhibit FGF-2-driven endothelial cell proliferation and neovascularization (*62,69*; Fig. 1). Perlecan, a secreted HSPG abundant in basement membranes, is a major accessory receptor for FGF-2, capable of inducing FGF-receptor interactions in vitro and angiogenesis in vivo *(70)*. Autocrine and paracrine functions of FGF-2 were suppressed by stable expression of a construct expressing antisense RNA to perlecan in mouse fibroblasts and human melanomas *(71)*.

3.5. Signal Transduction, Internalization, and Subcellular Localization

Binding of FGFs to their high-affinity receptors causes the activation of the intrinsic tyrosine kinase activity and a cascade of events, leading eventually to the induction of immediate early gene transcription, and to cell proliferation. Like all transmembrane tyrosine kinase receptors, FGF receptors dimerize upon ligand binding, and transphosphorylate at tyrosine residues *(40)*. Heterodimerization between different FGF receptors and heterologous transphosphorylation between FGFR-1 and FGFR-2 have been demonstrated, indicating that hetero- and homodimers of the types 1-1, 2-2, and 1-2 exist *(40)*.

Upon binding to their receptors, FGF-1 and FGF-2 are rapidly internalized, with concomitant downregulation of the surface receptors. However, they are not degraded through the lysosomal pathway like other growth factors; instead, fragments of various sizes are stabilized and accumulate within the cell *(72)*. FGF-2 is internalized by using both pathways, i.e., via binding to low-affinity receptors in the absence of high-affinity receptors, or by binding to high-affinity receptors in the absence of low-affinity receptors *(73)*. Some of the exogenously added FGF-1 and FGF-2 that is internalized upon binding to its receptors is taken up into the nucleus, where it resides within the nucleolus, the nucleoplasmic network, and the nuclear chromatin *(74)*. How FGF-1 or FGF-2 enter the nucleus, and how their nuclear translocation affects signal transduction, remains to be elucidated. HSPGs are known to cycle from the cell surface to the nucleus, suggesting that fragments of HS may act as natural chaperones to shuttle FGF-2 and other growth factors to different cellular compartments.

4. FGFS IN TUMOR ANGIOGENESIS

Formation of new blood vessels from pre-existing blood vessels (angiogenesis) is a multistep process. Stages in this process include the activation of quiescent endothelial cells in a pre-existing vessel, degradation of the basement membrane, migration of endothelial cells into the interstitial space and sprouting, endothelial cell proliferation at the migrating tip, lumen formation, generation of new basement membrane with the recruitment of pericytes, formation of anastomoses, and, finally, blood flow *(75)*.

FGF-1 and FGF-2 are mitogenic for endothelial cells and stimulate endothelial cell migration *(1,2,75)*. They induce endothelial cell production of collagenase and plasminogen activator that are capable of degrading basement membranes *(76)*. FGF-1 and FGF-2 also cause endothelial cells to migrate and form capillary-like tubes in three dimensional culture systems in vitro *(77)*. Both FGF-1 and FGF-2 exhibit potent angiogenic activity in all in vivo assays tested. In fact, they are usually employed in these bioassays as positive references. Endothelial cells derived from large or small vessels are not only a target for FGF-2, but they also synthesize high amounts of FGF-2 in vitro *(78,79)*. Most of the FGF-2 produced by the cells is found associated with the extracellular matrix and subendothelial basement membrane *(53)*. Spontaneous migration of

endothelial cells was inhibited by neutralizing antibodies to FGF-2, suggesting an autocrine role of FGF synthesized and released by the endothelial cells themselves *(76)*. A similar autocrine role of endothelial cell-produced FGF-2 has been shown to promote tube formation in collagen gels *(77)*.

Angiogenic sprouting from rat aorta specimen, cultured in collagen, was stimulated by addition of exogenous FGF-2, and was inhibited by neutralizing antibodies to FGF-2 *(80)*. Apparently, FGF-2 was released by experimental wounding, and the released FGF-2 mediated autocrine stimulation of angiogenesis after injury. FGF-2, packaged into polymer-based pellets and implanted under the kidney capsule, in the mouse cornea, the periadventitial space of rat carotid artery, or in the rat mesenteric window, caused a dose-dependent stimulation of angiogenesis *(81)*. The results summarized above clearly demonstrate that FGF-1 or FGF-2 are able to directly induce angiogenesis in vitro and in vivo. However, it is not known how FGFs interact with the target endothelial cells in vivo, or which of their pleiotropic activities is actually required for the induction of angiogenesis in vivo. For instance, it has not been demonstrated that FGF-1 or FGF-2 are directly involved in physiological or pathological angiogenesis, such as ovulation, implantation, wound healing, or vascular disease. Most of the accumulated data has been based predominantly on expression studies that attempted to correlate expression of FGFs with angiogenic processes. Although the involvement of members of the FGF family in embryonic development is undisputed, a functional role for FGFs in developmental angiogenesis has not been unequivocally demonstrated. Conclusions are based chiefly on the spatial and temporal correlation of FGF expression and neovascularization.

Since FGF-2 and, to a lesser extent, FGF-1 are expressed by many tumors in vivo, and by tumor cell lines in vitro, it has been assumed that they were responsible for the induction of angiogenesis during tumor progression. This assumption, however, is oversimplified, since, in some cases, other angiogenic factors, such as VEGF, have been shown to play a lead role. In some instances, expression of FGF-1 or FGF-2 in vivo correlates with the degree of vascularity of the tumors. However, at the same time, FGFs have other target cell specificities. In most cases, the targets are the tumor cells themselves, or cells within remodeling stroma, leading to an autocrine or paracrine stimulation of tumor-cell proliferation. Therefore, it has been difficult to dissect the pleiotropic activities of FGFs in functional terms, and to assess their actual role in tumor angiogenesis in vivo.

In order to induce tumor angiogenesis, FGFs produced by the tumor cells need to be released to bind FGF receptors on the surface of endothelial cells. However, both FGF-1 and FGF-2 lack a signal sequence for secretion, and, although several mechanisms for their release from cells have been proposed, their export pathway has not been elucidated (*see* Section 5.3.). It is possible that FGFs are produced by cells other than tumor cells. For example, an increase in FGF-2 synthesis in the tumor vasculature has been observed, indicating that endothelial cells themselves could be a source of FGF-2 *(81)*. Tumors also recruit macrophages, and activate them to secrete FGF-2 *(82)*. Furthermore, mast cells may also be recruited by tumors. They are loaded with heparin, which they might release in the tumor, and amplify the effects of FGF-1 or FGF-2 *(83)*.

In many naturally growing, as well as in experimentally induced, tumors, the extent of vascularization correlates with the expression of angiogenic FGF family members. For example, neural transplants that have been retrovirally transfected with the FGF-4 gene exhibit abundant capillary proliferation, and induce the formation of capillary angio-

sarcomas, suggesting that FGF-4 may have a direct role in angiogenesis and endothelial cell transformation *(84)*. Moreover, in glioblastoma and meningioma, the levels of expression of FGF-2 and FGFR-1 correlate not only with tumor cell proliferation, but also with vascularity of tumors *(15)*. FGF-1 and FGF-2 have been found to be highly expressed in KS lesions of AIDS patients. Indeed, FGF-2 injected into nude mouse elicited the formation of KS-like lesions regarding the extent of vascularization, the morphology of proliferating cells, and general pathology. In these experiments, FGF-2 synergized with the Tat gene product of human immunodeficiency virus and caused KS-like lesions. Fibronectin could replace Tat, suggesting that FGF-2 induced the lesions, and that Tat enhanced its activity by mimicking the effect of ECM molecules *(85)*.

Quantification of angiogenic proteins in the blood and urine of cancer patients may measure progression of disease and guide therapy. Based on detection of endothelial cell stimulators in the urine of cancer patients *(86)*, an immunoassay for FGF-2 was developed that revealed elevated levels of the angiogenic protein in the serum of patients with renal cell carcinoma *(87)*. Elevated levels of FGF-2, but not FGF-1, were found in the serum and urine of bladder and hepatocellular carcinoma patients, with the highest levels in patients with active metastatic disease *(87,88)*. High levels of FGF-2 were found in the serum of approx 10% of a wide spectrum of cancer patients *(89)*, and in the urine of more than 37% of cancer patients *(90)*. Biologically active FGF-2 was abnormally elevated in the CSF of children with brain tumors, but not in children with hydrocephalus or malignant disease outside of the CNS *(91)*. The FGF-2 level in CSF correlated with microvessel density in histologic sections, which itself provided a prognostic indicator of risk of mortality *(91)*. Also, FGF-2 levels in the urine of children with Wilms' tumor correlated with stage of disease and tumor grade *(92)*. In some tumors, tissue levels of angiogenic proteins have correlated with severity of disease or outcome. Immunohistochemical levels of FGF-2 in renal carcinoma correlated with the risk of death *(93)*. FGF-2 mRNA was detected in 80% of patients with renal cell carcinoma. FGF was suggested to induce microvessel tube formation in these patient tumors *(94)*. In infants with hemangiomas, urinary FGF-2 levels were abnormally elevated, and returned toward normal with involution of the lesions. In life-threatening hemangiomas treated with IFN-α-2a, quantification of urine FGF-2 has been a useful way of determining an effective dose, and of differentiating between hemangioma and vascular malformation *(95)*.

In an experimental setting, Balb/c 3T3 cells that expressed a mutant, but active, form of FGF-2 were transplanted into athymic mice, resulting in elevated levels of the mutant form of FGF-2 in the urine of tumor bearing mice *(96)*. These experiments suggest that the source of circulating FGF in the serum and urine originated from the tumor itself. However, in cancer patients, in addition to the export of FGF-2 from tumor cells, FGF-2 may also be mobilized from extracellular matrix by tumor-derived heparinases or collagenases. FGF-2 and other angiogenic peptides could also be released from host cells, such as macrophages, recruited into the tumor *(82)*. Another puzzle is why FGF-2 remains elevated in the serum of tumor patients when it is normally cleared within approx 30 min after intravenous injection *(97)*. The normal clearance mechanisms for FGF-2 *(98)* may be saturated or disturbed in cancer patients, but these systems remain to be studied. Circulating FGF-2 may be bound to soluble receptors. It is not known whether abnormally elevated levels of circulating FGF-2 maintained over prolonged periods of time may potentiate growth of dormant metastases. The presence of FGF-2 in body fluids of cancer patients and experimental animals correlated significantly with the extent of

vascularization and metastasis, suggesting that expression and release of FGFs might be involved in tumor angiogenesis. Based on these results, the detection of FGF in the urine of cancer patients might be a useful diagnostic and prognostic tool. The results also indicate that FGF-2 is released by producer cells, despite the lack of a signal peptide.

5. REGULATION OF FGF ACTIVITY

5.1. Inhibition of FGF Activity

Angiogenic growth factors, like FGF-2 and VEGF165, require interaction with HS in order to induce a proliferative signal through tyrosine kinase receptors. This represents a novel target for inhibiting growth factors involved in angiogenesis. Compounds that block the interaction of these angiogenic factors with HS were found to block endothelial cell proliferation and angiogenesis in vivo.

Suramin, a polyanion, disrupts binding of FGFs to their low- and high-affinity receptors *(99)*. It has been used in clinical trials on cancer patients, with some promising effects. However, suramin is not specific for FGFs and also interferes with binding of several other growth factors to their receptors. Also, treatment of KS derived cells with suramin or protamine had no inhibitory effect. Instead, it resulted in the upregulation of FGF-2, FGF-5, and FGF receptor, consistent with clinical observations that suramin caused stimulation of KS growth in patients *(100)*. A limitation on the clinical use of suramin is the narrow margin between the dose required to achieve antitumor effect and that leading to the onset of prohibitive toxic side effects. Several polysulfonated naphthylureas, with structures related to suramin, effectively blocked FGF-2-stimulated growth of capillary endothelium in vitro and FGF-2-driven angiogenesis in vivo *(101)*. These, together with their lower toxicity, offer the opportunity of widening the suramin therapeutic window.

Pentosan polysulfate, another polyanionic heparin analog, has been reported to inhibit proliferation of tumor cell lines derived from breast carcinoma, prostate carcinoma, lung carcinoma, epidermoid tumors, and rhabdomyosarcomas, in culture and in athymic mice *(102)*. Human adrenal cancer cell lines that have been transfected with FGF-4 were dramatically inhibited in their proliferation and tumor formation by pentosan polysulfate, when suramin or dextran sulfate exhibited only a slight inhibitory effect *(103)*. However, treatment of HIV-associated KS in patients with pentosan polysulfate did not reveal a significant tumor response *(104)*. The antitumoral effect of suramin, pentosan polysulfate, laminarin sulfate, and other polysulfated compounds was also attributed to their inhibition of heparanase activity, and hence tumor metastasis *(105)*.

Tecogalen (DS4152), a low-mol-wt peptidoglycan extracted from the bacterial wall of the bacterium *Arthrobacter,* appears to inhibit angiogenesis by interfering with binding of FGF-2 to endothelial cells. It is currently being evaluated in a phase I clinical trial for the treatment of refractory malignancies, including breast, lung, and head and neck cancers. In addition to its antiangiogenic activity in vivo, it also has antitumor activity in vitro and in vivo *(95,106)*.

5.1.1. Heparin-Mimicking Compounds

In an attempt to identify potent mimetics that can modulate abnormal FGF signaling, the authors have synthesized a series of negatively charged, nonsulfated aromatic compounds that mimic many of the effects of heparin *(62,107,108)*. These nontoxic,

nonsulfated polyanionic compounds were found to compete with HS on the cell surface and ECM, on FGF-2 binding (Fig. 1). Compound RG-13577 (polymer of 4-hydroxyphenoxy acetic acid and formaldehyde ammonium salt, Mr ~5,800) and related compounds were also found to revert the transformed phenotype of FGF-2-transfected cells through a disruption of FGF-2-mediated autocrine loop *(107)*. Direct interaction between compound RG-13577 and FGF-2 was suggested by the ability of the former to release FGF-2 from ECM or heparin-Sepharose *(107)*, and to compete with heparin on binding to FGF-2 *(62)*. Compound RG-13577 also inhibited FGF-2-receptor binding, as demonstrated in crosslinking experiments *(62)*. Unlike heparin, compound RG-13577 alone failed to induce dimerization of FGF-2. Moreover, it abrogated the dimerizing effect of heparin *(62)*. Similarly, dimerization of FGFR on the surface of HS-deficient CHO cell mutants was prevented when the cells were incubated with FGF-2 and heparin in the presence of excess compound RG-13577. These effects were associated with a profound inhibition of FGF-2-mediated signal transduction (tyrosine phosphorylation) and proliferation of vascular endothelial and smooth muscle cells *(62,108)*. Moreover, the antiangiogenic effect of compound RG-13577 was demonstrated by its ability to inhibit the outgrowth of microvessels from rat aortic rings embedded in a collagen gel *(62)*. Synthetic polyanionic compounds that block the interaction of FGFs and other heparin-binding growth factors with HS may therefore provide a potential avenue for pharmacological intervention with undesirable effects of heparin-binding growth factors in processes such as tumor angiogenesis, diabetic retinopathy, and restenosis of balloon injured blood vessels *(63)*.

Biological activities of FGFs can also be modulated through the respective high-affinity receptors. One approach is the utilization of dominant-negative receptor constructs, which, when co-expressed with FGF receptors, can block activation and signal transduction. Some of these tools have already been employed to study the functional role of FGFs and their receptors in airway epithelia development, and in wound healing *(109,110)*. In a similar manner, NIH 3T3 cells transformed by FGF-4 were suppressed by expression of tyrosine kinase-deficient dominant negative FGFRs *(111)*.

Another approach to interfere with tumor growth is the ligand-specific targeting of toxins to tumor cells expressing FGFRs. For example, a fusion protein consisting of FGF-2 and saporin, a cell toxin isolated from the plant *saponaria*, was specifically targeted to FGFRs, and exhibited antitumor activity in vitro and in vivo *(112)*. Recombinant versions of these fusion proteins expressed in *Escherichia coli* inhibited growth of B16-F10 melanoma cell lines in vitro, and retarded tumor growth and metastasis in vivo *(113)*. Alternatively, fusion of FGF-1 to Pseudomonas exotoxin A resulted in specific cytotoxicity to a variety of tumor cell lines expressing FGFRs, including those of prostate, colon, or breast carcinoma *(114)*. Systemic application in athymic mice grafted with several different tumor cell lines slowed tumor growth, but did not induce complete regression. Probably, the exotoxin attacked the tumor mass, rather than the tumor endothelium *(114)*. Pseudomonas exotoxin FGF-1 fusion proteins also inhibited endothelial cell proliferation and tube formation in vitro, by inducing dose-dependent cell death *(115)*.

Other inhibitory compounds have been developed that bind and inactivate FGF ligands. However, their specificity toward FGFs and/or other growth factors is not yet established. For example, using systemic evolution of ligands by exponential enrichment (SELEX), high-affinity RNA ligands to FGF-2 have been isolated that inhibit binding of FGF-2 to its receptor *(116)*. It has also been reported that phosphothioate oligodeoxynucleotides

bind to FGF-1, -2, and -4, thereby preventing their binding to FGFRs, and removing FGF ligands from low-affinity binding sites on the cell surface and ECM *(117)*. Based on a multitude of expression studies in tumor biopsies and cell lines, it is well accepted that FGFs and their receptors appear to play a role in the growth regulation of many cancers. Experiments utilizing tools that interfere with FGF ligand and receptor activity clearly demonstrated such a functional role. However, better and more specific reagents need to be developed in order to efficiently interfere with either tumor cell proliferation or tumor angiogenesis.

5.2. Modulation of Angiogenesis by ECM Constituents and Cell Matrix Interactions

Formation of a new microvessel requires a number of interactions that must be coordinated in a spatially and temporal manner. These adhesion events are mediated by endothelial cell-adhesion molecules and ECM molecules that provide instructions to the endothelial cells as they migrate into the perivascular space, and assemble into new vessels with surrounding pericytes. ECM components synthesized by endothelial cells (i.e., collagens, laminin, thrombospondin, fibronectin, secreted protein acidic and rich in cysteine [SPARC]) function to regulate endothelial cell growth, migration and shape *(118)*.

Integrins have been shown to function in vivo in vasculogenesis and angiogenesis. Injection of a neutralizing antibody against the β_1-subunit blocked formation of an aortic lumen in quail embryos *(119)*. Brooks et al. *(120)* have provided evidence that $\alpha_v\beta_3$ is required for blood vessel growth. An antibody (LM609) against the $\alpha_v\beta_3$ integrin complex inhibited normal vessel growth, and also FGF-2-stimulated or tumor-induced angiogenesis in the CAM assay, but did not disrupt pre-existing vessels. The mechanism by which anti-$\alpha_v\beta_3$-mAb disrupts angiogenesis appears to involve apoptosis. A single intravascular injection of a cyclic RGD peptide antagonist of $\alpha_v\beta_3$ integrin, or of the LM609 monoclonal antibody, led to the rapid regression of human tumors transplanted into the CAM *(121)*. Although antibody against $\alpha_v\beta_3$ inhibited angiogenesis induced by FGF-2, anti-$\alpha_v\beta_5$-antibodies inhibited angiogenesis induced by VEGF, suggesting that integrins used to produce new blood vessels can differ, depending on the angiogenic stimuli *(122)*.

In vitro studies suggest that endothelial cells use collagen fibrils to fold and align into tube structures. Co-expression of type I collagen and the calcium-binding protein SPARC is initiated when bovine aortic endothelial cells undergo sprouting angiogenesis *(123)*. Type VIII collagen is synthesized during sprouting, and has been localized to proliferating endothelial cells within endothelial cords *(124)*. It was also reported that, when endothelial cells are exposed to angiogenic hyaluronic acid (HA) oligosaccharides, synthesis of both type I and type VIII collagen is upregulated four- to sixfold within 12 h *(125)*. Several modulators of collagen metabolism were examined *(126)*. Regression of growing capillaries in the chick embryo was induced by proline analogs, such as L-azetidine-2-carboxylic acid, *cis*-hydroxyproline, *d*L-3,4,dehydroxyproline, and thioproline. These compounds and *a*,*a*-dipyridyl, an inhibitor of prolyl hydroxylase, all interfere with triple helix formation, and prevent collagen deposition. β-Aminopropionitrile, an inhibitor of collagen crosslinking, was also antiangiogenic *(127)*. These general inhibitors of collagen formation also inhibited tumor growth in mice, but were too toxic for long-term safe administration. A low mol wt (495), nontoxic, anticoccidial quinoazolinone derivative (halofuginone) specifically inhibits collagen type αl(I) gene

expression and synthesis *(128)*. Recent studies indicate that halofuginone also inhibits endothelial cell proliferation and tube formation *(*rat aortic ring assay), as well as FGF-2-driven angiogenesis (mouse corneal micropocket assay) and tumor formation in vivo (Miao et al., manuscript in preparation).

5.3. Export of FGF-1 and FGF-2

FGF-1 and FGF-2 lack a signal sequence, and the molecular pathway by which these growth factors are secreted from the cytoplasm to the extracellular space is not known *(129)*. Two steps for the release of FGF-1 and FGF-2 from cells have to be considered: first, the actual translocation through the plasma membrane into the extracellular space; and, second, the release of FGF-1 and FGF-2 from their sequestration sites in the extracellular matrix and basement membranes.

Several reports provide evidence that export of FGF-1 and FGF-2 may involve a novel secretory pathway. Stably transfected NIH 3T3 cells, which expressed high levels of FGF-1, released inactive homodimers of FGF-1 on heat shock, in a process that required *de novo* synthesis of protein. This export was not prevented by Brefeldin A and methyl amine, inhibitors of ER-Golgi transport and exocytosis, respectively *(130)*. Active export of FGF-2 in unusual high mol wt forms has been reported in cell lines derived from different stages of fibrosarcoma development in transgenic mice carrying the bovine papilloma virus genome *(131)*. Cell lines established from the β-cell tumors of Rip1Tag2 transgenic mice (βTC) constitutively secreted FGF-1 into the culture medium (*131a*). Treatment of this medium with high salt recovered FGF-1 as high mol wt (HMW) forms with reduced heparin-affinity and a molecular mass of approx 40 kDa. Brefeldin A, an inhibitor of conventional secretion, did not interfere with FGF-1 export by β-tumor cells. In other established tumor cell lines, including human breast carcinoma and murine fibrosarcoma, a similar export of FGF-2 as HMW forms, with reduced heparin affinity, was detected. This did not hold true for normal or premalignant cell lines that expressed high levels of FGF-2 *(131a).* Collectively, these data strongly suggest that a similar, nonconventional pathway is utilized by different cell lines for the export of FGF-1 and FGF-2. Alternatively, FGF-1 could be bound as a heterodimer to another unknown partner to produce the observed HMW forms. One candidate for a partner protein that could associate with FGF-1 and FGF-2, to produce the ~40 kDa HMW forms, is a recently discovered FGF binding protein (FGF-BP) *(132)*. Tumor cell growth and angiogenesis could be enhanced using gene transfection approaches to upregulate FGF-BP in tumor cells *(132)*. Moreover, suppression of FGF-BP expression significantly blocked the growth of squamous cell carcinoma and colon carcinoma in nude mice *(133)*. These data suggest that FGF-1 and FGF-2 are selectively exported by many tumor cell types, and also by normal cell types, under stress conditions or during physiological angiogenesis via a nonconventional secretory pathway. A switch in the subcellular localization of FGF may be a general mechanism of tumor promoted angiogenesis.

5.4. Extracellular Sequestration and Release of FGF-2

Another major issue associated with the release pathway of FGF-1 and FGF-2 is the nature of their sequestration by HSPG in the extracellular matrix and basement membrane of endothelial cells from different sources *(79,134–136)*. The model in which FGFs might be stored in a latent form that can be locally activated by heparin or heparanase,

or by exposure to their high-affinity receptors, offers an attractive possibility for the regulation of FGF activities in normal and pathological situations.

The authors' studies on the control of cell proliferation by its local environment focused on the interaction of cells with the ECM produced by cultured corneal and vascular endothelial cells *(53,79)*. This ECM closely resembles the subendothelium in vivo in its morphology and molecular composition. Vascular endothelial cells, plated in contact with the subendothelial ECM, no longer require the addition of soluble FGF in order to proliferate *(79)*. This observation, together with the presence of HS as a major glycosaminoglycan (GAG) in the subendothelial ECM, raised the possibility that ECM contains heparin-binding growth factors that are tightly bound and stabilized by the ECM-HS. Indeed, FGF-2 was extracted from the subendothelial ECM produced in vitro *(79)* and in vivo *(136)*, suggesting that ECM may serve as a reservoir for FGF-2. Immunoreactive FGF-2 was identified in BM of the cornea *(136)* and in BM underlying endothelial *(137)* and epithelial *(138)* cells. It was suggested that intracellular FGF may be released into the ECM in response to mild cell damage and certain stress conditions associated with tissue injury, irradiation, inflammation, shear force, heat shock, and tumor necrosis. High amounts of FGF-2 are contained in platelets and macrophages. Since these cells release their entire cell constituents upon activation, it is conceivable that FGFs are released by these cells during inflammation, hypoxia, or ischemia. The released factor may then be sequestered from its site of action by means of binding to HS *(139)*, and possibly to FGF-2 receptor proteins in the ECM *(47)*, and saved for emergencies, such as wound repair and neovascularization *(53,140)*. It appears that FGF-2 binds primarily to HS in ECM and basement membrane, since the majority of the bound growth factor was displaced by heparin, HS, or HS-degrading enzymes (i.e., heparanase), but not by unrelated GAGs or GAG-degrading enzymes *(139–141)*.

The involvement of sulfate groups in FGF-2 sequestration by the subendothelial ECM was studied by growing the ECM-producing cells in the presence of chlorate, a potent inhibitor of sulfation *(61)*. Both the FGF-2 content and growth-promoting activity of sulfate depleted ECM were less than 10% of native ECM, indicating that sulfate moieties of HS are involved in FGF-2 sequestration and growth promoting activity of the ECM. FGF-2 is also sequestered by HS on cell surfaces, as revealed by immunohistochemistry *(137)*, release by glycosyl phosphatidylinositol specific phospholipase C (PI-PLC) *(142,143)*, and displacement by heparin from the luminal surface of blood vessels *(144)*. Heparanase, an endoglycosidase that specifically degrades HS, was found to be a most efficient specific releaser of active FGF-2 from ECM *(145)*. The authors' studies suggest that heparanase activity expressed by metastatic tumor cells and activated cells of the immune system may not only function in cell migration and invasion, but at the same time may elicit an indirect neovascular response by means of releasing the ECM-resident FGF *(53,140)*. Apart from HS-degrading enzymes, active FGF-2 is released from ECM by thrombin *(146)* and by plasmin *(147)*, as a noncovalent complex with HSPG.

Despite the ubiquitous presence of FGF-2 in the ECM and basement membranes of tissues, EC proliferation in these tissues is usually very low, with turnover time measured in years. This raised the question of how FGF-2, and possibly other growth factors, are prevented from acting on the vascular endothelium continuously, and in response to what signals do they become available for stimulation of capillary EC proliferation? One possibility is that FGF-2 may be stored in a latent inactive form or bound to truncated high-affinity FGF receptors, identified in the BM of retinal vascular endothelial cells

(47). Restriction of FGFs in ECM and BM prevents their systemic action on the vascular endothelium, thus maintaining a very low rate of EC turnover and vessel growth. On the other hand, release of FGF-2 from storage in ECM may elicit a localized EC proliferation and neovascularization in processes such as wound healing, inflammation, and tumor development *(53,140,148)*.

Based on a recent study on the involvement of FGF-BP in tumor growth and angiogenesis *(133)*, it is conceivable that, during tumor progression, FGF-BP expression is upregulated, and the protein is secreted into the microenvironment. There it can bind FGF-2 molecules that are inactive because of their immobilization within the ECM. The displaced (soluble), and now biologically active FGF-2 molecules are free to mediate various functions, such as angiogenesis *(149)*. In other words, secretion of FGF-BP by tumors may flip the angiogenic switch during tumor progression. It should also be noted that, under the normal in vivo situation, the lack of EC proliferation in response to ECM-resident FGF-2 may simply be caused by the closely apposed and contact inhibited configuration of the cells. Once the cells are released from contact inhibition (i.e., in response to stress conditions and tissue injury), cells that remain bound to the ECM, but are no longer growth arrested, become susceptible to stimulation by the ECM-bound FGF-2, until they regain their characteristic contact inhibited cobblestone morphology.

6. CONCLUDING REMARKS

FGF-2, one of the first identified stimulators of angiogenesis, initially occupied a center stage in the angiogenesis field, but was edged out by VEGF. The lack of a characteristic signal sequence for secretion, and the low expression of FGF-2 receptors, raised doubts about its role as a major, direct acting angiogenic growth factor *(149)*. Nevertheless, elevated levels of soluble FGF-2 have been detected in the serum and urine of many cancer patients, and FGF-2 has been shown to be a potent inducer of angiogenesis in a variety of experimental systems.

This review has focused on the potential functional role of members of the FGF family and their receptors in tumor-cell proliferation, in physiological and pathological angiogenesis, and in tumor angiogenesis. Most, if not all, members of the FGF family seem to play an important role in tumor-cell proliferation in cancer patients, or in experimental systems. FGF-1, FGF-2, and FGF-4 are clearly angiogenic in vitro and in vivo. However, despite a vast and extreme experimental effort, their detailed functional role in physiological angiogenesis, and in tumor angiogenesis, remains to be elucidated. Recent studies on the involvement of FGF-BP in tumor growth and angiogenesis may explain earlier results, which demonstrated that angiogenesis is associated with a switch to the extracellular export of FGF-2 in the multistep development of fibrosarcomas in transgenic mice *(150)*. Clearly, these observations do not diminish the importance of VEGF. Several studies indicate that FGF-2 and VEGF cooperate in various ways to stimulate angiogenesis. For example, FGF-2 induces the expression of VEGF, and the two growth factors act synergistically, not just additively, to stimulate tumor angiogenesis *(151–153)*. Thus, targeting one may be tantamount to targeting the other.

Members of the FGF family exhibit a confusing multitude of biological activities, and, for this reason, their distinct functions on particular target cells cannot be easily identified in vivo. The situation becomes even more complicated by other growth factors that share some of the activities and target cells of FGFs. Novel experimental strategies need to be

utilized, including genetic manipulation in transgenic mice or the development of highly specific inhibitors for growth factor and growth-factor receptor activities, in order to clarify the detailed biological role of FGFs. Some of these tools are now available, and are also being used to unravel the functional role of FGF family members in tumor development, and, particularly, in tumor angiogenesis.

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8 Role of Vascular Endothelial Growth Factor in Regulation of Angiogenesis

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CONTENTS

1. INTRODUCTION

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, and so on *(3,4)*. The negative regulators so far identified include thrombospondin *(5,6)*, the 16-kDa N-terminal fragment of prolactin *(7)*, angiostatin *(8)*, and endostatin *(9)*.

This chapter discusses the molecular and biological properties of the vascular endothelial growth factor (VEGF) proteins. Over the past few years, several members of the VEGF gene family have been identified, including VEGF-B, VEGF-C, placenta growth factor, and VEGF-D *(10)*. This chapter focuses primarily on VEGF, referred to also as VEGF-A. Work done by several laboratories has elucidated the pivotal role of VEGF and its receptors in the regulation of normal and abnormal angiogenesis *(10)*. 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 *(11,12)*. Furthermore, VEGF-induced angiogenesis has been shown to result in

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a therapeutic effect in animal models of coronary *(13)* or limb *(14)* ischemia, and, more recently, in humans *(15)*.

2. 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 *(10)*. VEGF promotes angiogenesis in tridimensional in vitro models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures *(16)*. Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel *(17)*. VEGF also elicits a pronounced angiogenic response in a variety of in vivo models, including the chick chorioallantoic membrane *(18)*, and so on.

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 *(19)*. Moreover, VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells, but not in dermal fibroblasts *(20)*. Other studies have shown that VEGF promotes expression of urokinase receptor (uPAR) in vascular endothelial cells *(21)*. Additionally, VEGF stimulates hexose transport in cultured vascular endothelial cells *(22)*.

VEGF is known also as vascular permeability factor (VPF), based on its ability to induce vascular leakage in the guinea pig skin *(23)*. Dvorak *(24)* proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumors and wounds. 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 *(25)*. Recent studies have also suggested that VEGF may also induce fenestrations in endothelial cells *(26,27)*. 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 *(26,27)*.

Melder et al. *(28)* have shown that VEGF promotes expression of VCAM-1 and ICAM-1 in endothelial cells. 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. *(29)* reported that VEGF may promote monocyte chemotaxis. Broxmeyer et al. *(30)* have shown that VEGF induces colony formation by mature subsets of granulocyte-macrophage progenitor cells. 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. *(31)* have reported that VEGF may have an inhibitory effect on the maturation of host professional antigen-presenting cells, such as dendritic cells. VEGF was found to inhibit immature dendritic cells, without having a significant effect on the function of mature cells. These findings led to suggestion that VEGF may facilitate tumor growth also, by allowing the tumor to avoid the induction of an immune response *(31)*.

VEGF induces vasodilatation in vitro in a dose-dependent fashion *(32,33)*, and produces transient tachicardia, hypotension, and a decrease in cardiac output when injected intravenously in conscious, instrumented rats *(33)*. 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 *(33)*. Accordingly, VEGF has no direct effect on contractility or rate in isolated rat heart in vitro *(33)*. 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 *(34,35)*.

3. ORGANIZATION OF VEGF GENE AND CHARACTERISTICS OF VEGF PROTEINS

The human VEGF gene is organized in eight exons, separated by seven introns. The coding region spans approx 14 kilobases (kb) *(36,37)*. The human VEGF gene has been assigned to chromosome 6p21.3 *(38)*. 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; $VEGF_{121}$ 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 *(36)*.

$VEGF_{165}$ 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 *(36)*. In contrast, $VEGF_{206}$ is a very rare form, so far identified only in a human fetal liver cDNA library *(36)*. The genomic organization of the murine VEGF gene has also been described *(39)*. Similar to the human gene, the coding region of the murine VEGF gene encompasses approx 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 in 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 *(40)*. A frequently used site is about 1.9 kb further downstream from the previously reported transcription termination codon *(41)*. 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 *(40)*.

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa *(42)*. These properties correspond to those of $VEGF_{165}$, the major isoform *(43)*. $VEGF_{121}$ is a weakly acidic polypeptide that fails to bind to heparin *(43)*. $VEGF_{189}$ and $VEGF_{206}$ are more basic, and bind to heparin with greater affinity than $VEGF_{165}$ *(43)*. 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 *(44)*. 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

mol wt ~34,000 kDa *(43)*. 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. *(45)* have shown that the bioactive product of plasmin action is comprised of the first 110 NH_2-terminal amino acids of VEGF. 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 caused by 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 *(45)*. It has been suggested that the stability of VEGF–heparan sulfate–receptor complexes contributes to effective signal transduction and stimulation of endothelial cell proliferation *(45)*. Thus, VEGF has the potential to express structural and functional heterogeneity to yield a graded and controlled biological response. Recently, Poltorak et al. *(46)* have provided evidence for the existence of an additional alternatively spliced molecular species of VEGF 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 et al. *(47)* determined the crystal structure of VEGF at a resolution of 2.5 Å. 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.

4. REGULATION OF VEGF GENE EXPRESSION

4.1. 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 *(48,49)*. 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 *(50,51)*. 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 *(52)*.

Similarities exist between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo) *(53)*. 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 *(54,55)*. Such sequence reveals a high degree of homology and similar protein binding characteristics as the hypoxia-inducible factor 1 (HIF-1) binding site within the Epo gene *(56)*. HIF-1 has been identified as a mediator of transcriptional responses to hypoxia, and

is a basic, heterodimeric, helix-loop-helix protein *(57)*. When reporter constructs containing the VEGF sequences that mediate hypoxia inducibility were co-transfected with expression vectors encoding HIF-1 subunits, reporter gene transcription was much greater than that observed in cells transfected with the reporter alone, both in hypoxic and normoxic conditions *(58)*. However, transcriptional activation is not the only mechanism leading to VEGF upregulation in response to hypoxia *(40,59)*. Increased mRNA stability has been identified as a significant posttranscriptional component. Sequences that mediate increased stability were identified in the 3' untranslated region of the VEGF mRNA.

4.2. 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 *(60)* EGF also stimulates VEGF release by cultured glioblastoma cells *(61)*. 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 *(62)*. 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 *(62)*. Furthermore, IL-1-β induces VEGF expression in aortic smooth-muscle cells *(63)*. Both IL-1-α 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 *(64)*. IL-6 has also been shown to significantly induce VEGF expression in several cell lines *(65)*. 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 *(66)*.

4.3. Differentiation and Transformation

Cell differentiation has been shown to play an important role in the regulation of VEGF gene expression *(67)*. 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 nonmalignant, 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 *(68)*. Likewise, oncogenic mutations or amplification of ras lead to VEGF upregulation *(69,70)*. 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 VHL 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 cell 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 prolifera-

tions 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 that VHL regulates VEGF expression at a posttranscriptional level, and that VHL inactivation in target cells causes a loss of VEGF suppression, leading to formation of a vascular stroma. Despite fivefold differences in VEGF mRNA levels, VHL overexpression did not affect VEGF transcription initiation.

5. 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 and 100 p*M*, 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 *(29)*.

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)*.

5.1. Flt-1 and Flk-1/KDR Tyrosine Kinases

5.1.1. Binding Characteristics

Two VEGF receptor tyrosine kinases (RTKs) have been identified. The Flt-1 (fms-like tyrosine kinase) *(80)* and kinase domain region (KDR) *(81)* receptors bind VEGF with high affinity. The murine homolog 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 approx 10–20 p*M (80)*. KDR has a somewhat lower affinity for VEGF: The K_d has been estimated to be approx 75–125 p*M (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 p*M*), 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) *(89,90)*. VEGF-C/VRP has been shown to be a regulator of lymphatic angiogenesis *(91)*.

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 abol-

ished 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, in which 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 *(92)*.

One 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 *(93)*. Therefore, this agent may a useful tool to determine the role of endogenous VEGF in several in vivo models.

5.1.2. Signal Transduction

VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells *(94)*. PLC-γ, and two proteins that associate with PLC-g, were phosphorylated in response to VEGF. Furthermore, immunoblot analysis for mediators of signal transduction, which 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, the mitogenic effect of VEGF on cultured microvascular endothelium isolated from coronary venules *(95)*. 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 *(96,97)*. Porcine aortic endothelial cells, lacking endogenous VEGF receptors, display chemotaxis and mitogenesis in response to VEGF, when transfected with a plasmid coding for KDR *(96)*. In contrast, transfected cells expressing Flt-1 lack such responses *(96,97)*. Flk-1/KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, but Flt-1 reveals a weak or undetectable response *(96,97)*. Also, VEGF stimulation results in weak tyrosine phosphorylation that does not generate any mitogenic signal in transfected NIH 3T3 cells expressing Flt-1 *(97)*. 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 *(98)*. 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 *(99)*. These findings cast doubt on the role of Flt-1 as a truly signaling receptor. However, more recent evidence indicates that Flt-1 indeed signals, although understanding of these events is fragmentary. Cunningham et al. *(100)* 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 implicating elevated levels of PtdIns(3,4,5)P3 levels in this process *(100)*. Also, 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 *(96)*. Furthermore, it has been shown that a specific biological response, the migration of monocytes in response to VEGF (or PlGF), is mediated by Flt-1 *(101)*.

5.1.3. Regulation

The expression of Flt-1 and Flk-1/KDR genes is mostly 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 *(102)*. Likewise, a 4-kb 5' flanking sequence has been identified in the promoter of KDR that confers endothelial cell specific activation *(103)*.

Similar to 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 Flk-1/KDR genes in the lung vasculature *(104)*. Also, Flk-1/KDR and Flt-1 mRNAs were substantially upregulated throughout the heart, following myocardial infarction in the rat *(105)*. 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 *(106)*. Brogi et al. *(107)* 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. Recent studies have provided evidence for a differential transcriptional regulation of the Flt-1 and KDR genes by hypoxia *(108)*. 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-1 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 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 *(108)*. Also, recent studies have shown that both TNF-α *(109)* and TGF-β *(110)* have the ability to inhibit the expression of the KDR gene in cultured endothelial cells.

6. ROLE OF VEGF AND ITS RECEPTORS IN PHYSIOLOGICAL ANGIOGENESIS

6.1. 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 first detected within the first few days following implantation in the giant cells of the trophoblast *(79,111)*. At later devel-

opmental stages in 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 *(111)*. In the human fetus (16–22 wk), VEGF mRNA expression is detectable in virtually all tissues, and is most abundant in lung kidney and spleen *(112)*.

In situ hybridization studies have shown that the Flk-1 mRNA is expressed in the yolk sac and intraembryonic mesoderm, and later in angioblasts, endocardium, and small- and large-vessel endothelium *(113,114)*. 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 *(115)*. Flk-1 is then detectable in endocardial cell of heart primordia, and subsequently in the major embryonic and extraembryonic vessels *(115)*. These studies have indicated that Flk-1 may be the earliest marker of endothelial cell precursors. The Flt-1 mRNA is selectively expressed in vascular endothelial cells, both in fetal and adult mouse tissues *(116)*. Like 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 *(116)*.

VEGF expression is also detectable around microvessels in areas where endothelial cells are normally quiescent, such as kidney glomerulus, pituitary, heart, lung, and brain *(117,118)*. 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 *(117)*. In agreement with this hypothesis, Alon et al. *(119)* have shown that VEGF acts as a survival factor, at least for the developing retinal vessels. They propose that hyperoxia-induced vascular regression in the retina of neonatal animals is a consequence of inhibition of VEGF production by glial cells.

6.2. Flk-1/KDR, Flt-1, and VEGF Gene Knock-Outs 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 *(120,121)*. Mouse embryos, homozygous for a targeted mutation in the Flt-1 locus, died *in utero* between d 8.5 and 9.5 *(120)*. Endothelial cells developed in both embryonic and extra embryonic 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 d 8.5 and 9.5 *(121)*.

However, these findings do not necessarily imply that VEGF is equally essential, since other ligands might potentially activate the Flt-1 and Flk-1 receptors, and thus substitute VEGF action. Very recent studies *(11,12)* 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 d 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 the result of gene dosage, and not maternal imprinting.

Although several heterozygous phenotypes have been described *(122)*, 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, so that even reduced concentrations of VEGF are inadequate to support a normal pattern of development. Inactivation of the PlGF gene does not result in embryonic lethality, even in the homozygous state *(123)*. 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.

6.3. Role of VEGF in Corpus Luteum Angiogenesis

The development and endocrine function of the ovarian corpus luteum (CL) are dependent on the growth of new capillary vessels. Although several molecules have been implicated as mediators of CL angiogenesis, at present there is no direct evidence for the involvement of any. 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 that occurs in the female reproductive tract *(124–127)*. Very recently, the hypothesis that VEGF may be a mediator has been examined in a rat model of gonadotroin-induced ovulation *(128)*.

Treatment with truncated soluble Flt-1 receptors resulted in virtually complete suppression of CL angiogenesis. This effect was associated with inhibition of CL development and progesterone release. Failure of maturation of the endometrium was also observed. Areas of ischemic necrosis were demonstrated in the CL of treated animals. However, no effect on the pre-existing 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 for the treatment of ovarian disorders characterized by hypervascularity and hyperplasia, such as policystic ovary syndrome.

7. ROLE OF VEGF IN PATHOLOGIC ANGIOGENESIS

7.1. Tumor Angiogenesis

Many tumor cell lines secrete VEGF in vitro *(117)*. *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 *(129,130)*, breast *(131,132)*, gastrointestinal tract *(133,134)*, kidney *(135)*, bladder *(135)*, ovary *(136)*, endometrium *(137)*, and uterine cervix *(138)* carcinomas; angiosarcoma *(139)*; germ cell tumors *(140)*; and several intracranial tumors, including glioblastoma multiforme *(141–143)* and sporadic, as well as VHL-syndrome-associated, capillary hemangioblastoma *(144,145)*. In glioblastoma multiforme and other tumors with significant necrosis, the expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas *(141–143)*. A

correlation exists between the degree of vascularization of the malignancy and VEGF mRNA expression *(138,144,145)*. 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 *(133,146)*. These findings are consistent with the hypothesis that VEGF is primarily a paracrine mediator *(147)*. Immunohistochemical studies have localized the VEGF protein not only to the tumor cells, but also to the vasculature *(133,142)*. This localization indicates that tumor-secreted VEGF accumulates in the target cells *(148)*. Recent studies have suggested that the angiogenesis mediated by the HIV-1 Tat protein *(149)* requires activation of the KDR receptor *(150)*. Tat induces growth of Kaposi's sarcoma (KS) spindle cells, and has been implicated in the vascularity of the KS lesions *(150)*.

Elevations in VEGF levels have been detected in the serum of some cancer patients *(151)*. Also, a correlation has been noted between VEGF expression and microvessel density in primary breast cancer sections *(152)*. 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 *(153)*. A similar correlation has been described in gastric carcinoma patients *(154)*. 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 *(154)*.

The availability of specific monoclonal antibodies, capable of inhibiting VEGF-induced angiogenesis in vivo and in vitro *(155)*, made it possible to generate direct evidence for a role of VEGF in tumorigenesis. In a study published by Kim et al. *(156)*, such antibodies were found to exert a potent inhibitory effect on the growth of three human tumor cell lines injected subcutaneously in nude mice: SK-LMS-1 leiomyosarcoma, G55 glioblastoma multiforme, and A673 rhabdomyosarcoma. The growth inhibition ranged between 70% and >95%. Subsequently, other tumor cell lines were found to be inhibited in vivo by this treatment *(157–159)*.

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, compared with controls. Furthermore, neither the antibodies nor VEGF had any effect on the in vitro growth of the tumor cells *(156)*. Intravital videomicroscopy techniques have allowed a more direct verification of the hypothesis that anti-VEGF antibodies indeed block tumor angiogenesis *(160)*. Noninvasive imaging of the vasculature revealed a nearly complete suppression of tumor angiogenesis in anti-VEGF-treated animals, compared with controls, at all time-points examined *(160)*.

VEGF is a mediator of the in vivo growth of human colon carcinoma HM7 cells in a nude mouse model of liver metastasis *(157)*. 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 *(158)* or HT-1080 fibrosarcoma cells implanted in Balb/c nude mice *(159)*. Recent studies have shown that VEGF is also a mediator of stromal-induced enhancement of human prostate cancer LNCaP cell growth in vivo *(161)*.

Recently, Borgström et al. *(162)* have shown that a combination treatment, which 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 also been applied to address the important issue of the effects of VEGF on permeability and other properties of tumor vessels *(163)*. 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 *(163)*. 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 *(163)*.

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 *(164,165)*.

7.2. 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 *(166)*. All of these conditions are known to be associated with retinal ischemia *(167)*. In 1948, Michaelson *(168)* proposed that a key event in the pathogenesis of these conditions is the release by the ischemic retina into the vitreous diffusible angiogenic factor(s) (factor X) responsible for retinal and iris neovascularization. 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 humors of eyes with proliferative retinopathy have been described *(169–171)*. 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 nonneovascular disorders or diabetes without proliferative retinopathy *(169)*. 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 *(172,173)*. In the former, intraocular administration of anti-VEGF antibodies dramatically inhibits the neovascularization that follows occlusion of central retinal veins *(174)*. Likewise, soluble Flt-1 or Flk-1 fused to an IgG suppresses retinal angiogenesis in the mouse model *(175)*.

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 (exuda-

tive) 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 *(176,177)*. These findings suggest 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 in which angiogenesis plays a significant role *(178,179)*. The RA synovium is characterized by the formation of pannus, an extensively vascularized tissue that invades and destroys the articular cartilage *(180)*. Levels of immunoreactive VEGF were found to be high in the synovial fluid of RA patients; they were very low or undetectable in the synovial fluid of patients affected by other forms of arthritis, or by degenerative joint disease *(178,179)*. Furthermore, anti-VEGF antibodies significantly reduced the endothelial cell chemotactic activity of the RA synovial fluid *(178)*.

It has been shown that VEGF expression is increased in psoriatic skin *(181)*. 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 *(182)*.

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 *(183,184)*. 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 *(183,184)*. VEGF upregulation has been also implicated in the hypervascularity of the ovarian stroma that characterizes Stein-Leventhal syndrome *(185)*.

Moreover, Sato et al. *(186)* proposed that VEGF may be responsible for the characteristic hypervascularity of Graves' disease. TSH, insulin phorbol ester, dibutiryl cAMP, and Graves' IgG were found to stimulate VEGF mRNA expression in cultured human thyroid follicles *(186)*.

8. PERSPECTIVES

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 *(187)*, should help determine the timing 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 $\alpha_v\beta_5$ *(188)*. Furthermore, a ligand selective for the endothelial-cell-specific tyrosine kinase Tie-2 has been recently identified and named angiopoietin (Ang)-1 *(189)*. Gene knock-out studies have shown that Ang-1 is required for the correct assembly of the vessel wall *(190)*. 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. Very recent studies provide evidence for the existence of Ang-2, a natural antagonist for the tie-2 receptor *(191)*. 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.

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. $rhVEGF_{165}$ is already in clinical trials for the treatment of myocardial ischemia associated with coronary artery disease.

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 *(192)*, and is being tested in humans as a treatment for solid tumors, alone or in combination with chemotherapy.

In conclusion, in spite of the plurality of factors potentially involved in angiogenesis, one specific factor, VEGF, appears to play an irreplaceable role in a variety of physiological and pathological circumstances.

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9

Regulatory Aspects of Neovascularization

Regulation of Wound Angiogenesis by Metabolic Alterations

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Contents

1. INTRODUCTION

Angiogenesis is a critical component of tissue repair and involves a series of interconnected, interdependent events, among which are generation and reception of angiogenic signals, chemotaxis, proteolysis of extracellular matrix, cell replication, cell-matrix adhesion, tube formation, and ligation of the newly formed vascular sprouts. The discovery of angiogenic cytokines and growth factors has greatly contributed to understanding of the regulation of angiogenesis *(1–3)*. Although many angiogenic substances have been found that potentially upregulate most of the above mentioned angiogenic events, no unifying postulate for their synthesis or their mode of action has emerged. Perhaps a search for how these substances are elicited may prove to be a fruitful alternative. Only recently have research efforts touched on how the initiation of angiogenesis could be linked to the metabolic state *(4)*.

It has now been recognized that angiogenesis occurs when the energy demand of a given tissue exceeds the availability of substrates that supply energy *(3)*. Adair et al. *(5)* reviewed the evidence for the hypothesis that metabolic conditions regulate blood vessel growth, and left little doubt that metabolic demand creates circumstances conducive to angiogenesis during growth and development, wound healing, physical training, high-

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altitude exposure, diabetic retinopathy, and in some tumors. Healing wounds represent an excellent model to test the role of metabolism in angiogenesis. The assessment of cellular behavior in wounds is clearly useful in defining the angiogenic stimuli.

Injury obviously disrupts microvasculature, and thus impairs perfusion. As a consequence, the wound extracellular environment, which is easy to measure, contains clear evidence of unmet metabolic demand, in that it is hypoxic, hyperlactated, poor in glucose, and acidotic *(6–8)*. These characteristics are present in many tumors, muscles in physical exercise, high altitude exposure, and so on.

Wounds contain a number of angiogenic polypeptides, such as vascular endothelial growth factor (VEGF), interleukin-1 (IL-1), insulin-like growth factor-I (IGF-I), and others *(9–11)*. Considering that VEGF is specific for endothelial cells, and is a major contributor to many angiogenic phenomena *(12)*, the authors have asked the question whether the wound environment can stimulate the expression and the angiogenic activity of VEGF.

Hypoxia is an important stimulus for new vessel formation in wound healing *(13)*, and in pathological angiogenesis, such as coronary artery disease *(14)*, diabetic neuropathy *(15)*, and tumor progression *(16)*. In all these instances, hypoxia has been shown to stimulate the release of VEGF from many cell types *(16–18)*. Apparently, hypoxia represents the ultimate angiogenic stimulus. Note, however, that, biologically, hypoxia may not be an important regulator. First, hypoxia comes dangerously close to cell death, and thus prolonged hypoxia is difficult to conceive as a source of physiologic angiogenesis. Energy through respiratory metabolism is necessary for replication and migration of most mammalian cells, and oxygen is essential for construction of a collagenous basement membrane, which represents an obligatory component of angiogenesis. Second, some well-oxygenated tumors are highly angiogenic. Third, and most important, the authors have found that wound angiogenesis depends on oxygen supply, and is accelerated when increased oxygen is provided. Surgeons and those who use hyperbaric oxygen have long observed that angiogenesis proceeds only from well-perfused and oxygenated tissues. As found by Knighton et al. *(13)*, this limb of angiogenesis is directly dependent on the availability of oxygen at the response site. Furthermore, ambient hypoxia inhibits the angiogenic response to VEGF when it is placed in its active form, in vivo, and suspended in Matrigel plugs *(19)*. These considerations strongly imply a role for energy metabolism in angiogenesis, but also suggest that the operative signal need not be, and often is not, hypoxia.

2. ROLE OF LACTATE IN ANGIOGENESIS

Crucial evidence in support of this thesis is provided by the role of lactate in tissue repair. Wound spaces are also characterized by increased levels of lactate (5–15 m*M*) *(9)*. However, hypoxia only partially accounts for this. The excess of lactate is primarily the result of the influx of fibroblasts and macrophages, both of which contain few mitochondria, rely on aerobic glycolysis as their normal source of energy, and, therefore, consume small amounts of oxygen and large amounts of glucose. These cells secrete lactate, even when their oxygen supply is plentiful. The authors and others have previously shown that high concentrations of lactate enhance collagen synthesis, but only if oxygen is present *(6,20,21)*. Subsequently, the authors reported that lactate also enhances macrophage-mediated angogenesis *(4)*, primarily through the production of VEGF. This is not to say that hypoxia cannot play a role: It is clearly a strong stimulus for angiogenic factor production in cultured cells.

Given that both hypoxia and lactate can upregulate VEGF release, it occurred to the authors that hypoxia and lactate share a common chemical effect, namely, the reduction of NAD^+ to NADH, which is mediated by lactate dehydrogenase. Hypoxia favors anaerobic metabolism, and accumulates lactate through various means and reduces the NAD^+/NADH ratio. The authors' work with lactate revealed that NAD^+ supports ADP-ribosylation (ADPR), and, under conditions here, downregulates collagen and angiogenic expression *(4,21)*. It seemed that the decline in the NAD^+ pool, and hence the decline in ADPR products (ADP-ribose and polyADP-ribose), might, therefore, represent one of the signals of metabolic demand, and might support elevated production of VEGF, which, even in the presence of sufficient oxygen, could stimulate angiogenic response to support other cellular functions.

3. ADP-RIBOSYLATION AND ANGIOGENESIS

The idea that ADPR may regulate angiogenesis is intriguing. ADPR is the second most abundant modification reaction that regulates protein function; the most common is phosphorylation. Discovered independently by Chambon and Hayaishi, ADPR is involved in DNA repair in its polymeric form (pADPR), which anneals DNA strand breaks *(22,23)*. Subsequently, pADPR is implicated in gene expression as well. On the other hand, mono-ADPR has been found to modify the functions of many cytoplasmic proteins, including that of adenyl cyclase, by several bacterial toxins *(24)*. As early as 1989, Loestcher et al. *(25)* had postulated that ADP-ribosylation might prove to be a regulator that might translate energy status to cell function.

NAD^+ (not NADH) is the substrate for ADP-ribosylation reaction, during which the nicotinamide moiety from NAD^+ is removed. The resultant ADP-ribosyl portion attaches to the reactive groups of acceptor proteins, thereby regulating their functions. As NAD^+ diminishes, ADPR decreases with it. Metabolically, lactate is converted only to pyruvate, with the generation of NADH from NAD^+. Therefore, high concentrations of lactate decrease the NAD^+ pool, as would be expected by hypoxia. Depletion of NAD^+ depletes ADPR of both nuclear and cytoplasmic proteins *(20,24)*. Hypoxia can do this as well, but lactate alone, in the presence of oxygen, is capable of considerable changes in ADPR in many cells.

The authors found that a decrease in ADPR induces signals for angiogenesis and production of extracellular matrix support for the new vessels *(4,20)*. ADPR came to the authors' attention as a possible mediator of the lactate effect on prolyl hydroxylase. The authors reported that prolyl hydroxylase is inhibited by ADP-ribose in nanomolar concentration, thus providing a partial explanation of the lactate effect *(20)*. Ghani and Hussain *(21)* demonstrated that exposing fibroblasts to NAD^+ (which translocates into fibroblasts, but not macrophages) abrogated the effect of lactate on ADPR, prolyl hydroxylase, and collagen synthesis. It turned out that ADPR negatively controls collagen mRNA pool and prolyl hydroxylase activity. Inhibitors of polyADPR synthetase, such as nicotinamide and 3-aminobenzamide, have similar effect on collagen mRNA production by fibroblasts.

4. VEGF AND ANGIOGENESIS

VEGF is now regarded as a major stimulus for many physiological and pathological angiogenesis *(12)*. Shweiki et al. *(16)* found VEGF in the central hypoxic zone of tumor

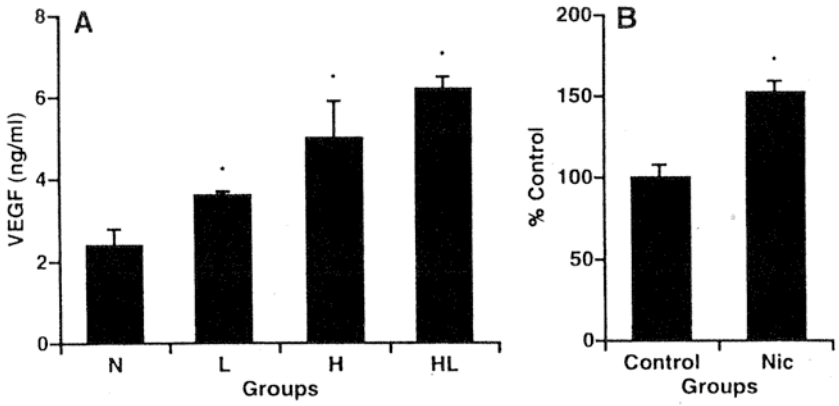

Fig. 1. Lactate, hypoxia, and nicotinamide stimulate macrophage VEGF expression. **(A)** N = normoxic, L = 15 m*M* lactate, H = hypoxic, HL = hypoxic plus 15 m*M* lactate. **p* < 0.01 by ANOVA, compared to normoxic cultures. Values are mean ± SEM (*n* = 7 per group). **(B)** Nic = 20 m*M* nicotinamide. **p* < 0.05 by Student's *t*-test, compared to control.

spheroids, and have demonstrated that VEGF formation is also responsive to glucose concentration. However, no metabolic or molecular mechanism for VEGF release emerged from these studies, but some of the molecular events leading to VEGF expression have been reported. Postulated contributors to VEGF transcriptional control include HIF-1, AP-1 transcription factor, and c-SRC *(26–31)*. Whether these mechanisms are somehow linked to metabolic alterations and cellular energy depletion is not known *(32)*.

The authors found that wound fluid contains large amounts of VEGF, and that it is produced by macrophages, and accounts for the majority of the angiogenic activity released by these cells. Experiments showed VEGF expression is greatly increased when macrophages are exposed to lactate or nicotinamide, which inhibits pADPR synthetase (Fig. 1). The fact that both lactate and nicotinamide reduce pADPR level by different mechanisms, and yet concurrently stimulate macrophages to synthesize VEGF, implicate polyADPR in the regulation of VEGF transcription. However, the molecular events in the repression of VEGF gene by polyADPR remain to be determined. The functions of a number of proteins, including some of those involved in transcription, are altered by pADPR *(22)*.

5. VEGF ANGIOGENIC ACTIVITY IS ALSO REGULATED BY ADP-RIBOSE

Little is known about how the angiogenic activity of VEGF is regulated, but observations indicating inactivity or angiogenically less potent VEGF in some conditions are beginning to emerge. This is true of VEGF produced by macrophages maintained in normoxic environment *(33)*. Furthermore, in the spheroid tumor model, VEGF found during the premalignant stage to the intermediate stage, is inactive, but, in the malignant stage, becomes highly angiogenic *(16)*. The biochemical reason for this behavior is not known.

The authors considered the possibility that posttranslational modification of VEGF polypeptide by mono-ADPR may regulate VEGF activity, after it was found that conditioned media derived from macrophage cultures exposed to 15 m*M* lactate enhanced

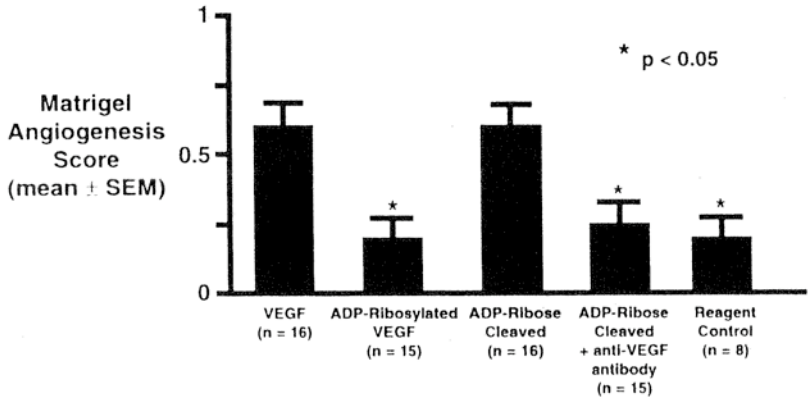

Fig. 2. Angiogenic activity of VEGF and modified VEGF. Recombinant human $VEGF_{165}$ was incubated in the presence of NAD^+ and cholera toxin for ADPR. The figure illustrates the angiogenic activity of unmodified VEGF and ADP-ribosylated VEGF in the mouse Matrigel angiogenesis assay. ADPR significantly decreases the original angiogenic activity of recombinant. Removal of ADP-ribose by hydroxylamine (NH_2OH) from ADP-ribosylated VEGF returns angiogenic activity to control levels. The increased activity following the removal of ADP-ribose is inhibited by anti-VEGF antibody.

Table 1
In Vivo ADPR of Macrophage VEGF

Treatment	*Radioactivity in VEGF (dpm × 10^4) ± SEM*
Untreated control	3.2 ± 0.3
Lactate (15 m*M*)	1.7 ± 0.2[a]
Lactate (15 m*M*) + oxamate (20 m*M*)	2.9 ± 0.3

[a]Macrophages (6×10^6) were labeled with 1 μCi/mL of ^{14}C-adenosine for 16 h, under the indicated conditions. VEGF was isolated from conditioned media using anti-VEGF antibody affinity column. Radiolabel in the VEGF fraction was characterized as ADP-ribose, and total radioactivity was measured.

angiogenesis in animal models, even when *de novo* VEGF synthesis was blocked by cyclohexamide. The functions of a number of cytoplasmic proteins are also altered by ADPR *(24)*. Subsequently, the authors found that human recombinant VEGF and VEGF from human macrophages are avid acceptors of ADP-ribose from NAD^+ in the presence of arginine-specific ADP-ribosyl transferases. The ADP-ribosylated VEGF is angiogenically less potent. The modification of VEGF is reversible, and the removal of ADPR from VEGF returns the angiogenic activity (Fig. 2). Further studies indicated that an appreciable amount of released VEGF from macrophages contained covalently bound ADP-ribose, which was significantly decreased when macrophage cultures were maintained in the presence of 15 m*M* lactate. However, the lactate effect was reversed on simultaneous addition of oxamate, which competes against lactate for the enzyme lactate dehydrogenase. In this case, the level of ADP-ribose on VEGF returns to that of control value (Table 1).

These observations led to postulation that postsynthetic VEGF undergoes reversible mono-ADPR which alters its angiogenic potential. It appears that the conformational

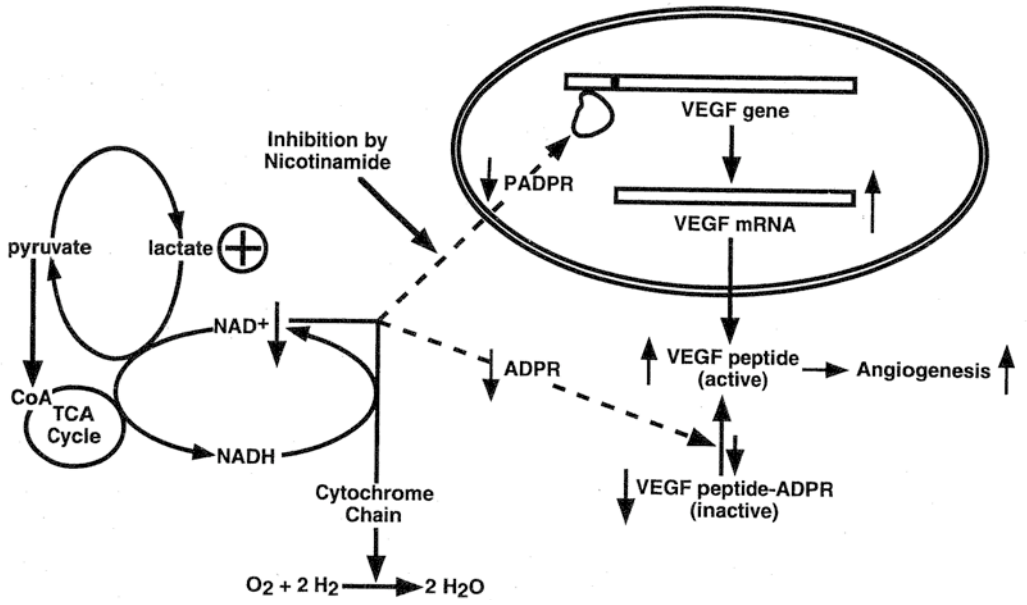

Fig. 3. Lactate effect in the regulation of VEGF synthesis and activity. High concentrations of lactate reduces NAD^+ through the lactate dehydrogenase (LDH) reaction. Thus, the synthesis of nuclear pADPR and cytoplasmic ADPR are decreased. This, in turn, causes the transcriptional activation of VEGF mRNA that are translated into an increased level of VEGF. Concurrently, high lactate lowers the levels of cytoplasmic ADPR, and reverses the inhibition of VEGF angiogenic activity. As a result, an enhanced VEGF-dependent angiogenesis occurs.

change of VEGF polypeptide caused by ADP-ribose linkage is sufficient to alter its angiogenic activity. The authors assume that the loss of angiogenic activity of ADP-ribosylated VEGF is related to an inadequate receptor binding and/or endothelial cell proliferation and migration. Studies are under way to evaluate which of these processes are adversely affected by ADPR. Results also predict that VEGF molecules normally exist as a mixture of free (angiogenic) and ADP-ribosylated (poorly angiogenic) forms. The ratio of these forms normally determines the angiogenic potential, and is sensitive to metabolic alterations involving a change in ADPR synthesis. The authors suggest this ratio to be different in different physiologic and pathologic conditions. For example, it is anticipated that highly angiogenic tissues have a greater proportion of the free form, and that normal tissue have a higher proportion of ADP-ribosylated form of VEGF.

6. CONCLUSIONS

The authors' concept of wound angiogenesis, thus, is as follows: Initiation of angiogenesis in wounds can be described as a response to a metabolic demand precipitated in an environment that has little oxygen and/or a high level of lactate. These conditions limit the supply of NAD^+, and, therefore, ADPR and pADPR. In response to this deficit, macrophages (and perhaps endothelial cells) elicit VEGF, and keep it in an active form, which stimulates new vessel growth. Figure 3 shows how metabolic alterations affect VEGF synthesis and angiogenic activity. However, endothelial cells do not respond well to VEGF in hypoxia, despite upregulation of VEGF receptors *(12)*. Enhanced perfusion

caused by new vessel growth subsequently re-establishes normoxia, and lowers the local lactate levels. Endothelial cells now actively respond to VEGF for blood vessel formation. Finally, the endothelial cell response terminates as the macrophage-derived angiogenic signals diminish.

The metabolic control of angiogenesis in wounds presents a unifying basis for angiogenic regulation. The apparent nonspecific feature of this mechanism is consistent with known facts of angiogenesis. It is remarkable that different forms of ADPR potentially regulate nuclear, as well as posttranslational controlling events related to VEGF expression and activity.

7. FUTURE TRENDS

It is interesting to note that ADP-ribose synthetase is stimulated by DNA strand breaks caused as a consequence of oxidative stress. The pADPR synthesized is utilized to repair the damaged DNA, resulting in a relative deprivation of pADPR. This opens up a possibility that angiogenesis may also respond to hyperoxia. This hypothesis is under investigation.

A number of growth factors induce lactate synthesis in various cells. It is logical to assume that these growth factors stimulate collagen deposition and angiogenesis through the ADPR mechanism.

The authors' findings with macrophages clearly pertain to wound angiogenesis, which has been conceived as different from tumor angiogenesis. However, intriguing observations suggest that the underlying mechanism may pertain to both. Many investigators have noted the exceptional reliance on aerobic glycolysis in cancers and their high lactate production *(34)*. Furthermore, rapidly growing tissues, such as fetal tissue *(35)*, regenerating liver *(36)*, and a variety of tumors *(37)*, all exhibiting pronounced angiogenesis, contain lower NAD^+ levels than the corresponding normal or adult tissues. These observations present interesting insight for the study of tumor angiogenesis in relation to ADPR.

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II ANTIANGIOGENIC AGENTS

10 Squalamine

A New Angiostatic Steroid

Jon I. Williams

Contents

1. INTRODUCTION

Squalamine is a natural aminosterol purified and characterized from several tissues of the dogfish shark. Originally identified as an antimicrobial substance, squalamine has now been shown to be an angiostatic steroid in several in vitro assays and in vivo. Squalamine differs in structure from previously described angiostatic steroids, does not interact with glucocorticoid or mineralocorticoid receptors, and operates by a previously undescribed mechanism for steroids that modulate angiogenesis. Squalamine has antitumor activity in animal models, with its greatest effects on inhibition of primary tumor growth being seen when squalamine is combined with various cytotoxic agents in treating xenograft or allograft solid cancers. Squalamine also has been shown to have low toxicity with repeated dosing in animals. The broad specificity of squalamine for solid tumors, and the margin of safety seen with long-term dosing regimens incorporating squalamine, combine to make this aminosterol an attractive development candidate for treating patients with advanced malignancies. Squalamine is now in phase I human safety clinical trials for cancer.

2. ANGIOGENESIS AND ANGIOSTATIC STEROIDS

Angiogenesis is the growth and maturation of new blood vessels from existing blood vessels *(1–3)*. Angiogenesis is a normal component of several important natural physiological activities, such as organismal growth, wound healing, fertility, and embryonic

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development *(4)*. Ordinary angiogenesis has been extensively reviewed, and can be summarized as requiring production of paracrine and autocrine growth factors by endothelial cells and surrounding tissue; endothelial cell activation and cell division, changes in local extracellular matrix under the influence of various proteases and subsequent matrix invasion by the sprouting vessel, attraction of accessory cells, such as pericytes and smooth muscle cells, and the laying down of basement membrane as the vessel reaches maturation *(1,2,5–7)*. Each step in angiogenesis is now considered the result of modifying and then restoring the balance between positive effectors, such as growth factors, and negative regulators, such as thrombospondin, tissue inhibitors of metalloproteineses (TIMPs), and plasminogen activator inhibitor (PAI-1). This balance is somehow disregulated in abnormal blood vessel development *(8,9)*.

Abnormal blood vessel development or neovascularization is seen in numerous diseases, of which the more important include solid tumors and ocular diseases such as diabetic retinopathy and macular degeneration *(10)*. Details for vascularization of tumors resemble those of normal vessel development, including such characteristics as expression of certain surface molecules on invasive cells *(11)*. The potential prevention and control of tumor development through attack on the tumor vasculature, in particular, have received increasing attention in the oncology community, especially because improvements in cancer diagnosis and conventional treatments over the past 30 yr have not produced corresponding improvements in age-adjusted patient survival for many tumor types, and new approaches are being sought *(12–14)*. Antiangiogenic therapy has emerged as one such important new approach in cancer biology, mostly through the efforts of Judah Folkman and his associates *(15)*. Following Folkman's original proposal that an angiogenesis inhibitor might control tumor growth *(16,17)*, it has been shown in animal studies that limiting the growth of blood vessels adjacent to and within tumors can lead to limitation of the size of primary or secondary tumors, and, in some instances, to regression of the tumor. Since the vasculature also serves as the entry point for metastatic spread of a tumor, antiangiogenic therapy is also considered one way to reduce metastases to vital organs for tumors in earlier-stage cancer patients *(14,18,19)*. A number of papers and reviews have recently been published that describe tumor vessel density as an important prognostic indicator positively correlated with poor outcomes in patients with breast cancer, lung cancer, and other solid tumors *(20–24)*. This work supports the concept that a reduction in tumor vessel density by some form of antiangiogenic therapy might lead to improved patient condition, and possibly to prolonged survival.

A number of antiangiogenic compounds are now known, several of which currently are in clinical trials. The first antiangiogenic compounds to be identified were steroids, including the progestin, medroxyprogesterone acetate (MPA), and the glucocorticoids, dexamethasone and cortisone *(25–27)*. These steroids were shown to be antiangiogenic by virtue of several properties: They caused reduction in new vessel growth in the chick embryo chorioallantoic membrane (CAM) assay, or in a rabbit corneal micropocket assay, in which an allogeneic tumor fragment was implanted in the cornea, and a polymer pellet containing the steroid was placed between the tumor and the adjacent limbus of the eye; they were not directly cytotoxic to tumor cells in tissue culture; and they were shown mechanistically to interfere with at least one step of angiogenesis, either collagenolysis associated with tissue invasion or interaction with heparin or heparin fragments. Heparin is a stimulus to angiogenesis, and promotes endothelial cell movement in vitro *(28)*.

Research on the basis of heparin–steroid synergy in reducing angiogenesis revealed that heparin, heparin fragments as small as a hexasaccharide or a synthetic pentasaccharide, the heparin analog hexuronyl hexosaminoglycan sulfate, suramin (a polysulfonated naphthylurea), and β-cyclodextrin tetradecasulfate all enhance the antiangiogenic activity of certain steroids *(26,29–33)*. It was also found that the most potent antiangiogenic steroids, such as epicortisol (11 α-hydrocortisone) and tetrahydrocortisol (a lipid-soluble metabolic product of cortisol), which were termed "angiostatic steroids" by Crum et al. *(29)* lacked any mineralocorticoid or glucocorticoid activity. The angiostatic nomenclature is possibly unfortunate, since Ingber et al. *(34)* identified morphologically, on the chick embryo CAM, actual capillary breakdown and capillary regression following exposure to angiostatic steroids, showing that vessel stasis is not the only possible outcome of exposure to angiostatic steroids. It was also noted in subsequent CAM experiments that angiostatic steroids or heparin enhance the effect of inhibitors of collagen deposition, such as proline analogs , suggesting a correlation can be drawn between decreased vessel formation in the presence of angiostatic steroids and modified extracellular matrix formation *(35)*. These observations led to the thesis that extracellular matrix could be a focus of attention for limiting angiogenesis and tumor growth *(36,37)*.

Certain principles were derived from studies with a number of angiostatic steroids that pointed to structural features considered important for antiangiogenic activity. The 4,5-double bond in the steroid A-ring and the C-11 hydroxyl group are not required, as was noted by comparing the antiangiogenic activity of, e.g., corticosterone vs desoxycorticosterone, and hydrocortisone vs tetrahydrocortisone (tetrahydro S) *(29)*. A positive relationship was found between the presence of a C-17 hydroxyl or its esters, or C-20 and C-21 carbonyls on the steroid D-ring, and increased antiangiogenic activity of the steroid *(29,38)*. More recently, it has been suggested that steroid 17-β carboxylic acids are angiostatic, a 1,2-double bond is important for angiostatic activity, and a 16-β methyl group increases steroid angiostatic activity *(39)*. Efforts to identify angiostatic steroids that did not require potentiation with heparin or a heparin substitute have yielded few leads *(38)*. However, it has been reported that the endogenous estrogen metabolite 2-methoxyestradiol is antiangiogenic in vitro, and has antitumor activity when given orally to tumor-bearing mice without heparin cotreatment *(40,41)*.

Investigations in tumor models with angiostatic steroids generally have been promising as treatment for limiting primary tumor growth, or number and size of tumor metastases *(26,42–47)*, notably when combined with large oral doses of heparin or a heparin substitute, but there is disagreement in the literature on whether angiostatic steroid plus heparin was more active than steroid alone *(48,49)*. In some instances, angiostatic steroid plus heparin was not effective, and toxicity from daily steroid treatment has been noted. There are also reports of angiostatic steroids leading, under certain conditions, to no change in tumor metastases, or to increased numbers of tumor metastases, although primary tumor growth was inhibited, but these observations are restricted to steroids retaining glucocorticoid function *(49–52)*. The significance of these findings for the general class of angiostatic steroids is not clear, since inhibition of metastatic spread of tumors in animal systems has been inhibited in other experiments with angiostatic steroids and heparin *(44)*, suggesting the relationship between tumor metastases and angiostatic steroids may be strongly dependent on the choice of animal model. Complete tumor regressions were seen following treatment with angiostatic steroids in multiple tumor types (B16 melanoma, Lewis lung carcinoma, V2 rabbit carcinoma, M5076 reticu-

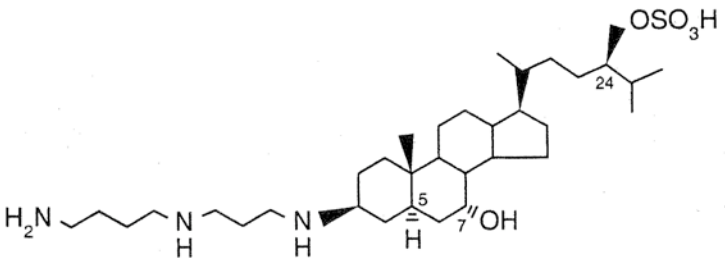

Fig. 1. Structure of squalamine.

lum cell sarcoma, MB49 bladder carcinoma, and a human colon carcinoma xenograft) by Folkman et al. *(26)*, but this work has not been reproduced. This is quite possibly because of differences in the quality of heparin used; Folkman et al. reported that Panheprin (Abbott Laboratories, North Chicago, IL) was the most active commercial heparin preparation *(26)*, but this heparin source was subsequently discontinued by the manufacturer.

Beyond the use of corneal micropocket assays for accessing antiangiogenic activity of compounds, it has been found that angiostatic steroids are also active in ocular disease in which neovascularization is important. The angiostatic steroids, hydrocortisone, 6-α-fluoro-17,21-dihydroxy-16-β-methyl-pregna-4,9,(11)-diene-3,20-dione, or tetrahydrocortisol-S, in combination with heparin or beta-cyclodextrin tetradecasulfate, all reduced endotoxin-induced corneal neovascularization in the eyes of rabbits *(53)*. Further, this work showed that the neovascularization could be reduced in the absence of any anti-inflammatory responses brought about by steroid treatment. No further progress in this field of application for angiostatic steroids has been published.

MPA is the only angiostatic steroid that has undergone human clinical trials *(54–56)*. This may be partly because of the historical weight given to co-administering angiostatic steroids with heparin, where equivocal antitumor results and confusion over the effect of different lots of heparin may have given drug developers pause. It may also be a result of the failure to obtain positive results with MPA in clinical studies. MPA was initially tested as second-line hormonal treatment in metastatic breast cancer, after anecdotal observation that high-dose MPA was more effective than low-dose MPA. Response rates with MPA have varied, with some potential for efficacy emerging from uncontrolled clinical trials, but a key randomized trial conducted in Denmark and Sweden failed to provide statistical significance for MPA treatment *(55)*. In 1989, Ashino-Fuse et al. *(57)* reported MPA was an inhibitor of plasminogen activator from bovine cortical capillary endothelial cells in tissue culture at $10^{-8} – 10^{-7}$ *M*, but was not inhibitory to endothelial cell growth up to 10^{-5} *M*, suggesting that MPA may act on tumor angiogenesis at the stage of tumor tissue penetration by nascent capillaries.

3. THE AMINOSTEROL SQUALAMINE: CHEMICAL AND BIOLOGICAL PROPERTIES

Squalamine was initially described by Moore et al. *(58)* as an antimicrobial substance isolated from the dogfish shark *Squalus acanthias,* and was successfully demonstrated to be a broad spectrum antibiotic. Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to a spermidine at C-3 (Fig. 1). Key features of the chemical

Table 1
Percentage Inhibition of Angiogenesis in 3-d Chick Chorioallantoic Membrane (CAM) Assay Scored in the Presence of Different Concentrations of Squalamine

Squalamine concentration (μg/disk)	*% Embryos with inhibited developmental angiogenesis (n = number of embryos scored)*
0.43	28% (*n* = 25)
0.87	18% (*n* = 22)
1.75	35% (*n* = 31)
3.5	91% (*n* = 22)
14.0	52% (*n* = 21)
28.0	50% (*n* = 12)

Inflammatory reactions were present on the CAMs of embryos exposed to 14.0 and 28.0 μg/disk squalamine.

structure for squalamine are the presence of the polyamine segment and the sulfate-containing side chain at C-20. In light of the previously described structural features thought important for angiostatic steroids, it should be noted that squalamine lacks any double bonds, carries a hydroxyl group on the main steroid nucleus only at C-7 (7α), and more closely resembles a cholesterol derivative than any of the adrenocortical steroids, or their derivatives, that have been classified as angiostatic steroids. Although these differences would not lead one to expect antiangiogenic properties for squalamine, previous work had highlighted a role for polysulfonated compounds, such as suramin, and polyamines, such as protamine, as inhibitors of angiogenesis *(28,59,60)*. Squalamine, therefore, in one sense resembles a molecular composite of chemical agents discovered in three threads of research on antiangiogenic compounds.

Squalamine was initially tested as an antiangiogenic compound because of its structural similarities to the angiostatic steroids. In a chick CAM assay carried out with squalamine on 3-d-old chick embryos, squalamine inhibited angiogenesis (defined as the presence of a >4 mm avascular zone around the methylcellulose disk) in a dose dependent manner over the concentration range 0–3.5 μg/mL, when scored 72 h after placement of the disk on the chick CAM (Table 1). At doses of 14 and 28 μg/mL, inflammatory reactions were evoked that induced new blood vessel growth, and provided a lower frequency of angiogenesis inhibition. The effect on the 3-d-old chick CAM with squalamine did not require heparin, and was comparable to the effects seen with angiostatic steroids that require heparin or suramin for optimal activity *(32)*, as well as for the strongly antiangiogenic fungal compound fumagillin *(61)*. To eliminate the possibility that inhibition of angiogenesis with squalamine was nonspecific, the author compared the effect of CAM treatment with spermidine, squalamine, or a combination of the two. It was found that spermidine had essentially no effect on its own, and did not enhance the degree of inhibition seen with squalamine on the 3-d-old CAM (data not shown). Squalamine also had no effect on 13-d-old chick embryo vessels, which hints that squalamine affects growing and newly formed microvessels but not established blood vessels. Failure to obtain an effect with squalamine on 13-d-old CAM vessels is similar to the previous report that angiostatic steroids, in combination with heparin, also did not elicit a response on the mature CAM, but were highly active on the 4-d and 6-d CAM.

To evaluate the antiangiogenic potential of squalamine in vivo, squalamine was prepared in an ethylene vinyl acetate (EVAc) sustained-release polymer, and implanted in

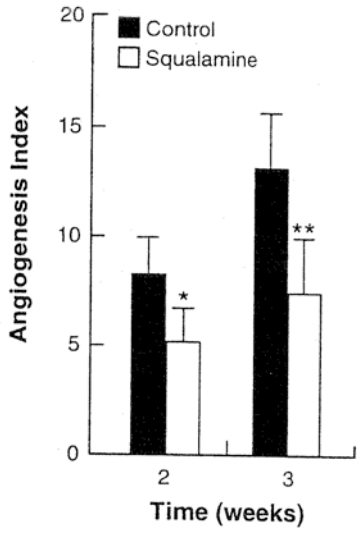

Fig. 2. Rabbit cornea assay with squalamine. Squalamine shows significant reduction in the angiogenesis index of treated eyes when compared with controls at both 2 and 3 wk after tumor implantation (*$p < 0.05$; **$p < 0.01$).

rabbit eyes in which the rabbit VX2 rectal carcinoma was present as an angiogenic stimulus. The contralateral eye of each rabbit was implanted with empty EVAc polymer pellets to provide internal controls. Squalamine reduced both the density and length of new vessels, as measured by slit-lamp stereomicroscopy; this is expressed as an angiogenesis index, which is the product of the vessel density and the length of the longest observed vessel in each eye (Fig. 2). The degree of reduction of angiogenesis was found to be dose-dependent, and persisted for the entire 3 wk of the study *(62)*. To discriminate between antiangiogenic effects of squalamine and possible reduced angiogenic response to decreased production of growth factors and other diffusible factors from tumor tissue resulting from toxic effects of squalamine, histopathology was conducted on several control and squalamine-treated corneas. Histology of the corneas of control and squalamine-treated eyes revealed that the squalamine-treated eyes were poorly vascularized compared to the controls, but viable, dividing tumor was present without any significant degree of tumor necrosis in either corneal group. This result would be expected if squalamine is an angiostatic steroid; in other words, fewer vessels would be expected to cross the cornea if squalamine is a specific inhibitor for endothelial cell growth, and is not a general cytotoxic compound. This supposition is supported by observations that therapeutic combinations containing angiostatic steroids, such as cortisone plus heparin, cortexolone plus β-cyclodextrin, or $\Delta^{9(11)}$ 11-deoxycortisol plus heparin, all inhibit the growth of new blood vessels from the corneal limbus under an angiogenic stimulus implanted in the rabbit eye, but are not inherently cytotoxic to the stimulus if it is a tumor cell line. However, unlike all known angiostatic steroids previously described, except for 2-methoxyestradiol, squalamine does not require heparin or a heparin substitute for significant antiangiogenic activity in vivo.

Angiogenesis can be followed in vivo in tumor explants. Squalamine was tested in one such model, an alginate angiogenesis model in mice. Swiss nu/nu mice were injected with 100 μL alginate beads containing 6×10^4 human MCF-7 mammary carcinoma cells, and

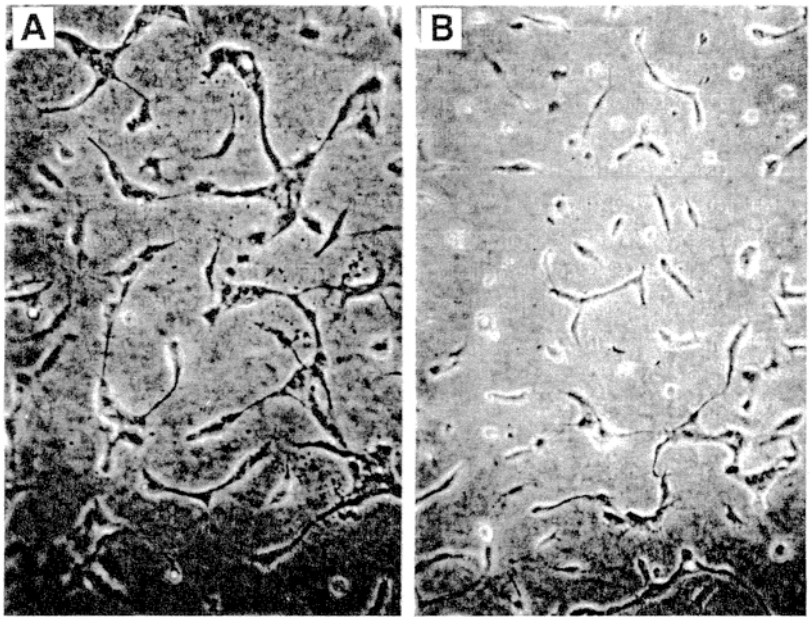

Fig. 3. Effect of squalamine on growth of human umbilical venous endothelial cells (HUVECs) in three-dimensional collagen matrix. HUVECs were grown between two collagen layers in the presence of VEGF and bFGF for 10 d in medium lacking squalamine **(A)**, or in medium with 0.7 μg/mL squalamine present **(B)**. Note that the endothelial cells aggregate and have begun to form three-dimensional branching networks in the absence of squalamine, but that endothelial cell interactions are minimized when squalamine in present.

then treated subcutaneously on a daily basis with 15 mg/kg squalamine for 12 d. Fluorescent microbeads were then injected in the mice 20 min prior to removal of the alginate implants, and quantitation of the fluorescence recovered with the implants was carried out. A maximum of 55% inhibition of fluorescence was obtained in the mice bearing MCF-7 alginate implants. This degree of inhibition is comparable to that seen with scoring of microspheres in 2-methoxyestradiol-treated mice bearing meth-A sarcoma or B16 melanoma tumors *(41)*.

Vessel-like formations of organized endothelial cells, grown in a three-dimensional matrix in culture following stimulation by various growth factors, has frequently been used to study endothelial cell differentiation and coordinated endothelial cell–cell interactions *(63,64)*. In matrices such as Matrigel® or collagen, vessel-like structures can be produced that are collections of endothelial cells, and which have a distinct lumen. Human umbilical vein endothelial cells (HUVECs; Clonetics [San Diego, CA]) were grown in a collagen matrix containing 50 ng/mL VEGF and 20 ng/mL bFGF and 0–7 μg/mL squalamine, as previously described *(65)*. After 10 d of growth, control cultures displayed elongated endothelial cell aggregates, but concentrations of squalamine as low as 0.7 μg/mL disrupted the endothelial cell connections to each other, and to the collagen matrix. At concentrations as low as 7 μg/mL, squalamine also induced visible rounding up of many of the endothelial cells (Fig. 3). The influence of squalamine on endothelial cell aggregates and endothelial cell shape, consequently, occurs at lower concentrations than that required for reduction of endothelial cell proliferation, which in turn occurs at lower squalamine concentrations than required for inhibition of endothelial cell migration.

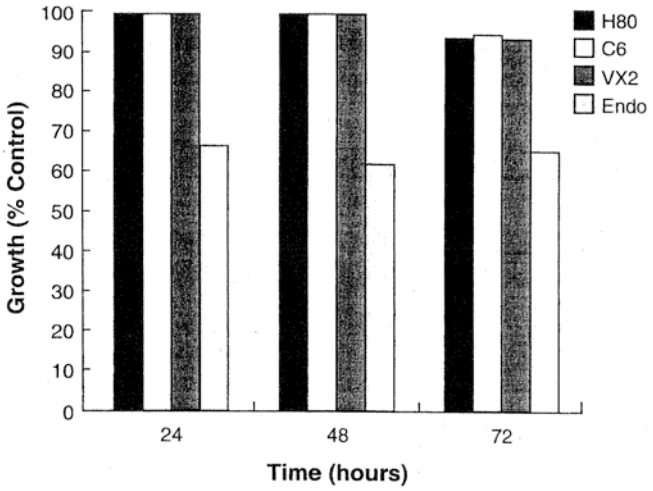

Fig. 4. Squalamine effects on tumor cell lines and BREC cells in vitro. Squalamine at 7 μg/mL shows almost no inhibition in tissue culture growth of human H80 glioma, rat C6 glioma, or rabbit VX2 carcinoma cells for up to 72 h of exposure, but BREC cells stimulated with VEGF (20 ng/mL) are significantly inhibited.

Because of recent research correlating endothelial cell shape and cell interaction with the extracellular matrix elements to endothelial cell viability and growth, it is not too surprising that an agent like squalamine to which endothelial cells respond subtly through cell shape change, can also reduce endothelial cell proliferative capacity.

4. SQUALAMINE AND ENDOTHELIAL CELLS

The specificity of squalamine for endothelial cells was evaluated in culture with bovine retina (BREC) and rat brain (RBE-4) endothelial cells at different concentrations, and compared to results with rat (9L glioma), rabbit (VX2 carcinoma), and human brain tumor (H80) cells *(66)*. The endothelial cell lines were grown in DMEM media containing 10% fetal bovine serum, 1% L-glutamine, 25 m*M* HEPES, and genticine and 20 ng/mL vascular endothelial growth factor (VEGF) in the presence or absence of squalamine. The growth factor was added to the cells prior to the addition of squalamine to the cell cultures. Squalamine exposure was observed to specifically inhibit VEGF-stimulated endothelial cell proliferation for BREC cells, but not the growth of any of the tumor lines (Fig. 4). The degree of inhibition of VEGF-stimulated BREC cells, or RBE-4 cells found with squalamine, increased with increasing squalamine concentration. For example, the net growth of BREC cells was inhibited by more than 30% with 7 μg/mL squalamine, and, at 21 μg/mL, squalamine inhibition was more than 70% (Fig. 5). By contrast, squalamine had no effect on the survival or growth of unstimulated endothelial cells (Fig. 5). Restricted growth of endothelial cells in the presence of 7–70 μg/mL squalamine was found to be independent of growth factor *(67)*. Squalamine was inhibitory for endothelial cell division in media, including VEGF (20 ng/mL), basic fibroblast growth factor (bFGF; 10 ng/mL), platelet-derived growth factor ($PDGF_{bb}$; 10 ng/mL), hepatocyte growth factor or scatter factor (5 ng/mL), tumor-conditioned media from the rat 9L glioma, or human hemangioblastoma cyst fluid. Exemplary data for scatter factor are shown in Fig. 6.

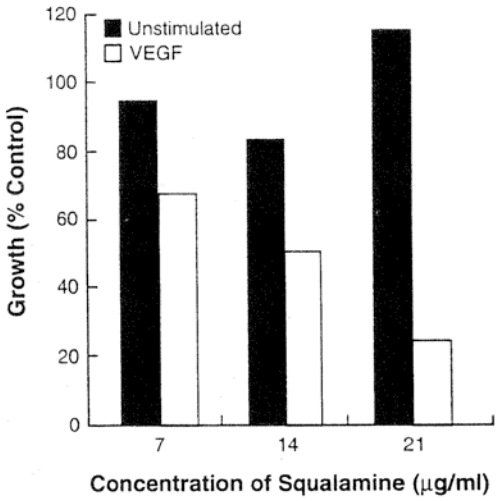

Fig. 5. Inhibition of mitogen-stimulated BREC cells after 48 h exposure to squalamine at doses of 7, 14, and 21 μg/mL. A dose-dependent inhibition is seen in those cells stimulated with VEGF (20 ng/mL), but not in unstimulated endothelial cells. Cells were trypsinized and counted at the end of the experiment, using a Coulter counter. All concentrations were determined.

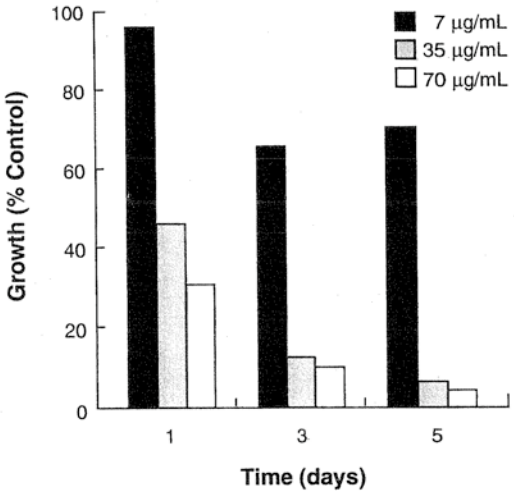

Fig. 6. Percent inhibition of endothelial cell growth for RBE-4 cells exposed to scatter factor and various concentrations of squalamine. RBE-4 cells (10^4 cells per well) were plated and cultured overnight in fibronectin-coated tissue-culture plates, in DMEM media containing 10% fetal bovine serum, 1% L-gluatamine, 10 m*M* HEPES, and geneticine. The media were then removed and replaced with fresh media supplemented with 5 ng/mL scatter factor. Cell proliferation was determined for trypsinized cells by Coulter counter 1, 3, and 5 d after stimulation.

Squalamine's effect on endothelial cell motility was also assessed in a wounding/migration experiment. BREC cells were grown and scraped with a sterile razorblade. The degree of cell migration across the edge of the scraped cell zone, as a function of squalamine concentration was then assessed by image analysis. Squalamine at 35 or

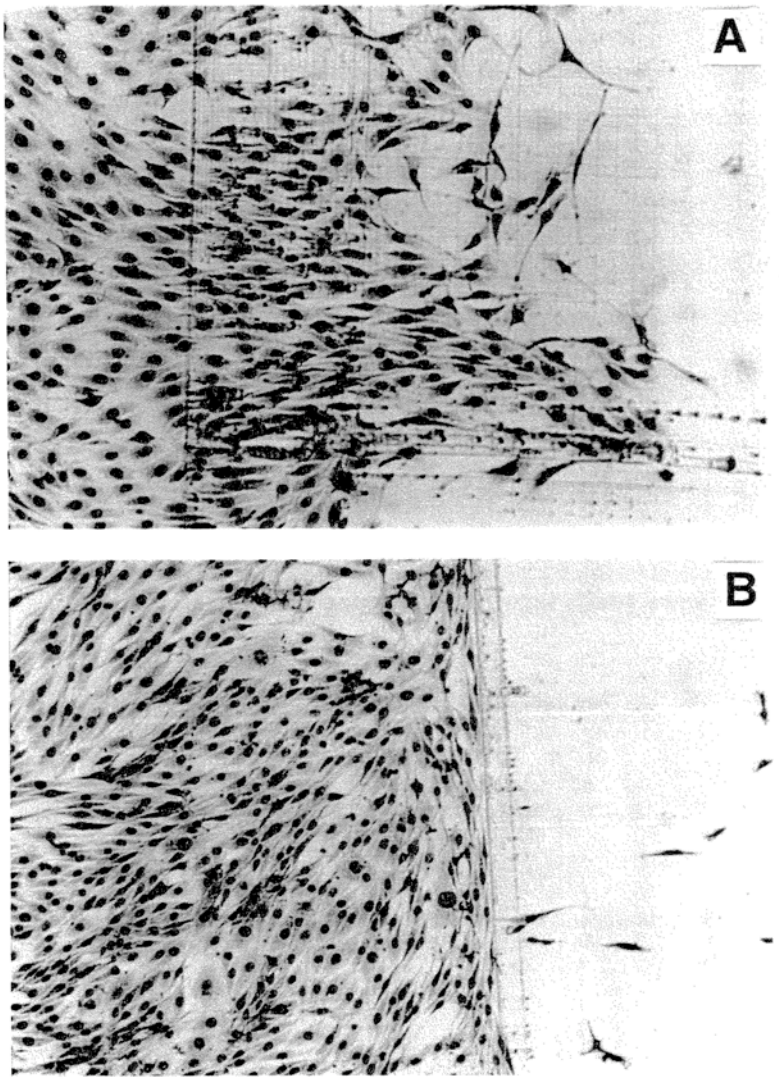

Fig. 7. Squalamine influence on BREC cell motility. BREC cells were grown to confluence on gridded plastic tissue-culture dishes coated with fibronectin. When the cultures reached 70% confluency (2–3 d), a zone of cells was scraped away with a sterile razorblade, and fresh supplemented DMEM medium, containing 20 ng/mL bFGF and 0–70 μg/mL squalamine, was added. The cell cultures were fixed when further cell migration appeared negligible (i.e., after 4–7 d of squalamine exposure), and stained with Wright's stain (Sigma). The images of cells growing with no squalamine added **(A)**, or in the presence of 35 μg/mL squalamine **(B)**, were then analyzed on a Macintosh model 8100/80AV computer, using the public domain NIH Image program (developed at the U.S. National Institutes of Health, and available on the Internet by anonymous FTP from zippy.nimh.nih.gov).

70 μg/mL, but not 7 μg/mL, was effective in reducing endothelial cell migration across the scraping boundary by more than 50% (Fig. 7). This response is similar in magnitude to that seen for inhibition of endothelial cell growth for RBE-4 endothelial cells, when stimulated by various mitogens.

Table 2
Growth of BREC Endothelial Cells
on Extracellular Matrix Protein Coatings in the Presence
of Various Amounts of Calf Serum (CS), and on Different Coated Culture Matrices

Matrix protein coating	*Growth factor added (A_{570} value)*			
	No cytokine	*VEGF*	*bFGF*	*10% CS*
Polylysine	0.608	0.703	0.844	1.011
Fibronectin	0.653	0.728	0.875	0.887
Collagen IV	0.635	0.760	0.891	0.922
Laminin	0.607	0.825	0.970	0.934
Vitronectin	0.578	0.597	0.749	0.811

Cell density is expressed as A_{570} values, determined with the CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega).

To gain further insight into the relative specificity of squalamine for interruption of growth stimulation by the major endothelial growth factors VEGF and bFGF, BREC cells were plated in a 96-well dish previously coated with purified extracellular matrix (ECM) proteins fibronectin, laminin, type IV collagen, or vitronectin. The BRECs had been incubated the previous 24 h in media containing 0.5% calf serum (CS) and no bFGF; BREC cells are normally grown in media containing 10% CS and 10 ng/mL bFGF. The cells were then plated on the above ECM proteins in either media with 0.5% CS, 0.5% CS plus 20 ng/mL bFGF, 0.5% CS plus 20 ng/mL VEGF, or 10% CS. Six hours later, 20 μg/mL squalamine was added to half the wells, and the cultures were maintained for 48 h before cell density (expressed as A_{570} optical absorbance) was assessed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). The results on various matrices for cells not exposed to squalamine (Table 2) indicate that BREC cells grew on all the matrices tested, with laminin the best substrate in the presence of either added cytokine. By contrast, squalamine addition to cytokine-treated cultures containing 0.5% CS resulted in a total suppression of cell growth; the BREC cells in these cultures remained rounded, and did not spread. However, in the presence of 10% CS, squalamine treatment had no effect on BREC cell morphology or proliferation (results not shown). Squalamine appears to block the growth stimulatory effects of the cytokines VEGF or bFGF, but growth induced by uncharacterized serum factors found in CS is not sensitive to squalamine treatment.

A surprising observation with squalamine emerged during preliminary studies of microvessels in the chick embryo CAM assay. Adjacent to the CAM are the yolk-sac vessels, which transport nutrient to the developing embryo. The yolk sac vessels are simple in structure, comprised of endothelial cells, with no apparent basal lamina or supporting connective tissue. They arise initially through vasculogenesis, and then elongate as the vasculature becomes more complex. It was noticed that, beyond any influence on CAM vessels, squalamine caused a rapid change in the architecture of the yolk-sac vessels. Many yolk-sac vessels, especially the smaller vessels, seemed to occlude or disappear under the light microscope within 20–40 min after applying 0.3 mL of a 70 ng/mL squalamine solution in 30% Ficoll 400 (Pharmacia) to the CAM (compare Fig. 8A,B). The explanation for this phenomenon became apparent when the yolk-sac vessels, after

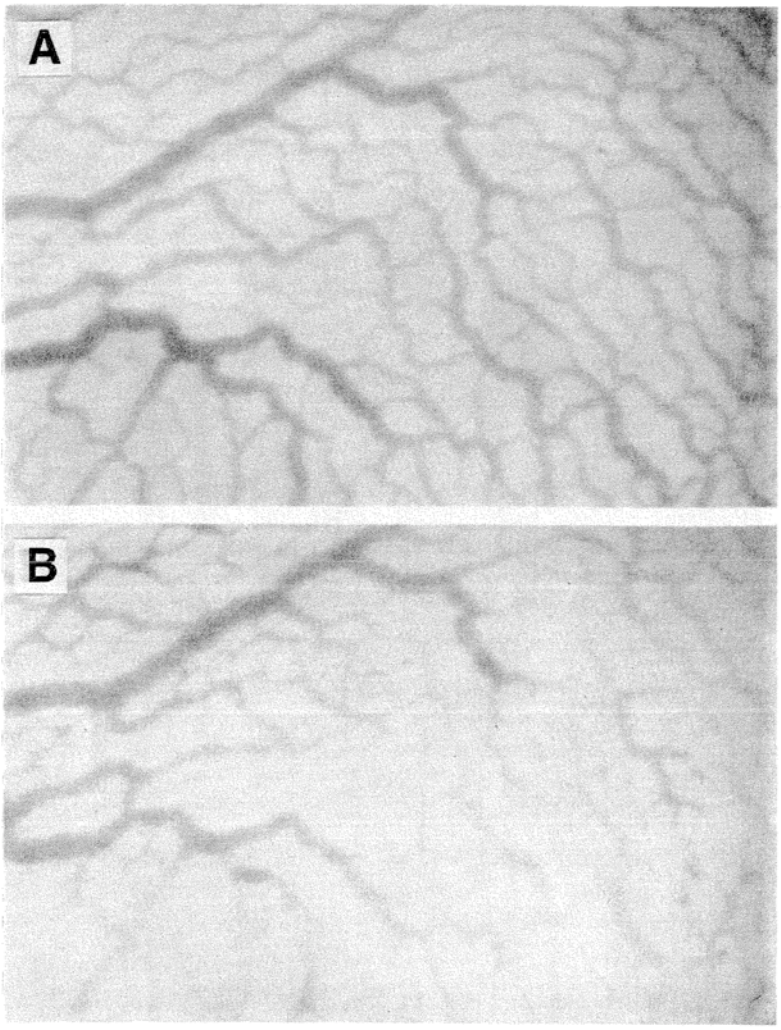

Fig. 8. Changes seen in CAM yolk-sac vessels after exposure to squalamine. A solution of 70 ng/mL squalamine was applied to the CAM and a section of the yolk-sac vessels was photographed immediately **(A)**, or after 35 min **(B)**. The larger blood vessels are unaffected by squalamine, but the smaller vessels have constricted or disappeared.

squalamine application, were fixed in 10% phosphate-buffered formalin, and examined by high-power light microscopy. It was found that the yolk-sac vessels occluded because the endothelial cells that surrounded the lumen of the small vessels constricted (Fig. 9). The shrinkage of the smaller yolk-sac vessels was partially reversible in time, but there was a distinct repatterning of the yolk-sac vessels after 2 h, as blood flow was redirected to the more major vessels. The changes seen in the yolk-sac microvessels may reflect the early stages of the developing CAM vessel response to squalamine, which result in an attenuation of CAM vessel development, as seen at later times. It is not known if prolonged exposure to squalamine leads to a failure of CAM vessel development or vessel growth and regression, as previously seen with angiostatic steroids in the presence of heparin *(34)*. It has occasionally been noted in tumor-dependent angiogenesis that some

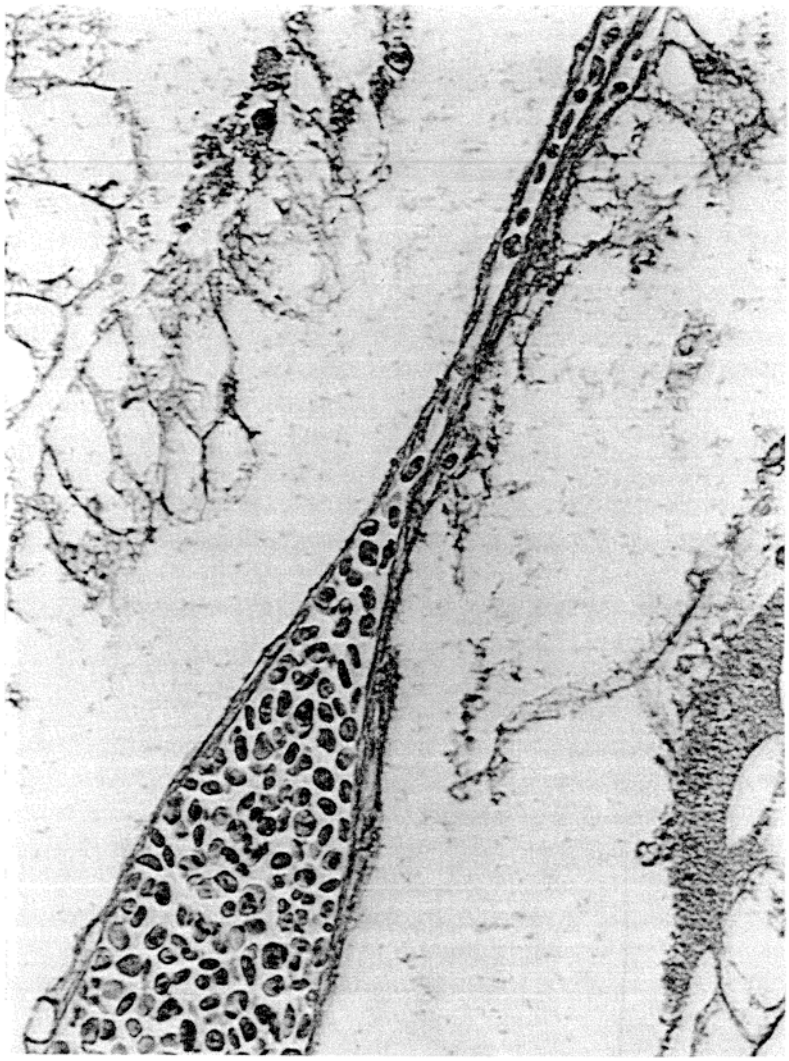

Fig. 9. Chick yolk-sac vessel constriction seen following squalamine treatment. Yolk-sac membranes exposed to 70 ng/mL squalamine for 1 h were fixed overnight *in situ* in Formalin, and the yolk sac was embedded in paraffin, sectioned, and stained with hematoxylin-eosin. A constricted vascular segment is shown in lateral view (×100); collapsed structures such as these are not seen in untreated embryos.

or all of the developing microvasculature lacks basal lamina and/or pericytes *(68,69)*. This class of microvessels in tumors may be an embryonic type similar to that seen in the CAM yolk sac vessels, and may well be a principal target for the inhibitory effects of squalamine.

5. MECHANISM OF ACTION FOR SQUALAMINE

Steroid hormone function is well understood, including considerable knowledge that has accumulated in the past 20 yr on the interaction of steroid hormones and hormone

Table 3
Percent Decrease in V_{max} for Rabbit or Human NHE-3 Transfected in Chinese Hamster Lung Cell Line PS120 (an NHE-Negative Cell Line)

Squalamine concentration (μg/mL)	*Percent decrease in V_{max1}*	
	Rabbit NHE-3	*Human NHE-3*
0.7	79	
3.5	53	75
5.0	43	

The V_{max} values used to calculate this table were determined from measurements of proton efflux rates, using transfected cells loaded with the pH-sensitive dye 2',7'-*bis*(carboxymethyl)5-6-carboxyl-fluroscein (BCECF-AM, 5 μ*M*). The degree of inhibition of NHE-3 function is dose-dependent, and is slightly greater with human NHE-3 than with rabbit NHE-3 at an equivalent dose.

receptors in mammalian systems, and their role in gene transcription *(70)*. It is surprising that angiostatic steroids, by comparison, are poorly defined in their effects on cells. Angiostatic steroids appear to exhibit multiple diverse effects on cells and vascular tissues. They have been implicated in the direct blocking of in vitro and in vivo endothelial cell growth *(47)*, inhibition of collagenolysis and of plasminogen activator production *(25,42,57,71–73)*, altered regulation of PAI synthesis *(72,74)*, induction of basement membrane dissolution and regulation of collagen metabolism *(27,34,35)*, direct antitumor activity *(75)*, and, in the case of 2-methoxyestradiol, inhibition of tubulin polymerization *(40)*. This is a remarkable collection of cellular and biochemical functions for angiostatic steroids, the diversity of which suggests much remains to be learned about how these functions interrelate. There is no evidence whether or not angiostatic steroids have specific receptors that they influence.

Squalamine adds a new dimension to understanding of the mechanisms by which angiostatic steroids may act. Squalamine is selectively inhibitory to endothelial cells. Changes following squalamine treatment in endothelial cell morphology in collagen gels, or in chick embryo yolk sac microvessels, initially drew attention to membrane-bound ion transporters that govern cell volume. A major family of transmembrane antiporters, which modulate cell pH and cell volume, are the sodium-proton (Na^+/H^+) exchangers (NHEs). The influence of squalamine on cloned mammalian rabbit and human NHEs (NHE-1, NHE-2, and NHE-3) has been studied in stably transfected PS120 fibroblasts *(76)*. Na^+/H^+ exchange was measured both by spectrofluorometric methods using the pH-sensitive dye BCECF, and by amiloride-sensitive isotopic Na^+ uptake. Squalamine decreased the V_{max} of rabbit and human NHE-3 in a concentration-dependent manner (Table 3), blocking both the basal NHE-3 rate of ion exchange and, with pretreatment, the bFGF-stimulated NHE-3 activity in transfected PS120/NHE-3 cells. Reduced NHE-3 function with exposure to squalamine was specific; squalamine had no effect on function of rabbit and human NHE-1 or rabbit NHE-2 (data not shown). Squalamine also inhibited ileal brush border membrane vessel NHE function at a comparable concentration, with a time lag of 30–60 min. The delay in NHE inhibition, following exposure to squalamine, suggests that squalamine, unlike amiloride and its analogs, does not interact directly with the NHE-3 antiporter. On the other hand, the response time to squalamine treatment is sufficiently rapid that squalamine cannot resemble known steroid hormones

in requiring new gene transcriptional and translational activity. One can speculate that squalamine may act through a membrane-based steroid receptor, possibly one of the many known orphan receptors with unidentified functions, since it does not bind to the aldosterone, progesterone, glucocorticoid, or estradiol receptors (data not shown).

Reduced NHE-3 function following squalamine treatment does not appear to be connected with cytotoxicity to transfected PS120/NHE-3 cells, as indicated by the inability to detect levels of lactate dehydrogenase release from transfected cells >30% at concentrations up to 35 μg/mL *(76)*. The evidence on brush border exchanger function hints that squalamine may act indirectly through an intracellular signaling pathway, or even as an intracellular modulator of cell activation. Additional information on intracellular signaling was obtained by examining inhibition of NHE-3 function in PS120/NHE-3 transfected cells, using a series of C-terminal truncation mutants of rabbit NHE-3 *(77)*. It was found that the inhibitory activity of squalamine mapped to the C-terminal 76 amino acids of rabbit NHE-3, a region of the NHE-3 antiporter known to contain a tyrosine kinase phosphorylation site and a calmodulin-binding site. It is not known if either of these molecular signaling sites is involved with squalamine inhibition, although it was found that 3.5 μg/mL squalamine was much more potent than the tyrosine kinase inhibitor genistein (at 100 μg/mL) in reducing Na^+/H^+ exchange, and squalamine plus genistein was very similar to squalamine alone in inhibitory action *(76)*. Since it has been elegantly shown that NHE phosphorylation correlates with activation of NHE ion exchange *(78)*, these data appear to increase the likelihood that squalamine influences NHE-3 phosphorylation, and thereby reduces the activity of NHE-3 by inhibiting a tyrosine kinase pathway common to genistein. The effect of squalamine on NHE-3 may or may not be relevant to endothelial cell activation, unless certain types of endothelial cells carry NHE-3.

Mechanistic data that also implicate squalamine in NHE function were obtained using human microvascular endothelial cells (HMVECs; Clonetics) in assays relying on the extracellular acidification rate (ECAR), as detected in a Cytosensor microphysiometer (Molecular Devices, Mountain View, CA). It was found for HMVECs, which were pretreated with 4 μg/mL squalamine or buffer for 60 min and then stimulated in culture with 20 ng/mL VEGF, that squalamine provoked a 50% decrease in the VEGF-stimulated ECAR for HMVECs. However, squalamine was ineffective in suppressing the ECAR in HMVECs stimulated with acid activation. By contrast, it was found with human melanoma cells that 4 μg/mL squalamine reduced the basal ECAR and the acid-stimulated ECAR, but 10 μ*M* methyl-isobutylamiloride (MIA), which is a known NHE inhibitor highly specific for NHE-1, had no effect. This suggests that NHE-3 or closely related NHEs (possibly NHE-5, which is structurally similar to NHE-3), which are sensitive to squalamine, are involved in maintaining basal ECAR in melanoma cells, but NHE-1 is not. Since many tumors, such as melanomas, produce autocrine growth factors and divide rapidly in response, it is also possible that the basal ECAR in melanoma cells is elevated over that found in normal cell types, and more closely resembles the ECAR in growth-factor-stimulated normal cells.

Are the squalamine effects at a cellular level, as studied with melanoma cells, at odds with the lack of cytotoxicity seen when gliomas were treated with squalamine (Fig. 4)? Possibly not, since, in the authors' experience, human wm1617 melanoma cells are unusual, in that, unlike any other tumor line evaluated, they have been comparable in sensitivity in vitro to squalamine inhibition of cell proliferation to endothelial cell lines, such as bovine pulmonary artery endothelial cells, and more sensitive than human coro-

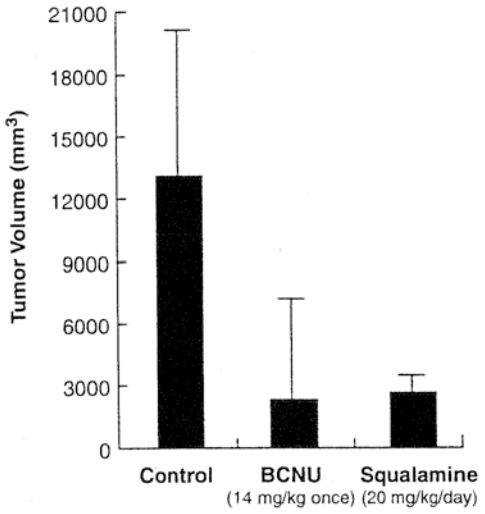

Fig. 10. Squalamine inhibition of growth of the rat 9L glioma implanted in the rat flank. Animals received 14 mg/kg 1,3-*bis*(chloroethyl)-1-nitrosourea (BCNU; $n = 6$), daily injections of squalamine ($n = 6$), or daily injections of saline ($n = 4$), beginning 5 d after the tumors were implanted.

nary artery endothelial cells. More data would need to be collected to clarify if squalamine truly has some degree of activity against certain cancer cell types. There is precedence for antitumor activity of antiangiogenic compounds in vitro, such as has been seen with the antiangiogenic agent AGM-1470 (TNP-470) *(79,80)*.

6. DISEASE MODELS INVOLVING NEOVASCULARIZATION AND SQUALAMINE

To determine if squalamine, like the angiostatic steroids previously studied, was effective in disease models involving neovascularization in animals, squalamine was evaluated in two tumor models, the rat 9L glioma allograft and human lung tumor xenografts in mice, and in a neonatal mouse model for corneal neovascularization, which employs hyperoxic exposure *(81)*. The conditions of this model simulate the ocular condition of retinopathy of prematurity (ROP). Although rare, ROP in premature human infants is important, because it can result in blindness.

The rat 9L glioma is widely used as a model for human brain tumors, and for evaluation of the consequences of chemotherapy *(82–85)*. Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU) is an approved agent for the treatment of human brain tumors, and is active in the rat 9L glioma model. BCNU displays cumulative toxicity in animals and humans, so that, when given systemically in animals, a single dose is the common dosage. Systemic squalamine was compared in the rat 9L glioma model to BCNU treatment, when the 9L glioma was transplanted either in the rat brain or in the rat flank. In the latter instance, the blood–brain barrier is not encountered, and drug delivery is less in doubt.

It was found that squalamine given daily by sc or ip injection, beginning on d 3 after transplantation of the 9L glioma, had limited influence on rat survival when the 9L glioma was in the rat brain, but control of 9L glioma growth in the rat flank was similar for BCNU (14 mg/kg, single dose) and squalamine (20 mg/kg daily; Fig. 10). When tumor

microvessel density was evaluated by histochemistry with an antibody to the endothelial cell integrin CD34, it was found that squalamine decreased tumor microvessel density in the rat flank model, in concert with tumor volume reduction, but the tumor microvessel density was comparable between control animals and animals treated with BCNU (data not shown). It was also found in this experiment that combined use of BCNU and squalamine was even more effective than either agent alone.

These results suggest that, if the blood–brain barrier is not an issue in drug delivery, squalamine may potentiate the activity of BCNU in restricting the growth of solid tumors, such as the 9L glioma. The degree of tumor control that squalamine has on the 9L glioma in the rat flank was investigated in a subsequent experiment, by varying the frequency of squalamine dosing. It was observed in this study that dosing as infrequently as twice a week considerably reduced the mean and median 9L tumor volume, when scored after 2 wk of treatment, and that tumor volume control improved as the frequency of squalamine dosing was increased *(86)*.

Another study, which further defined possible interaction between squalamine and cytotoxic agents, was conducted with human lung tumor xenografts in nude mice *(87)*. The human lung tumor lines H460 and Calu-6 were implanted subcutaneously in Balb/c athymic nude mice (d 0), and then treated with cisplatin at a maximally tolerated dose (on d 3), squalamine at 10 or 20 mg/kg daily (beginning on d 4), or with cisplatin on d 3 followed with daily squalamine treatment beginning on d 4. Squalamine as a single agent had no significant effect on the growth of either lung tumor line, but squalamine did significantly improve the response of both tumors to cisplatin. This observation remained true for the aggressive H460 tumor growth, even when squalamine was administered only on d 4, suggesting that squalamine has a persistent effect, as was seen with the rat 9L glioma in the rat flank.

Unwanted neovascularization is common to many diseases, of which solid tumors are perhaps the most devastating. Ocular neovascularization is another area of research in which there is interest in limiting angiogenesis to modify the disease outcome. A neonatal mouse model of hyperoxia-induced retinopathy was recently developed by Smith et al. *(81)*, for purposes of understanding the contribution of neovascularization to ultimate eye damage, and for testing the effects of antiangiogenic therapy on the course of the disease. Squalamine was studied in hyperoxygenated mouse neonates, to determine if squalamine could inhibit development of retinopathy without affecting normal retinal vessel growth. Newborn C57Bl/6J mice were exposed to 75% oxygen from postnatal d 7–12, and then allowed to recover in room air. Mice were then given sterile water or 30 mg/kg squalamine subcutaneously daily from d 12–17. Mice were sacrificed at postnatal d 17, and retinal flatmounts were analyzed following fluoroscein-conjugated dextran angiography. It was found that squalamine administration inhibited oxygen-induced retinopathy ($p < 0.001$), compared to controls *(88)*. Squalamine had no effect on ocular development in room-air-raised control neonates, and also did not have an impact on normal weight gain. These results can be interpreted to mean that squalamine did not have an appreciable effect on normal angiogenesis associated with growth of the animals.

7. SUMMARY

Squalamine is a new and natural angiostatic steroid. Multiple lines of evidence underline its ability to reduce new blood vessel development by a previously undescribed mechanism of action for angiostatic steroids, one which, like 2-methoxyestradiol, does

not require heparin or a heparin substitute. It is interesting that another inhibitor of human NHEs, the classical diuretic amiloride which preferentially blocks the activity of human NHE-1, has previously been shown to limit tumor growth and metastatic spread in mice and rats *(89,90)*.

The results described in this chapter, using animal disease models with squalamine, show that this new angiostatic steroid offers considerable promise in the treatment of solid tumors, probably because of its antiangiogenic character, as well as in other diseases characterized by undesirable neovascularization. Antiangiogenic agents have been suggested for over a decade as likely to be effective as adjunctive therapy in cancer, but to date no previous description, in animal tumor models, of the combined activity of a cytotoxic compound and an angiostatic steroid without heparin or a heparin substitute, has been published. Squalamine is unique, because it has been shown to have optimal activity in animal models of tumor growth when used in combination with BCNU or cisplatin. Additional data has recently been obtained that squalamine is also effective in a metastatic tumor model when used in combination with cyclophosphamide or 5-fluorouracil *(91)*. The spectrum of cytotoxic drugs that can be supplemented by the addition of squalamine treatment is therefore broad.

A number of antiangiogenic therapies have recently entered clinical trials in cancer *(92)*, and it is not clear which, if any, of them will be clinically effective. Squalamine has also entered clinical trials as a candidate drug for cancer therapy. It will be interesting to see to what extent preclinical research on squalamine will translate into clinical efficacy. It is possible that squalamine will eventually prove effective in treating advanced malignancies in humans, either as a single agent or in combination with one or another treatment modalities (chemotherapy, radiation, or hormonal therapy) now in clinical use. It is also conceivable that squalamine eventually will work in clinical applications in combination with other antiangiogenic agents with distinctly different mechanisms of actions, a concept first described by Folkman and Ingber *(15)*. Such combinations of antiangiogenic compounds, with different mechanisms that have been successful in preclinical animals models, include combinations of AGM-1470 (TNP-470) and minocycline *(93)* or AGM-1470 and α/β interferon *(94)*. The effective use of squalamine with other antiangiogenic agents remains to be demonstrated until more of these agents are readily available.

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11 Cartilage as a Source of Natural Inhibitors of Angiogenesis

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CONTENTS

1. INTRODUCTION

Ever since tumor-induced neovascularization was recognized as one of the key parameters that control tumor growth, considerable effort has been expended to identify ways to inhibit angiogenesis *(1–3)*. Two approaches have been attempted.

1. Inhibit the production or activity of angiogenic molecules produced by cancer cells;
2. Disable the endothelial cells from responding to the angiogenic signals coming from the malignant cells.

This second approach has been, to date, the most successful in identifying agents that block tumor neovascularization *in vivo* in model animals, resulting in an arrest of tumor growth. Several molecules are actively being studied and have already reached preclinical or clinical trials *(4)*. These include angiostatin *(5–7)* and endostatin *(8)*, two endogenous inhibitors resulting from the degradation of plasminogen and type XVIII collagen, respectively, TNP-470 *(5)*, a fumagilin derivative, as well as several pharmaceutical agents. Extracts of cartilage have also been suggested to bear antiangiogenic and antitumor growth activities, and companies, claiming potential antitumor activities, have proposed several solid-cartilage extracts as food supplement for cancer patients. However, for most of these non–well-characterized extracts, there is no scientific or clinical evidence of either antiangiogenic activities or benefit for the patient. Nevertheless, a liquid extract of shark cartilage, namely Æ-941 has been developed. This cartilage derivative

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is said to exhibit in vitro and in vivo antiangiogenic activities as well as antitumor and anti-inflammatory properties, as suggested by results obtained in mouse models. It has been engaged in phase I/II clinical trials, and phase I data indicated full tolerance of the compound as well as a preliminary efficacy response.

2. COMPOSITION OF CARTILAGE

Cartilage is a specialized connective tissue consisting of cells, chondrocytes and chondroblasts, and an extracellular matrix composed of fibers embedded in an amorphous, gel-like solution. The extracellular components of cartilage largely predominate the cells, which are isolated in small cavities within the matrix. Three kinds of cartilage, hyaline, elastic, and fibrocartilage, are distinguished on the basis of the amount of amorphous matrix and the relative abundance of collagenous and elastic fibers. Hyaline cartilage is the most common and characteristic type. Its capacity to grow rapidly while maintaining its stiffness make this tissue a particularly favorable skeletal material for the embryo. Indeed, most of the axial and appendicular skeleton is first formed in cartilage models, which is later replaced by bone. Cartilage is restricted in postnatal life but continues to play an important role in the growth of long bones. In adults, it persists on the articular surface and represents in mammals <1% of total body weight. In some species however, like fish, cartilage remains the unique skeleton component. For example, cartilage contributes up to 6–8% of the total body weight of the shark *(9)*.

A unique characteristic of cartilage is the absence of nerves or blood vessels, both of which are present in other connective tissues. The colloidal properties of the cartilage matrix are therefore important for the nutrition of chondocytes and chondroblasts. The cartilage matrix is composed of water (up to 80%), type II collagen and proteoglycans. These latter molecules contribute up to 50% of the dry weight of cartilage. Three major types of proteoglycans are described: chondoritin-4 –sulfate, chondroitin-6-sulfate, and chondroitin keratosulfate.

3. CARTILAGE: A TISSUE THAT RESISTS VASCULARIZATION

Skeletal development depends on the concomitant stimulation and inhibition of *in situ* angiogenesis. Thus, capillaries in the prechondrogenic areas regress to allow cartilage differentiation *(10–12)*, whereas during endochondral bone formation, proliferative cartilage undergoes vascularization and erosion, leading to bone deposition *(13,14)*. The avascular status of resting cartilage could be owing either to absence of proangiogenic molecules or to the overproduction of inhibitors of angiogenesis by chondrocytes. Indeed, the resistance of cartilage to capillary invasion seems to result from a balance between angiogenic and angiostatic molecules, the latter being in excess in resting cartilage, whereas the transformation of cartilage into bone is initiated by the switch toward an angiogenic phenotype. Such regulation of the angiogenesis switch in cartilage resembles the regulation of tumor angiogenesis where induction of neoformation of blood vessels is accompanied by upregulation of angiogenic growth factor, metalloproteinase activity, and downregulation of tissue inhibitors of metalloproteinases *(15,16)*. In cartilage, a number of angiogenic growth factors, such as basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGFβ) *(17,18)*, the low-mol-wt factor known as endothelial cell-stimulating factor (ESAF) *(19)* and transferin *(20)* have been shown to be present in growth-plate cartilage. On the other hand, among the angiogenesis inhibitors

present in cartilage, proteases inhibitors appear to play an important and preponderant role. Several TIMP-like molecules, among them at least two elastase inhibitors have been isolated from cartilage.

The possibility of using cartilage and more particularly shark cartilage, as a source of antiangiogenic activity has been the subject of intense discussion. The presence of diffusible angiogenesis inhibitors in normal cartilage was first reported by Brem and Folkman, who showed that normal cartilage fragments inhibit the proliferation of capillaries induced by tumors *(21)*. In these experiments, fragments of V2 carcinoma fragments were implanted into the intracorneal pockets of adult rabbits. The tumor induced a clear neovascularization visible through the cornea. Cartilage from the scapula of neonatal rabbits was implanted between the V2 tumor and the limbus of the eye. The control consisted of a fragment of lyophilized cartilage, boiled in distilled water and reconstituted in saline solution before implantation. The rate of capillary growth into the cornea was drastically decreased in the rabbit eyes implanted with fresh cartilage fragment but not in the controls. The same results were obtained when the experiments were performed using the chorioallantoid membrane of chick embryos as a source of neovascularization (CAM assay). A liquid extract of this cartilage was also found to inhibit the growth of new blood vessels. Thereafter, Langer et al. *(22)* reported that a cartilage fraction isolated by guanidine extraction and purified by affinity chromatography inhibited tumor-induced vascular proliferation and, as a consequence, restricted tumor growth. Three days after tumor implantation, regional infusion with a partially purified cartilage extract was initiated. This treatment induced a significant slowdown of capillaries and tumor growth. Since the cartilage extract had no direct effect on tumor cell proliferation, the antitumor action of the cartilage derivative was likely because of its antiangiogenic activities. Note that the infusion of cartilage extracts into rabbits or mice did not induce toxic effects.

Attempts to identify the active antiangiogenic compounds present in the cartilage extracts led to the observation that the angiostatic potential resides in molecular fractions below 50 kDa. Hence, cartilage-derived TIMP-1-like protein with a molecular weight of 27.7 kDa inhibits endothelial cell proliferation and migration in vitro and angiogenesis in vivo *(23,24).* An equivalent inhibitor was also isolated from the conditioned media of scapular chondrocytes grown in serum-free medium *(25)*. TIMP-2 was also isolated from cartilage extracts *(26)*. This protein, from which a partial sequence was obtained, exhibits inhibitory activities toward mammalian collagenases *(24)*. Therefore, the antiangiogenic activities of cartilage extracts were attributed at least in part to their anticollagenolytic activities. However, several authors showed that the cartilage contains other molecules that inhibit specifically endothelial cell proliferation, such as chondromodulin-1 and TGF-β *(27,28)*.

Because cartilage is present in a limited amount in mammalian species, much interest was directed toward the use of shark cartilage. In 1983, Lee and Langer *(9)* demonstrated that a shark cartilage extract, prepared using a more modified protocol than the one used for calf scapular cartilage, contains a substance that strongly inhibits tumor angiogenesis and restricts tumor growth. The authors found that the shark cartilage contains an inhibitor of type I collagenase. In addition, a low-mol-wt (<10 kDa) molecule was isolated from shark cartilage *(29)*. These data led to the development of a variety of commercial derivatives of shark cartilage proposed as antiangiogenic preparations. To date, only one of these extracts, Æ-941 has been characterized for its antiangiogenic properties in vitro and

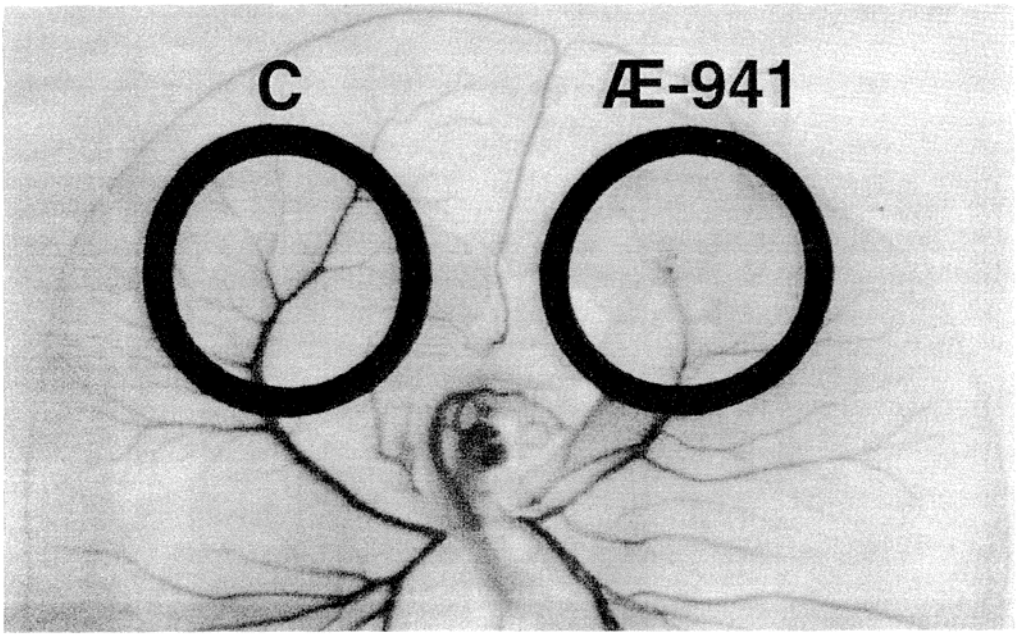

Fig. 1. Antiangiogenic activity of Æ-941. Æ-941 blocks the formation of vitelline blood vessels of chicken embryo (EVT).

in vivo and is engaged in clinical in-patient trial for cancer but also in patients suffering from angiogenic-dependent diseases, such as psoriasis and age-related macular degeneration.

4. Æ-941, A LIQUID SHARK CARTILAGE EXTRACT WITH ANTIANGIOGENIC PROPERTIES

Æ-941 is a liquid shark cartilage extract prepared by washing and scrubbing fresh shark cartilage under water to remove any residual tissue. The cleaned cartilage is then homogenized in demineralized water and stirred for 1 h to allow extraction of water-soluble molecules. After centrifugation, the liquid fraction is ultrafiltrated and the fraction below 500 kDa is retained. The ultrafiltrate is further filtered through a 0.22-μm membrane, dispensed into sterile glass vials, and stored at –20°C until use *(30–32)*.

The anti-angiogenic potential of Æ-941 was studied in a variety of in vitro and in vivo assays. Among them, this cartilage liquid extract was tested for its ability to inhibit blood vessel formation in the *ex ovo* chick chorioallantoid membrane assay. This assay is based on the development of a vascular system in the vitelline membrane, which is, upon opening of the egg shell, directly accessible for observation. When placed onto the vitelline membrane, antiangiogenic substances can inhibit angiogenesis. Methylcellulose disks containing Æ-941 were placed on the external border of the vascular perimeter of the vitelline membrane where the angiogenic process is the most active. The effect of the Æ-941-containing disk on angiogenesis was always compared to that of the control disk containing an equimolar amount of NaCl (Fig. 1). As shown in Fig. 2, Æ-941 inhibits in a dose-dependent fashion the growth of capillaries in the chorioallantoid membrane.

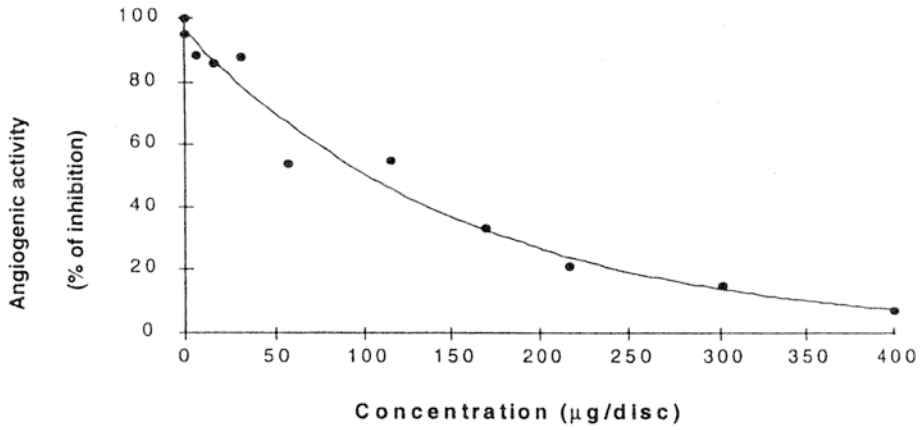

Fig. 2. Antiangiogenic activity of Æ-941. Dose-response inhibition of blood vessel formation by Æ-941 (EVT).

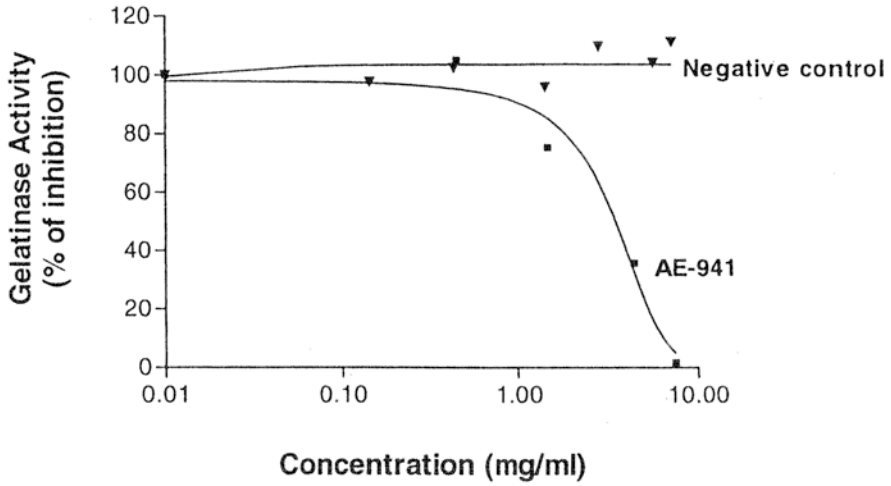

Fig. 3. Antimetalloproteinase activity of Æ-941. The enzymatic activity of gelatinase is inhibited by Æ-941 in a dose-response manner.

These results indicates that the liquid shark cartilage extract contains compounds that block angiogenesis. In addition to its ability to inhibit angiogenesis, the potential presence of metalloproteinase inhibitors in Æ-941 was explored based on previous reports of the presence of such molecules in cartilage extracts. A collagenase inhibition assay was developed using a fluorogenic peptide substrate. This test led to the demonstration that the cartilage extract induces a dose-dependent inhibition of MMP-2 activity (Fig. 3) *(33)*. Further in vitro assays are under investigation to determine the mechanism of action through which Æ-941 exercises its antiangiogenic and anticollagenolytic activities.

The possibility that Æ-941 may retain its antiangiogenic properties in tumor-bearing animals and potentially stop or slow down cancer progression or inflammatory phenomena was examined in vivo. Among the various animal models for solid tumors where Æ-941 have been tested, the results obtained with the DA3 adenocarcinoma model are the

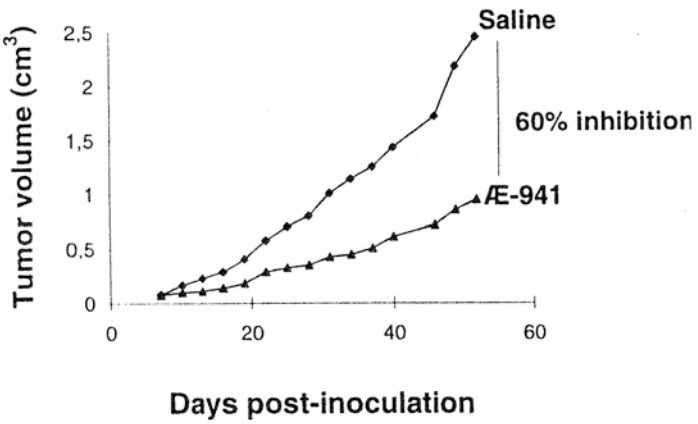

Fig. 4. Antitumor activity of Æ-941. Æ-941 induces a 60% decrease of primary tumor growth (DA3 model).

most representative *(34,35)*. DA3 cells are murine mammary carcinoma cells obtained by the treatment of mouse 7,12-dimethylbenzanthracene (DMBA). Injected subcutaneously into mice, the cells form a slow growing tumor with low metastatic potential. Mice injected with the DA3 cells received a daily oral administration of Æ-941 or a saline solution for the control group 7 d following cell inoculation. Tumor growth was monitored every third day by determination of the relative tumor volume. The mice were sacrificed on d 54 after tumor inoculation. The tumors were then dissected and weighed. Oral administration of 100 mg/kg d of Æ-941 as compared to the control group resulted in a statistically significant inhibition of 60% of tumor volume ($p < 0.02$) (Fig. 4) and of 47% of tumor weight ($p < 0.01$) (data not shown). Similar antitumor effects of Æ-941 have also been observed in metastatic models, like the Lewis lung carcinoma.

The evolution of chronic inflammatory phenomena being tightly linked to the formation of new vessels, the anti-inflammatory activity of Æ-941 has been tested in a human model of skin irritation *(33)*. The degree of redness of the skin was measured with a chronometer and compared with a positive and a negative control. The value of the negative control was estimated as 100% of irritancy. The results showed that administration of Æ-941 was efficient in reducing skin irritation in a dose-dependent manner (Fig. 5). Therefore, the above data are the first pharmacological demonstration that an orally administered cartilage extract is able to slow down the progression of a variety of pathological angiodependent situations. In addition, they demonstrate that, when given orally, the active component(s) of Æ-941 is (are) bioavailable and nontoxic. The absence of toxicity has further been verified in different species. Æ-941 was administered orally in mice, rats, dogs, and primates at various doses. Until now no toxic effects directly associated with Æ-941 administration have been reported.

Because it has virtually no toxic effect, Æ-941 has been engaged in phase I and phase II clinical trials in Canada and the United States for the treatment of breast, prostate, and lung carcinoma, as well as for psoriasis and age-related macular degeneration. Up to 430 cancer patients for more than 25 mo have to-date received a daily oral dose of Æ-941. No serious adverse reactions associated with the use of Æ-941 have been reported thus far. Most interestingly, preliminary data of clinical efficacy from lung and prostate cancer as

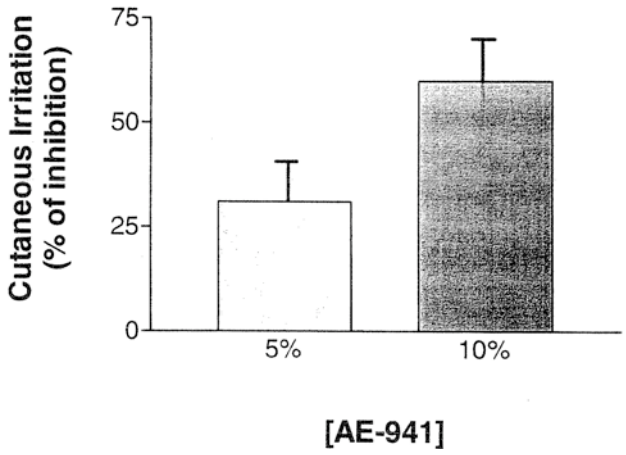

Fig. 5. Anti-inflammatory activity of Æ-941. Dose-dependent inhibition of cutaneous irritation in humans by Æ-941.

well as psoriasic patients have been obtained. Despite the small number of patients per dose, these data showed a significant trend in favor of a dose/response effect on the patients' clinical benefit, body weight loss, and analgesic consumption ($p < 0.01$) and allowed scientists to determine the most effective dose to be administered in phase III clinical trials, planned for early 1999.

5. CONCLUSIONS

Although there are still intense discussions on the value of cartilage as antiangiogenic agents, there are now sufficient data that support that the derivatives of Æ-941 extract from shark cartilage contain antiangiogenic compound(s) that is (are) active in vitro and in vivo. The bioavailability of these compounds appears to be efficient since biological response was demonstrated in animals receiving the derivative orally. Appropriate phase III clinical trials should demonstrate the therapeutic value of Æ-941 in human cancer. It is also crucial to isolate, purify, and characterize the active molecule(s) that is (are) responsible for the biological properties of Æ-941. Intense research engaged in answering these questions is currently being done and should in the near future the confirmation that may make Æ-941 a unanimously recognized tool to be added to the batteries of new emerging anticancer therapies.

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12 Thrombospondin as an Inhibitor of Angiogenesis

David W. Dawson and Noël P. Bouck

Contents

1. INTRODUCTION

Most normal, healthy adult tissues exist in a state of vascular quiescence that is sustained by a combination of influences that include embryonic factors *(1)*, associated cells *(2)*, and an overall balance of angiogenic factors in which inhibitors predominate over inducers *(3)*. Thrombospondin (TSP) is one of a small number of naturally occurring inhibitors of angiogenesis that is well-positioned to contribute to this maintenance of vascular quiescence in normal tissues, and whose loss in pathologic conditions, particularly cancer, contributes to increased neovascularization. First identified over two decades ago as a secretory product of thrombin-stimulated platelets *(4,5)*, TSP is now known to be secreted by a wide array of cell types, including endothelial, fibroblast, smooth muscle, glial, keratinocyte, and inflammatory cells, and to participate in diverse biological processes, including coagulation, fibrinolysis, neurite outgrowth and nerve regeneration, tumor growth and metastasis, embryonic development, differentiation, inflammation, and angiogenesis *(6)*.

Thrombospondin-1 (TSP-1) is the prototype for the thrombospondin family, which consists of five related proteins encoded by separate genes. All family members assemble into homotrimers or homopentamers, and their primary sequences share key structural domains (Fig. 1; *7*). TSP-1 is a 450-kDa trimeric glycoprotein made up of three identical 180-kDa subunits associated by interchain disulfide bonds. Each subunit consists of globular domains at its amino and carboxy termini, connected by a large central stalk, which can be further subdivided into a region of procollagen homology, a region of three properdin (type 1) repeats, a region of three EGF-like (type 2) repeats, and a region of seven calcium-binding (type 3) repeats *(8)*.

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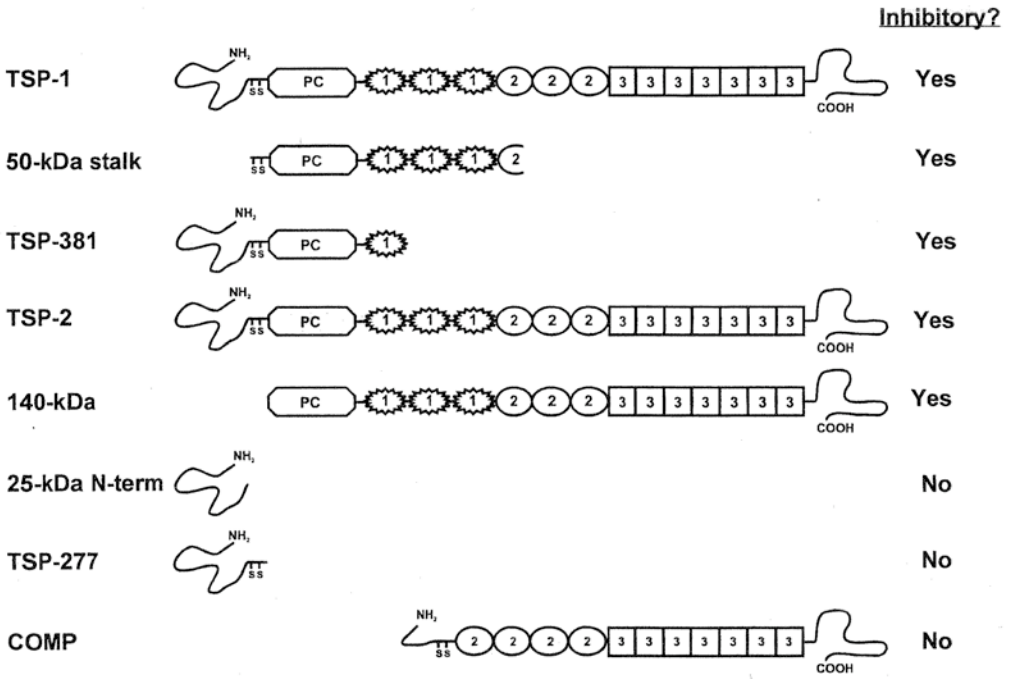

Fig. 1. The angioinhibitory activity of various fragments of thrombospondin. The 180-kDa subunit of TSP-1 and its domains are depicted roughly to scale in relationship to generated TSP-1 fragments and family members. The ability of each fragment to inhibit both in vitro and in vivo angiogenesis is listed. Domains are represented by different shapes and labels, and include procollagen homology (PC, octagon), type-1 repeats (1, star), type-2 repeats (2, oval), type-3 repeats (3, square), and region of disulfide bonding (noted by S). Shaded domain indicates lack of sequence homology to other fragments.

This chapter focuses on the role of TSP-1 as an inhibitor of angiogenesis, a function that has generated much controversy, and has been a topic of many reviews *(9–13)*. Current data suggest that, although multifunctional TSP-1 can have both stimulatory and inhibitory effects on endothelial cells, and on angiogenesis-dependent processes, at normal physiological concentrations, its inhibitory influences seem to predominate.

2. INHIBITION OF ANGIOGENESIS BY THROMBOSPONDIN-1

2.1. TSP-1 Is a Potent Inhibitor In Vivo

2.1.1. TSP-1 Inhibits Neovascularization in Multiple Experimental Assays

Purified TSP-1 protein, isolated from human platelets or from BHK hamster cells, has been tested in a variety of in vivo assays, in which, with a single exception, it has been a potent inhibitor of new blood vessel growth. When incorporated into Hydron pellets, along with an inducer, and implanted into the corneas of rats *(14–16)* or mice *(19)*, TSP-1 is consistently inhibitory (concentrations from 0.65 to 2.5 μg/mL have been tested) *(16)*. It is effective against pure angiogenic agents bFGF, TNFα, VEGF, and TGF-β; against angiogenic media conditioned by activated macrophages, and by transformed epithelial or fibroblastic cell (*15*; O. V. Volpert, N. Bouck, and P. J. Polverini, unpub-

lished data), and against scatter factor *(17)*. The observation that the inhibition of corneal neovascularization by TSP-1 is sensitive to two different monoclonal anti-TSP-1 antibodies *(16)* suggests that inhibition is caused by the TSP-1 molecule itself, and not a contaminant. TSP-1 also inhibits angiogenesis that is stimulated by VEGF on the chick chorioallantoic membrane *(18)*, and that accompanying granulation tissue invading polyvinyl sponges *(14)*.

TSP-1 is effective at inhibiting angiogenesis induced by living tumor cells in several settings. Small pieces of mouse melanoma, or human colon carcinoma cut from tumors growing in nude mice and implanted directly into rat corneas, induce vessel ingrowth that is blocked when a pellet containing TSP-1 is interposed between the tumor fragment and the vascular limbus (P. J. Polverini and N. Bouck, unpublished data). Systemic treatment of mice with purified TSP-1 can also be antiangiogenic and inhibit tumor growth. High levels of TSP-1 in the circulation render mice unable to mount a corneal angiogenic response to a pellet containing stimulatory bFGF and halt the growth of experimental lung metastases *(19)*.

There is a single report of TSP-1 failing to inhibit angiogenesis in an in vivo assay *(20)*. When incorporated into Elvax-40 pellets, human platelet thrombospondin was slightly angiogenic when tested alone, and enhanced, rather than inhibited, angiogenesis stimulated by bFGF or by LPS. This assay seems to be accompanied by significant inflammation, as indicated by its sensitivity to antibodies that neutralize inflammatory mediators, and its insensitivity to those that neutralize the implanted inducers *(21)*. Thus, it is possible that the stimulation attributed to TSP-1 is caused by the presence of TGF-β, which often contaminates TSP-1 preparations *(22)*, and may enhance the already significant inflammation present in this model. In contrast, similar concentrations of TSP-1 from three different sources tested in Hydron pellets in rat and mice corneas have not stimulated vessel ingrowth, and have always been inhibitory when combined with inducers (*14,16*; O. V. Volpert and N. Bouck, unpublished data).

2.1.2. Endogenously Produced TSP-1 is a Natural Inhibitor in a Variety of Different Tissues

TSP-1 is produced by many normal cells growing in vitro (*see* ref. *13*), and by many tissues in vivo *(23,24)*. It is present in human plasma at about 0.5 n*M* (i.e., ref. *25*), a level roughly equivalent to its ED_{50} for inhibition of endothelial cell migration *(14)*. TSP-1 is the major inhibitor of angiogenesis secreted by primary cultures of cells derived from human glia *(26)*, or foreskin fibroblasts *(27)*, or bladder urothelium *(27a)*. TSP-1 is produced by these cells at such high levels that their conditioned media are inhibitory, and underlying inducing activity is masked.

TSP-1 also has an inhibitory influence on the development of the normal vasculature in organs that are initially vascularized by angiogenesis, and in tissues undergoing remodeling. When vessel counts are compared between wild-type mice and those null for TSP-1, a fivefold increase is seen in the number of vessels traversing the chambers of the eye in the null animals *(28)*, and a threefold increase is seen in the number of small capillaries of the cerebral cortex of null animals *(29)*. As might be expected from the fact that antiangiogenic TSP-1 is the major protein released when platelets degranulate during plasma clotting to form serum, the sera of wild-type animals are antiangiogenic at concentrations where sera from TSP-1 null animals are angiogenic *(28)*. TSP-1 null mice also show prolonged neovascularization of skin wounds *(30)*, and an increased vessel density in regressing mammary glands *(18)*.

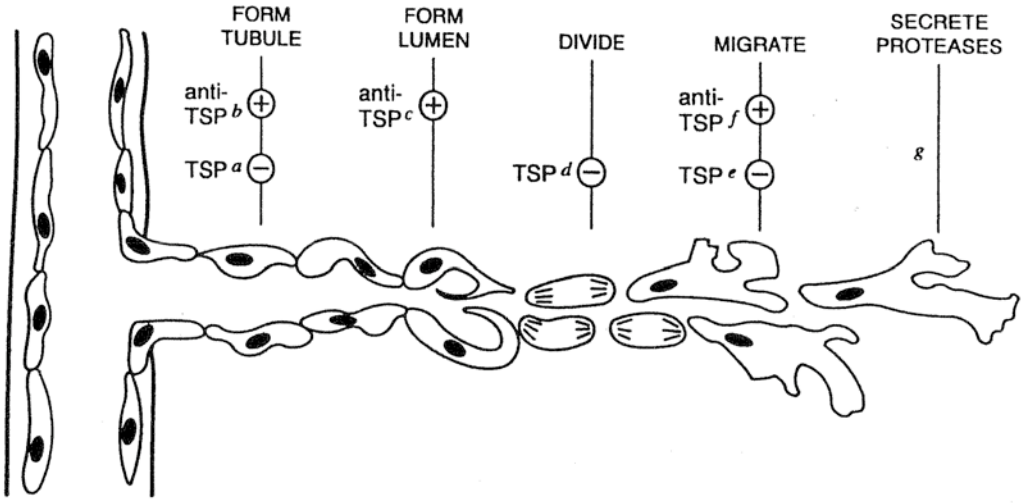

Fig. 2. Summary of how increases and decreases in TSP-1 activity influence in vitro functions of endothelial cells that contribute to neovascularization in vivo. **(A)** *33*; **(B)** *106–108*; **(C)** *33*; **(D)** *16,32,40,44,48,109,110*; **(E)** *14–16,38–40*; **(F)** *33,107*; **(G)** *33*. Symbols: +, stimulatory; –, inhibitory; o, no change detected. Updated with permission from ref. *33*.

2.2. TSP-1 Acts Directly on Endothelial Cells

2.2.1. TSP-1 Inhibits Most Proangiogenic Activities of Cultured Endothelial Cells

TSP-1 acts directly on endothelial cells. Exogenous TSP-1, at concentrations from 1 to 10 n*M* inhibits the chemotactic migration of cultured capillary endothelial cells (*see* Fig. 2 and references therein). At 5–10-fold higher concentrations, it also inhibits mitogenesis and tube formation (Fig. 2). Although TGF-β is an effective inhibitor of migration and mitogenesis in endothelial cells and is a frequent contaminant of TSP-1 preparations, and TSP-1 is able to activate latent TGF-β released by endothelial cells *(31)*, the inhibition of mitogenesis and migration by TSP-1 can occur independently of this cytokine *(14,32)*.

TSP-1 produced by endothelial cells also has an autocrine inhibitory effect on both migration and tube formation, because neutralizing TSP-1 antibodies increase the sensitivity of endothelial cells to inducers that stimulate these activities (Fig. 2). Such antibodies also stimulate sparsely plated endothelial cells to develop vacuole-like structures that fuse to form cylindrical holes in the cell, which appear to be sealed by thin membranes at each end *(33)*. Because these vacuole-like structures stain for the luminal marker angiotensin converting enzyme, they may represent incipient lumens, whose formation is usually prevented by endogenous TSP-1. Such an interpretation is in concert with the observation of Sheibani and Frazier *(34)* that clones of immortal endothelial cells made to express very high levels of TSP can organize into cords, but fail to form lumens.

The only in vitro phenotype of activated endothelial cells that has not been shown to be influenced by TSP-1 treatment is the production of proteases. Although the migration of endothelial cells requires both serine proteases and metalloproteinases, TSP-1 does not cause measurable changes in protease activities of endothelial cells. Neither overall secreted metalloproteinase activity nor induced cell-associated urokinase plasminogen activator activity is altered by the presence or absence of TSP-1 *(33)*.

2.2.2. TSP Inhibition of Migration Is Specific for Vascular Cells

Although TSP-1 can stimulate the migration of a variety of different cell types at μ*M* concentrations (i.e., refs. *35* and *36*), when it is tested at lower, n*M* concentrations, only the migration of small-vessel endothelial cells or vascular smooth muscle cells is inhibited *(33)*. Endothelial cells from large vessels, like the human umbilical vein, are often insensitive *(37)* because of lack of CD36 (*see* Section 4.1.3.), although bovine endothelial cells from the pulmonary artery are responsive *(15)*. Levels of TSP in the inhibitory n*M* range have no effect on the migration of fibroblasts, neutrophils, or keratinocytes *(15,33)*.

2.2.3. TSP Inhibition Is Effective Against a Variety of Inducers

TSP-1 is effective at inhibiting chemotaxis in vitro and neovascularization in vivo that is induced by a wide variety of angiogenic stimuli. These include proteins that act via tyrosine kinase receptors (VEGF, bFGF, aFGF, PDGF, SF), via G proteins (IL-8), via serine/threonine kinase receptors (TGF-β), and also lipids (PGE-1; lysophosphatidic acid) *(17,32,38)*. Additionally, TSP-1 is effective against mixtures of these inducers, including natural mixes derived either from tumors or from activated macrophages or inflammatory cells *(14)*. This broad specificity makes questions about its mechanism of action especially intriguing.

2.2.4. At High Concentrations TSP-1 Can Stimulate Endothelial Cells In Vitro

Although TSP-1 is an effective inhibitor of endothelial cell migration at doses in the lower n*M* range, when its concentration rises above 20 n*M*, it becomes stimulatory and additive with the inducer bFGF (*14,15,39–41*; Fig. 3). As detailed in Section 4.1.4., this stimulation can be attributed in part to increased random migration caused by the N-terminal heparin-binding region of TSP-1 *(14,39)*, an effect that seems unlikely to contribute to angiogenesis, because this region of the molecule is inactive when tested in vivo *(14)*. It is also caused in part by the engagement of a second receptor (IAP) *(41)*, by the C-terminal globular domain of TSP-1 (*see* Section 4.1.4.). As predicted, no stimulation of migration is observed when the central stalk region of TSP-1, which lacks the N- and C-terminal domains, is used *(14)*.

High concentrations of TSP-1 can stimulate tube formation in an aortic ring assay, but in this case the TSP-1 is acting, not on the endothelial cells directly, as it is in the migration assays described above, but instead is stimulating myofibroblasts. In this assay, tubules of endothelial cells grow out from the wounded surface of the aorta explant in response to the continuous production of potent angiogenic factors by the aortic myofibroblasts *(42)*. A similar proangiogenic influence of myofibroblastic cells has also been seen in cells cultured from fat pads *(43)*.

3. DOMAINS OF TSP-1 PROTEIN INVOLVED IN ANGIOGENESIS

3.1. TSP-1 Proteolytic Fragments and TSP-2 Inhibit Angiogenesis

The antiangiogenic activity of TSP-1 is thought to reside in its 50-kDa central stalk region (*see* Fig. 1), because this chymotrypsin-resistant core fragment of TSP-1, composed of the procollagen domain and the type-1 repeats, has the same specific activity as intact TSP-1 in the migration assay *(14)*. Two independent antiangiogenic regions seem to exist within this 50-kDa stalk, because angiogenesis is inhibited by a recombinant fragment of the first 381 residues of TSP-1 (TSP-381) *(14)*, which contains only the procollagen homology domain, and also by TSP-2 *(38)*, which contains type 1 repeats

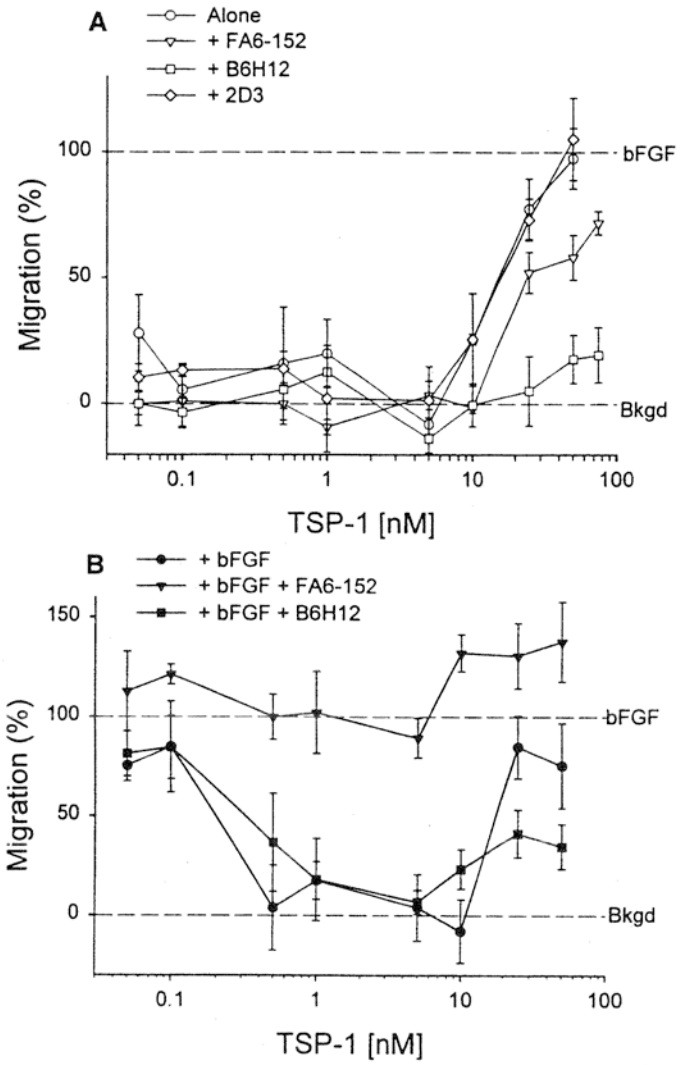

Fig. 3. Biphasic response of endothelial cell migration to TSP-1 explained by two receptors. Human microvascular endothelial cells were migrated, as previously described *(37)*, toward increasing concentration of TSP-1, in the absence **(A)** or presence **(B)** of inducer (10 ng/mL bFGF), either alone (circle) or in combination with a blocking antibody to CD36 (FA6–152, triangle), a blocking antibody to IAP (B6H12, square), or a nonblocking antibody to IAP (2D3, diamond). Data from seven experiments were normalized and reported as a percentage of the response of endothelial cells to bFGF alone (100%). Random background migration in the absence of inducer was subtracted and set at 0%. Antibodies were without effect when tested alone or in combination with bFGF. Bars indicate standard error.

similar to those of TSP-1, but has little homology to antiangiogenic sequences of TSP-1 in its procollagen domain *(14)*. TSP-2 is only half as potent as TSP-1 in inhibiting endothelial cell migration *(38)*, although no discrepancy in potency is seen using a ^{3}H-thymidine incorporation assay *(32)*. Trimerization of TSP-1 is not essential for antiangiogenesis, since a monomeric 140-kDa fragment of hamster TSP-1 is fully active *(14,16)*.

Table 1
TSP-1 Peptide Fragments with Angiogenic Activity

			Angiogenic activity	
Peptide	*TSP residues*	*Active motif*	*In vitro*	*In vivo*
Col overlap	303–309	GVXXR	Inhibitory	Inhibitory
WSXW	412–428	WSXWSXW	Inhibitory	?
Mal III	481–489	GVXXR	Inhibitory	Inhibitory
4N1K	1016–1024	RFYVVM	Stimulatory	Weakly stimulatory

The location of the peptide in TSP-1, its essential amino acid motif, its angiogenic activity in endothelial cell proliferation, and/or migration assays in vitro and in cornea assays in vivo are indicated.

Regions outside the 50-kDa stalk seem unlikely to play an important role in TSP-1 inhibition of angiogenesis. The N-terminal globular domain does not contribute to this activity. Both a proteolytically derived 25-kDa N-terminal piece and a recombinant fragment encompassing the first 277 residues of TSP-1 (TSP-277) fail to inhibit endothelial cell migration or corneal neovascularization *(14)*. There is a report *(44)* of a fragment containing the first 174 residues of TSP-1 inhibiting bFGF-induced endothelial cell proliferation, but concentrations 500–1000-fold over those sufficient for intact TSP-1 activity were required. In this situation, the heparin-binding regions of TSP-1 are probably blocking bFGF access to heparin-binding sites.

Regions C-terminal to the 50-kDa stalk also appear to lack antiangiogenic activity. COMP (TSP-5), which contains the type-2 and type-3 repeats and C-terminal globular domain, but not the procollagen homology or type 1 repeat domains, fails to inhibit endothelial cell migration or corneal neovascularization *(38)*.

3.2. Small Peptides from the Stalk Region of TSP-1 Inhibit Angiogenesis

Four peptides derived from the procollagen and properdin repeat regions of TSP-1 have allowed the further localization of TSP-1's antiangiogenic activity (Table 1). Two of these peptides act directly through a surface receptor on the endothelial cell, and two act indirectly to influence endothelial cells.

Receptor-dependent peptides have been derived from the procollagen homology region (Col overlap), and from the third type 1 repeat (Mal III). They inhibit in vitro endothelial cell chemotaxis and in vivo neovascularization in corneas and sponge implants *(14)*. Both peptides act directly on endothelial cells via the CD36 receptor *(37)* (*see* Section 4.1.1.). Mal III contains the well-documented CD36-binding motif CSVTCG *(45–47)*, but this motif is not sufficient to inhibit angiogenesis (S. Tolsma, personal communication). Nor is this motif essential for the inhibitory activity of Mal III, because substitution of CSVTCG with CSTSCG does not alter inhibitory activity of the peptide in vitro or in vivo (*14*; S. Tolsma, personal communication). Mal III and Col overlap share the common motif, GVXXR, which is necessary for their inhibitory activity *(14)* and thus probably defines the residues able to activate CD36.

Although a peptide from the second type 1 repeat, Mal II, was originally reported to be antiangiogenic *(14)*, that activity has since been found to be caused by a minor contaminant that is identical in primary sequence to Mal II, but contains an L- to D-amino acid substitution *(47a)*. Mal II containing all L-amino acids is inactive in an endothelial cell

migration assay, but a Mal II analog with a single D-Ile is inhibitory at concentrations as low as 10 n*M*, within a log of the effective concentration of the intact TSP-1 protein *(47a)*. The active, substituted Mal II acts through CD36, and apparently derives its activity from an increase in CD-36 receptor affinity *(47a)*. The low n*M* potency of the D-Ile-substituted Mal II peptide suggests that small, CD36-interacting TSP-1 peptides or their mimetics have therapeutic potential.

Two additional sets of inhibitory peptides, derived from the 50-kDa stalk of TSP-1, act indirectly to block proangiogenic activities of endothelial cells. Peptides derived from a sequence in the second type-1 repeat, containing the heparin-binding motif WSXWSXW, block bFGF-induced endothelial cell proliferation and chemotaxis, apparently by competing with bFGF for cell surface heparin-binding sites *(44,48)*. The ability of these heparin-binding peptides to block angiogenesis in vivo has yet to be demonstrated.

Peptides also derived from the second type 1 repeat, which contain the RFK motif, are able to activate TGF-β *(48,49)*, which in turn can influence endothelial cell behavior and angiogenesis (*see* Section 4.2.2.). A retroinverso mimetic derived from TSP-1, containing both heparin-binding and TGF-β-activating sequences, is able to slow the in vivo growth of a breast carcinoma xenograft *(48)*, but it is not yet clear if the peptide is acting directly on the tumor cells to slow their growth, or indirectly via halting angiogenesis, or both.

3.3. Stimulatory Peptides from C-Terminal Domain of TSP-1

Other peptides derived from TSP-1 have localized the stimulatory activity that is seen when endothelial cells are exposed to high concentrations of TSP-1 to the C-terminal globular region. Peptides derived from the TSP-1 C-terminal cell-binding domain, and containing the sequence motif RFYVVM (*see* Table 1), stimulate endothelial cell migration through an interaction with integrin-associated protein (IAP) *(41)*, and one of these peptides can stimulate endothelial cell chemotaxis, and is weakly positive in a rat cornea assay *(41,50)*. When inhibitory peptides Col overlap, or Mal III are mixed in equimolar amounts with a stimulatory RFYVVM peptide, the mix is inhibitory in a migration assay. However, when the whole TSP-1 molecule is at high concentrations, inhibitory sequences lose their predominance, and endothelial cell migration is stimulated through an interaction of C-terminal sequences of TSP-1 with IAP on the endothelial cells (*41,50*; *see* Section 4.1.3.).

4. MECHANISMS BY WHICH TSP-1 MODULATES ANGIOGENESIS

4.1. Cd36 Mediates TSP-1 Angioinhibitory Activity

4.1.1. Experimental Evidence

CD36 (glycoprotein IV) is an 88-kDa transmembrane protein that binds several molecules, including TSP-1, and is involved primarily in adhesion and scavenging (for reviews, *see* refs. *51* and *52*). A variety of experiments indicate that CD36 is necessary for the angioinhibitory activity of TSP-1 both in vitro *(37)* and in vivo *(52a)*. First, CD36 fusion proteins block the inhibitory activity of whole TSP-1 and of TSP-1 peptides in endothelial cell migration assays, and the potency of TSP-1 angioinhibitory peptides correlates directly with their relative binding affinity for CD36. Second, anti-CD36

antibodies, which block binding of TSP-1 to CD36, also abolish TSP-1 inhibition of endothelial cell migration. Third, TSP-1-unresponsive CD36-deficient human umbilical vein endothelial cells become responsive to TSP-1 inhibition of their migration and tube formation after transfection with CD36 *(37)*. Finally, bFGF-induced corneal neovascularization cannot be inhibited by TSP-1 in CD36 null mice, although angiostatin continues to inhibit well *(52a)*. CD36 alone may be sufficient for the antiangiogenic activity of TSP-1. A multivalent anti-CD36 IgM antibody itself also inhibits endothelial cell migration, presumably through oligomerization of the receptor *(37)*. Although TSP-1 can induce dimerization of membrane-bound CD36 *(53)*, this dimerization does not appear to be essential for its inhibition of endothelial cell migration, since TSP-1 peptides as small as seven residues, which seem unlikely to induce dimerization, also inhibit via a CD36-dependent mechanism *(37)*.

The ability of TSP-1 to directly bind certain inducers of angiogenesis, such as scatter factor *(17)* and bFGF *(54)*, has led to the suggestion that TSP-1 may inhibit their activity by sequestering them. In other experiments, TSP-1 competes with bFGF for binding to cell surface proteoglycans, which has led to the suggestion that TSP-1 may inhibit the activity of some inducers by masking their binding to proteoglycans *(44)*, an essential preliminary step for subsequent receptor binding *(55)*. Although the possibility exists that such competitive binding interactions might occur in vivo, no such effects have yet been demonstrated. In vitro TSP-1 inhibition of endothelial cell migration toward either scatter factor or bFGF is sensitive to anti-CD36-blocking antibodies *(37)*, and in vivo TSP-1 is unable to block bFGF-induced corneal neovascularization in CD36 null mice *(52a)*, suggesting there is no significant interference with inducer–receptor binding in these settings.

4.1.2. Potential Mechanisms for TSP-1 Inhibition of Angiogenesis Through CD36

Several models can be envisioned to explain why TSP-1 engagement of CD36 blocks the action of many different inducers of angiogenesis. Although the cytoplasmic domain of CD36 does not have any motifs that suggest it possesses enzymatic activity, it does co-precipitate from endothelial cell lysates with the src-family kinase Fyn, and possibly other src-related kinases *(12,52a,56)*. In microvascular endothelial cells, engagement of CD36 by TSP-1, or by its angioinhibitory peptides, leads to an increased tyrosine phosphorylated fyn associated with CD36 *(52a)*. Although subsequent downstream signaling events remain unclear, Fyn may be necessary for TSP-1 inhibition of angiogenesis, because TSP-1 fails to inhibit corneal neovascularization in Fyn null mice *(52a)*.

TSP-1 binding to CD36 on microvascular endothelial cells can also lead to an association of FAK with CD36 (Jimnéz et al., personal communication), and a peptide representing the C-terminal cytoplasmic domain of CD36 is able to co-immunoprecipitate focal adhesion kinase (FAK) from endothelial cell lysates *(12)*. Thus, TSP-1 may act through CD36 to modulate the activity of FAK, and thus inhibit endothelial cell migration, which is necessary for angiogenesis. FAK is involved in cell motility in general *(57,58)*. In migrating endothelial cells, there is an increase FAK phosphorylation *(59,60)* and recruitment to new focal adhesions *(61)*, and inhibition of FAK impairs migration, proliferation, and cord formation *(59,62,63)*.

In addition to being essential for TSP-1 inhibition of endothelial cell migration, CD36 can also mediate the induction of apoptosis by TSP-1 that the authors and others have

observed *(52a,64)*. TSP-1 induction of apoptosis in capillary endothelial cells is prevented by blocking antibodies to CD36, and a CD36-activating antibody itself is able to induce apoptosis in these cells *(52a)*. This raises the intriguing possibility that inhibition of endothelial cell migration may be the consequence of a commitment to apoptosis. Induction of apoptosis by peptides with the WSXWSXW motif from the second type-1 repeat *(64)* seems likely to occur by an alternate mechanism, because these peptides do not appear to possess a functional CD36-interacting motif. TSP-1 also inhibits neovascularization in vivo by a mechanism that involves apoptosis *(52a)*.

4.1.3. CD36 as Modulator of Endothelial Cell Responsiveness to TSP-1

Endothelial cell response to TSP-1 is affected by variations in CD36 expression and its posttranslational modifications. CD36 is absent from large-vessel endothelial cells derived from umbilical and portal veins *(65,66)*, and HUVECs are resistant to TSP-1 inhibition of migration and tube formation *(37)*. CD36 is present, although its expression may be heterogeneous *(67)*, on microvascular endothelial cells in various tissues *(65,66,68,69)*, and microvascular cells are generally sensitive to TSP-1, unless they have lost CD36 during extended culturing.

One might also predict that endothelial cell responsiveness to TSP-1 would be altered by factors that regulate CD36. Interferon-γ upregulates CD36 expression on endothelial cells *(66,70)*, suggesting that the ability of interferon-γ to inhibit angiogenesis may in part be the result of its ability to sensitize endothelial cells to endogenous TSP-1. Phosphorylation of CD36 on an extracellular domain decreases its affinity for TSP-1, and increases its affinity for collagen, with dephosphorylation having the opposite effects *(71)*. The action of extracellular kinases, such as platelet ectoprotein kinase A *(72)*, or phosphatases, seen in inflammation, could modulate inhibition of endothelial cells by TSP-1 (or collagen) by altering their CD36 phosphorylation state.

4.1.4. Engagement of Two Distinct Receptors May Underlie Biphasic Response of Endothelial Cells to Increasing Concentrations of TSP-1

The combined effects of stimulatory IAP and inhibitory CD36 provide a satisfactory explanation for the biphasic dose-response curve in which whole, intact TSP-1 first inhibits, and then stimulates, endothelial cell migration (*see* Fig. 3; *14,39,41*). At low TSP-1 concentrations, the action of CD36 predominates, and migration is inhibited. At high TSP-1 concentrations, CD36 becomes ineffective, either because it is depleted from the cell surface, as might be predicted from its role as a scavenging receptor, or because IAP signals predominate.

A TSP-1 dose response curve performed in the absence of inducer, showed TSP-1 was neutral at concentrations <20 n*M*, and then began to induce endothelial cell migration (Fig. 3A). This induction was, in large part, caused by IAP as a blocking anti-IAP antibody *(41)* that greatly reduced TSP-1-induced migration, but a blocking anti-CD36 antibody had no effect. The remaining TSP-1-induced migration seen in the presence of the anti-IAP antibody may represent residual chemokinetic activity contributed by the N-terminal heparin-binding domain of TSP-1 *(14,73)*. A second TSP-1 dose-response curve performed in the presence of the inducer bFGF showed that TSP-1 doses between 0.5 and 10 n*M* inhibited migration, and this inhibition was completely blocked by an antibody to CD36 (Fig. 3B). The blocking antibody against IAP had no effect on inhibition, but did

reduce stimulation of migration seen at high concentrations of TSP-1 in the presence of bFGF.

4.2. Biochemical Activities of TSP-1 That Could Influence Angiogenesis

Several biochemical activities of the TSP-1 protein have been defined in vitro that could ultimately impinge on angiogenesis in vivo. Proteolysis is essential for angiogenesis, and TSP-1 has been shown to bind and inactivate the serine proteases, neutrophil elastase and cathepsin G, which are found at sites of inflammation or injury *(74)*. Cathepsin G is also present in new vessels forming in glioblastomas and prostate carcinomas *(75)*. TSP-1 also binds to, and may inhibit the activity of, urokinase plasminogen activator and plasmin *(74)*, which clearly play important roles in extracellular matrix degradation by endothelial cells, as well as by tumor and stroma *(76)*.

TSP-1 could also modulate angiogenesis through its ability to activate TGF-β. In vitro, TGF-β stimulates endothelial cell migration at picomolar concentrations; at nanomolar concentrations, it inhibits endothelial cell migration *(14)*, as well as endothelial cell growth, proteolysis, and tube formation *(77–79)*. TSP-1 binds and activates latent TFG-β in vitro, and such activation can contribute to a portion of TSP-1's ability to inhibit the mitogenesis of cultured endothelial cells *(31)*. Because cultured endothelial cells produce latent TGF-β, its activation by TSP-1 can be hard to avoid. However, using neutralizing antibodies for TGF-β or TSP family members unable to activate the cytokine, the ability of TSP-1 to inhibit endothelial cell migration *(14)*, mitogenesis *(32)* and apoptosis *(52a)* have been shown to occur independently of the activation of latent TGF-β.

TSP-1 can also play a significant role in the activation of TGF-β in vivo *(79a)*. It is doubtful that this activation contributes to the ability of TSP-1 to inhibit neovascularization, because, when tested in vivo, TGF-β always appears to induce, rather than inhibit, angiogenesis *(78,80)*, probably because of its proinflammatory activity, and TSP-1 can block angiogenesis induced in the cornea by TGF-β *(15)*.

4.3. Physiological Activities of TSP-1 That May Influence Angiogenesis

TSP-1 can induce nonendothelial cells to proliferate, and to secrete growth factors that act in paracrine fashion on endothelial cells. In rat aortic explants placed in fibrin or collagen matrices, TSP-1 at high concentrations stimulates the proliferation of myofibroblasts, which in turn secrete inducers that stimulate the formation of capillary outgrowths *(42)*. High concentrations (0.5–3.0 μ*M*) of TSP-1 can also directly stimulate monocyte migration *(35)*, and permit macrophages to cross endothelial cell barriers *(81)*. TSP-1 at such high levels might be encountered in a fresh wound bed, where it could promote angiogenesis directly through its activation of IAP on endothelial cells, or indirectly via recruitment of inflammatory cells and activation of stromal cells or TGF-β.

5. INFLUENCE OF TSP-1 ON TUMOR ANGIOGENESIS

5.1. Varying TSP-1 Secretion by Tumor Cells Influences Angiogenesis and Tumor Growth

Changing the level of TSP-1 secreted by tumor cells themselves can regulate tumor growth rate and tumor-induced angiogenesis in vivo. The forced expression of high levels of TSP-1 in several tumor lines including a human breast carcinoma *(82)*, a human skin

carcinoma *(83,83a)*, a mouse hemangioma *(34)*, and a src-transformed NIH/3T3 *(84)*, has little effect on the growth rate of the cells in vitro, but changes their angiogenic phenotype, and causes a sharp decline in tumor growth rate and in tumor angiogenesis. The introduction of a normal chromosome 10q in human glioblastoma lines causes them to lose the ability to grow as tumors and simultaneously to become antiangiogenic because of the production of high levels of thrombospondin *(26)*.

Lowering TSP-1 production can speed tumor development. Some tumors produce high levels of TSP-1, yet are still angiogenic because of their ability to elaborate extremely high levels of inducers *(19)*. When transfection of antisense TSP-1 constructs is used to lower the level of TSP-1 secreted by one such fibrosarcoma line, its latent period is reduced in proportion to the degree to which TSP-1 secretion is depressed *(19)*.

5.2. Circulating TSP-1 Can Slow Tumor Growth and Produce Concomitant Tumor Immunity

Circulating levels of TSP-1, whether produced endogenously by tumors releasing high amounts of the protein, or exogenously by daily injections of purified protein, render mice unable to mount an angiogenic response, and also halt the growth of experimental lung metastases *(19)*. When the human fibrosarcoma HT1080, producing high levels of TSP-1, is grown subcutaneously in a nude mouse, the animal develops a high circulating level of human TSP-1 that can reach ~10 μg/mL in plasma. This circulating TSP-1 is able to dramatically reduce the growth of B16 melanoma lung metastases seeded by tail vein injection. Inhibition is probably caused by the inhibition of angiogenesis, because animals treated with TSP-1 become antiangiogenic, in that they are unable to mount a corneal angiogenic response to bFGF; TSP-1 has no effect on the in vitro growth rate or cloning efficiency of the melanoma cells forming the metastases; and the inhibition of the growth of metastases in vivo is reversible if TSP-1 treatments are halted.

This example of concomitant immunity, when a large tumor generating a circulating inhibitor of angiogenesis holds smaller tumors in check *(85,86)*, is the first in a human tumor in which the inhibitor is identified. The patient from whose primary tumor the HT1080 line was derived died within 3 mo of surgery, from widespread metastases *(87)*. TSP-1 in the human circulation has a half-life of 9 h *(88)*, making it reasonable to suggest that, in this patient, TSP-1 produced by the primary tumor may have been holding metastases in check by blocking angiogenesis, and that this control was lost upon removal of the primary, TSP-1-producing tumor. Exceptionally high levels of circulating TSP-1, detected in the sera of a few patients with other malignancies *(89,90)*, suggests that this may not be an isolated incident of concomitant tumor immunity dependent on TSP-1.

In other contexts, TSP-1 has been reported to stimulate metastases. When large amounts of TSP-1 are mixed with tumor cells prior to their injection into the blood stream, or are injected within a few minutes of injection of tumor cells, the clumping of tumor cells and platelets is enhanced *(91,92)*. This increases the size of emboli, and thus favors their arrest in the lung, and increases the frequency of lung metastases *(92,93)*. This effect requires large numbers of tumor cells, and TSP-1 concentrations of 50–100 μg/mL. This stimulation is an acute effect on the rate of seeding, not a chronic influence on the growth of the tumors. At the lower concentrations of TSP-1 present in experiments demonstrating concomitant immunity, no enhancement of metastasis seeding was observed *(19)*.

5.3. Modulation of TSP-1 Production During Tumor Development

TSP-1 is produced by a number of normal cells, and, as a result, their secretions inhibit angiogenesis *(3)*. As such normal cells develop into malignant tumors whose growth and survival depends on vigorous neovascularization, they must develop an angiogenic phenotype *(94)*. Tumors developing in tissues that elaborate high levels of TSP-1 usually downregulate TSP-1 secretion. This is seen in glioblastoma multiforme *(26)*, some fibrosarcomas *(95)*, some breast carcinomas *(96,97)*, and bladder carcinomas *(27a)*.

Essential changes in secretion of TSP-1, as well as in the production of other inducers and inhibitors of angiogenesis, are a result of the same genetic changes that drive carcinogenesis, the activation of oncogenes, and the inactivation of tumor-suppressor genes *(3)*. TSP-1 production can be regulated by a variety of oncogenes and tumor-suppressor genes, and, in contrast to the mitogenic effects of oncogene activation and tumor suppressor gene loss, which often can have a broad tissue range, the effects of these mutations on angiogenic agents are often strikingly tissue-specific. The activation of a variety of oncogenes can depress the production of TSP-1, and thus presumably enhance the angiogenic potential of a developing tumor. Activated ras is effective in several different cells *(97,98)*, as is src *(98,99)*. V-myc *(100)* and c-jun *(101)* also downmodulate TSP-1. Only in the case of v-myc has it been shown that the angiogenic phenotype of the cells switches from antiangiogenic to angiogenic as a result of the oncogene modulation of TSP-1 (O. V. Volpert, unpublished data).

Some tumor-suppressor genes support the production of TSP-1. When they are inactivated, the secretion of inhibitory TSP-1 falls off, and, as a result, the cells become more angiogenic. Wild-type p53 supports TSP-1 production in some breast cells *(96)* and in fibroblasts *(27,95)*, although not in glioma cells *(102)*, where TSP-1 production depends, instead, on an unidentified tumor-suppressor gene on chromosome 10p *(26)*, possibly PTEN/MCMAC *(103,104)*. In each of the above cases, the phenotype of the cells switches from antiangiogenic to angiogenic upon loss of the tumor-suppressor gene, because of a decrease in the secretion of inhibitory TSP-1. In addition, expression of NM-23 can increase TSP-1 production in melanoma cells *(97)*, suggesting that this metastasis suppressor could in some settings regulate angiogenesis via TSP.

It is also possible that the TSP-1 gene, located on human chromosome 15, could eventually come considered a tumor-suppressor gene in some contexts. Mice null for TSP-1, now about 2 yr old, do not yet have an increased number of tumors (J. Lawler, personal communication). However, loss of some tumor suppressor gene on chromosome 15 is associated with progression to a metastatic stage in breast cancer *(105)*, and a human squamous cell carcinoma line deficient for chromosome 15 is suppressed for both tumor growth and angiogenesis, upon return of either a normal human chromosome 15 or the TSP-1 gene *(83,83a)*.

6. SUMMARY

Thrombospondin-1 is one of only a handful of molecules identified to date found naturally in tissues in the absence of disease that contribute to the quiescence of the vasculature. Although, at high concentrations, such as those that might be found in regions of extensive platelet degranulation, it is capable of a number of activities that can be proangiogenic, the primary role of TSP-1 in normal physiology is that of a potent inhibitor of neovascularization. At low n*M* concentrations, it inhibits angiogenesis by

engaging the CD36 receptor on endothelial cells, rendering them refractory to all stimulants tested to date. TSP-1 is also an obstacle to be overcome, as normal cells, producing high levels of this inhibitory protein, attempt to progress to angiogenic tumors. The identification of potent peptide derivatives of TSP-1 raises the possibility that such molecules may be developed into nontoxic agents that will block angiogenesis, and thereby be useful in controlling the progression of a variety of angiogenesis-dependent diseases.

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13 Matrix Metalloproteinase Inhibitors

Peter D. Brown and Mark Whittaker

CONTENTS

1. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of structurally related enzymes that are capable of degrading proteins of the extracellular matrix. One of the earliest descriptions of MMPs was as depolymerizing enzymes, which, it was proposed, could facilitate tumor growth by making connective tissue stroma, including that of small blood vessels, more fluid *(1)*. Subsequent research has shown that these enzymes play a central role in the tissue remodeling associated with both physiological and pathogenic processes.

The human MMP family is now known to include at least 15 enzymes (Table 1). Three collagenases have been identified: interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase 3 (MMP-13). These enzymes can degrade the generally proteolytic-resistant fibrillar collagens, making a characteristic three-fourths length break in the α-chain *(2,3)*. There are two type IV collagenases *(4,5)*, now termed gelatinase-A (MMP-2) and gelatinase-B (MMP-9), which, as described by Liotta et al. *(6)*, can degrade type IV collagen of basal laminae, as well as other nonhelical collagen domains and proteins, such as fibronectin and laminin. The gelatinases have also been shown to degrade native insoluble elastin *(7)*. Three enzymes have been classified as stromelysins, although only stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) are closely related functionally, degrading various proteoglycan components of the extracellular matrix, as well as fibronectin and laminin *(8,9)*. Stromelysin-3 (MMP-11) was identified relatively recently in the tissue surrounding invasive breast carcinoma *(10)*. Its preferred substrate remains a matter of debate. It does not appear to breakdown known extracellular matrix proteins, but it is effective in degrading the serine proteinase inhibitor (serpin) α-1 antitrypsin, and in doing so may potentiate the action of serine proteinases, such as urokinase-type plasminogen activator (uPA) *(11)*. This serpinase activity is also dis-

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Table 1
Human Matrix Metalloproteinase Family

Enzyme	*Number*	*Principal substrates*[a]
Interstitial collagenase	MMP-1	Fibrillar collagens, types I, II, III
Neutrophil collagenase	MMP-8	Fibrillar collagens, types I, II, III
Collagenase-3	MMP-13	Type I collagen
Gelatinase A	MMP-2	Nonfibrillar collagens, fibronectin, laminin
Gelatinase B	MMP-9	Nonfibrillar collagens, types IV and V
Stromelysin-1	MMP-3	Nonfibrillar collagens, proteoglycan, laminin
Stromelysin-2	MMP-10	Nonfibrillar collagens, proteoglycan, laminin
Stromelysin-3	MMP-11	Serine protease inhibitors (serpins)
Matrilysin	MMP-7	Nonfibrillar collagens, fibronectin, laminin
Metalloelastase	MMP-12	Elastin, nonfibrillar collagen
MT1-MMP[b]	MMP-14	Progelatinase-A
MT2-MMP	MMP-15	Not defined
MT3-MMP	MMP-16	Progelatinase-A
MT4-MMP	MMP-17	Not defined
– –	MMP-18	Not defined

[a]The principal substrates listed are only helpful as a guide; in practice, the substrate specificity shown in vitro is broad with considerable overlap between MMPs.
[b]MT stands for membrane type.

played by other MMPs, and supports the hypothesis that the metallo- and serine proteinase families act in an interdependent manner.

Two enzymes have been identified, which, on the basis of sequence homology, do not belong in the three subgroups described above: matrilysin (MMP-7, formerly known as Pump) *(12)* and metalloelastase (MMP-12) *(13)*. Matrilysin is a short, truncated proteinase, which can degrade nonfibrillar collagen, fibronectin, and laminin; metalloelastase, as the name suggests, is capable of degrading elastin. In the past 2 yr, the MMP family has grown by the addition of a new subgroup, the membrane-type (MT)-MMPs. Currently, four members have been identified (MMP-14–MMP-17) *(14–17)*. These proteinases have a C-terminal transmembrane domain that allows them to be anchored in the cell membrane. The substrates for most of these enzymes have yet to be established; however, MT1-MMP and MT3-MMP (MMP-14, MMP-16) appear to be specific activators of latent gelatinase A *(16,18)*, and MT1-MMP has been shown to degrade a variety of matrix molecules, including collagen *(19)*. More recently, MMP-19, an enzyme with some homology to the stromelysins, has been added to the family *(20)*, and it is likely that other members will be discovered in the next few years.

Another recent development is the finding that MMPs can hydrolyze the membrane-bound precursor form of tumor necrosis factor-α (TNF-α), as well as various other cytokines *(21)*. Several members of the MMP family show some activity in this form of cytokine processing, and a novel metalloproteinase disintegrin that processes precursor TNF-α has recently been cloned *(22,23)*. The physiological and pathological relevance of this activity remains to be determined.

MMPs share several highly conserved domains, including a zinc atom binding domain [VAAHEXGHXXGXXH] in the active site, and an activation locus [PRCGXPD] in the

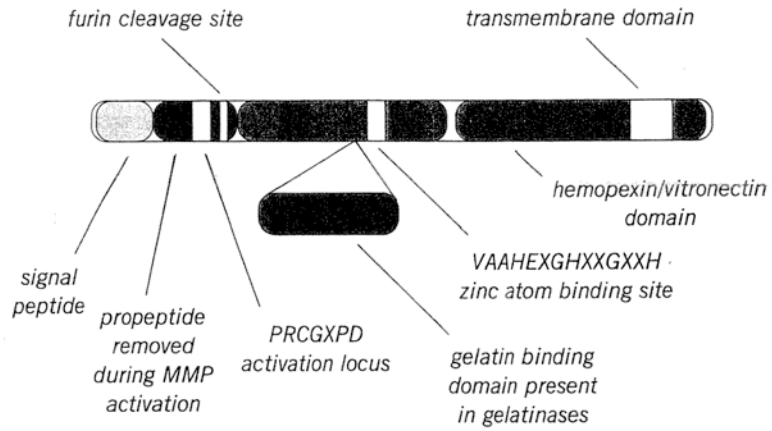


Fig. 1. Generic domain structure of matrix metalloproteinases. The hemopexin–vitronectin carboxy-terminal domain is severely truncated in the smallest matrix metalloproteinase, matrilysin. This domain also contains a transmembrane domain in the MT-MMP subfamily. The gelatin binding domain is unique to the two gelatinases, and the furin cleavage site is only present in stromelysin-3 and the MT-MMPs. Latency is conferred through co-ordination of the zinc atom by the cysteine residue in the PRCGXPD activation locus in the amino-terminal propeptide.

amino-terminal domain (Fig. 1). The cysteine residue in the activation locus co-ordinates the active-site zinc atom thereby conferring latency. This link must be broken by proteolytic cleavage of the amino-terminal domain or conformational modification, before the metalloproteinase can degrade matrix proteins. The activation step is initiated by several possible mechanisms involving either an enzymatic activation cascade and/or cell-surface regulation. In the case of stromelysin-3 and MT1-MMP, the presence of a furin-processing motif (Arg-Xaa-Lys-Arg) suggests that these enzymes can be processed intracellularly in the Golgi vesicles *(10,14)*. Once activated MMPs are subject to inhibition by endogenous proteinase inhibitors, such as α2-macroglobulin, and, more importantly, the family of tissue inhibitors of metalloproteinases, TIMPs 1–4 *(24–27)*.

These negative regulatory controls are clearly important for a family of enzymes with such destructive potential. Finely regulated MMP activity is associated with processes of ovulation *(28)*, trophoblast invasion *(29)*, skeletal *(30)*, and appendageal development *(31)* and mammary gland involution *(32)*. Unfortunately, it appears that these controls do not always operate as they should, and there is now a substantial body of observational and experimental data that indicates that inappropriate expression of MMP activity constitutes part of the pathogenic mechanism in several diseases. These include the destruction of cartilage and bone in rheumatoid and osteoarthritis *(33,34)*, tissue breakdown and remodeling during invasive tumor growth and tumor angiogenesis *(35)*, degradation of myelin-basic protein in neuroinflammatory diseases *(36,37)*, opening of the blood–brain barrier following brain injury *(38)*, increased matrix turnover in restenotic lesions *(39)*, loss of aortic wall strength in aneuryms *(40)*, and tissue degradation in gastric ulceration *(41)*.

In these pathogenic conditions, it seems that, rather than a complete uncoupling of inhibitory controls, there is a reprogramming of enzymes and inhibitors involved in tissue remodeling. This allows the construction of new tissue, including new blood vessels. This is perhaps best illustrated by cancer, in which simple tissue destruction would not be of advantage to the growing malignancy. In effect, the tumor must remodel the local tissue

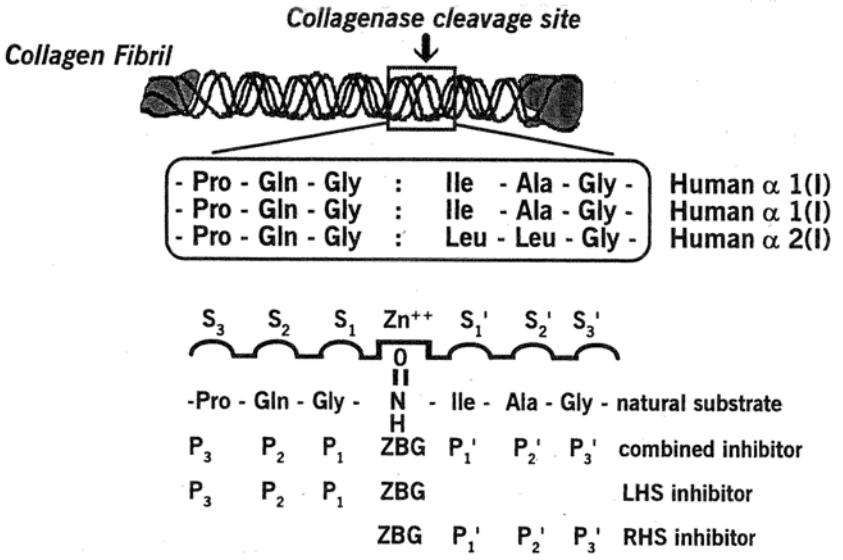


Fig. 2. Substrate-based design of MMP inhibitors. Many of the inhibitors currently being studied are derived from the peptide structure of the α-chain of type I collagen, at the point at which collagenase first cleaves the molecule. The peptide backbone is retained although groups are modified to give different inhibitory and physicochemical properties. Left-hand side (LHS) and right-hand side (RHS) and combined inhibitors have been prepared. The zinc binding group (ZBG) binds the zinc atom in the active site of the matrix metalloproteinase enzyme.

to suit its own needs. The generation of a modified and increased vasculature is perhaps the most obvious feature of this remodeling, but associated with this must be the generation of supportive connective tissue.

As the role of MMPs in disease has become better understood, interest in the therapeutic use of MMP inhibitors has increased. This chapter describes the development of synthetic inhibitors and examines the experimental studies and observations that support their clinical use, with a particular focus being given to cancer.

2. DEVELOPMENT OF MMP INHIBITORS

Since the early 1980s, there has been considerable interest in the design of MMP inhibitors, but it is only recently that clinical evaluation of compounds of this class has commenced *(42–47)*. A key structural feature of all potent MMP inhibitors is the presence of a chemical functional group that is capable of chelating the active site zinc(II) ion, which is a ubiquitous feature of the MMP enzymes. The first inhibitors to be identified were peptide derivatives designed on the basis of the sequence around the glycine–isoleucine and glycine–leucine at the site in the collagen α-chain, which is initially cleaved by collagenase (Fig. 2). In these pioneering studies, the zinc-binding group (ZBG) was incorporated into peptide analogs of the sequence on the left-hand side, right-hand side, or both sides of the cleavage site (Fig. 2). Other workers followed the serendipidous approach of screening compound libraries and/or natural products, and succeeded in identifying novel compounds and others that are structurally related to inhibitors obtained by substrate-based design. Although considerable insight into MMP ligand interactions has been obtained from the study of inhibitor structure–activity relation-

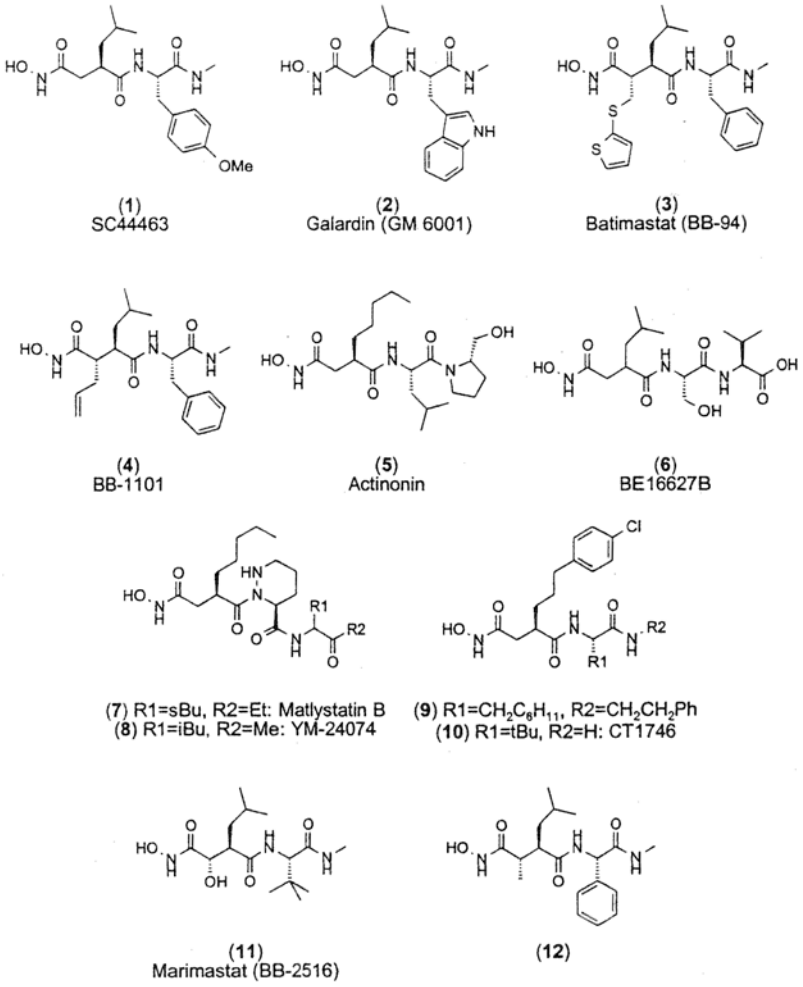
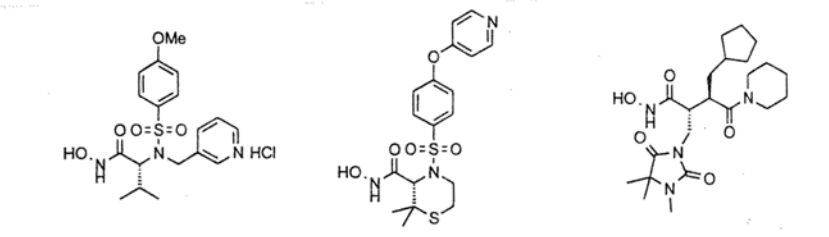


Fig. 3. Chemical structures of pseudopeptide hydroxamic acid MMP inhibitors.

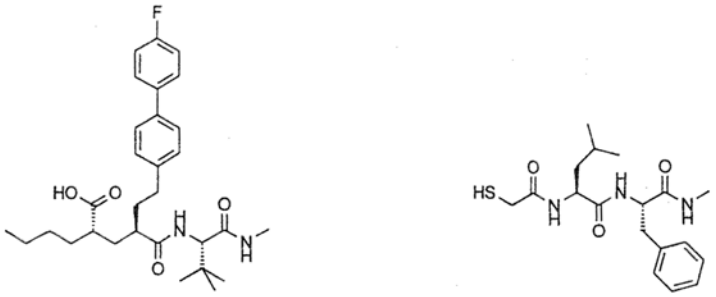
ships (SAR) *(48)*, and elegant studies of enzyme substrate specificity *(49–51)*, it is the recent advent of high-resolution X-ray *(52–62)* and NMR *(63)* structures of MMP-inhibitor complexes that has provided new paradigms for inhibitor design. To date, the new technology of combinatorial chemistry has had little impact on MMP-inhibitor design *(64–67)*.

Medicinal chemists have been faced with the dilemma of whether to design broad-spectrum or selective inhibitors, and the difficulty of obtaining oral activity. In principle, selective inhibitors should provide greater specificity and, hence, safety than broad-spectrum MMP inhibitors. However, human and animal studies have revealed that a number of the MMP family members are often coexpressed in disease states, making it difficult to single out a particular MMP as being casual *(43)*. Although it has proved possible to obtain satisfactory oral activity for selected pseudopeptide MMP inhibitors, it has generally been considered that acceptable pharmacokinetics can be more readily obtained for nonpeptidic compounds. Structures for the compounds discussed below in the text are shown in Figs. 3–6 and in vitro enzyme activities are given in Table 2.



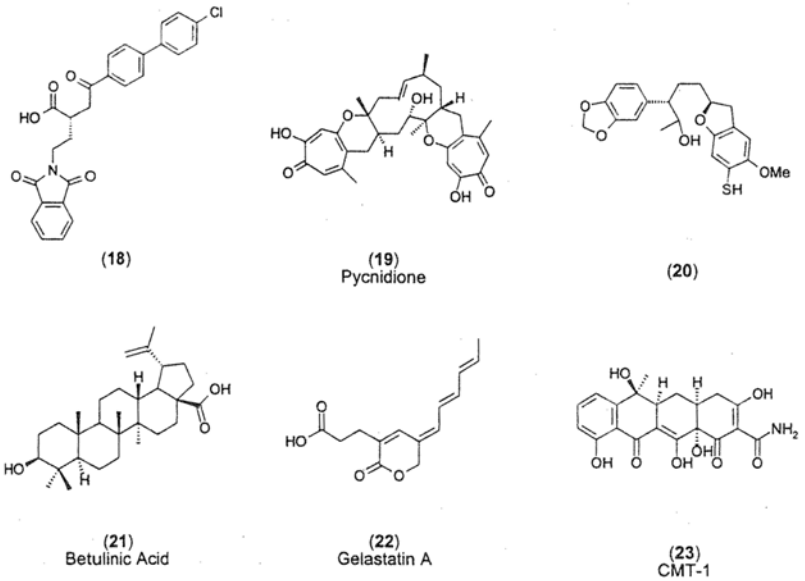
(13) CGS 27023A (14) AG-3340 (15) Ro 32-3555

Fig. 4. Nonpeptidic hydroxamic acid MMP inhibitors.



(16) L-758,354 (17)

Fig. 5. Nonhydroxamic acid pseudopeptide MMP inhibitors.



(18) (19) Pycnidione (20) (21) Betulinic Acid (22) Gelastatin A (23) CMT-1

Fig. 6. Nonpeptidic hydroxamic acid MMP inhibitors.

2.1. Pseudopeptide Hydroxamic Acid MMP Inhibitors

At an early stage, it was found that MMP inhibitors that feature a hydroxamic acid ZBG and mimic the sequence to the right-hand side (RHS) of the cleavage site (Fig. 2), such

Table 2
In Vitro Activities of MMP Inhibitors

HFC compound	*HNC MMP-1*	*72-kDa Gel. MMP-8*	*92-kDa Gel. MMP-2*	*Strom-1 MMP-9*	*MMP-3*	*Ref.*
1	6[a]	–	–	–	–	*76*
2	0.4[a]	0.1[a]	0.5[a]	0.2[a]	27[a]	*68*
3	3	10	4	1	20	*43*
4	10	3	5	3	30	*115*
5	190	300	–	330	1700	*71*
6	–	–	590	850	650	*72*
7	–	–	1700	570	350	*73*
9	2440[a]	–	0.03[a]	–	7.31[a]	*75*
10	122[a]	–	0.04[a]	0.17[a]	10.9[a]	*102*
11	5	–	6	3	200	*43*
12	3	–	–	3.9	–	*78*
13	33[a]	–	20[a]	8[a]	43[a]	*79*
14	8.2[a]	–	0.083[a]	–	0.27[a]	*80*
15	3[a]	4.4[a]	154[a]	59.1[a]	527	*84*
16	–	–	150[a]	–	4[a]	*87*
17	–	50	–	90	2600	*88*
18	–	–	1.15	0.91	5.73	*46*
19	–	–	–	–	31,000	*89*
20	75%[b]	–	–	44%[b]	600	*90*
21	1300[a]	–	–	–	2200[a]	*91*
22	–	–	630	–	–	*92*
23	510,000	31,000	–	–	–	*94*

Notes: IC_{50} n*M* values except; [a]K_i n*M* values; [b]% inhibition at 10 μ*M*. Because of different assay methods care should be taken in making comparisons between the results from different research groups.

as SC44463 (**1**; Searle), are particularly potent in terms of their in vitro activity *(48)*. Indeed, the hydroxamic acid group has proved to be a particularly effective ZBG, and the majority of inhibitors currently in clinical testing contain this group. Recent X-ray crystallography studies have shown that the hydroxamate acts as a bidentate ligand, with each oxygen an optimal distance (1.9–2.3 Å) from the active-site zinc(II) ion, and the position of the hydroxamate nitrogen suggests that it is protonated and forms a hydrogen bond with a carbonyl oxygen of the enzyme backbone of neutrophil collagenase *(54,57,59)*, fibroblast collagenase *(56)*, matrilysin *(58)*, and stromelysin-1 *(60,61,63)*. Investigations of the SAR for RHS inhibitors, such as **1** and analogs, has been extensively reviewed *(43,48)*, and led to the discovery of important prototype inhibitors Galardin™ (**2**; Glycomed) *(68)*, Batimastat® (**3**; British Biotech; *69,70*), and analogs (e.g., **4**; BB-1101 British Biotech). These compounds show broad specificity in their inhibition of the members of the MMP family, but display little detectable activity against other classes of metalloproteinases, such as angiotensin converting enzyme and enkephalinase *(68)*. It is of interest to note that the screening of natural products has led to the discovery of structurally related MMP inhibitors, namely actinonin (**5**; Rhone-Poulenc; *71*), BE16627B (**6**; Banyu; *72*), matlystatin B (**7**; Sankyo; *73*), and YM-24074 (**8**; Yamanonchi; *74*). Systematic structural modifications to the pseudopeptide hydroxamic acid derivatives resulted in two key discoveries. The first, made by workers at Celltech,

was that a degree of enzyme selectivity could be obtained; particularly, that the introduction of larger P1' substituents (e.g., the 3-phenylpropyl P1' substituent in **9** and **10**) enhances inhibition of the gelatinases and stromelysin-1, at the expense of activity against fibroblast collagenase and matrilysin *(42,75)*. The second was made by the group at British Biotech, who discovered that the combination of certain substituents gave orally active broad spectrum compounds *(43)*. Previous work from the groups at Sterling Winthrop and Celltech had shown that the introduction of basic substituents at P3' increased plasma half-life, and enhanced oral activity *(76,77)*. The British Biotech group found that the combination of an α-hydroxy group with tert-butylglycine at P2', as in Marimastat **11**, was particularly advantageous. The precise reasons for Marimastat's improved bioavailability are not clear, but the substitutions made may reduce the compound's susceptibility to peptidases, improve absorption, or reduce first-pass metabolism *(43)*. Recent results from Kanebo indicate that a P2' phenylglycine substituent (e.g., **12**) can also lead to oral activity *(78)*.

2.2. Nonpeptidic Hydroxamic Acid MMP Inhibitors

Novartis (formerly Ciba Geigy), by screening of libraries of synthetic compounds, identified a novel nonpeptide inhibitor of stromelysin-1 that was subsequently elaborated to give the orally active development compound CGS 27023A **13** *(79)*. This series of compounds has served as a lead for new inhibitor design by medicinal chemists at a number of pharmaceutical companies. In particular, workers at Agouron have used the Novartis compounds as the starting point for a structure-based inhibitor design program that made extensive use of X-ray crystallography *(62)*. The advent of high resolution X-ray crystal structures of MMP inhibitor–enzyme complexes has confirmed the presumed binding mode for inhibitors, such as batimastat *(59)* and, very importantly, together with homology modeling, provided insight into differences in the active site for the different MMPs *(62)*. The most significant difference is in the size and shape of the S1' pocket for the various MMPs, and it appears to offer the greatest opportunity for selective inhibitor design. For fibroblast collagenase and matrilysin, it is occluded, respectively, by an arginine or tyrosine residue; for other enzymes, such as neutrophil collagenase, collagenase-3, the gelatinases, and stromelysins, it is a much deeper pocket. This observation of the difference in the size of the S1' pocket between the MMPs explains the selectivity that the workers at Celltech obtained by the incorporation of large P1' groups. The Agouron team used X-ray crystallography to determine the binding mode of a Novartis inhibitor to human fibroblast and neutrophil collagenases *(62)*. It was found that the ring of the aryl sulfonamide bound into the P1' pocket, and structural analysis of the enzyme–inhibitor complex, suggested that further substitution of the aryl sulfonamide ring might enhance binding *(80)*. This led to the discovery of a potent series of inhibitors that are selective for the deep-pocket over the shallow-pocket enzymes (fibroblast collagenase and matrilysin), from which the orally active development candidate AG-3340 **14** was selected *(80)*.

It is notable that both CGS 27023A **13** and AG-3340 **14**, because of their nonpeptidic nature, have fewer hydrogen bond donors and acceptors than are present in the pseudopeptide derivatives, such as compounds **1–4**. Indeed, early SAR derived for RHS MMP inhibitors had suggested that the backbone amide bonds are important for inhibition *(81)*, and it has subsequently been discovered from structural studies of such inhibitors that the P1'–P2' C=O and N-H, and the P2'–P3' C=O and N-H, are all involved in

H-bonding interactions with the enzyme. However, the group at Roche found that the P2' amino acid could be replaced by a nitrogen heterocycle, and potent activity against the collagenases could be maintained, if a cyclic imide group was introduced at P1 *(82,83)*. From these studies, the orally active hydantoin derivative Ro 32–3555 **15** was identified as a development candidate for the treatment of arthritis *(84)*.

2.3. Nonhydroxamic Acid Pseudopeptide Derivatives

The use of alternative ZBGs to the hydroxamic acid moiety has been driven by both intellectual property considerations *(85)* and unmet fears of potential toxic liability. A variety of different ZBGs (carboxylate, aminocarboxylate, sulfhydryl, and derivatives of phosphorus acids) have been identified *(48,81)*. A comparative study of different ZBGs has suggested the following preference in terms of inhibition of fibroblast collagenase; hydroxamate >> formylhydroxylamine > sulfhydryl > phosphinate > aminocarboxylate > carboxylate *(86)*. Recent findings in this area of note are a series of carboxylic acid derivatives from Merck (e.g., **16**; L-758,354) selective for the deep-pocket enzymes *(87)*, and sulfhydryl derivatives from Chiroscience (e.g., **17**) with broad spectrum activity *(88)*.

2.4. Nonhydroxamic Acid Nonpeptidic MMP Inhibitors

Bayer found that the known anti-inflammatory agent, fenbufen, possesses modest inhibitory activity against gelatinase-A, and has prepared more potent analogs, such as **18** *(46)*. This compound features a carboxylic acid zinc binding group and a cyclic imide P1 substituent. A large P1' biphenyl group ensures selectivity for the deep-pocket MMPs. A variety of nonpeptidic natural-product MMP inhibitors have been discovered by screening. These include pycnidione (**19**; Merck; *89*), futoenone derivatives (e.g., **20**; OAS-1148; OsteoArthritis Sciences) *(90)*, betulinic acid (**21**; Sterling Winthrop; *91*), gelastatins (e.g., **22**; *92*), and tetracyclines such as aranciamycin and minocycline, for which chemical modification (e.g., **23**; CMT-1) has enabled the separation of MMP activity from antibiotic activity *(93,94)*. Two of these compounds feature a carboxylic acid that may serve as a ZBG; for others, it is possible that a ring hydroxyl and/or carbonyl chelates the active site zinc(II) ion. In the case of the futoenone derivatives, replacement of a ring oxygen substituent by a sulfhydryl, as in **20**, enhances inhibition of stromelysin-1, presumably as a result of stronger zinc(II) ion chelation *(90)*.

A number of potent MMP inhibitors of different structural types and enzyme inhibition profiles are now in development (Table 3). The preclinical studies that support the development of some of these compounds are reviewed in the next section.

3. ACTIVITY OF MMP INHIBITORS IN MODELS OF DISEASE

3.1. Tumor Invasion and Metastasis

The first studies with MMP inhibitors in cancer models examined their ability to block organ colonization or experimental metastasis. Marked inhibition of colonization was demonstrated for both TIMP-1 and the hydroxamate SC44463 (**1**; Searle) in mice inoculated with B16 murine melanoma cells *(95,96)*. Similarly, TIMP-2 was shown to inhibit colonization by 4R transformed rat fibroblasts *(97)*. These models only examine one discreet part of the the multistep process of tumour invasion and spread. More recently, MMP inhibitors have been tested in more complex and more clinically relevant cancer models.

Table 3
Matrix Metalloproteinase Inhibitors in Development (Pharmaprojects 1997)

Company	*Compound*	*Therapeutic target(s)*	*Clinical status*
Agouron	AG-3340	Cancer	Phase I
Bayer	Bay 12–9566	Arthritis/cancer	Phase II
British Biotech	Marimastat (BB-2516)	Cancer	Phase III
	BB-3644	Multiple Sclerosis	Phase I
Chiroscience	D5140	IBD[a]	Phase I
	D1927	Cancer	Preclinical
	D2163	Cancer	Preclinical
Novartis	CGS 27023A	Arthritis/cancer	Phase I
RocheRo 32–3555	Arthritis	Phase I	

[a]IBD, inflammatory bowel diseases.

In a study of a rat mammary carcinoma, Eccles et al. demonstrated effective suppression of micrometastatic disease with the MMP inhibitor Batimastat **3**. Animals were treated with short (7 d) and long (58 d) courses of Batimastat, starting just prior to the removal of the primary tumor grown adjacent to the mammary fat pad. Animals treated with the short course developed significantly fewer lung metastases than animals receiving a vehicle control, but approximately one-half of these animals developed local and distant lymph node metastases, which led to significant morbidity. These lymphatic metastases, however, remained as silent micrometastases in animals receiving the long course of Batimastat **3** *(98)*. This is a finding with obvious relevance for the adjuvant treatment of breast cancer.

Other studies have shown inhibition of the growth of human carcinomas established as xenografts, either subcutaneously or by orthotopic implantation in the tissue equivalent to the human primary site. Both Batimastat **3** and CT1746 (**10**; Celltech) have been shown to inhibit the local invasive growth and spread of orthotopically implanted human colorectal carcinoma *(99,100)*. Batimastat **3** was also shown to inhibit the local regrowth of MBA-MD-435 human breast carcinoma, following resection in nude mice *(101)*.

Both TIMPs and Batimastat **3** are broad-spectrum MMP inhibitors blocking the activity of most, if not all, MMPs. However, CT1746 **10** shows selectivity for the gelatinases over stromelysin, and particularly fibroblast collagenase. In addition to the inhibitory effects in colorectal cancer, this compound inhibited the growth of the murine Lewis lung carcinoma, and appeared to give additive effects when used in combination with cisplatin or cyclophosphamide *(102)*. Further studies with other selective inhibitors should help to define which of the MMPs are the most important targets in the development of these compounds as anticancer agents.

The effects of MMP inhibitors on experimental tumors, as observed through histopathological techniques, are quite varied. Some tumors show evidence of increased fibrotic stroma or a capsule of fibrotic tissue *(103,104)*; others show little evidence of change, other than being smaller than comparative tumors grown in untreated animals *(101)*. Other tumors have shown enlarged necrotic centers *(105,106)*, which may be an

indication of increased hydrostatic pressure *(107,108)*. It therefore appears that MMP inhibitors can act in different ways on different tumors.

3.2. Rheumatoid Arthritis and Osteoarthritis

Rheumatoid arthritis was an early target in many MMP-inhibitor development programs. The disease is characterized by the destruction of the articular cartilage and underlying bone. MMP levels are elevated in effected joints *(109)* and MMP inhibitors have been shown to be effective in models of the disease *(84)*. Similar relationships appear to exist for osteoarthritis *(34,110)*.

MMP inhibitors may also be active in these models by virtue of their ability to inhibit the cellular processing of tumor necrosis factor-α (TNF-α), mediated by the recently identified metalloproteinase disintegrin *(22,23)*. Antibodies to TNF-α have been shown to give symptom relief in patients with rheumatoid arthritis *(111)*. Several companies have developed MMP inhibitors for the treatment of rheumatoid arthritis, and trials with two compounds, Ro 32–3555 (**15**; Roche) *(112)* and D5140 (Chiroscience), have started. More recently trials with D5140 have been curtailed because of insufficient oral absorption.

3.3. Neurodegenerative Disease

MMP inhibitors have also been considered a treatment for neurodegenerative or neuroinflammatory diseases. Interest has focused on multiple sclerosis (NS) in which the active phase of the disease is characterized by degradation of the blood–brain barrier, demyelination, and axonal loss. Studies have shown elevated levels of MMPs in the cerebrospinal fluid of MS patients, in particular gelatinase B *(36)*, and MMPs have been shown to degrade myelin basic protein, releasing encephalogenic fragments *(37,113)*.

Experimental autoimmune encephalomyelitis (EAE) serves as a model for some elements of MS. Induced by injection of myelin basic protein in Freunds adjuvant, EAE displays lesions in the spinal cord and axonal degeneration. Several broad-spectrum MMP inhibitors, such as BB-1101 (**4**), have been shown to reduce the symptoms and weight loss associated with EAE *(114,115)*. The target enzyme in this model is not clear. Expression of matrilysin has been shown to be elevated in the spinal cord of EAE rats *(115)*, and TNF-α is known to exacerbate the condition *(116)*. Anti-TNF-α antibodies have been shown to be effective at reducing the severity of the experimental disease *(117)*. BB-1101 **4** is capable of blocking both the action of matrilysin and the cellular processing and release of TNF-α. Again, the relative contribution of these different pathways in the model will become clearer with the testing of selective inhibitors. Clinical trials with MMP in patients with MS are expected to start in 1998.

3.4. Other Diseases

Inappropriately regulated MMP expression has been proposed as a mediator of tissue breakdown in the chronic phase of several other disease states. In models of cerebral hemorrhage, increased MMP expression is observed approx 24 h after the initiating injury, and may be responsible for degradation of the blood–brain barrier *(118)*. The MMP inhibitors, Batimastat **3** and BB-1101 **4** were shown to reduce the damage to the blood–brain barrier, as monitored by ^{14}C-sucrose uptake *(38,118)*. It has been proposed that such inhibitors may be useful in the treatment of the clinical deterioration that occurs 1–2 d after hemorrhagic brain injury.

In coronary balloon angioplasty, increased extracellular matrix deposition frequently occurs, leading to restenosis. Studies have shown increased type I collagen production following experimental angioplasty, as well as decreased MMP activity *(119)*. The broad-spectrum MMP inhibitor Galardin **2** reduced both collagen synthesis and degradation in an iliac artery model of restenosis following balloon angioplasty. Increased matrix turnover has also been linked to the destabilization of atherosclerotic plaques *(120)*, and elevated gelatinase-B activity has been implicated as a causative factor in the enlargement of abdominal aortic aneurysms *(40)*. Other diseases that have been considered as targets for MMP inhibitor therapy include emphysema *(121)*, gastric ulcers *(122)*, and inflammatory bowel disease *(123)*. In each case, there is evidence to suggest that MMPs secreted by inflammatory or stromal cells are responsible for the tissue remodeling and degradation that occurs in these conditions.

4. ANTIANGIOGENIC ACTIVITY

Although functional analysis clearly demonstrates the requirement for proteolytic activity in the process of angiogenesis, the relative contributions of the various classes of extracellular matrix degrading proteinases have not been easy to establish. This is almost certainly the result of the interdependence of the different proteinase systems that must act in concert during the processes of endothelial cell migration, invasion, and vessel formation. Early studies with bovine corneal endothelial cells demonstrated that in vitro invasion through human amniotic membrane can be blocked both by serine protease inhibitors, such as aprotinin, and by TIMP-1 *(124)*. Antigelatinase A antibodies also blocked invasion. In each case, invasion was inhibited by 80–90% independently for each inhibitor, supporting the hypothesis that invasion may require the coordinate expression of both classes of proteinase. A role for the cysteine proteinase, cathepsin B, has also been suggested *(125,126)*.

Recent evidence for the participation of MMPs in angiogenesis comes from studies with synthetic and endogenous MMP inhibitors. Batimastat **3** was shown to reduce the angiogenic response in vivo to heparin-Matrigel implants to levels comparable to controls, without added heparin *(127)*. The same study showed that Batimastat **3** inhibited the invasion of human umbilical vein endothelial cells through Matrigel in vitro, but did not significantly alter endothelial cell proliferation, haptotaxis, or chemotaxis. It appears that endogenous MMP inhibitors may differ in their antiangiogenic activity from synthetic inhibitors, since TIMPs also appear to block endothelial cell proliferation and/or migration. Studies with a cartilage-derived MMP inhibitor have demonstrated the ability of this TIMP-related protein to block both capillary endothelial cell proliferation and migration, as well as in vivo angiogenesis in the chick chorioallantoic membrane assay *(128)*. Similarly, TIMP-2 was shown to inhibit the proliferation of human microvascular endothelial cells in conditions in which neither batimastat nor antigelatinase-A antibodies were effective *(129)*. This suggests that TIMP-2 may modulate the proliferation of these human endothelial cells in a protease-independent fashion. TIMP-1 did not slow endothelial cell proliferation in this study, but did inhibit endothelial cell chemotaxis and angiogenesis in a more recent study *(130)*.

An immunohistochemical study of angiogenesis in skin during fetal development, and in adult cutaneous tumors, has identified interstitial collagenase as being the principal MMP expressed in developing microvessels. Expression of stromelysin, matrilysin,

gelatinase-A, and gelatinase-B was not detected *(131)*. However, earlier studies have shown that antigelatinase-A antibodies are able to inhibit endothelial cell invasion in vitro *(124)*. Activation of gelatinase-A has also been observed in tubule formation in vitro in co-cultures of glial cells and central nervous system microvascular endothelial cells *(132)*. As has been suggested by Pepper *(133)* and others, the proteinase(s) used in the process of angiogenesis may change according to the type of tissue being vascularized.

As has been seen, synthetic MMP inhibitors have been widely tested in animal models of cancer. Unfortunately, effects on microvessel density have rarely been assessed in these models. In one of the first studies with Batimastat **3**, Davies et al. *(103)* reported that treatment of nude mice bearing human ovarian cancer malignant ascites resulted in the formation of dense avascular nodules with small islands of tumor cells. However, this is a specialized setting in which vascularization is not a major factor. More recently, Sledge et al. *(101)* used antibodies to the endothelial cell antigen CD31 to analyze blood vessel density in MDA-MB-435 xenografts treated with Batimastat **3**. The tumors were grown in the mammary fat pads of nude mice, and resected after 9 wk. The mice were then treated with Batimastat or vehicle to investigate the ability of this inhibitor to inhibit regrowth. The rate of regrowth was significantly reduced in the Batimastat **3**-treated animals, but the microvessel density appeared unchanged in the tumors that did develop. The authors note that, by definition, the tumors that do develop in these animals are resistant to the effects of the MMP inhibitor, and it seems likely that a different class of proteinase is acting in a compensatory manner.

5. CLINICAL TRIALS

The pharmaceutical development and clinical testing of new drugs takes many years. To the layman, it must seem an overly drawn out and mysterious process. However, the MMP inhibitor programs provide good examples of the milestones and obstacles of drug development: the isolation and characterization of MMPs and TIMPs, the understanding of their role in different diseases, the synthesis of the first pseudopeptide inhibitors, the disappointment of poor oral bioavailability, the difficulty of using TIMP proteins as drugs, and the challenges of long-term preclinical toxicology. The first MMP inhibitors began clinical trials in 1993, and were the product of at least 7 yr of research. Four years later, several inhibitors are being tested in patients (Table 3). Preliminary results from some of these compounds are described in Chapter 26. Trials over the next 2–3 yr should at last define the therapeutic value of these new inhibitors, and provide some indication of the impact that they are likely to make in the treatment of disease.

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14 Angiostatin Protein and Other Plasminogen Fragments

B. Kim Lee Sim

CONTENTS

1. INTRODUCTION

In some experimental and clinical presentations of metastatic disease, a primary tumor can inhibit the growth of its metastases. This phenomenon of the inhibition of tumor growth by tumor mass had been hypothesized to be caused by concomitant immunity (a resistance to second tumor graft in the presence of the first growing tumor) in early publications *(1,2)*. Several other hypotheses have been proposed (reviewed in refs. *3* and *4*), but, similarly, had not explained the mechanism of this phenomenon. O'Reilly and Folkman more recently proposed that the phenomenon is the result of the specific inhibition of metastatic growth by the primary tumor. They proposed that a primary tumor initiates its own neovascularization by generating angiogenesis stimulator(s) in excess of angiogenesis inhibitor(s). The positive regulators act most effectively around the primary tumor site, stimulating angiogenesis and primary tumor growth. By virtue of its longer half-life in circulation, negative regulators circulate to other distal sites and inhibit endothelial cell growth, which in turn causes inhibition of metastatic growth *(5,6)*. The hypothesis proposed by O'Reilly and Folkman was validated with the identification of Angiostatin™ protein (AP) generated by the primary tumor, which inhibits angiogenesis and growth in a secondary metastasis *(6)*. Data accumulated to date on the novel protein Angiostatin and another potent endogenous antiangiogenesis inhibitor, Endostatin™

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protein, show that, indeed, tumor growth is dependent on angiogenesis (first proposed by Folkman *[7]*), and show that the inhibition of angiogenesis can in fact block the growth of metastases, as well as maintain tumors in a dormant state *(6,8,9)*.

AP is a potent endogenous, specific inhibitor of endothelial cells that inhibits the growth of metastases and regresses primary tumors to dormant microscopic foci. The purpose of this review is to examine understanding of AP and other fragments of plasminogen as they relate to AP. Accordingly, this review discusses the experiments that led to the discovery of AP, the evidence that AP is a fragment of plasminogen, the specificity of AP for cells of endothelial origin and its effect in vivo, the proposed mechanism of AP generation in vivo, and the role of plasminogen fragments in endothelial cell inhibition.

2. DISCOVERY OF ANGIOSTATIN PROTEIN

2.1. Mouse Model Exhibiting the Phenomenon of Inhibition of Tumor Growth by Tumor Mass

An animal model that exhibited the phenomenon of the inhibition of tumor growth by tumor mass was developed *(5,6)*. Several passages of the Lewis lung carcinoma (LLC) in C57B6 mice, with constant selection for the occurrence of low numbers of metastases in the lungs, resulted in an LLC-low metastatic (LLC-LM) phenotype. This LLC-LM tumor typically grew from an inoculum of 1×10^6 cells in the subcutaneous dorsum of mice, to a 1500-mm^3 primary tumor in 2 wk. Within 13–21 d after resection of the primary LLC-LM tumors, the lungs of resected mice filled with neovascularized metastases. Lung weights, which correlated with tumor burden, were increased by >400%, compared with mice with intact primary tumors. Lungs of mice with unresected primary tumors were free of metastases *(6)*. Thus, in this model, the presence of the primary tumor is associated with suppression of the growth of its remote metastases. Comparable results were seen with SCID mice lacking both T- and B-cell populations, indicating that inhibition of metastases in this model was not dependent on intact immune system metastases *(6)*. O'Reilly et al. *(6)* fractionated biological fluids of animals with LLC-LM, to determine if these fluids contained factors that inhibited the proliferation of bovine capillary endothelial (BCE) cells stimulated by bFGF in vitro. Using this system, mouse AP, a 38-kDa protein, was identified and isolated from both serum and urine of LLC-LM-bearing mice.

3. EVIDENCE THAT ANGIOSTATIN PROTEIN IS A FRAGMENT OF PLASMINOGEN

3.1. Plasminogen

Plasminogen is the proenzyme form of the serine protease plasmin, which represents the major fibrinolytic enzyme in humans *(10)*. It is synthesized in the liver, and is maintained at a stable plasma concentration of about 1.6 μ*M (11)*. The complex structure of plasminogen includes five triply disulfide bonded, highly homologous domains, termed kringles (K), at the N-terminal half of the protein. Cleavage of the Arg^{561}–Val^{562} bond, with urokinase or tissue plasminogen activator, forms the active two-chain plasmin linked by two-chain plasmin linked by two disulfide bonds (Fig. 1).

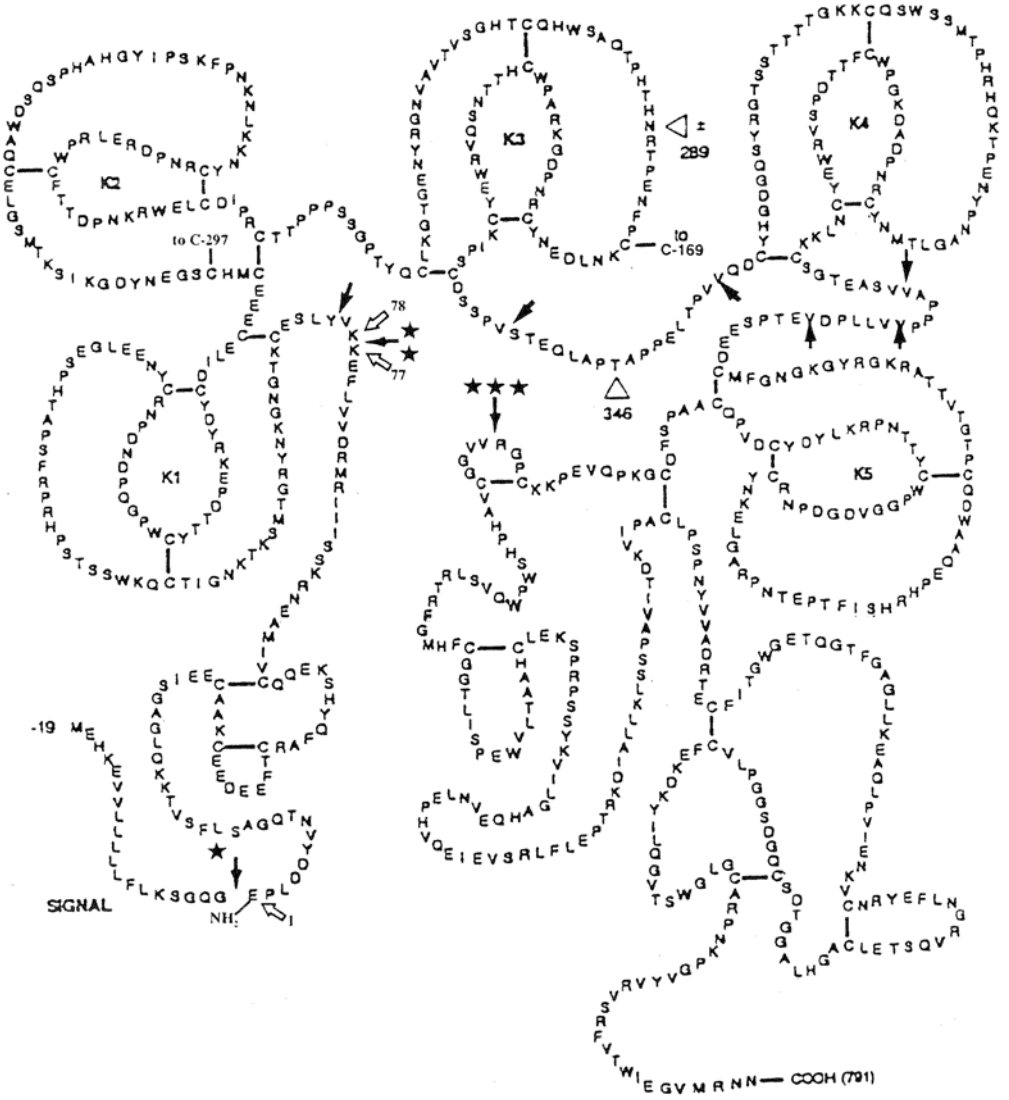

Fig. 1. Schematic representation of the structure of human plasminogen depicting the five K domains. Plasminogen consists of 791 amino acids with a single N-linked glycosylation at N^{289} (only present on about 40% of plasminogen) and a single O-linked glycosylation at T^{346}. Open arrows indicate specific amino acids. Filled arrows denote some of the various putative and known elastase cleavage sites in plasminogen. The filled arrow with a single star is the signal peptide cleavage site and E1 is the first residue of the mature protein. The filled arrow with two stars depicts the amino termini of AP derived from elastase cleavage of plasminogen, and consists of the first four K domains *(6)*. Cleavage between L^{77} and L^{78} is also the proteolytic site for plasmin on plasminogen. The filled arrow with three stars depicts the site for TPA/urokinase-generating active plasmin. Figure courtesy of Dr. Francis J. Castellino.

3.2. Endogenous Mouse Angiostatin Protein

Microsequencing of the 38-kDa AP isolated from mice bearing LLC-LM tumors, revealed that the N-terminus was Val^{98}, at the start of K1 of mouse plasminogen. Tryptic

cleavage of the 38 kDa fragment yielded fragments with N-termini within K2 and K3 *(6)*. The predicted carboxy terminus of mouse AP, based on an amino terminus of Val^{98} and a size of 38 kDa places it at residues in the middle of K4. The characteristics of glycosylation that can result in an upward shift on SDS-PAGE mobility is known for human plasminogen, but not for mouse plasminogen. Thus, the mouse 38 kDa AP must include a minimum of K1–3.

Several explanations can be suggested for why the cleavage of a protein yielded fragments with activity unrelated to the parent molecule. Cleavage of the Ks from plasminogen must cause a conformation change in the Ks, generating AP. This conformational change must include exposure of previously inaccessible sites that are involved in endothelial cell inhibition. It is also possible that the C' terminus of AP, previously inaccessible in plasminogen, is required for antiangiogenesis.

3.3. Human Angiostatin Protein Derived from Elastase Cleavage of Human Plasminogen

A corresponding fragment of human plasminogen, including K1–3 obtained by elastase cleavage of human plasminogen, was highly effective at inhibiting the bFGF-stimulated proliferation of BCE cells, in vivo reduction of metastases, and inhibition of growth of primary tumors. In fact, all in vivo studies reported with native human AP was with material derived from elastase-cleaved human plasminogen *(6,8)*. There are several known and putative elastase sites within K1–4 of human plasminogen (Fig. 1): at Tyr^{80}, at the beginning of K1, two sites in the K3–4 interkringle region, and three sites in the K4–5 interkringle region. The limited elastase digestion, followed by affinity purification over lysine-sepharose, described by O'Reilly *(6,8,12)*, resulted only in fragments containing K1, K4, or both, since only K1 and K4 binds lysine at high affinity. An additional dialysis step, using a 15-kDa mol wt cutoff membrane, removed most of K4, that is, about 10–12 kDa. Depending on the efficiency of elastase cleavage, a mixture of fragments can be obtained. O'Reilly describes the AP derived from elastase cleavage of human plasminogen as three fragments, 40, 42.5, and 44 kDa, on SDS-PAGE *(6)*.

Such multiple-banding patterns, seen on SDS-PAGE analysis, are typical of the K1–3 or K1–4 region of plasminogen. Plasminogen is the product of a single gene, a single transcript, but posttranslational modifications differentially glycosylates the gene product. Human plasminogen exists in two major glycoforms: type I, which possesses an N-linked high-mannose-type carbohydrate chain at Asn^{289}, and an O-linked carbohydrate chain at Thr^{346}; and type II, which contains a carbohydrate chain only on Thr^{346}. In each individual, about 33% of plasminogen molecules are type I and rest type II. Type II plasminogen can be further resolved into at least six glycoforms that differ only in their sialic acid content *(13)*. Recently, a novel O-linked sialylated trisaccharide on Ser^{250} was reported for plasminogen type II, which provided a structural basis for some of the observed microheterogeneity of type II plasminogen *(14)*. Since the carbohydrate chains are all localized on plasminogen fragments that are included in AP, an important question arises: What role do these different glycoforms of plasminogen play in the activity of AP? If activity of AP derived from elastase-cleaved plasminogen is affected by these different glycoforms, then is it possible that activity of different preparations of plasminogen-derived AP is dependent on the plasminogen donor pool?

The fact that mouse and human recombinant AP expressed in *Escherichia coli* is active in vivo (Lin et al.; Zhou et al., unpublished) argues against this possibility, because *E. coli*-derived proteins are expressed without posttranslational modifications, including glycosylation. However, the role of the carbohydrate chains, with respect to the specificity of activity, and in vivo clearance time, among others, remains to be defined.

3.4. Recombinant K1–4 Confers Angiostatin Protein Activity

Recombinant AP has been shown to confer in vitro and in vivo activities similar to that of AP derived from elastase cleavage of human plasminogen. A recombinant protein expressed in *Pichia pastoris,* a methylotropic yeast, representing the K1–4 region of human plasminogen (amino acids 93–470 of human plasminogen, starting with the first amino acid methionine; or Leu^{74} to Ala^{444}, shown in Fig. 1), shared physical properties with AP derived from elastase cleavage of human plasminogen. This recombinant AP possessed the physical characteristics of AP derived from plasminogen, in that it bound lysine-sepharose, and was recognized by a monoclonal antibody that reacted with a conformationally dependent epitope on plasminogen *(15)*. Systemic administration of this recombinant protein, at doses of 1.5 mg/kg/d, potently suppressed the growth of metastases in C57BL/6 mice with murine LLC-LM tumor, and suppressed the growth of primary LLC at a dose of 100 mg/kg/d *(15)*. The reactivity of antibodies against von Willebrand factor, which is specific for endothelial cells on lung sections from these animals, suggested that *P. pastoris*-expressed recombinant AP directly inhibited angiogenesis, and that suppression of metastases resulted from decreased angiogenesis in the metastases of mice treated with recombinant AP *(15)*. Similarly, baculovirus-expressed recombinant mouse AP (K1–4) inhibited the proliferation of BCE cells in vitro, and suppressed the growth of primary LLC in vivo, at a dose of 6 mg/kg/d, T/C = 0.08 *(16)*. The apparent requirement for a higher dose of human AP, when used to inhibit mouse endothelium in the mouse, albeit derived from native or recombinant sources, suggests that species specificity may play a role in the in vivo activity or clearance time of in vivo-administered AP in mice *(8,15–17)*. There was no evidence of toxicity in the treated mice in all studies reported to date. However, formal toxicity studies have yet to be performed. Findings provided by studies with recombinant K1–4 show without ambiguity that the antiangiogenesis activity of AP resides within the K1–4 region of plasminogen, and that in vivo activity of AP can be conferred by K1–4. Questions regarding the subsequent processing of this recombinant K1–4 in vivo should be addressed. Cell-derived proteases cleave plasminogen to generate AP (discussed in the following subheading). Whether intact K1–4 confers activity in vivo, or further processing of K1–4 occurs in vivo, to result in antiangiogenesis, is unknown.

4. SPECIFICITY OF ANGIOSTATIN PROTEIN FOR CELLS OF ENDOTHELIAL ORIGIN AND ITS EFFECT IN VIVO

4.1. Inhibition of Endothelial Cell Growth In Vitro

Native (from enzymatic cleavage of plasminogen) or recombinant AP have been studied in in vitro BCE cell cultures (Table 1). AP does not inhibit cells that are not of endothelial origin (Table 1). The results of these initial experiments, and data generated from further in vitro studies, emphasize an important finding: The inhibition caused by AP is specific for endothelial cells.

Table 1
Specific Inhibition of Endothelial Cells by Angiostatin Protein and Inhibition of Tumor Growth In Vivo with Angiostatin Protein Therapy

Source	Cell Type		Tumors affected by inhibition of angiogenesis	Reference
	Inhibited	Not inhibited		
Mouse Angiostatin™ protein from serum or urine of tumor bearing mouse	Bovine capillary endothelial Bovine aortic endothelial EOMA (murine hemangio-endothelioma)	Lewis lung carcinoma Mink lung epithelium 3T3 fibroblasts Bovine aortic smooth muscle Bovine retinal pigment epithelium MDCK (canine renal epithelium) WI38 (human fetal lung fibroblasts) EFN (murine fetal fibroblasts) LM (murine connective tissue)		O'Reilly et al., 1994
Human Angiostatin™ protein derived from elastase cleavage of human plasminogen	Bovine capillary endothelial Human umbilical vein endothelial Bovine aortic endothelial		Lewis lung carcinoma T_{241} fibroscarcoma Reticulum cell sarcoma Prostate carcinoma* Colon carcinoma* Breast carcinoma*	O'Reilly et al., 1994 Cao et al., 1996 EntreMed, Inc. Unpubl. Menendez et al., 1997 O'Reilly et al, 1996
Human Angiostatin™ protein derived from macrophage metalloelastase digested human plasminogen	Bovine capillary endothelial			Dong et al., 1997
Human Angiostatin™ protein derived from serine proteinase activity expressed by human prostate carcinoma cells	Human umbilical vein endothelial		Lewis lung carcinoma	Gately et al. 1996
Human Angiostatin™ protein generated from plasminogen by urokinase and free sulfhydryl donors	Bovine capillary endothelial			Gately et al., 1997
Human Angiostatin™ protein genereated from reduction of plasmin and cofactor to maintain free sulfhydryl groups.	Human dermal microvascular endothelial	Bovine aortic endothelial Human umbilical vein endothelial		Stathakis et al., 1997
Recombinant human Angiostatin™ protein expressed in *Pichia pastoris*	Bovine capillary endothelial		Lewis lung carcinoma	Sim et al., 1997
Recombinant mouse Angiostatin™ protein expressed in baculovirus system	Bovine capillary endothelial			Wu et al., 1997

[a]Tumors of human origin.

4.2. Inhibition In Vivo

Since the target of AP is the endothelial cell, it is expected that an indiscriminate variety of tumor types can be inhibited by AP in vivo (Table 1).

Studies reported by O'Reilly et al. *(8)* show that systemic administration of AP leads to regression of malignant tumors and maintenance of the tumors in a dormant state. These studies also show that their findings are associated with a prolonged blockade of angiogenesis *(8)*. AP is not cytotoxic, but cytostatic, for cultured endothelial cells in vitro. The question arises: What causes the regression and dormancy of micrometastases and solid tumors? The elegant work by Holmgren et al. *(18)* may provide an explanation for the effects on micrometastases. In that study, it was shown that dormancy of micrometastases is explained by a state of high tumor-cell proliferation balanced by an equally high cell death rate. Holmgren et al. suggested that angiogenesis inhibitors, such as AP, control metastatic growth by indirectly increasing apoptosis in tumor cells.

O'Reilly et al. *(8)* showed that, in fact, the proliferative index for tumors treated with AP was the same as that of untreated tumors. It was the apoptotic index that was more than fourfold higher for tumors treated with AP, compared to that of untreated tumors. The studies reported by O'Reilly et al. *(8,9)* show that AP can induce a state of harmless dormancy that has not been possible with conventional treatments so far. These works pave the way to begin addressing the feasibility of dormancy therapy of tumors.

The therapeutic regression of primary tumors without toxicity has not been previously documented. In the studies with human AP, aggressive murine primary tumor growth was inhibited by 81–87% at doses of 50 mg/kg given subcutaneously every 12 h, seen for the duration of the experiment of 60 d in some studies *(8)*. There was no weight loss, bleeding, hair loss, growth abnormalities, or other toxicity in the treated animals, including those receiving 100 mg/kg/d, the maximum dose tested, of either human AP derived from elastase cleavage of plasminogen or recombinant human AP *(8,15)*. It should be noted, however, that formal toxicity studies on AP have yet to be reported.

Most standard chemotherapy regimens are associated with significant toxicity and development of resistance with continued use. There have been no formal immunogenicity studies reported to date on AP. Treatment of immunocompromised mice transplanted with a human breast carcinoma, with human AP delivered at 50 mg/kg twice daily, maintained the tumor in a dormant state, with no evidence of resistance for the duration of the experiment of 60 d *(8)*.

5. PROPOSED MECHANISM OF ANGIOSTATIN PROTEIN GENERATION

What is the mechanism for the phenomenon of the inhibition of tumor growth by tumor mass? The working hypothesis put forward by O'Reilly and Folkman proposes that the presence of the primary tumor inhibits the growth of metastases. This occurs because the primary tumor generates the inhibitors of metastases. Attempts at detecting AP transcripts in LLC-LM cells, freshly resected from mice or after 4 passages in vitro by RT-PCR, or by Northern blotting, have been unsuccessful, indicating that primary LLC-LM tumors do not express AP *per se* (Sim et al., unpublished). Recent work by Gately et al. *(19)* and Dong et al. *(20)*, provided an explanation for the hypothesis that tumors could generate their own inhibitors of angiogenesis. Metalloelastase produced by tumor-infil-

trating macrophages can generate AP from plasminogen *(20)*. Also, several human prostate carcinoma cell lines were shown to express a serine protease(s) that could convert plasminogen to AP *(19)*. More recently, it was shown that AP activity could be generated by the proteolysis and reduction of plasmin *(21)*. In the generation of plasmin, plasminogen is first converted to the active serine protease plasmin by hydrolysis/cleavage of the Arg^{561}–Val^{562} peptide bond, and an autoproteolytic release of the amino-terminal peptide by cleavage of the Lys^{77}–Lys^{78} peptide bond (Fig. 1). The five K domains (heavy chain) of plasmin are covalently attached to the serine proteinase module (light chain) by two disulfide bonds. Stathakis et al. *(21)* suggest that a cell-derived plasmin reductase, generated, in their case, by Chinese hamster ovary or HT1080 cells, then reduces the disulfide bonds and releases the heavy from the light chain. They then show that this reduction of plasmin disulfide bond(s) is followed by the autoproteolysis of the heavy chain, resulting in the K1–4, active AP *(21)*. Several sequential steps must occur for the generation of active AP: the generation of plasmin, the reduction of plasmin, the auto proteolysis to generate K1–4 from the heavy chain that is released, and the constant presence of free sulfydryls. The generation of plasmin can occur by a variety of methods: Urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), or urokinase cleave the Arg^{561}–Val^{562} peptide bond to generate plasmin. Recent studies by Gately et al. *(17)* show that human prostate carcinoma cells (PC-3) release uPA, which functions this way. They also show that PC-3 cells produce sulfydryl donors, which, essentially in combination with released uPA, can by themselves generate AP *(17)*. This work provides the explanation for the working hypothesis that the primary tumor, even though not expressing AP itself, controls the production of AP by being responsible for the mechanism that generates AP.

What is the in vivo mechanism for the inhibition of endothelial cells by AP? Does the resultant K1–4 then bind to a specific endothelial cell receptor(s) and/or is further processing and/or internalization required? It is known that plasminogen can bind to a variety of cell-surface receptors, including alpha enolase, a glycolytic enzyme on monocytes *(22)*; receptors on lymphocytes; granulocytes, as well as on adherent fibroblasts *(23)*; and platelets *(24)*. Annexin II, a phospholipid-binding protein, has also been identified as an endothelial cell receptor for plasminogen *(25,26)*. The K domains have been implicated in the receptor-binding interactions of plasminogen *(25)*. The role of the K domains of plasminogen, in the regulation of activation and overall conformational changes of plasminogen, has been well documented (reviewed in ref. *27*), but the role of the K domains in the proteolytic cascade caused by plasmin, has not been established. Can proteolysis and reduction of plasminogen to generate AP occur on receptor-bound plasminogen? Or is there a multistep process of further processing of smaller K fragments that bind to separate cellular receptors and stimulate other signal processes, to bring about endothelial cell inhibition?

6. ROLE OF PLASMINOGEN FRAGMENTS IN ENDOTHELIAL CELL INHIBITION

6.1. Fragments of Plasminogen Generated by Elastase Digestion

The number of elastase sites between K3 and K4 of human plasminogen would yield several possible fragments upon elastase digestion: K1–3 with the O sugar, K1–3 with the O and N sugar, K1–3 without sugars, and K1–3 with the N sugar (Fig. 1). The N-terminus of AP, generated by elastase digestion of human plasminogen, was defined

as Lys^{78} or Tyr^{80} *(6)*. Once formed, plasmin converts the circulating zymogen Glu-plasminogen (N-terminus is Glu^{1}) to Lys-plasminogen (N-terminus is Lys^{78}) by releasing a 77-amino acid preactivation peptide. Lys-plasminogen is 10–20-fold more readily activated by tPA and uPA, and has higher affinity for cell surfaces *(28–30)*.

It can be deduced that fragments with amino termini beginning with Lys^{78} resulted from elastase cleavage of Lys-plasminogen, since elastase cleavage does not result in N-terminal lysines. But what is the C-terminal sequence of AP generated by elastase digestion of plasminogen? Partial elastase digestion could yield K1–4 with at least three possible carboxy termini (Fig. 1). Questions arise: Are all these fragments antiangiogenic? If they are, are they all equipotent in vivo?

In studies of individual or combined Ks, Cao et al. *(31)* have shown that K1, 2, and 3, as separate K entities, and K2–3, 1–3, and 1–4, existing as tandem arrays, can inhibit BCE cells in vitro. K1–3 derived from elastase cleavage of native plasminogen was most potent, and even more potent than K1–4 derived from elastase cleavage of native plasminogen, at inhibiting BCE cells *(31)*. K4 alone has been shown to be ineffective against BCE cells in vitro, or, at best, to have a low stimulatory activity *(6,31)*. More recently, K5 was shown to potently inhibit BCE cells in vitro *(32)*. Although these studies show that K domains can inhibit endothelial cells in vitro as individual units, and need not necessarily require the Ks to be tandemly linked, and although these studies compare the role of K domains on the antiproliferative effect on endothelial cells in vitro, some caution must follow the derived conclusions. The correlation between in vitro BCE inhibition and in vivo tumor inhibition has not been fully studied, and is not established. Also, endothelial cells have also notoriously been known to be very sensitive and potently inhibited by endotoxin *(33,34)*. In this study, fragments derived from native plasminogen were compared to recombinant Ks generated from *E. coli.* Such fragments as those generated by Cao et al. *(31)*, when tested in in vivo tumor models, should allow a better understanding of the in vivo situation.

6.2. Plasminogen and Plasminogen Activator Assembly on Endothelial Cell

Circulating Glu-plasminogen is converted to Lys-plasminogen upon high-affinity binding to its endothelial cell receptor annexin II *(25,26)*. Tissue plasminogen activator, which is synthesized and secreted be endothelial cells, can bind to Lys-plasminogen on the endothelial cell surface at a separate domain. Such an assembly provides an efficient method of plasmin generation at the endothelial cell surface (*see* review in ref. *35*). Lipoprotein(a), a low-density lipoprotein with extensive homology to K4, possesses multiple copies of this K4 domain, and competes with Lys-plasminogen for the annexin II receptor *(35)*. Does AP also compete for this receptor site? The fact that Lys-plasminogen did not inhibit metastatic tumor growth in the LLC-LM model argues against this *(6)*. Studies have indicated that specific enhancement of plasmin generation occurs in the presence of annexin II, when annexin II-bound Lys-plasminogen efficiently converts to plasmin *(26)*. This may trigger a chain of proteolytic events involving cleavage of extracellular matrix-bound $VEGF_{189}$ or $VEGF_{206}$, to release a diffusable active $VEGF_{110}$ *(36)*, and activate procollagenase, which promotes migration and sprouting of endothelial cells and angiogenesis (*see* review in ref. *37*). Thus, it can also be argued that the K domains in AP have a higher affinity and compete for the same site(s) on annexin II as Lys-plasminogen, preventing plasmin generation on the endothelial cell membrane, resulting in blocking of angiogenesis. However, there is no evidence to date that indicates annexin

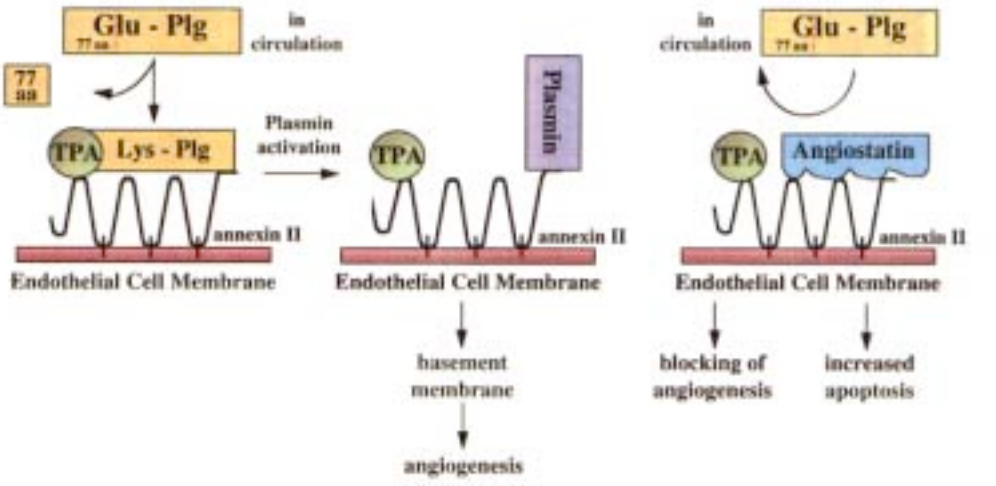

Fig. 2. Working model for assembly of plasminogen and AP on the endothelial cell. TPA and Lys-plasminogen-bound annexin II is shown associated with the endothelial cell surface. Circulating Glu-plasminogen (Glu-Plg) is converted to the truncated, noncirculating Lys-plasminogen (Lys-Plg), through the proteolytic release of a 77-amino acid preactivation peptide (77 aa). In this binding arrangement, plasminogen is efficiently activated to form plasmin (adapted with permission from ref. *25*). Plasmin may cleave extracellular matrix-bound VEGF ($VEGF_{189}$ or $VEGF_{206}$) to release a diffusible proteolytic fragment ($VEGF_{110}$) that stimulates angiogenesis. In this working model, annexin II-associated AP may block this process, and may trigger apoptosis of tumor cells in close proximity, by other mechanisms.

II functions as the receptor for AP, nor is there evidence that AP binds annexin II. Figure 2 is a proposed schematic of plasminogen and plasminogen-activator assembly on the endothelial cell, and postulates scenarios for AP interaction. The resultant increase in apoptosis of tumors treated with AP is inferred to be caused by the direct inhibition of endothelial cells of microcapillaries supplying the tumor *(8,18)*. AP interaction with the endothelial cell may stimulate caspace activity, which could directly affect tumor-cell gelsolin, causing apoptosis *(38)*. Whether apoptosis occurs as a consequence of direct endothelial cell inhibition, or if apoptosis is a consequence of a complex multistep process, is currently unknown.

7. CONCLUDING REMARKS

The discovery of AP, and the biological data generated by its use, provide substantial support for the hypothesis first proposed by Folkman *(7)*, that tumor growth is dependent on neovascularization. There is much interest in the field for better understanding AP, and also Endostatin protein, another potent endogenous endothelial cell inhibitor that is a fragment of collagen XVIII *(8)*, as members of a novel class of proteins: fragments of larger proteins with activities distinct from that of the parent protein.

The method of generation, the regulation of the generation, and the mechanism of action of native AP is unknown. Can the activity of AP be localized to a minimal fragment of the protein, and what is the receptor(s) for AP? What are the downstream components of the receptor-signaling pathway affecting the endothelial cell? With the availability of AP derived from proteolytic cleavage of plasminogen, and recombinant sources of AP, the answers to some questions may be imminently forthcoming. The impressive in vitro

and animal data on this specific endogenous angiogenesis inhibitor should pave the way for its assessment in the clinic.

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15 Dietary and Nutritional Modulation of Tumor Angiogenesis

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CONTENTS

1. INTRODUCTION

Tumor angiogenesis is intimately linked to the dynamic interactions between the nutrient status of the host and the tumor. It is well recognized that a growing tumor requires a vascular supply to provide oxygen, and to remove metabolic waste *(1–4)*. The tumor vasculature also provides the pathway for the delivery of energy-yielding compounds to fuel metabolism, required vitamins and minerals to serve as catalysts and critical cofactors for enzymes, essential building blocks for cellular macromolecules, and substrates for intracellular signaling pathways that cannot be synthesized by cells. Therefore, from the perspective of the tumor microenvironment, it is probable that cancer cells may alter the synthesis or balance of angiogenic and antiangiogenic factors in response to nutritional and metabolic needs. Furthermore, the nutritional status of the host modulates concentrations of circulating hormones and growth factors, or the availability of nutrients that may directly influence tumor cell proliferation and metabolism, as well as

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the response of the tumor vascular compartment to regulatory signals in the local environment. Although direct evidence for these concepts, derived from carefully designed experiments, is only beginning to emerge, the rapid development of laboratory models and tools for the evaluation of tumor angiogenesis provides the foundation for scientific inquiry *(5)*.

2. DIET, NUTRITION, AND CANCER: HISTORICAL PERSPECTIVES

It is abundantly clear that diet and nutrition are important contributors to carcinogenesis, both in humans and laboratory animals *(6–10)*. Evidence documenting the critical role of nutrition in cancer risk began to accumulate after the separate disciplines of carcinogenesis and experimental nutrition were established in the first half of this century. During this period, the essential nutrients were identified, requirements of rodents and humans were defined, and human deficiency syndromes corrected in industrialized nations. Purified nutrients and components of foods were characterized for the preparation of carefully controlled experimental diets in laboratory animals. Scientists proceeded to examine the effects of specific nutrients on tumorigenesis in the rapidly expanding list of newly characterized rodent cancer models that were originally based on carcinogens found in the environment, such as the workplace. The meticulous studies from the laboratories of Tannenbaum *(11–13)*, Boutwell *(14)*, and McCay, during the 1930s and 1940s *(15)*, characterized the important roles of fat and energy intake, as well as many other nutrients, in the genesis of experimental cancers *(13)*. Although the relevance of the observations from rodent models to human cancer was frequently discussed and debated, the epidemiologic methodology needed to investigate nutrition and cancer hypotheses in humans would not be refined for several decades.

The development of food availability data and dietary surveys coupled with statistics on cancer incidence and mortality in many nations around the globe provided the first examples of provocative associations between human cancer risk and dietary intake *(6–10)*. The observations that human cancers exhibit 10- to over 100-fold variations in incidence among different geographic and cultural areas suggested to some investigators that diet and nutrition was a critical etiologic factor. This concept was greatly strengthened when studies of migrant populations from low-risk areas to high-risk areas frequently showed dramatic increases in cancer incidence, suggesting that environment and lifestyle, not genetics, plays the major role in the origins of most common cancers *(16–18)*. Variations in dietary patterns and the intake of specific nutrients were among the factors most frequently found to change in migrant populations experiencing increases in several malignancies, especially those of the gastrointestinal tract, breast, and prostate *(6–10)*. The continuing refinement of food-frequency questionnaires for the assessment of individual food selection and consumption patterns has led to the rapid proliferation of case-control studies and several large prospective cohort studies that are providing important data concerning diet, nutrition, and cancer risk *(7,19)*. As we move toward the end of the century, several national and international organizations have prepared diet and nutritional guidelines for the prevention of cancer, based upon the accumulated fund of knowledge *(7,20,21)*. In contrast to the public health approach, investigators are beginning to target high-risk subgroups for rigorous intervention studies, based upon nutrition and chemoprevention *(22,23)*. Most investigators agree that the field of nutrition and cancer is only beginning to mature. New investigators are trained within the area of

nutrition and cancer to extend knowledge via diverse studies, ranging from epidemiology to molecular biology. Although many associations between diet and cancer risk have been described, the mechanisms that underlay these relationships are typically unknown. The critical role of tumor angiogenesis in the cancer cascade suggests that these processes may be a target for diet and nutritional interventions.

3. RELEVANCE OF NUTRITION FOR INVESTIGATION OF TUMOR ANGIOGENESIS IN THE LABORATORY AND IN CLINICAL STUDIES

Hypotheses concerning tumor angiogenesis are ultimately evaluated and characterized in animal models prior to moving into human intervention studies *(5)*. All investigators working with animal models of tumor angiogenesis should be familiar with several basic concepts of diet and nutrition in rodent models of carcinogenesis. Simply expressed, each investigator should be confident that the diets employed in their studies are reportable and reproducible, in the same fashion that the animal model is described and the laboratory techniques documented in publications. The selection of the diet for the animals is as important as many other variables that can influence outcome, such as species, strain, age, sex, and tumor cell line. Control of the diet and careful collection of data, regarding the effects of treatment on growth and food intake, will allow laboratory investigators to improve consistency between their own experiments over time and these different laboratories.

Diet and nutritional status may also prove to be relevant to the outcome of clinical studies of antiangiogenic agents for cancer therapy. Patients enrolling in phase I and phase II trials are often extensively pretreated with chemotherapy, radiotherapy, and surgery. Each of these interventions can have significant impact on nutritional status. In addition, the majority of cancer patients consume some type of nonprescription diet or nutritional supplements during the course of their illness. This information is typically unknown to the clinical investigator, since physicians rarely inquire about these products. The control of dietary variables, or the incorporation of various measures of nutritional status into clinical studies of antiangiogenic agents, may ultimately assist efforts to maximize efficacy and minimize toxicity for the patients.

4. CHOOSING APPROPRIATE DIET FOR STUDIES IN RODENT MODELS

The effects of nutrients, and their interactions with anticancer agents, on tumorigenesis can be rigorously tested in animal models. Although the information derived from these investigations must be extrapolated to humans, with appropriate caution, these studies provide important evidence for the biologic plausibility of relationships and mechanisms of action. Most rodent studies employ cereal-based commercial laboratory diets, often referred to chows. Some natural ingredient diets are open formula, meaning that the ingredients have been published, such as the NIH-07 formulation *(24)*. In contrast, closed formula cereal-based diets are formulated on proprietary recipes (i.e., Purina 5010, St. Louis, MO). Although these formulations provide adequate nutrition for maximal growth and reproduction, they vary over time and between companies in the sources of natural ingredients included in the final product. Chow diets may include varying concentrations of cereals, vegetables, legumes, fish meal, milk products, and other components that are a function of local market availability and cost to the manufacturer.

Although the content of these diets satisfies the established minimum requirements for most nutrients in mice and rats, the concentrations of individual nutrients may vary substantially. For example, the vitamin A and β-carotene content of different batches of the NIH-07 chow diet were found to vary over 6- and 20-fold, respectively *(25)*. Natural ingredients may also exhibit marked variation in trace elements. For example, selenium found in corn and soybean meal from the Great Plains is typically 0.5–1.0 ppm. In contrast, corn and soy from the Ohio valley or Northeastern states may be <0.1 ppm selenium. Furthermore, many manmade and natural toxins are detected in chow diets, such as: aflatoxins, nitrosamines, pesticides, herbicides, and heavy metals *(25,26)*. Occasionally, cadmium levels in cereal diets have been reported in the range that has effects on the renal vasculature *(26,27)*. Most importantly, many undefined substances found in grains, fruits, and vegetables exhibit anticarcinogenic activity *(28)*. Data is beginning to emerge showing that natural substances of plant origin, incorporated into cereal-based diets, can exhibit antiangiogenic activity *(29–33)*.

It is the authors' opinion that most investigators should consider using standardized semipurified diets in their short-term studies of angiogenesis, which adds very little to the overall costs of in vivo investigations, and can help improve the quality of data and their exchange among laboratories *(34–37)*. The nutrient requirements of most laboratory animals have been precisely defined, and purified or semipurified ingredients can be used to formulate diets for cancer studies *(37,38)*. Investigators can purchase these formulations from a number of reputable suppliers. Researchers should recognize that many of the dietary formulations, particularly vitamin and mineral mixes, published prior to 1975 may be inadequate in some nutrients *(39)*.

Consistent, reproducible results in laboratory studies of angiogenesis require control over as many variables as possible, including the diet. Investigators should always report details about the diets employed in their work. A citation of the primary reference is adequate for published semipurified or purified diets. The manufacturer and trade name of commercial, natural-ingredient diets should be specified, if possible.

5. INTERACTIONS BETWEEN FOOD (ENERGY) CONSUMPTION, TUMOR GROWTH, AND ANGIOGENESIS

A strong positive correlation between energy intake and the incidence or growth of tumors has been reported in virtually every animal model system evaluated *(6,10,40,41)*. Many studies have shown that an imposed restriction of total food intake or energy inhibits tumorigenesis (Fig. 1; *12*). In addition, groups of mice or rats with free access to food, which is typical in most rodent studies, also show a striking positive correlation between self-selected energy intake and risk of cancer (Fig. 2; *40,42,43*). Recent examination of data derived from the National Toxicology Program shows that chemicals reducing body weight, and presumably reducing self-selected energy intake, are associated with a lower cancer risk *(44)*. Subsequent studies show that energy intake can have a profound influence upon the sensitivity of the bioassays used to identify health risks from environmental chemicals and define regulatory policy *(45)*.

Readers of cancer therapy literature, including studies of antiangiogenic agents, must carefully evaluate the data, try to ascertain if the drug or therapeutic intervention alters food intake and body weight, and determine if energy intake has confounded the interpretation of the data. If food consumption is altered by the growth of a tumor, or changed by the anticancer treatments employed, it may be difficult to disentangle the results of the

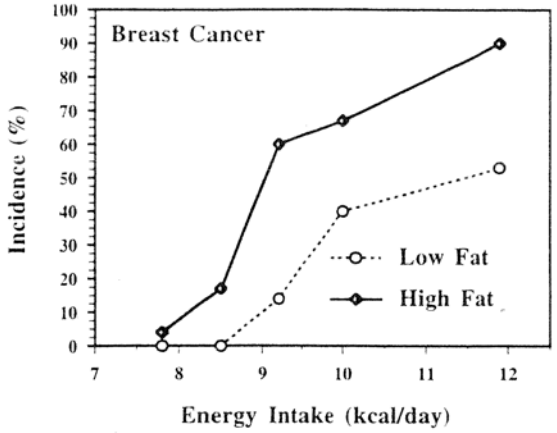

Fig. 1. The effects of high-fat or low-fat diets fed at controlled levels of energy intake on spontaneous mammary carcinoma in C3H mice *(12)*. Both dietary fat intake and energy intake exhibit significant effects on tumorigenesis.

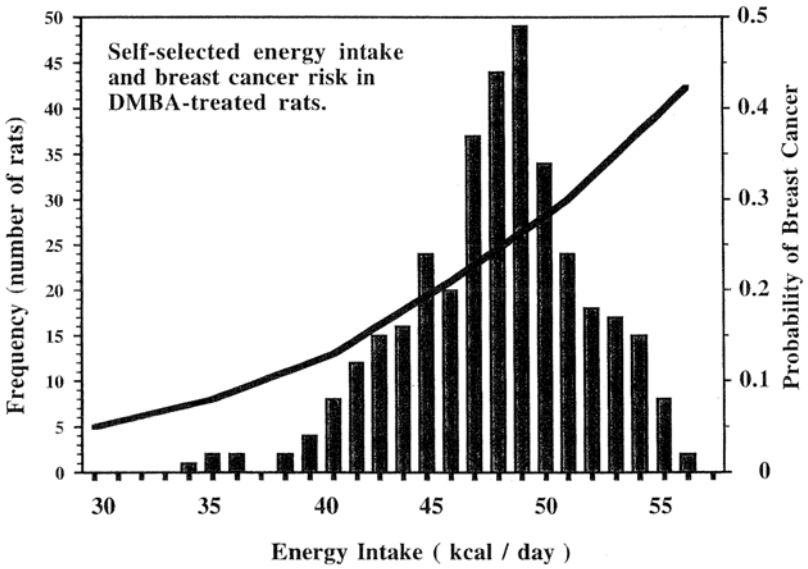

Fig. 2. The effects of self-selected energy intake on risk of developing breast cancer in female Sprague-Dawley rats previously exposed to the mammary carcinogen 7,12-dimethylbenz(a)anthracene. The frequency distribution shows the number of rats self-selecting a specific average daily energy intake. Regression analysis generated the curve illustrating the odds of exhibiting a breast tumor over the range of self-selected energy intake. The odds of having a tumor was increased by approx 10% for each 1-kcal increase in self-selected energy intake *(43)*.

study. It is highly desirable to monitor the effects of anticancer agents on food consumption and body weight of the experimental animals. In some cases, pair-feeding and other methods of compensating for treatment-induced differences in energy intake can be used to assist the investigator in understanding the results of studies in which food intake is a covariable of treatment.

Thus far, few studies have focused on the role of energy intake and tumor vascular biology. A recent report showed that the transplantable Englebreth-Holm-Swarm (EHS) sarcoma *(46)*, grown in young mice fed reduced-energy intake, contained fewer blood vessels and increased matrix, compared to those fed ad libitum *(47)*. These observations are supported by the authors' recent unpublished experiments. The authors have completed a series of studies employing total diet or energy restriction with a variety of rodent tumor models, and assessed tumor growth and its relationship to microvessel density, based on factor VIII staining. These studies show that energy or diet restriction significantly reduces microvessel density in several transplantable tumor models, including murine MB49 bladder cancer, RT2 rat glioma, Dunning R3327H rat prostate cancer, and LNCaP human prostate carcinoma grown in nude mice *(48)*.

Since the inhibition of tumor growth by energy restriction appears to be associated with a reduction in microvessel density, one can begin to speculate on the potential mechanisms involved. The endocrine system plays a significant role in the integration of nutritional status with the coordinated function of tissues and organs in mammals. Possible mediators of changes in tumor angiogenesis during dietary restriction are several hormones and growth factors that show changes with energy balance, and have been linked to angiogenesis.

5.1. Steroid Hormones

One of the hormonal alterations associated with diet restriction is the elevation of serum adrenal corticosteroids *(49–52)*. This phenomenon has been termed a "caloric stress," and may be one component of an adaptive response by the host to reduced energy availability, and perhaps contributes to some of the beneficial biological effects observed *(53)*. Recent studies of skin carcinogenesis in the Sencar mouse model suggest that energy restriction inhibits tumor formation, enhances morning glucocorticoid concentrations, and alters expression of transcription factors in the developing skin lesions *(54)*. Metabolites of adrenal steroids exhibit inhibition of angiogenesis *(55)*. Several metabolites, including tetrahydrocortisol, 17-α-hydroxyprogesterone, and hydrocortisone, are active antiangiogenic molecules in the presence of heparin *(55)*. Tumor angiogenesis was inhibited when the steroids, with heparin as a pellet, were inserted into the rabbit cornea between the implanted tumor (V2 carcinoma in rabbit) and the vascular bed. Capillary growth was almost completely inhibited only in the corneas implanted with both heparin and the angiostatic steroid. One mechanism of action may be via induction of plasminogen activator inhibitor, thereby reducing matrix degradation associated with angiogenesis and tumor invasion *(55,56)*. Additional efforts, focusing on how diet-induced alterations in adrenal hormone metabolism may modulate angiogenesis, are necessary.

Additional endogenous steroid hormone metabolites appear to modulate tumor angiogenesis. For example, the endogenous estrogen metabolite, 2-methoxy-estradiol, inhibits endothelial cell proliferation and migration, as well as vessel formation in collagen gels *(57)*. Further in vivo studies show that 2-methoxy-estradiol inhibits tumor growth and angiogenesis *(57)*. Since dietary factors are major regulators of hormonal secretion, metabolism, and biological activity, it is reasonable to hypothesize that nutrition may indirectly influence angiogenesis via alterations in the steroid hormone metabolism *(58)*.

5.2. Prolactin

Several studies have suggested that the 16 kDa fragment of prolactin is a modulator of angiogenesis *(59–61)*. Prolactin is synthesized in the anterior pituitary and processed

into biologically active fragments by secreting cells and target tissues. For example, the prostate and mammary gland contain enzymes that can convert intact 23 kDa rat prolactin into a variant form that is cleaved in the large disulfide loop *(62,63)*. A 16-kDa fragment is generated after reduction of a disulfide bridge linking the 16- and 8-kDa fragments. The cleaved form is normally found in the anterior pituitary and plasma of rodents and humans *(64,65)*. The 16-kDa form, but not the intact prolactin, blocks bFGF-stimulated endothelial cell proliferation *(59)* via a novel receptor *(60)*, and reduces organization of endothelial cells into capillary-like structure in collagen gels *(61)*. Furthermore, 16 kDa prolactin inhibits capillary formation in the chick embryo chorioallantoic membrane assay *(61)*. The authors have recently reported that protein-energy restriction inhibits prostate cancer progression in rats and reduces pituitary prolactin secretion, alters systemic prolactin metabolism, and reduces the concentration of prolactin receptors in the prostate *(66)*. The possibility that energy intake, or other dietary variables, modulates angiogenesis via changes in prolactin metabolism and homeostasis will be examined in future studies.

5.3. Endothelin

Endothelins are a family of 21-amino acid peptides synthesized by endothelial cells and some tumors *(67,68)*. Endothelins are potent regulators of vascular tones *(69)*. In addition, mitogenic activity of endothelin on vascular smooth muscle and stromal fibroblasts have been demonstrated *(68,70,71)*. Short term fasting or chronic dietary restriction in rats has been found to decrease plasma endothelin levels *(72)*, in contrast to many other types of pathophysiologic stressors previously shown to increase plasma endothelin level *(73–77)*. Perhaps altered endothelin expression may participate in homeostatic adjustments related to the need for overall host energy conservation during chronic restriction. How endothelin may modulate tumor vascularity and blood flow under conditions of dietary restriction remains to be explored.

5.4. VEGF

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), appears to be one of the key growth factors participating in physiologic and pathologic angiogenesis. VEGF is present in many human tumors, and may contribute to vascular hyperpermeability and enhanced angiogenesis *(78,79)*. Blocking VEGF expression by antibodies or antisense strategies has been shown to inhibit the growth of some tumors; overexpression enhances tumorigenesis. A major regulator of VEGF expression is hypoxia *(80–83)*. VEGF immunohistochemical staining is often noted in proximity to necrotic, and presumably hypoxic, regions *(84)*. In addition of hypoxia, there is some evidence suggesting that VEGF and other angiogenic factors may be induced by lower local concentrations of key nutrients, such as glucose *(85)*. For example, expression of a glucose transporter, GLUT-1, and VEGF, at the mRNA level, increase in response to both glucose deficiency and hypoxia, in studies with the C6 glioma cell line in vitro *(85)*. The interrelationships between glucose concentrations and hypoxia in the regulation of specific genes involved in the stress response was examined in normal and ES cells having a targeted disruption of the aryl hydrocarbon receptor nuclear translocator (ARNT) gene *(86)*. ARNT is a nuclear transcription factor that forms a heterodimer with hypoxia-inducible factor-1a (HIF-1a) to regulate genes involved in the response to oxygen deprivation. The Arnt–/– ES cells failed to respond to a decrease in glucose con-

centrations, indicating that ARNT is crucial in the response to hypoxia and hypoglycemia. Furthermore, embryonic failure caused by defective angiogenesis was noted in Arnt–/– embryos when tested *in vivo*, an result similar to that observed in mice bearing defective VEGF genes. These studies suggest that ARNT and HIF-1a may be key transcriptional regulators of downstream genes controlling angiogenesis in response to hypoxia or changes in nutrient concentration.

The authors' recent studies with transplantable rodent and human prostate tumors provide evidence that dietary restriction inhibits tumorigenesis, in association with an inhibition of tumor VEGF expression. Rats bearing the Dunning R3327H well-differentiated prostate adenocarcinoma, and fed diets restricted in total diet or energy, show an inhibition of tumor growth, reduced microvessel density, and significantly less VEGF *(48)*. Additional in vivo and in vitro studies concerning the regulation of VEGF expression by dietary and nutritional factors are clearly indicated.

5.5. IGF-I

Several lines of evidence suggest that IGF-I may mediate some of the proangiogenic effects of energy intake on tumor angiogenesis and other pathologic processes. IGF-I receptors are present on endothelial cells, and activation stimulates proliferation *(87,88)*. IGF-I also directly stimulates migration and tube formation by vascular endothelial cells in vitro *(89)*. In experimental models, myocardial ischemia following vascular occlusion stimulates the expression of IGF-I, which is temporally followed by capillary sprouting *(90–92)*. Angiogenic factors such as IGF-I are also implicated in the angiogenesis associated with diabetic retinopathy *(93)*. In osteoblast cell lines, IGF-I stimulates VEGF expression, which can subsequently act on endothelial cells to stimulate angiogenesis for bone growth and remodeling *(94,95)*. IGF-I also enhanced VEGF expression by colorectal carcinoma cells, which may promote tumor progression by facilitating the development of new blood vessels *(96)*.

The synthesis of IGF-I and circulating concentrations are very sensitive to nutritional variables, particularly protein and energy consumption *(97–101)*. Fasting or chronic food restriction reduces plasma IGF-I concentrations, and are maintained at lower concentrations under conditions of chronic energy restriction. Lower serum concentrations of IGF-I have been associated with reduced risk of cancer in human and rodent studies *(102)*. For example, Hursting et al. *(103)* provided strong evidence that dietary restriction modulates mononuclear cell leukemia progression in Fischer 344 rat via suppression of growth hormone-IGF-I axis. Parallel in vitro studies showed that the leukemia cell line was very sensitive to IGF-I concentrations, and that supplementation of IGF-I to energy-restricted rats restored rapid growth of the leukemia. Energy restriction inhibits DMBA-induced mammary carcinogenesis in rats, with parallel reductions in circulating IGF-I *(104)*. IGF-I is a established growth factor for breast cancer *(105–107)*. Energy restriction also reduces carcinogen-induced bladder tumorigenesis, in association with reduced IGF-I concentrations in p53-deficient mice *(108)*. Although the interrelationships among dietary energy intake, the IGF-I axis, and tumor angiogenesis have not been examined in focused studies, the data described above suggest very strong interactions.

6. DIETARY LIPIDS AND FATTY ACID PATTERNS

The role of dietary lipid concentration, sources of fat, and specific fatty acid patterns on the incidence and progression of many cancers has been the source of much specula-

tion and investigation *(6,7,21)*. The human and laboratory evidence is most convincing for a stimulatory effect of dietary lipid concentration, particularly a diet rich in saturated fats, on cancers of the colon and rectum *(6,7,21)*. Less certain, but supported by many studies, are the positive relationships between diets rich in fat and cancers of the prostate, breast, lung, ovary, and endometrium *(6,7,21)*. The possibility that diets rich in omega-3 fatty acids from marine sources have inhibitor properties for cancers has been postulated, and is currently an area of active investigation *(6,7,21,109–114)*.

Dietary lipid intake is primarily in the form of triacylglycerols (triglycerides), with one molecule of glycerol bound to three fatty acids. Dietary fatty acids vary in chain length (typically even numbered, 4–22 carbon atoms in length) and the degree of saturation, which is relevant to biological functions in vivo. Most fatty acids are in a *cis* configuration in nature, but current food industry techniques, involving the hydrogenation of polyunsaturated vegetable oils for the preparation of variably saturated fats, results in a significant intake of *trans* fatty acids. Hydrogenated fats are used in cooking and table fats, and as an ingredient in many manufactured foods. The effects of *trans* fatty acids on metabolism and disease processes remains very controversial. The only specific dietary requirement for lipids in the diet is for essential fatty acids, linoleic acid (18:2) and α-linolenic acid (18:3), which cannot be synthesized by humans.

A linkage between dietary fat and tumor angiogenesis has not been firmly established. However, circumstantial evidence, based on the role of prostaglandins and leukotrienes in modulating endothelial and vascular smooth muscle function, blood vessel dilation/constriction, and blood clotting, suggests several pathways whereby dietary lipids may influence tumor angiogenesis and related phenomena. Linoleic, arachidonic, eicosapentanoic, and related fatty acids are the precursors to a prostaglandin network, in which substrate availability, as well as enzymatic activity, critically regulate the synthesis of specific metabolic products. One example of how dietary lipids can modulate tumor growth and prostaglandin metabolism is derived from the breast cancer literature. Several studies have shown increased breast tumorigenesis with diets higher in total fat concentration *(11,43,115)*. Other reports have documented that the growth of breast cancer cells in culture or in vivo can be modulated by fatty acid patterns *(113,114,116–118)*. Cycloxygenase and lipoxygenase are the two critical enzymes responsible for producing the precursors of eicosanoids. The use of pharmacologic inhibitors of these pathways has clearly demonstrated a role of eicosanoids in breast cancer. Indomethacin treatment, an inhibitor of these two enzymes, suppressed growth and metastasis of mammary cancer, which was stimulated by a high-fat, high linoleic acid rich diet *(119)*. Furthermore, the effect of indomethacin was correlated with lower PGE_2 concentrations, one of the principal cyclooxygenase products, in tumors.

The role of endogenous prostaglandins in angiogenesis has been demonstrated by several studies. The inhibitory effect on neovascularization of tumors by several prostaglandin synthesis inhibitors, including indomethacin, diclofenac, and aspirin, were demonstrated by microangiography studies *(120)*. A recent study examined the ability of a topical application of diclofenac to inhibit prostaglandin metabolism, angiogenesis, and tumorigenesis in a colon carcinoma model *(121)*. Daily treatment with topical diclofenac resulted in a significant inhibition of basal cell skin tumor growth, or subcutaneous colon tumor growth accompanied by a retardation of vascularization development. There was an 80% inhibition of tumor PGE_2 synthesis following treatment in the same experiment.

The inhibition of tumor growth, and antimetastatic effects of marine fish oils rich in long-chain omega-3 fatty acids, have been observed in several rodent studies *(111,113,122–131)*. An antiangiogenic effect of omega-3 fatty acids, perhaps via alterations in the synthesis and metabolism of prostaglandins or leukotrienes, is one of several mechanistic hypotheses. McCarty *(132)* hypothesized that ingestion of omega-3 rich fish oils may impede angiogenesis, and reduce tumor invasiveness by downregulating hormonal activation of protein kinase C (PKC) and by modulating eicosanoid metabolism. Rabbit diets supplemented with sardine oil resulted in a significant 25% inhibition of neovascularization in the corneal alkali-burn injury model *(133)*. Other studies show reduced vascularization and inflammation in immunogenic keratitis of the rabbit cornea by the topical application of eicosapentaenoic acid in eyedrops *(134)*. Further in vitro studies with endothelial cells showed that eicosapentaenoic acid, but not arachidonic or docosahexaenoic acid, was able to inhibit tube formation by endothelial cells in collagen gels, without effects on PGE_2 or PGI_2 *(135)*. These studies provide indirect evidence supporting the hypothesis that dietary omega-3 fatty acids may inhibit tumor angiogenesis.

One additional mechanism by which dietary fatty acid profiles may modulate angiogenesis is via modulation of cellular signal transduction pathways. Activation of cellular PKC by proangiogenic growth factors and hormone receptors appears to contribute to the synthesis and secretion of collagenase and other lytic enzymes produced by endothelial cells, to enable them to migrate through basement membrane and matrix during angiogenesis *(136–140)*. Omega-3 fatty acid supplementation of endothelial cells in vitro can inhibit activation of PKC *(132)*. In many studies, activation of PKC occurs in conjunction with stimulation of phospholipase C-β, which is downregulated by EPA *(141–146)*. Additional studies of dietary fatty acid profiles and intracellular signal transduction pathways involved in endothelial proliferation and function are clearly warranted.

7. DIETARY PROTEIN AND AMINO ACIDS

It is not possible to establish recommendations for cancer prevention based upon dietary protein level or source. Dietary protein can increase, decrease, or have no effect on tumorigenesis in various tumor models, suggesting that protein may exert very specific mechanisms of action that are unique to each target tissue and initiating agent *(42,147–149)*.

Very little research has focused on the role of dietary protein or amino acids in tumor angiogenesis. One mechanism whereby dietary protein intake may influence angiogenesis is via dietary intake of the amino acid L-arginine, and subsequent modulation of nitric oxide (NO) production. NO is synthesized from L-arginine by the enzyme family, NO synthase (NOS). Recent investigations revealed that the NOS pathway is frequently upregulated in solid tumors of experimental animals *(150–153)*, and the expression of different isoforms of NOS has been characterized in several tumor systems *(150–152,154)*. The synthesis of NO by endothelial cells is blocked by L-arginine analogs such as N^{ω}-mono-methyl-L-arginine (L-NMMA) and L^{ω}-nitro-L-arginine methyl ester (L-NAME); D-isomers are ineffective. Systemic administration of L-NAME to rabbits bearing corneal implant blocked VEGF-, but not bFGF-induced angiogenesis *(155)*. Furthermore, L-NAME was very effective in blocking angiogenesis induced by VEGF-transfected MCF-7 breast cancer cells *(155)*. Stimulation of monocytes with LPS in absence of L-arginine greatly reduced their production of angiogenic activity; the NOS

inhibitors L-NMMA and L-NAME reduced the angiogenic activity, suggesting that this enzyme has a key role in controlling the pathway of angiogenesis *(156)*. In another model of angiogenesis, rat microvascular endothelial cells grown in three-dimensional collagen gels exhibited extensive tube formation in presence of TGF-B1, which was blocked by NOS inhibitors *(157)*. Excess L-arginine in this model reversed the inhibitory effect of L-NAME on vascular tube formation *(157)*.

The connection between NO and enhanced vascular permeability in solid tumors was explored, and found to be suppressed by NO synthatase inhibitors and augmented following the administration of L-arginine *(153)*. The ability of VEGF and bFGF to enhance vascular permeability and tone is also coupled to NO production and blocked by NOS inhibitors *(153, 159–161)*. Another potential mechanism whereby dietary arginine and NO may modify angiogenesis is via upregulation of urokinase-type plasminogen activator and participation in matrix degradation *(162)*. These studies clearly indicate that increased NO within tumors may enhance angiogenic pathways, as well as vascular permeability and blood flow. How changes in dietary arginine and metabolic antagonists, such as L-lysine, may modulate NO synthesis and alter tumor vascular tone, blood flow, and angiogenesis, remains to be clearly defined.

8. VITAMIN A

The role of vitamin A nutrition in the risk of human cancer is complex and does not warrant generalizations *(7,163)*. Simplistic conclusions and recommendations for intakes at specific dosages beyond the recommended daily intake is not possible. The possibility that vitamin A, its metabolites, and analogs may modulate angiogenesis is based on studies showing that molecular and functional properties of endothelial cells are influenced by these compounds. Both proliferation and shape of capillary endothelial cells are altered by retinoids in vitro *(164,165)*. Several genes, including transcription factors, tissue-type plasminogen activator (tPA), and thrombomodulin, are regulated in endothelial cells by retinoids *(166–168)*. Both excess and deficiency of retinoic acid can inhibit the normal development of the vascular system in chick and mammalian embryo models *(169,170)*. Additional retinoic acid also inhibited angiogenesis in the chorioallantoic membrane assay *(171)*. The pharmacologic supplementation of mice bearing a variety of transplantable tumors with 13-cis retinoic acid or 9-*cis* retinoic acid, in combination with 1,25(OH)2D3 or interferon-α, decreased tumor-cell-induced angiogenesis, which could be counteracted by a selective antagonist of the nuclear retinoic acid receptor α *(172,173)*. In vitro studies with all-*trans* retinoic acid or 13-*cis* retinoic acid at physiologic concentrations, administered to cultured squamous cell carcinomas, enhanced the expression of angiogenesis inhibitors *(174)*. Studies with a human cervical squamous cell carcinoma cell line, grown as xenografts in athymic nude mice treated with pharmacologic doses of all-*trans*-retinoic acid, has been examined *(175)*. All-*trans*-retinoic acid therapy was associated with reduced angiogenesis, increased apoptosis, and reduced tumor growth rate, as well as a reduced expression for an FGF-binding protein *(175)*. However, it is premature to generalize that retinoids are antiangiogenic under all conditions. For example, 13-*cis* retinoic acid and β-carotene (pro-vitamin A carotenoid) were associated with enhanced tumor vascularity in the DMBA hamster cheek pouch model *(176)*. Clearly, much more research needs to be undertaken regarding the role of vitamin A nutrition and metabolism in tumor angiogenesis. Furthermore, the possibility that synthetic

retinoids may have antiangiogenic properties at pharmacologic doses, in specific clinical situations, warrants additional effort *(177)*.

9. VITAMIN E (α-TOCOPHEROL)

Many benefits, including antitumor effects, have been proposed for increased consumption of vitamin E at concentrations in excess of recommended intake. The possibility that vitamins E, C, and carotenoids interact with other oxidative defense systems to modulate the angiogenic switch warrant investigation. It has been suggested that oxidative stress within the tumor microenvironment may be one modulator of the balance between angiogenic and antiangiogenic factors. In vitro studies suggest that vitamin E protects human endothelial cells from oxidative damage *(178)*. In 1996, Shklar and Schwartz *(179)*, in their study with DMBA-induced oral carcinogenesis in hamsters, reported that inhibition of carcinogenesis, following vitamin E supplementation, was associated with significant reductions in tumor microvessel density. An interesting study by Kunisaki et al. *(180)* showed that D-α-tocopherol improved abnormalities in retinal hemodynamics in diabetic animals, using computerized video-based fluorescein angiography. Parallel in vitro studies with retinal vascular cells showed that vitamin E modulated PKC signal transduction. Many investigators have focused on the effects of vitamin E on endothelial cells in the context of atherosclerotic vascular disease. For example, dietary vitamin E protects animals from oxidized LDL-induced endothelial dysfunction and changes in hemodynamics *(181)*, further supporting a possible role in tumor vascular biology.

Eales' disease is a perivasculititis of the retina associated with accumulation of reactive oxygen intermediates and increases in lipid peroxidation. It has been postulated that the oxidative damage is a key stimulus for the associated neovascularization and retinal damage. A significant decrease in serum vitamin E, in parallel with increased lipid peroxidation of red cell membranes, was noted during the perivasculitis stage of Eales' disease, compared to normal healthy volunteers *(182)*.

Overall, the data derived from a variety of sources suggests that vitamin E nutritional status can influence endothelial cell function, probably within the context of the antioxidant defense system. However, too few studies of tumor angiogenesis have been completed to make any general conclusions about how vitamin E may influence tumor blood flow and the cytokine and growth factor networks modulating these processes in vivo.

10. VITAMIN C (ASCORBIC ACID)

Anticancer properties of ascorbic acid have received extensive speculation, but relatively little evidence for cancer prevention has been generated to support consumption of quantities in excess of the recommendations *(7)*. Some interesting data relative to ascorbic acid and angiogenesis has emerged from the literature, characterizing its important role in synthesis of extracellular matrix, cartilage, and bone *(183)*. Chondrocytes at various stages of differentiation release molecules that inhibit endothelial cell migration and prevent neovessel formation, which contributes to the relative avascular state of cartilage. During differentiation to hypertrophic chondrocytes, and then to osteoblasts, a condition leading to the formation of mineralized and calcified cartilage, the cells switch to production of angiogenic factors *(184)*. Ascorbic acid was found to be a potent stimulus for the switch from antiangiogenic to angiogenic activity in suspension cultures of hypertrophic chondrocytes *(185)*. Ascorbic acid also enhances the barrier function of cultured

endothelial cells by stimulation of collagen synthesis *(186)*. These studies suggest that ascorbic acid may contribute to the formation of tumor matrix, and perhaps modulate vascular function or angiogenesis, but no in vivo data has yet accumulated to support this hypothesis.

11. VITAMIN D AND CALCIUM NETWORK

Evidence supporting a role for dietary vitamin D in the risk and prevention of human cancer remains insufficient for generation of recommendations concerning optimal intake *(7)*. However, a rapidly emerging field, focusing on synthetic analogs of vitamin D, takes advantage of the potent range of biological effects vitamin D may have on proliferation and differentiation in a variety of cell types and tissues *(187)*. Endothelial cells are also sensitive to vitamin D, and biological effects include the inhibition of cell migration *(188)*. One of the main obstacles to the use of vitamin D or analogs in vivo is the hypercalcemia associated with administration. Newer synthetic compounds exhibit lower activity for the intestinal mucosa, with little effect on calcium uptake, less effect on serum calcium, and greater specificity for modulating biological outcomes in other tissues *(189,190)*.

Rickets, a disease of impaired skeletal growth and bone remodeling, is the classic syndrome of vitamin D deficiency. The role of angiogenesis in bone formation is only beginning to be understood. Angiogenesis may be a central event in endochondral ossification. New vessels from the developing bone plate, at the junction of the metaphysis with the hypertrophic zone of the growth plate, grow into and degrade the hypertrophic cartilage, to define the scaffold upon which osteoblasts adhere and produce new bone. A number of angiogenic growth factors, such as bFGF, have been identified at the growth plate. In vitro studies of chondrocytes show that the expression of angiogenic factors is enhanced by both 1,25-$(OH)_2$ vitamin D_3 and 24,25-$(OH)_2$ vitamin D_3 *(184)*.

Many tumor types express vitamin D receptors, and are inhibited by natural and synthetic ligands in vitro and in vivo *(189–193)*. Few studies have focused attention on tumor angiogenesis *(172,194,195)*. A recent study reported an inhibition of retinal tumorigenesis in the retinoblastoma transgenic murine model by vitamin D analogs *(196)*. The inhibition of tumor growth was associated with reduced tumor vascularity.

Redistribution of intracellular calcium is a common signaling mechanism used to transfer information delivered by receptors at the cell surface into appropriate intracellular compartments, for the regulation of metabolisms, gene expression, differentiation, apoptosis, and proliferation. The regulation of intracellular calcium is controlled by many complex and interacting nutritional and endocrine networks, including vitamin D pathways. Intracellular calcium is an important regulator of many endothelial functions related to angiogenesis, based upon studies with inhibitors of ligand-stimulated calcium influx, such as carboxyamidotriazole *(197)*. For example, proliferation in response to bFGF, adhesion to basement membrane proteins, and expression of matrix degradation enzymes were significantly reduced following inhibition of intracellular calcium signaling. What role dietary calcium and vitamin D may play in modulating intracellular calcium, and participating in tumor angiogenesis, remains to be further explored beyond the cell culture systems.

12. PHYTOCHEMICALS

The term phytochemicals is used generically to describe substances derived from plants that have biological activity in mammals. Plants have been a rich source of pharma-

ceutical agents, including those for the treatment of cancer. It also appears that plants may serve as a bountiful resource for cancer chemopreventive agents. The incidence of many human cancers is less in groups consuming higher quantities of fruits and vegetables *(7,198)*. Furthermore, rodents in carcinogenesis studies fed diets composed of plant materials typically develop fewer cancers than those fed diets containing purified ingredients *(28,199–201)*.

The inhibition of angiogenesis is one potential target of chemopreventive agents derived from fruits and vegetables, although little effort has thus far been devoted to this line of investigation. The most extensive evaluation has focused on the isoflavones that comprise a large group of naturally occurring diphenolic compounds (Fig. 3), with at least 230 types chemically characterized *(202)*. Fotsis et al. *(29–31)* fractionated the urine of humans consuming diets rich in plant foods for the presence of antimitotic and antiangiogenic compounds, and found that fractions containing soy isoflavones and metabolites (genistein, daidzein, *O*-desmethylangolensin, or equol) inhibited bFGF-stimulated proliferation of bovine brain-derived capillary endothelial cells *(29,30)*. Further studies indicated that pure genistein exhibited a potent and dose-dependent inhibitory effect on proliferation of endothelial cells at a concentration of 5 μ*M* for half-maximal inhibition *(30)*. Genistein also inhibited the proliferation of other vascular endothelial cells, such as that derived from bovine adrenal cortex and aorta *(30)*. Isoflavonoids are present principally in legumes, and are at particularly high levels in certain legumes that are regularly consumed by humans and animals *(203)*. Soybeans and soy products, containing high levels of isoflavones (0.1–1.5 mg isoflavones/g) *(204)*, are the most commonly consumed plant products by humans, and thus contribute a major source of dietary isoflavones. The most abundant soy isoflavones are genistein, daidzein, and, in less content, glycitein. They exist mostly in glycoside forms in the soybean called genistin, daidzin, and glycitin, respectively. Upon intestinal bacterial action, the glycosides are converted to their biologically active aglucone forms. The excretion of genistein in the urine of vegetarians is 30-fold higher than that of omnivores *(205)*. These observations support additional epidemiologic and laboratory evidence suggesting that the consumption of diets rich is soy products is associated with low risk of several cancers *(206,207)*.

Fotsis et al. *(31)* have extended their earlier work by investigating the antiangiogenic properties of a diverse array of flavonoids that are more widely distributed in the plant kingdom. Several flavonoids, including 3-hydorxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone, fisetin, apigenin, and luteolin, inhibited in vitro angiogenesis in the micromolar range (Fig. 3). Both bFGF-induced endothelial proliferation and bFGF+VEGF-induced vasculogenesis in three-dimensional collagen gels were inhibited by several flavonoids *(31)*.

The mechanisms by which flavonoids inhibit angiogenesis in vitro is not well understood, and almost no work has been completed in vivo. Genistein may inhibit angiogenesis via regulating the expression and activity of angiogenic factors. Genistein has been shown to inhibit VEGF expression via posttranscriptional regulation in vitro *(208)*. VEGF stimulates endothelial cell proliferation and phosphorylation of a number of proteins involved in signaling pathways, including phosphorylation of phosphatidylinositol 3-kinase, ras GTPase activating protein (GAP), and the oncogenic adapter protein NcK *(209)*. Genistein can inhibit VEGF-promoted endothelial cell proliferation *(209,210)*, activation of PKC *(210)*, and tyrosine phosphorylation of signaling molecules in vitro

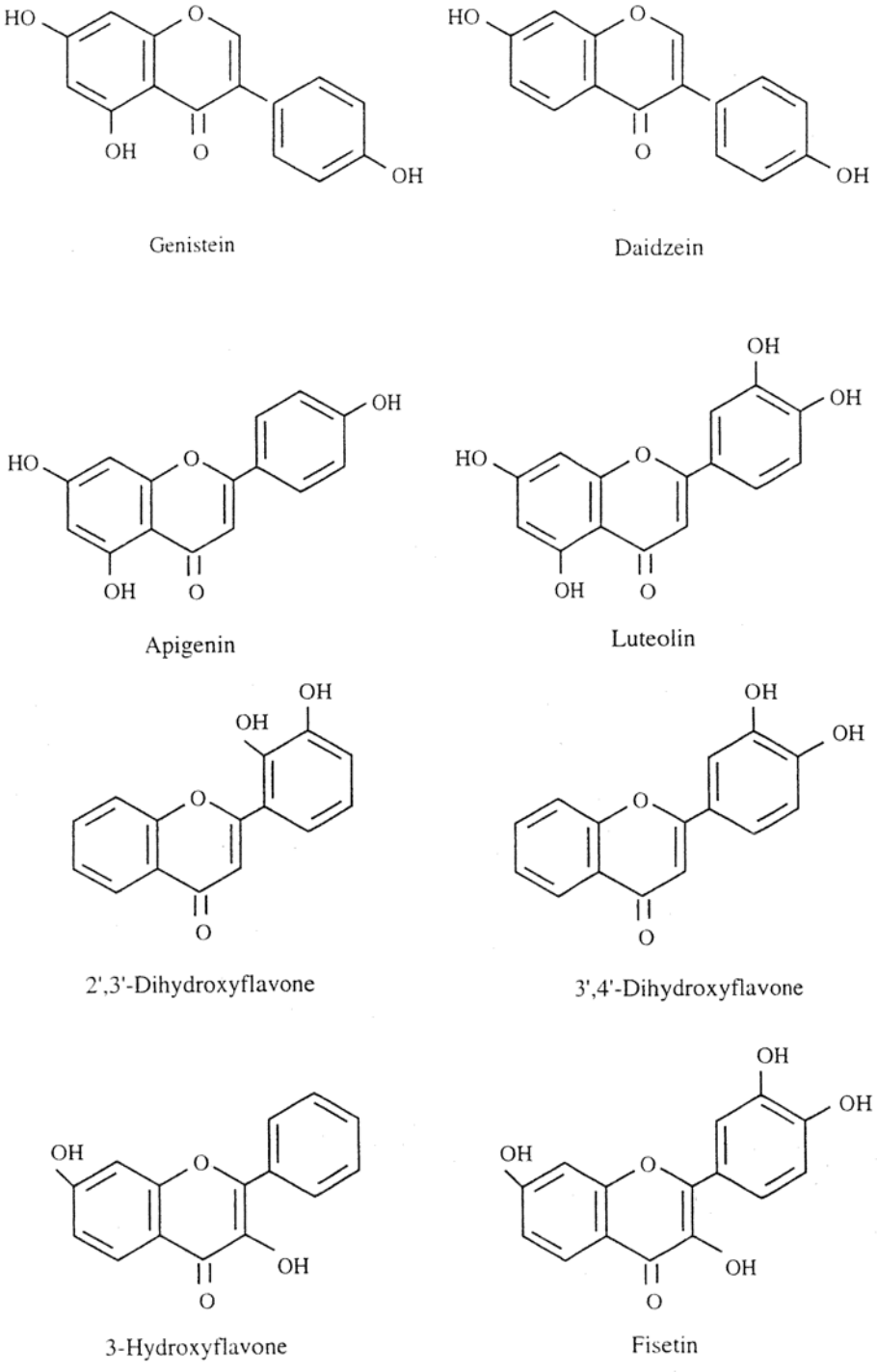

Fig. 3. Chemical structures of a variety of phytochemicals exhibiting antiangiogenic activity in vitro and in vivo *(29–32)*.

(209). Genistein and other flavonoids also inhibited bFGF-mediated vascular endothelial cell proliferation *(29–31,211)*.

The presence of mast cells at sites of angiogenesis has been observed in some experimental models and human tissues *(212)*. The local accumulation is believed to facilitate new vessel formation through complex cell–cell interactions that have yet to be clearly defined *(213)*. Genistein effectively dampened directed mast cell chemotaxis in response to several angiogenic growth factors, such as PDGF, VEGF, and bFGF *(213)*.

Genistein may inhibit angiogenesis by regulating the activity of proteolytic enzymes and their inhibitors: Proteolytic degradation of the extracellular matrix by endothelial cells is controlled by angiogenic factors, such as bFGF, which induces the production of urokinase-type plasminogen activator *(214)* and its physiological inhibitor, plasminogen activator inhibitor-1 *(215)*. Genistein markedly reduced both bFGF-stimulated and basal levels of plasminogen activator and plasminogen activator inhibitor-1 in vitro *(215)*.

Preliminary studies in this laboratory suggest that genistein, or diets rich in soy products, inhibit the growth of murine bladder cancer or human prostate cancer xenografts in rodents, in association with a reduction in tumor microvessel density *(32,33)*. Further efforts designed to better understand the role of plant-derived substances in tumor angiogenesis in vivo are clearly necessary.

13. SUMMARY AND CONCLUSIONS

The relationships between required nutrients, as well as the diverse array of other substances in the diet, and angiogenesis is a relatively new area of scientific investigation that is undergoing rapid expansion. The critical roles of angiogenesis in many disease processes, including wound healing, arthritis, vasculatitis, retinopathy, tissue responses to ischemia, and cancer, are beginning to be appreciated *(2)*. Many of these disease processes in experimental animals and humans are also profoundly modulated by diet and nutrition *(6,9,10)*. Future studies will undoubtedly reveal many steps in the angiogenic process modulated by substances contained in foods. The additional knowledge generated will be relevant to optimizing experimental models employed by many angiogenesis investigators. Furthermore, the incorporation of dietary assessment and controlled nutritional intake into clinical trials of angiogenesis modulators will reduce heterogeneity, and improve the power and sensitivity of the studies.

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16 Thalidomide and Analogs

Taturo Udagawa, Henk M. W. Verheul, and Robert J. D'Amato

Contents

1. BACKGROUND

Thalidomide was developed in 1958 by Chemie Grunenthal as an oral sedative. Subsequently, McBride *(1)* and Lenz *(2)* described the association between maternal usage of thalidomide and fetal limb defects. The effect of thalidomide as a teratogen was experimentally confirmed in a number of species *(3–8)*. By the time thalidomide was withdrawn from the market in 1962, thousands of babies worldwide were estimated to have been born with thalidomide-related defects. As a consequence, the mechanism by which thalidomide causes birth defects has been intensively investigated. Nevertheless, the mechanism has remained elusive *(9)*.

Numerous theories have been proposed regarding thalidomide's mechanism of action. This included the existence of a toxic arene metabolite *(10)*, a mutagenic effect resulting from intercalation of thalidomide into DNA *(11,12)*, glutamate toxicity based on structural resemblance to glutamic acid *(13)*, antagonism of B vitamins, and chelation of essential bivalent cations (for reviews of proposed mechanisms, *see* refs. *14–16*). Many of these hypotheses have remained largely unproven and controversial *(14,17)*, and arguments based on a mutagenic or a toxic effect are unlikely since the window of susceptibility is narrow, and the defects caused by thalidomide on the developing fetus are specific. In adults thalidomide exhibits little toxicity.

In 1961, McBride reported that the effect of thalidomide was a result of specific injury to the mesenchyme of the developing limb *(1)*. A cytologic study of chicken embryos treated with thalidomide, which resulted in defects similar to those seen in humans *(8)*, revealed early morphologic changes in endothelial cells *(18)*. These changes were speculated to influence the development of the nonvascular part of the mesoblast tissue. Further

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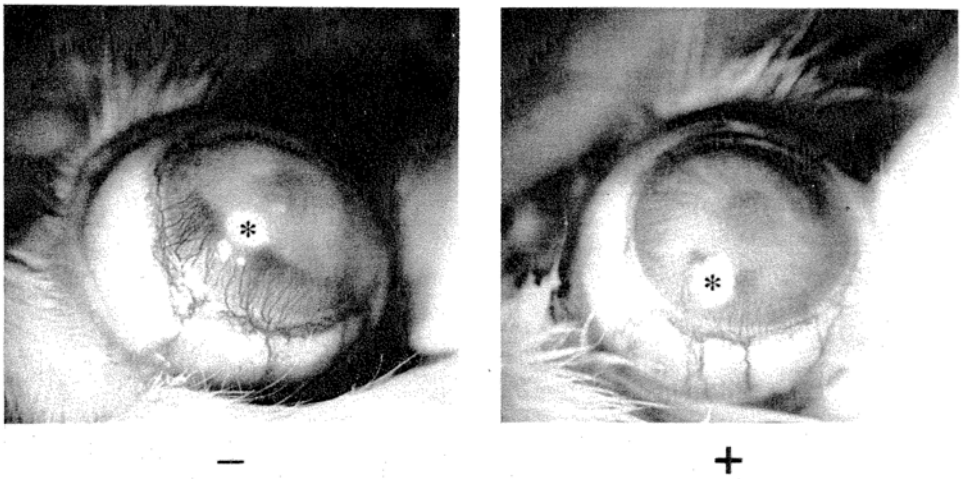

Fig. 1. Angiogenesis in the rabbit cornea induced by slow release of bFGF from implanted polymers (indicated by asterisks) in control animals (–) and in rabbits treated with 200 mg/kg of orally administered thalidomide (+).

insights into the mechanism of its teratogenic action may be revealed from recent studies investigating the effect of thalidomide on growth-factor-induced angiogenesis in the rabbit cornea *(19)*. Thalidomide administered orally to rabbits, at doses that induced malformations, inhibited angiogenesis induced by basic fibroblast growth factor (bFGF) in a rabbit cornea micropocket assay (Fig. 1). Thalidomide and related analogs also inhibited angiogenesis in the mouse cornea induced by vascular endothelial growth factor (VEGF) as well as bFGF *(20)*.

These observations led to the hypothesis that a primary cellular target of thalidomide is the endothelium, and that the birth defects caused by thalidomide usage were secondary to inhibition of blood vessel growth in the developing limb bud. Studies of the developing limb bud have revealed intense angiogenic activity *(21)*, and this developmental process may be particularly sensitive to inhibition by thalidomide. In the adult, angiogenesis is usually limited to the female reproductive cycle *(22)* and wound healing *(23)*, and the proliferation index for endothelial cells is very low *(24,25)*. Agents that interfere preferentially with angiogenesis would thus be predicted to have little toxicity in adults. Consistent with this hypothesis, the potent angiogenesis inhibitor TNP-470 has been demonstrated to have little toxicity *(26)*. However, pregnant mice were exquisitely sensitive to TNP-470, although the effects were different from that of thalidomide. A single injection of TNP-470 into pregnant mice resulted in complete blockade of early embryonic development, possibly because of inhibition of placental and fetal angiogenesis *(27)*. Conventional chemotherapy, which has little specificity, is generally toxic to both adults and fetuses.

The investigation of thalidomide as an inhibitor of angiogenesis may shed new light on a poorly understood mechanism for a widely recognized human teratogen. In addition, there are clear implications for thalidomide's use in the treatment of pathologic angiogenesis that occurs in cancer, macular degeneration, and diabetic retinopathy *(28,29)*. A drug like thalidomide, which is readily available and can be administered by oral route,

is advantageous. Thalidomide is also relatively safe, with few side effects in the absence of pregnancy. For these reasons, there has been a renewed interest in the use of thalidomide and the development of more potent and specific analogs for use in the clinic to treat cancer and other angiogenesis-dependent diseases.

2. THALIDOMIDE AND TERATOGENESIS

Initial studies showed that thalidomide and EM-12 (a more teratogenic analog of thalidomide) given orally to rabbits inhibited angiogenesis induced by bFGF in the cornea. Interestingly, neither thalidomide nor EM-12 inhibited angiogenesis when placed directly on the chick CAM. However, metabolites of thalidomide and EM-12, pthaloyl glutamic acid (PG acid) and EM-138, respectively, inhibited angiogenesis in the chick CAM assay *(19)*. For this reason, it is thought that the metabolism of thalidomide is required for its antiangiogenic effect, which correlates with evidence that metabolism of thalidomide is required for teratogenicity.

Extensive structure function studies of thalidomide and its breakdown products on teratogenicity have been performed. Thalidomide is a small-mol-wt organic compound composed of a pthalimide and a glutaramide moiety (Fig. 2). Thalidomide is a chiral molecule, and the R(+) and S(–) enantiomers of thalidomide have been reported to produce different teratogenic effects when administered by intraperitoneal route to mice and rats *(30)*. Only the S(–) enantiomer of thalidomide or its metabolites caused a dose-dependent teratogenicity in the offspring *(31,32)*. At neutral or higher pH, the glutaramide and the phthalimide rings of thalidomide are susceptible to hydrolysis, and thalidomide spontaneously decays into at least 12 products *(33)* (Fig. 3A). Successive hydrolysis of the glutaramide ring yields a dicarboxylic acid, PG acid. PG acid may play a central role in thalidomide teratogenicity since PG acid has been shown to be teratogenic when injected into the peritoneum of pregnant mice *(34)*. Cleavage of the phthalimide ring inactivates teratogenicity and also renders it susceptible to further hydrolysis, yielding phthalic and glutamic acids as stable end products. Cleavage of the glutaramide ring, in contrast, does not inactivate teratogenicity *(35)* and may even be necessary for its action *(36)*.

The phthalimidine ring of EM-12, with only one carbonyl group, is sterically less strained, and resonance with the neighboring nitrogen atom confers resistance to hydrolysis. Only the glutaramide ring is susceptible, and thus EM-12 exhibits a much simpler hydrolysis profile with only three main products (Fig. 3B). Unlike PG acid, which is further hydrolyzed, EM-138 is a stable product of EM-12 hydrolysis. The greater teratogenic potential of EM-12 in rabbits is attributed in part to the stability of the phthalimide ring *(13)*. EM63 (Fig. 1) is a nonteratogenic analog that also contains a pthalimidine group. Unlike EM-12, however, hydrolysis of the glutaramide ring of EM63 is not observed, which suggests that hydrolysis of the glutaramide ring is necessary for thalidomide's teratogenic activity *(36)*. Thus, the dicarboxylic acids PG-acid and EM-138 may be downstream metabolites that mediate either directly or indirectly the actions of thalidomide and EM-12, respectively.

The effectiveness of thalidomide depends on the species and route of delivery, which complicates interpreting the effects of thalidomide. For example, the actions of thalidomide on fetal development could be reproduced by oral administration in maternal rabbits, but failed to produce malformations in rodents when given orally *(1,2)*. Later studies show that hydrolytic metabolites of thalidomide given intraperitoneally, but not orally,

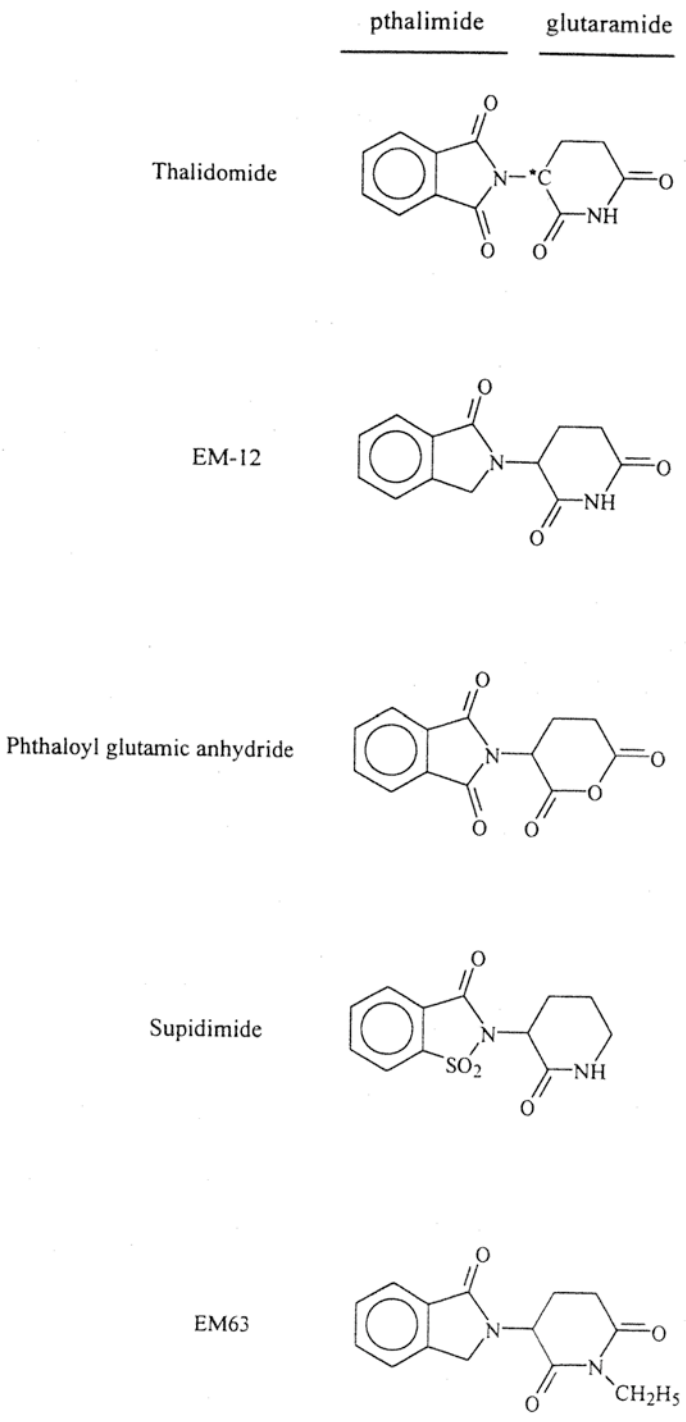

Fig. 2. Structure of thalidomide and related parent compounds. The chiral carbon atom of thalidomide is shown with an asterisk (*).

induced malformations in mice as well as rats *(34,37)*. Although PG acid was initially thought to be nonteratogenic in rabbits *(38)*, feeding experiments showed that PG acid was rapidly excreted and penetrated to such a limited extent that hardly any of the drug reached the embryo. Thalidomide is more hydrophobic than PG acid and showed greater penetration *(35)*. Thus, thalidomide may serve to carry the drug across the placenta, where it is later metabolized to an active product *(35)*. The absorption, distribution, excretion, and metabolism of thalidomide were different across species following oral doses *(35,39)*. Interaction of thalidomide with serum proteins, such as α1-acid glycoprotein *(40)*, may also affect clearance time.

3. THALIDOMIDE AND ANALOGS IN ANGIOGENESIS AND CANCER

Thalidomide and related compounds, including PG acid, EM-12, and EM-138 were tested in mice, and each of these related compounds inhibited bFGF- and VEGF-induced corneal neovascularization *(20)*. In these studies, intraperitoneal, but not oral, administration of thalidomide inhibited angiogenesis in mice. The route-dependence of thalidomide as an antiangiogenic agent in mice was similar to the reported susceptibility of rodents to congenital malformations induced by intraperitoneal injections of thalidomide *(31,34)*. Therefore, the route of delivery is again a critical parameter when evaluating the effects of thalidomide on angiogenesis *(20)* and tumor growth *(41)*.

Gordon et al. provided evidence for the conversion of thalidomide to a toxic arene oxide metabolite by liver microsomes *(10)*, possibly by the action of cytochrome P-450 *(42)*. Since both a 3- and a 4-hydroxy metabolite of thalidomide have been detected, it was hypothesized that a reactive 3,4-epoxy form of thalidomide may mediate its teratogenic action *(10)*. Interestingly, tetrafluorothalidomide, a fluorinated analog of thalidomide that cannot be hydroxylated or converted into an epoxide *(43)*, inhibited angiogenesis comparable to thalidomide at its maximum tolerated dose (unpublished data). This suggests that neither epoxide formation nor hydroxylation of the pthalimide ring is essential for inhibiting angiogenesis. Other metabolites of thalidomide and EM-12, including 3-hydroxyphthalmide, 4-hydroxythalidomide, EM-27, and EM-356, were also tested but did not inhibit corneal neovascularization *(20)*. Since PG-acid and EM-138 inhibited angiogenesis in both the chick CAM assay and in mice, PG acid and EM-138 may be metabolites of thalidomide and EM-12, respectively, which mediate their antiangiogenic effect.

Experiments examining the effect of thalidomide enantiomers on angiogenesis in mice revealed that the S(–) enantiomer had the strongest antiangiogenic activity in VEGF- and bFGF-induced corneal neovascularization, which further supported a link between thalidomide's antiangiogenic and teratogenic activities *(30,32,44)*. The antiangiogenic activity of thalidomide correlated with the teratogenic but not the sedative or mild immunosuppressive properties of thalidomide. Supidimide (Fig. 2), an analog of thalidomide that retains sedative but not teratogenic activity, did not inhibit angiogenesis.

Negative results using thalidomide have been reported in rodent cancer models *(41,45)*. These studies did not, however, take into consideration that thalidomide is neither teratogenic nor antiangiogenic in rodents when administered orally. In contrast, other studies in mice have shown that thalidomide had small antitumor effects, and in combination with 5,6-dimethylxanthenone-4-acetic acid, an analog of flavone acetic acid, resulted in complete cures in mice *(46)*. Mice treated with thalidomide administered by intraperitoneal route in combination with cytoxan and adriamycin

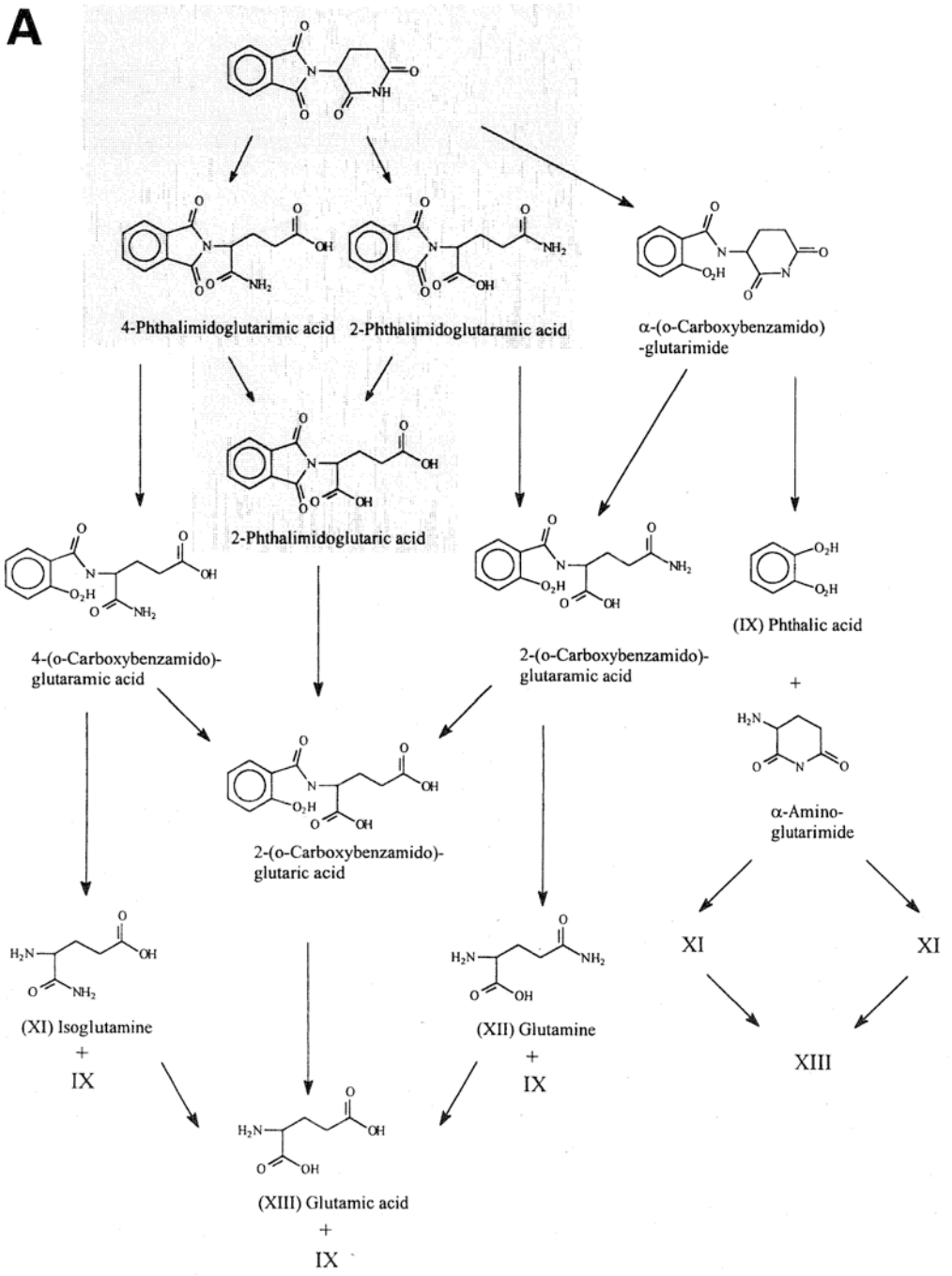

Fig. 3. Hydrolysis of thalidomide **(A)** and EM-12.

showed significantly smaller tumors than those given the two chemotherapeutic agents alone in a model of breast cancer *(47)*. Since the effects of thalidomide may be more accurately reflected in rabbits than rodent models, thalidomide was tested

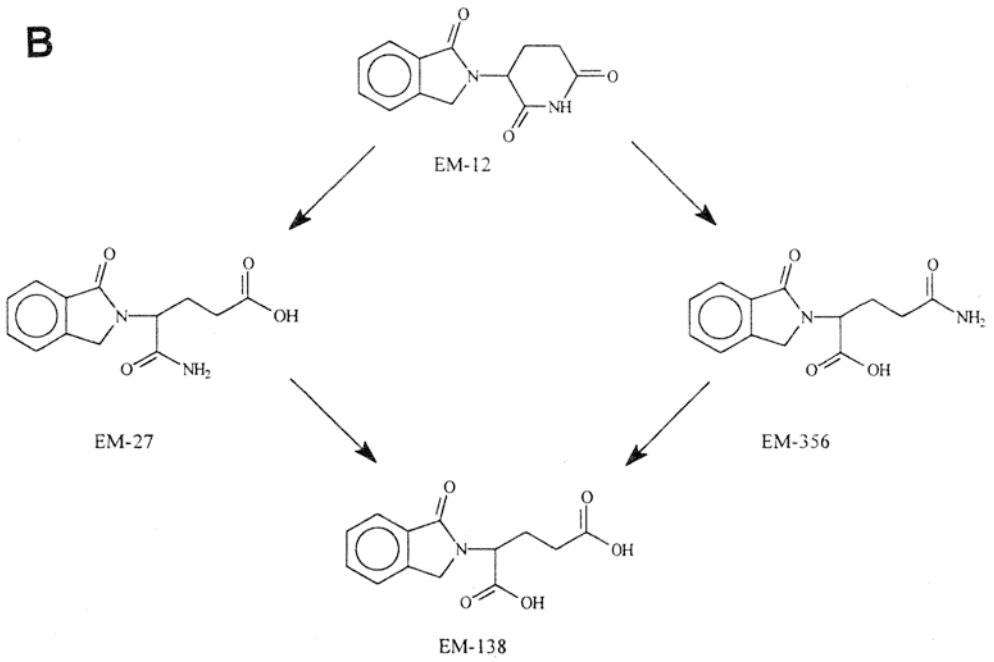

Fig. 3. *(continued)* **(B)** Thalidomide and its products corresponding to EM-12 hydrolysis are shaded.

for inhibition of rabbit tumors. In agreement with its effect on angiogenesis, oral administration of thalidomide alone inhibited the growth of V2 carcinoma by 50% (Verhuel, H. M., in press). Sulindac, a nonsteroidal anti-inflammatory drug reported to cause regression of colon polyps in humans and experimental models *(48,49)*, also has been shown to exhibit antiangiogenic activity. When thalidomide was combined with sulindac, there was an additive inhibition (approx 70%) of tumor growth (Verheul, H. M., in press). Recent studies have shown favorable preliminary clinical results using thalidomide in the treatment of AIDS-related Kaposi's sarcoma *(50)*, as well as glioblastoma, breast, and prostate cancer. In the future, thalidomide in combination with other angiogenesis inhibitors may further improve treatments. The identification of the active thalidomide metabolite and its receptor may lead to the development of more potent, orally administered analogs in the treatment of cancer and other angiogenesis-dependent diseases.

4. OTHER EFFECTS OF THALIDOMIDE

In addition to its antiangiogenic activity, other effects of thalidomide have been reported. Thalidomide has been used in the treatment of lepromatous reaction *(51–54)*, graft-versus-host disease *(55–60)*, lupus erythematosis, Behcet's syndrome *(61)*, rheumatoid arthritis *(62,63)*, and Crohn's disease *(64,65)*. Thalidomide has been shown to prolong graft survival in rat cardiac transplants *(66,67)* and to inhibit HIV replication *(68)*. Macrophages appear to be a cellular target of thalidomide, since the drug has been shown to inhibit lipopolysaccharide-induced tumor necrosis factor-α production *(69)* by selective degradation of TNF-α mRNA *(70)*, a pleiotropic cytokine that plays a central

role in immunity *(71)*. Thus, some of the effects of thalidomide on immunologic function may be caused by suppression of TNF-α production.

Studies investigating the relationship between the effect of thalidomide on TNF-α production and suppression of angiogenesis have failed to find a correlation. Agents that inhibit TNF-α production, such as pentoxifyllin (100 mg/kg), and Macronex 160 (120 mg/kg) do not inhibit angiogenesis *(72)*. TNF-α is only weakly angiogenic in vivo and may act by inducing VEGF expression *(44,73)*. Histologic examination of corneal angiogenesis *(74)* induced by bFGF and VEGF demonstrated paucity of macrophages and the absence of inflammation *(72,75)*. These observations argue that endothelial cells and macrophages are distinct cellular targets of thalidomide, and that thalidomide inhibits angiogenesis by directly inhibiting endothelial cell response to growth factors.

Amenorrhea is an interesting side effect of thalidomide *(62,76)* since in adults, the female reproductive cycle and wound healing *(23)* are dependent on angiogenesis. Indeed, this effect of thalidomide was exploited as a criteria for candidate antiangiogenic agents to be tested in the laboratory. Other effects of thalidomide may also be caused by inhibition of angiogenesis. For example, chronic inflammation may be angiogenesis-dependent, since an angiogenesis inhibitor could suppress collagen arthritis *(77)*, and neutralizing antibodies to VEGF inhibited the development of granulomateous inflammation *(78,79)*. The therapeutic effects of thalidomide observed in such disorders as arthritis *(62)* and Crohn's disease *(64,65)* may therefore be speculated to be caused in part by suppression of an angiogenic component that contributes to progression of the disease.

Thalidomide has been reported to modulate the expression of adhesion molecules on both lymphocytes *(80–82)* and endothelial cells *(83)*. Geitz et al. has shown that thalidomide inhibits the induction of VCAM-1 and E-selectin expression on endothelial cells in response to TNF-α *(83)*. Adhesion molecules expressed in response to inflammatory mediators participate in the recruitment of leukocytes from circulation into tissue. Thus, inhibition of adhesion molecule expression by thalidomide may contribute to the immunosuppressive effect of the drug.

Neubert and colleagues reported that the treatment of pregnant primate *Callithrix jacchus* with the thalidomide analog EM-12 resulted in decreased expression of integrin receptors, including CD29/CD49d (α4β1 integrin) *(80)*, its receptor CD106/VCAM-1 *(83)*, and CD61 (β3 integrin subunit) *(85,90)*. These adhesion molecules have been implicated to participate in the development of the vasculature *(84,88,89)*. Neubert et al. hypothesized that downregulation of adhesion receptors on cells of primate embryos is a possible mechanism for the teratogenic action of thalidomide *(90)*.

With regard to angiogenesis, Brooks has shown that the integrins αvβ3 and αvβ5 play an important role in angiogenesis. These integrins are induced in cultured endothelial cells in response to bFGF *(91–93)* and VEGF *(94)*, and in vivo are selectively expressed on angiogenic vessels. Antagonists of these integrins induce apoptosis of endothelial cells and inhibit angiogenesis in the chick CAM assay, in tumors, and in ocular neovascular diseases *(85–87,95)*. Thus, thalidomide may inhibit angiogenesis by antagonizing the expression of αvβ3 and αvβ5 or by interfering with integrin signaling. The role of adhesion molecules as part of the mechanism of thalidomide's antiangiogenic action is under investigation.

In conclusion, thalidomide is an orally administered, potent antiangiogenic molecule that inhibits ocular angiogenesis and cancer in animal models. Current clinical studies aim to establish the role of thalidomide in the treatment of macular degeneration and

cancer. With proper vigilant efforts to prevent exposure to pregnant women, thalidomide may experience a resurgence of use.

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III IN VIVO STUDIES AND CLINICAL TRIALS

17 Potentiation of Cytotoxic Cancer Therapies by Antiangiogenic Agents

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CONTENTS

1. INTRODUCTION

Cancer cure requires eradication of all malignant cells. Cancer growth, however, requires proliferation of malignant cells and normal cells. The several anticancer treatment modalities currently available, including surgery, chemotherapy, radiation therapy, and immunotherapy, have been envisioned to target primarily the malignant cell. Research over the past 35 yr has reinforced the hypothesis put forth by Folkman that, without the proliferation of normal cells, especially endothelial cells, a tumor cannot grow beyond the size of a colony *(1)*. The consequence of this finding is that both the normal cells and the malignant cells involved in tumor growth, as well as the chemical and mechanical signaling pathways that interconnect them, are valid targets for therapeutic intervention. The integration of therapeutics directed toward the vascular components, extracellular matrix components, and stromal and infiltrating cells, with classical cytotoxic anticancer therapies, may be regarded as a systems approach to cancer treatment *(2)*.

Although new noncytotoxic agents directed toward normal cells and extracellular enzymatic activities target processes critical to tumor growth, it is highly unlikely that treatment with these new agents alone will lead to tumor cure. The question arises of how to integrate these new therapeutic agents into existing cancer treatment regimens. Thus, by choosing multiple cellular and process targets for therapeutic attack, a systems approach to anticancer therapy regimen development may lead to the cure of systemic malignant disease.

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2. PRECLINICAL THERAPEUTIC STUDIES: SMALL MOLECULES

2.1. Steroids, Tetracyclines, and Heparins

Several studies have been reported using the Lewis lung carcinoma (LLC) growing subcutaneously in C57BL mice, with standard cytotoxic anticancer therapies, along with one or more new agents directed toward the normal cellular and extracellular compartments of the tumor *(3–9)*. The Lewis lung tumor is relatively resistant to many cancer therapies, and, because this tumor metastasizes avidly to the lungs from subcutaneous implants, it is a suitable model for both primary and metastatic disease.

The angiostatic activity of several steroids was discovered some years ago; however, the mechanism by which these steroids inhibit vessel growth and/or produce regression of growing vessels is only now being elucidated *(10–13)*. The most effective angiostatic steroid discovered was tetrahydrocortisol (THC), which lacks the 4,5 double bond in its A-ring, and, for this reason, lacks all of the known functions of cortisone.

In 1983, Folkman et al. *(14)* reported that heparin or a heparin fragment, administered in combination with cortisone, inhibited angiogenesis in the chorioallantoic membrane (CAM) assay and inhibited the growth of several solid murine tumors. The same group found that β-cyclodextrin tetradecasulfate, in combination with hydrocortisone, was 100–1000× more effective than heparin in combination with hydrocortisone in inhibiting capillary formation in the CAM assay, and in preventing neovascularization induced by endotoxin in the rabbit cornea *(15)*.

For tumor growth-delay studies, THC and 14-sulfated-β-cyclodextrin 14(SO_4)βCD were prepared in a 14-d osmotic pump, and implanted subcutaneously in the animals on d 4 posttumor cell implantation, by which time neovascularization of the tumors had begun *(16,17)*. Administration of minocycline ip daily was also initiated on d 4 posttumor cell implantation, and continued until d 18. Neither the 14-d continuous infusion of THC–14(SO_4)βCD nor daily im injection of minocycline for 2 wk altered the growth of the LLC (Table 1). The three modulators administered together (THC–14(SO_4)βCD–minocycline) produced a modest tumor growth delay of 1.2 d in the LLC. Single-agent chemotherapy or radiation therapy was administered to the tumor-bearing animals beginning on d 7, when the tumors were about 100 mm^3. Each treatment agent was administered at a standard dosage and schedule.

The combination of the three modulators (THC–14(SO_4)βCD–minocycline) was most effective at increasing the response of the Lewis lung tumor to these cytotoxic therapies (Table 1). The tumor growth delay produced by the antitumor alkylating agents was increased by about 5.8-, 3.9-, 3.8-, and 2.3-fold for CDDP, melphalan, and single and multiple doses of cyclophosphamide, respectively. Five of 12 animals treated with the three modulator combination and multiple doses of cyclophosphamide were long-term survivors. The tumor growth delays produced by single-dose and multiple-dose radiation therapy were increased about 2.2- and 2.8-fold, respectively, in the presence of the three-modulator combination. The increases in tumor growth delay observed with adriamycin and bleomycin, with the addition of the three modulators to the regimen, were about 1.7- and 1.5-fold, compared with the antitumor agents alone.

Untreated control animals bearing the Lewis lung tumors survived 21–25 d posttumor implantation and succumbed to disease metastatic to the lungs. The number and size of lung metastases in untreated and treated animals were scored on d 20 posttumor implant (Table 2). Treatment with THC–14(SO_4)βCD by continuous infusion from d 4 through

Table 1
Growth Delay of the Lewis Lung Tumor Produced by Various Anticancer Treatments Alone or in Combination with β-Cyclodextrin Tetradecasulfate–Tetrahydrocortisol, Minocycline, or the Combination of Modulators

Treatment group	*Dose*[a]	*Tumor growth delay, days*[b]			
		Alone	*+14(SO_4)βCD–THC*	*+ Minocycline*	*+14(SO_4)βCD–THC–Mino*
14(SO_4)βCD–THC	1000 mg/kg/125 mg/kg over 14 d		0.6 ± 0.3		
Minocycline	14 × 5 mg/kg		0.6 ± 0.3		
14(SO_4)βCD–THC–Mino	(As above)	1.2 ± 0.4			
CDDP	1 × 10 mg/kg	4.5 ± 0.3	2.2 ± 0.3	5.0 ± 0.3	26.2 ± 2.5
Melphalan	1 × 10 mg/kg	2.7 ± 0.3	1.1 ± 0.3	4.3 ± 0.3	10.5 ± 0.9
Cyclophosphamide	1 × 150 mg/kg	7.2 ± 0.4	16.2 ± 1.2	24.7 ± 2.7	27.6 ± 2.8
	3 × 150 mg/kg	21.5 ± 1.7	36.8 ± 3.4	45.2 ± 2.9[c]	48.8 ± 3.3[c]
Radiation	1 × 20 gy	6.2 ± 0.5	8.3 ± 0.5	11.9 ± 1.4	13.8 ± 1.3
	5 × 3 gy	4.4 ± 0.3	7.1 ± 0.7	7.8 ± 0.6	12.6 ± 1.2
Adriamycin	5 × 1.75 mg/kg	7.0 ± 0.6	–	9.8 ± 0.8	11.7 ± 1.2
Bleomycin	4 × 10 mg/kg	8.5 ± 0.6	–	12.0 ± 1.2	12.9 ± 1.3

[a]β-Cyclodextrin tetradecasulfate (1000 mg/kg) and tetrahydrocortisol (125 mg/kg) were administered in a 1:1 molar ratio by continuous infusion over 14 d in an Alzet osmotic pump from d 4–18 posttumor implant. Minocycline (5 mg/kg) was administered ip on d 4–18 posttumor implant. CDDP (10 mg/kg), melphalan (10 mg/kg), and cyclophosphamide (150 mg/kg) were administered ip on d 7 posttumor implant. Cyclophosphamide (150 mg/kg), was also administered on d 7, 9, and 11 posttumor implant. Radiation was delivered locally to the tumor-bearing limb as 20 gy on d 7 or 3 gy daily on d 7–11. Adriamycin (1.75 mg/kg) was administered ip daily on d 7–11. Bleomycin (10 mg/kg) was administered ip on d 6, 10, 13, and 16.

[b]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3, compared with untreated controls tumor. Untreated control tumors reach 500 mm^3 in about 14 d. Mean of 15 animals ± SE.

[c]Four of 12 animals were long-term survivors (>120 d).

[d]Five of 12 animals were long-term survivors (>120 d).

Table 2
Numbers of Lung Metastases on d 20 from sc Lewis Lung Tumors After Various Anticancer Treatments or in Combination with β-Cyclodextrin Tetradecasulfate/Tetrahydrocortisol, Minocycline or the Combination of Modulators

		Mean number of lung metastases (number and % of vascularized metastases)[a]			
Treatment group	*Dose*[b]	*Alone*	*+14(SO4)βCD–THC*	*+ Minocycline*	*+14(SO4)βCD–THC/Mino*
Untreated controls	15 (10; 66%)				
14(SO_4)βCD–THC	1000 mg/kg/125 mg/kg over 14 d	14.5 (10; 69%)			
Minocycline	14 × 5 mg/kg	12 (5; 43%)			
14(SO_4)βCD–THC–Mino	(As above)	13 (6; 46%)			
CDDP	1 × 10 mg/kg	12	15 (10: 67%)	10.5 (5; 48%)	6 (2.5; 39%)
Melphalan	1 × 10 mg/kg	8	8 (4; 48%)	6 (3; 50%)	5 (2.5; 47%)
Cyclophosphamide	1 x 150 mg/kg	6.5	6 (2; 32%)	3 (1; 33%)	4 (2; 44%)
	3 × 150 mg/kg	3.5	3 (0.5; 16%)	0.5 (0; 18%)	0.5 (0; 20%)
Radiation	1 × 20 gy	8	7 (3; 40%)	8 (1; 25%)	7 (3; 46%)
	5 × 3 gy	7	7 (2.5; 35%)	6.5 (2; 30%)	7 (3; 47%)
Adriamycin	5 × 1.75 mg/kg	8	–	7.5 (5; 63%)	5 (3; 57%)
Bleomycin	4 × 10 mg/kg	7	–	7 (4.5; 64%)	4 (2; 45%)

[a]The number of external lung metastases on d 20 posttumor implant were counted manually and scored as ≥3 mm^3 in diameter. The data is shown as the means from 6–12 pairs of lungs. Parentheses indicate the number of large (vascularized) metastases and percentage of the total number of metastases that were large.

[b]The schedules of drug administration were as shown in Table 1.

d 18 posttumor implant did not alter the number of lung metastases or the percent of large metastases observed in these animals. Treatment with minocycline or with the three-modulator combination (THC–14(SO_4)βCD–minocycline), over the same time-period, had little effect on total number of metastases; however, only 40–50% of the metastases were large, compared with about 70% in the untreated control animals. The cytotoxic antitumor treatments reduced the number of lung metastases in many cases to about one-half the number observed in the untreated control animals. Treatment with THC–14(SO_4)βCD did not alter the lung metastases produced in animals treated with CDDP, melphalan, cyclophosphamide or radiation therapy. The combination of minocycline with these cytotoxic therapies did not alter the number of lung metastases produced in animals treated with CDDP, melphalan, adriamycin, bleomycin, or radiation therapy; however, in animals treated with cyclophosphamide and minocycline, the number of lung metastases was reduced to 50 and 14%, respectively, of those treated with single-dose or multiple cyclophosphamide alone. The three-modulator combination (THC–14(SO_4)βCD–minocycline), along with the cytotoxic therapies, was more effective against metastatic disease, except when the cytotoxic therapy was radiation therapy to the primary tumor. In most cases, the number of lung metastases was reduced to about 50% of the number observed with the cytotoxic therapy alone, and the number of large metastases was 40–50% of those. The lowest number of large metastases was found in animals treated with cyclophosphamide and minocycline or the three-modulator combinations; in fact, with multiple doses of cyclophosphamide in combination with minocycline, three had no large lung metastases present on d 20.

2.2. TNP-470

TNP-470, a synthetic derivative of fumagillin, an antibiotic which has little antibacterial or antifungal activity, but does have marked amebicidal activity *(18,19)*, is a potent inhibitor of endothelial cell migration *(20)*, endothelial cell proliferation *(21)*, and capillary tube formation *(22)*. TNP-470 also inhibits angiogenesis as demonstrated in chick CAM, the rabbit, and rodent cornea *(22)*. TNP-470 has been shown to inhibit the growth of primary and metastatic murine tumors, as well as human tumor xenografts *(23–31)*. When administered to animals bearing the LLC, subcutaneously on alternate days beginning on d 4, and continuing until d 18, TNP-470 was a moderately effective modulator of the cytotoxic therapies (Table 3). TNP-470 was most effective with melphalan, BCNU, and radiation, increasing the tumor growth delay produced by these treatments 1.8–2.4-fold. TNP-470, along with minocycline, administered intraperitoneally daily on d 4–18, comprised a highly effective antiangiogenic agent combination. The increases in tumor growth delay produced by the modulator combination TNP-470–minocycline, along with the cytotoxic therapies, ranged from two to fourfold. In the treatment group receiving TNP-470–minocycline and cyclophosphamide, approx 40% of the animals were long-term (>120 d) survivors. Each of the cytotoxic therapies (including radiation, which was delivered locally to the tumor-bearing limb) produced a reduction in the number of lung metastases found on d 20 (Table 4). Neither TNP-470, minocycline, or the combination of antiangiogenic agents altered the number of lung metastases or the percentage of large (vascularized) lung metastases on d 20. The modulators did not alter the number of lung metastases from those obtained with the cytotoxic therapies, except in the case of cyclophosphamide, in which many animals treated with the drug and antiangiogenic agent combination had very few metastases on d 20, and most of those were very small.

Table 3
Growth Delay of Lewis Lung Tumor Produced by Various Anticancer Treatments Alone or in Combination with Potential Antiangiogenic Modulators

	Tumor growth delay, d[a]			
Treatment group	*Alone*	*+ Minocycline*[b]	*+ TNP-470*	*+ TNP-470–MINO*
–	–	1.2 ± 0.4	2.1 ± 0.4	1.8 ± 0.4
CDDP (10 mg/kg)	4.5 ± 0.3	5.0 ± 0.3	6.0 ± 0.5	10.9 ± 0.8
Cyclophosphamide (3 × 150 mg/kg)	21.5 ± 1.7	32.4 ± 1.8	25.3 ± 2.2	44.8 ± 2.8[c]
Melphalan (10 mg/kg)	2.7 ± 0.3	4.3 ± 0.3	6.0 ± 0.5	8.5 ± 0.6
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	5.2 ± 0.4	6.3 ± 0.5	14.6 ± 1.0
X-rays (5 × 3 gy)	4.4 ± 0.3	7.8 ± 0.6	10.6 ± 1.1	15.3 ± 1.2

[a]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3, compared with untreated controls tumor. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 15 animals.

[b]Minocycline (10 mg/kg) was administered ip daily on d 4–18. TNP-470 (30 mg/kg) was administered sc on alternate days for 8 injections, beginning on d 4. CDDP and melphalan were administered ip on d 7. Cyclophosphamide and BCNU were administered ip on d 7, 9, and 11. X-rays were delivered daily on d 7–11 locally to the tumor-bearing limb.

[c]5 of 12 long-term survivors (>180 d).

Table 4
Number of Lung Metastases on d 20 from sc Lewis Lung Tumors, After Various Anticancer Therapies Alone or in Combination with Potential Antiangiogenic Modulators

	Mean number of lung metastases (% large)			
Treatment group	*Alone*	*+ Minocycline*	*+ TNP-470*	*+ TNP-470–MINO*
–	20 (62)	20 (50)	21 (51)	18 (54)
CDDP (10 mg/kg)	13 (58)	11 (48)	14.5 (34)	14 (50)
Cyclophosphamide (3 × 150 mg/kg)	12 (40)	6 (33)	6 (30)	2 (25)
Melphalan (10 mg/kg)	13 (48)	11 (50)	15 (47)	15 (45)
BCNU (3 × 15 mg/kg)	16 (53)	15 (38)	15.5 (45)	13 (38)
X-rays (5 × 3 gy)	15 (40)	13 (30)	10 (40)	12 (42)

The efficacy of the modulator combination of TNP-470–minocycline against the primary Lewis lung tumors is compared with that of other potential antiangiogenic modulator combinations in Table 5. The most effective combination with cisplatin (CDDP) was 14(SO_4)βCD–THC–minocycline; with the other cytotoxic therapies, each of the three antiangiogenic agent combinations were approximately equally effective. Each of the three antiangiogenic agent combinations, along with cyclophosphamide, were highly effective therapies, resulting in 40–50% long-term survivors. None of the antiangiogenic agent combinations alone was effective against metastatic disease, although in each case the percent of large metastases on d 20 was reduced (Table 6). There was a trend toward the combination of 14(SO_4)βCD–THC–minocycline being the most effective antiangiogenic agents of cytotoxic therapies against metastatic disease.

In the data presented in Tables 3–6, antiangiogenic agent treatment was begun on d 4, when the primary tumor was palpable, and continued through d 18, when the tumor had

Table 5
Growth Delay of Lewis Lung Tumor Produced by Various Anticancer Treatments Alone or in Combination with Potential Antiangiogenic Modulators

	Tumor growth delay, d			
Treatment group	*Alone*	*+ 14(SO4)βCD–THC–mino*	*+ 14(SO4)βCD–THC–TNP-470*	*+ MINO/TNP-470*
–	–	1.2 ± 0.4	1.5 ± 0.3	1.8 ± 0.4
CDDP (10 mg/kg)	4.5 ± 0.3	26.2 ± 2.5	10.6 ± 0.7	10.9 ± 0.8
Cyclophosphamide (3 × 150 mg/kg)	21.5 ± 1.7	48.8 ± 3.3 (5/12)[a]	49.2 ± 3.4 (6/12)[a]	44.8 ± 2.8 (5/12)[a]
Melphalan (10 mg/kg)	2.7 ± 0.3	10.5 ± 0.9	12.2 ± 1.4	8.5 ± 0.6
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	9.8 ± 0.8	10.6 ± 1.1	14.6 ± 1.0
X-rays (5 × 3 gy)	4.4 ± 0.3	12.6 ± 1.2	10.3 ± 0.9	15.3 ± 1.2

[a]Lived a normal lifespan (~2 yr).

Table 6
Number of Lung Metastases on d 20 from sc Lewis Lung Tumors After Various Anticancer Therapies Alone or in Combination with Potential Antiangiogenic Modulators

	Mean number of lung metastases (% large)			
Treatment group	*Alone*	*+ 14(SO4)βCD–THC–mino*	*+ 14(SO4)βCD–THC–TNP-470*	*+ MINO–TNP-470*
–	20 (62)	17 (46)	18 (50)	18 (54)
CDDP (10 mg/kg)	13 (58)	8 (42)	15 (40)	14 (50)
Cyclophosphamide (3 × 150 mg/kg)	12 (40)	1 (0)	2 (50)	2 (25)
Melphalan (10 mg/kg)	13 (48)	7 (50)	15 (40)	15 (45)
BCNU (3 × 15 mg/kg)	16 (53)	14 (45)	14 (43)	13 (58)
X-rays (5 × 3 gy)	15 (40)	9 (43)	11 (36)	12 (42)

Table 7
Growth Delay of Lewis Lung Tumor Produced by Various Anticancer Treatments Alone or in Combination With TNP-470 and Minocycline Administered on Various Schedules

	Tumor growth delay, d				
		TNP-1470 + minocycline			
Treatment group	*Alone*	*d 4–11*	*d 7–11*	*d 7–18*	*d 4–18*
–	–	1.4 ± 0.3	0.6 ± 0.3	0.9 ± 0.3	1.8 ± 0.4
Cyclophosphamide (3 × 150 mg/kg)	21.5 ± 1.7	37.1 ± 2.7	28.8 ± 2.4	32.1 ± 2.9	44.8 ± 2.8
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	11.8 ± 1.4	10.4 ± 1.7	10.6 ± 1.5	14.6 ± 1.6
X-rays (5 × 3 gy)	4.4 ± 0.3	10.4 ± 1.6	6.6 ± 1.2	6.7 ± 1.0	15.3 ± 1.7

fully matured *(16,17)*. To determine the efficacy of the modulator combination against established disease, TNP-470 and minocycline were administered on different schedules; the cytotoxic treatments remained as previously described (Table 7). The antiangio-

Table 8
Number of Lung Metastases on d 20 from sc Lewis Lung Tumors After Various Anticancer Therapies Alone or in Combination with TNP-470 and Minocycline Administered on Various Schedules

	Mean number of lung metastases (% large)				
		TNP-470 + minocycline			
Treatment group	*Alone*	*d 4–11*	*d 7–11*	*d 7–18*	*d 4–18*
–	20 (62)	17 (57)	20 (52)	20 (48)	18 (54)
Cyclophosphamide (3 × 150 mg/kg)	12 (40)	2 (25)	4 (18)	4 (28)	2 (25)
BCNU (3 × 15 mg/kg)	16 (53)	17 (41)	12 (42)	14 (25)	15 (45)
X-rays (5 × 3 gy)	15 (40)	15 (38)	17 (34)	15 (38)	12 (42)

genic agent administration schedule (d 4–11) began when the tumors were palpable, and extended through the cytotoxic treatments, the second antiangiogenic agent schedule (d 7–11) allowed modulator administration during the same period as the cytotoxic therapies; and the third antiangiogenic agent schedule (d 7–11) allowed initiation of modulator administration at the same time as initiation of the cytotoxic therapy, and extended the modulators for 1 wk after completion of the cytotoxic therapies. As would be expected, the most effective therapies were those begun on d 4, when the tumor burden was smallest. However, both the d 7–11 and d 7–18 modulator treatment schedule resulted in enhanced tumor growth delays, compared with the cytotoxic therapies alone. In combination with cyclophosphamide, the antiangiogenic agent schedules beginning on d 4 resulted in 1.7- and 2.1-fold increased tumor growth delay, and the antiangiogenic agent schedules beginning on d 7 resulted in 1.3- and 1.5-fold increased tumor growth delay, compared with cyclophosphamide alone. BCNU on d 7, 9, and 11, along with the antiangiogenic agent schedules beginning on d 4, produced 3.3- and 4-fold increases in tumor growth delay; the antiangiogenic agent schedules beginning on d 7 produced a 2.9-fold increase in tumor growth delay. Finally, the antiangiogenic agent schedules beginning on d 4 resulted in 2.4- and 3.5-fold increases in tumor growth delay; the antiangiogenic agent schedules beginning on d 7 resulted in a 1.5-fold increase in tumor growth delay. Varying the modulator administration schedules did not appear to affect response of the metastatic disease to the therapies (Table 8). Only in the case of cyclophosphamide was it evident that beginning the antiangiogenic agent administration on d 7 led to decreased efficacy of the therapy against metastatic disease.

Given the relatively modest impact of established combination chemotherapy regimens on survival in advanced nonsmall-cell lung cancer, the development of new treatments for this very common malignancy is imperative. Among the newer chemotherapeutic agents, the taxane paclitaxel has demonstrated significant activity against metastatic nonsmall-cell lung cancer as a single agent, with much improved median survival *(32,33)*. Since platinum-based therapeutic combinations have been historically important in the treatment of nonsmall-cell lung cancer, several phase II studies were conducted combining administration of paclitaxel and carboplatin *(34–38)*. These phase II studies produced promising results, showing that the combination of paclitaxel and carboplatin is an active and generally well-tolerated regimen for nonsmall-cell lung

Table 9
Growth Delay of LLC, and Number of Lung Metastases on d 20 After Treatment of the Animals with Paclitaxel and/or Carboplatin with or Without Antiangiogenic Agents

Treatment group	*Tumor growth delay,[a] (d)*	*Number of lung metastases*
Controls	–	40 ± 7
TNP-470 (30 mg/kg) sc, alt. d 4–18 + minocycline (10 mg/kg) ip, d 4–18	1.0 ± 0.3	27 ± 5
Paclitaxel (36 mg/kg) iv d 7–11	4.6 ± 0.3	22 ± 4
TNP–mino–paclitaxel	6.4 ± 0.4[b]	20 ± 4
Carboplatin (50 mg/kg) ip, d 7	4.2 ± 0.3	25 ± 4
TNP–mino–carboplatin	7.8 ± 0.5[c]	21 ± 3
Paclitaxel/Carboplatin	6.6 ± 0.4	13 ± 2
TNP/MINO/Paclitaxel/Carboplatin	10.5 ± 0.6[b]	8 ± 1

[a]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 12.4 ± 0.3 d. Mean ± SE of 15 animals.

[b]Significantly increased tumor growth delay, compared with the cytotoxic therapy alone, $p < 0.01$.

[c]$p < 0.005$.

cancer. This two-drug regimen produced response rates between 30 and 50%, and prolonged median survival >1 yr. Paclitaxel–carboplatin is not curative in advanced nonsmall-cell lung cancer, and complete responses are rare. Paclitaxel administered by intravenous injection on d 7 through 11 after tumor cell implantation produced 4.6 d of tumor growth delay which was increased 1.4-fold to 6.4 d of tumor growth delay when administered along with TNP-470 and minocycline *(39)*. A single intraperitoneal injection of carboplatin on d 7 after tumor cell implantation produced a tumor growth delay of 4.2 d. When carboplatin was administered along with TNP-470 and minocycline a tumor growth delay of 7.8 d resulted, a 1.9-fold increase, compared with carboplatin alone. The combination of the cytotoxic anticancer drugs, paclitaxel and carboplatin, was well tolerated by the animals and produced a tumor growth delay of 6.6 d. The complete regimen including TNP-470 and minocycline along with paclitaxel and carboplatin produced a tumor growth delay of 10.5 d, a 1.6-fold increase compared with the cytotoxic drug combination alone.

Treatment with the antiangiogenic agent combination decreased the number of lung metastases on d 20 to 68% of the number found in untreated control animals (Table 9; *39*). Both of the cytotoxic chemotherapeutic agents also decreased the number of lung metastases on d 20. Paclitaxel administration decreased the number of lung metastases to 55% of controls, which was not significantly altered by the addition of co-administration of TNP-470–minocycline. Treatment with carboplatin decreased the number of lung metastases to 63% of the number in the untreated control animals. Addition of TNP-470–minocycline administration to treatment with carboplatin did not significantly alter the number of lung metastases, compared with carboplatin alone. The combination of the cytotoxic drugs reduced the number of lung metastases to 33% of the number in the control animals. With the addition of treatment with TNP-470–minocycline to the combination of cytotoxic anticancer drugs, the number of lung metastases was reduced to 20% of the number in the untreated control animals.

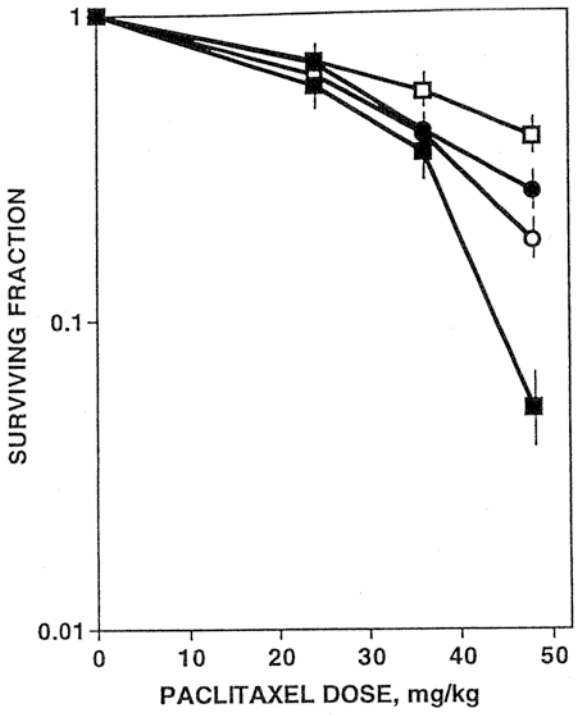

Fig. 1. Survival of EMT-6 tumor cells (●, ■) and bone marrow CFU-GM (❍,❑) from animals treated in vivo with single intravenous doses of paclitaxel alone on d 8 (●,❍), or after pretreatment with TNP-470 (30 mg/kg) and minocycline (10 mg/kg) on d 4–8 (■,❑). Points are the means of three independent determinations; bars are the SEM.

In animals bearing the EMT-6 mammary carcinoma, treatment with TNP-470–minocycline for 4 d prior to, and at the time of, a single dose of paclitaxel increased the tumor cell killing by the drug only at the highest dose of paclitaxel tested, at which there was a fivefold increase in tumor cell killing in animals receiving TNP-470–minocycline (Fig. 1). The survival of bone marrow CFU-GM was used as a representative sensitive normal tissue. At the highest dose of paclitaxel tested, there was a twofold diminution in the killing of bone marrow CFU-GM when the animals received TNP-470–minocycline. Therefore, the therapeutic index, as determined by the ratio of tumor cell killing to bone marrow CFU-GM killing of paclitaxel, was improved by the administration of TNP-470–minocycline.

Administration of TNP-470–minocycline on d 4–8, along with single doses of carboplatin on d 8 resulted in increased EMT-6 tumor cell killing in animals receiving the lower two doses of carboplatin, but not the highest dose of the drug (Fig. 2). There was 5.5- and 3.8-fold greater killing of EMT-6 tumor cells in animals pretreated with TNP-470–minocycline along with carboplatin at doses of 100 and 300 mg/kg, compared with animals receiving carboplatin alone, respectively. Pretreatment with TNP-470–minocycline had no effect on the toxicity of carboplatin to the bone marrow CFU-GM.

2.3. Genistein, Suramin, and Others

A high soy food consumption has been hypothesized to have a factor in the lower incidence of certain human cancers in Asians than in others *(40–42)*. Genistein is a

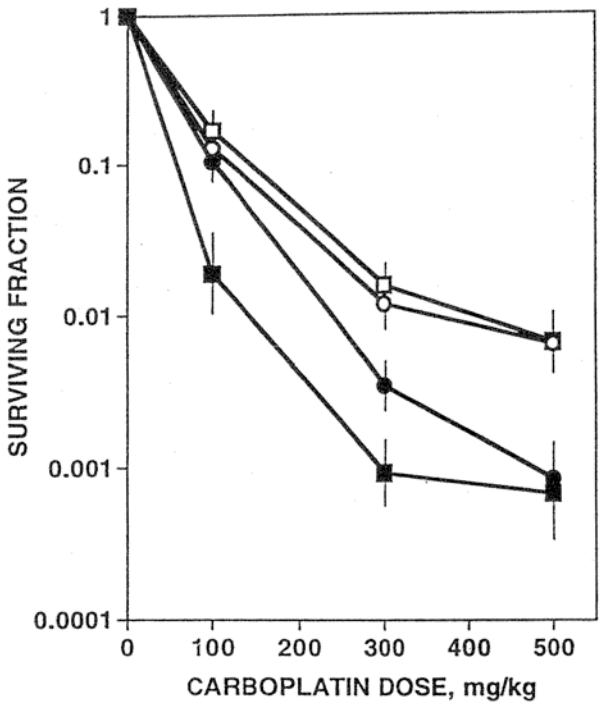

Fig. 2. Survival of EMT-6 tumor cells (●, ■) and bone marrow CFU-GM (❍,❑) from animals treated in vivo with single intraperitoneal doses of carboplatin alone on d 8 (●,❍), or after pretreatment with TNP-470 (30 mg/kg) and minocycline (10 mg/kg) on d 4–8 (■,❑). Points are the means of three independent determinations; bars are the SEM.

principal isoflavone in soybeans, and is a potent inhibitor of the activity of tyrosine protein kinases, such as epidermal growth factor receptors *(43)*. Tyrosine phosphorylation is important in cell proliferation and transformation *(44)*. Genistein specifically inhibits growth of *ras* oncogene-transfected NIH 3T3 cells *(45)*, and diminishes the platelet-derived growth factor-induced c-*fos* and c-*jun* expression in CH310T1/2 fibroblasts. Genistein inhibited endothelial cell proliferation and in vitro angiogenesis at concentrations giving half-maximal inhibition at 5 and 150 μ*M*, respectively *(46)*. Suramin, which, like heparin, is a polysulfonated molecule, interferes with binding of many growth factors (bFGF), including basic fibroblast growth factor, to their receptors. Suramin is an inhibitor of angiogenesis, a suppressor of endothelial cell growth and migration *(47–50)*. Elegant studies by Takano et al. *(51)* showed that suramin inhibited multiple control points of angiogenesis, including those stimulated by bFGF.

In tumor growth delay studies, genistein had some activity as a single agent, but suramin did not have a significant antitumor effect (Table 10). Two cytotoxic anticancer drugs, cyclophosphamide and adriamycin, were selected for the initial comparison of the potential of the various antiangiogenic treatments within a therapeutic regimen. Suramin and TNP-470–genistein were effective in increasing the tumor growth delay produced by cyclophosphamide. TNP-470–genistein and minocycline–genistein were effective in increasing the tumor growth delay produced by adriamycin.

Table 10
Growth Delay of Lewis Lung Tumor Produced by Potential Antiangiogenic Agents Alone or Along with Standard Regimen of Cyclophosphamide or Adriamycin

	Tumor growth delay, d[a]		
Treatment group	*Alone*	*+ Cyclophosphamide*[b]	*+ Adriamycin*
–	–	19.3 ± 1.7	5.3 ± 0.4
Suramin (20 mg/kg × 14)[c]	1.4 ± 0.3	31.5 ± 2.7[e]	6.6 ± 0.7
Genistein (100 mg/kg × 14)	2.4 ± 0.4[d]	23.5 ± 1.8	6.0 ± 0.6
TNP-470–suramin	0.4 ± 0.3	25.7 ± 2.5[d]	6.6 ± 0.7
TNP-470–genistein	1.9 ± 0.3	36.1 ± 2.8[c]	9.8 ± 1.1[e]
Mino–suramin	0.5 ± 0.3	21.2 ± 1.8	6.6 ± 0.5
Mino–genistein	2.4 ± 0.4[d]	21.3 ± 1.9	9.1 ± 0.9[d]
Suramin–genistein	1.2 ± 0.3	19.6 ± 1.8	7.4 ± 0.5

[a]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 15 animals.

[b]Cyclophosphamide (150 mg/kg) was administered by ip injection on d 7, 9, and 11 posttumor cell implantation. Adriamycin (1.75 mg/kg) was administered by intraperitoneal injection daily on d 7–11.

[c]Minocycline (10 mg/kg), suramin (20 mg/kg), and genistein (100 mg/kg) were administered intraperitoneally daily on d 4–18. TNP-470 (30 mg/kg) was administered sc on alternate days for 8 injections, beginning on d 4.

[d]Significant tumor growth delay compared with untreated control, or significantly increased tumor growth delay, compared to cyclophosphamide or adriamycin, $p < 0.01$.

[e]$p < 0.005$.

Table 11
Number of Lung Metastases on d 20 from Subcutaneous Lewis Lung Tumors Produced by Potential Antiangiogenic Agents Alone or Along with Standard Regimen of Cyclophosphamide or Adriamycin

	Mean number of lung metastases (% large)		
Treatment group	*Alone*	*+ Cyclophosphamide*	*+ Adriamycin*
–	21 (63)	12 (40)	18 (39)
Suramin (20 mg/kg × 14)	19 (47)	5 (33)	15 (38)
Genistein (100 mg/kg × 14)	22 (41)	10 (29)	15 (40)
TNP-470–suramin	23 (43)	7 (27)	14 (45)
TNP-470–genistein	21 (60)	11.5 (33)	22 (49)
Mino–suramin	21 (47)	9 (23)	16 (52)
Mino–genistein	19 (53)	12 (35)	22 (32)
Suramin–genistein	21 (54)	12 (40)	15 (40)

None of the potential antiangiogenic agents, single or in two-agent combinations, reduced the number of lung metastases on d 20 in animals implanted subcutaneously with the Lewis lung tumor (Table 11). However, genistein significantly decreased the percent of large lung metastases (>3 mm in diameter) potentially vascularized on d 20. Cyclophosphamide treatment decreased the number and percent of large lung metastases on d 20, from that in untreated controls. Suramin,

Table 12
Number of Intratumoral Blood Vessels as Determined by Immunohistochemistry in LLC Tumors After Treatment of Animals with Various Potential Antiangiogenic Agents[a]

	1st Exp.	*2nd Exp.*	*3rd Exp.*	*Means ± SD*
CD31				
Control	31.4	59.3	97.0	62.6 ± 39.6
TNP-470[b]	9.2	21.9	21.9	17.7 ± 14.6
TNP–mino	7.6	39.7	33.7	27.0 ± 25.3
Suramin	11.0	23.2	41.2	25.1 ± 17.9
TNP–suram	6.0	8.3	52.2	22.2 ± 28.6
Genistein	20.4	18.4	53.8	30.9 ± 21.8
TNP–genist.	16.6	13.5	33.4	21.2 ± 15.5
Factor VIII				
Control	13.4	11.6	19.2	14.7 ± 6.9
TNP-470	3.5	7.1	7.1	5.9 ± 6.0
TNP–mino	8.2	7.1	3.8	6.4 ± 4.9
Suramin	3.6	7.1	6.6	5.8 ± 4.0
TNP–suram	6.2	5.3	7.9	6.5 ± 4.9
Genistein	12.9	9.5	6.1	9.5 ± 6.3
TNP–genist.	6.9	4.0	9.9	6.9 ± 6.0

[a]Tumor tissue sections from paraffin-embedded blocks were deparaffinized and stained with monoclonal mouse anti-human endothelial cell antibody (CD31, DAKO, Carpinteria, CA) or monoclonal mouse antihuman von Willebrand factor (Cell Biology Boehringer Mannheim, Indianapolis, IN) using avidin-biotin complex (ABC) method (DAKO LSAB Kit, DAKO). For the counting of blood vessels, the most vascular area of the tumor was located at low magnification, and vessels were counted on 10 × 200 fields. Data are the means of 10 high power fields at ×200.

[b]TNP-470 (30 mg/kg) was administered sc on alternate days, d 4–10. Minocycline (10 mg/kg), suramin (20 mg/kg), and genistein (100 mg/kg) were administered ip daily on d 4–10.

administered along with cyclophosphamide further decreased the number and percent of large lung metastases from treatment with cyclophosphamide. TNP-470–suramin produced significant decreases in the number of lung metastases when administered along with cyclophosphamide, compared with cyclophosphamide alone. Treatment of the Lewis lung tumor-bearing animals with adriamycin as a single agent did not decrease the number of lung metastases on d 20, but did decrease the percent of lung metastases large enough to be vascularized on d 20, compared with untreated control animals (Table 11).

The number of intratumoral vessels in the Lewis lung carcinoma was determined by immunohistochemical staining with anti-CD31 or anti-factor VIII, after treatment of the tumor-bearing animals with various potential antiangiogenic agents on d 4–10 after tumor cell implantation (Table 12). Each of the potential antiangiogenic therapies decreased the number of stainable intratumoral vessels to one-half to one-third of the number in the untreated control tumors. This effect was evident with both of the immunohistochemical stains. These data indicate that there are fewer stainable endothelial cells in the tumors of the animals treated with the potential antiangiogenic therapies, but do not address the issue of tumor blood flow or permeability.

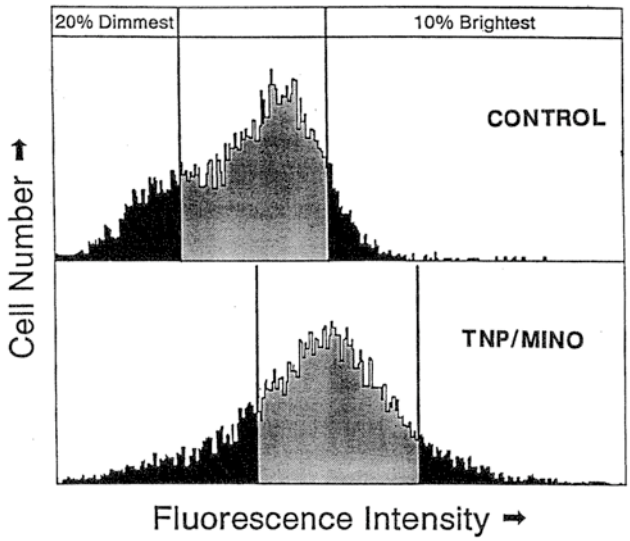

Fig. 3. Fluorescence distribution in FSaIIC tumor cells after iv injection of tumor-bearing animals with Hoechst 33342 (2 mg/kg). The data shown are for an untreated control tumor and a tumor treated with TNP-470 (3 × 30 mg/kg, sc) and minocycline (5 × 10 mg/kg, ip).

3. MECHANISM(S) OF INTERACTION: TNP-470/MINOCYCLINE

The antiangiogenic combination of TNP-470 and minocycline administered for 2 wk did not alter the growth of the LLC, the EMT-6 mammary carcinoma, or the 9L gliosarcoma or the FSaII fibrosarcoma *(3,4,6,8,52–54)*. However, when TNP-470 and minocycline were added to treatment with cytotoxic anticancer therapies, tumor response was markedly increased. When C3H mice bearing the FSaIIC fibrosarcoma were treated with TNP-470–minocycline for 5 d prior to iv injection of the fluorescent dye Hoechst 33342, there was a shift toward greater brightness of the entire tumor cell population, so that the 10% brightest and the 20% dimmest cell subpopulations were composed of cells containing much more dye than the same subpopulations in the control tumor (Fig. 3; *3*). The TNP-470–minocycline-treated tumors were more easily penetrated by the lipophilic dye *(3)*. This was the first indication that TNP-470 and minocycline treatment might allow greater distribution of small molecules into tumors. To determine if the TNP-470–minocycline affected cyclophosphamide tissue distribution, animals were injected ip with [^{14}C]-cyclophosphamide on d 8, then killed 6 h later, and tissue levels of ^{14}C were determined (Fig. 4). There was an increased level of ^{14}C in all of the tissues from TNP-470–minocycline-treated animals, except blood, compared with [^{14}C]-cyclophosphamide-only treated animals. The largest increases were 2.6-fold in the tumor, 2.3-fold in the kidney, 3.2-fold in the heart, 5.6-fold in the gut, and 7.9-fold in skeletal muscle *(4)*.

In a similar study, Lewis lung tumor-bearing mice pretreated with TNP-470–minocycline, or untreated, were injected intraperitoneally with a single dose of cisplatin on d 8, then killed 6 hr later, and tissue levels of Pt were determined (Fig. 5). There were increased levels of Pt in all of the tissues taken from animals treated with TNP-470–minocycline (except blood), compared with animals that were not pretreated. The largest increases were 5.2-fold in the tumor, 3.8-fold in the gut, 3.0-fold in the skin, and 2.5-fold in the skeletal muscle *(4)*.

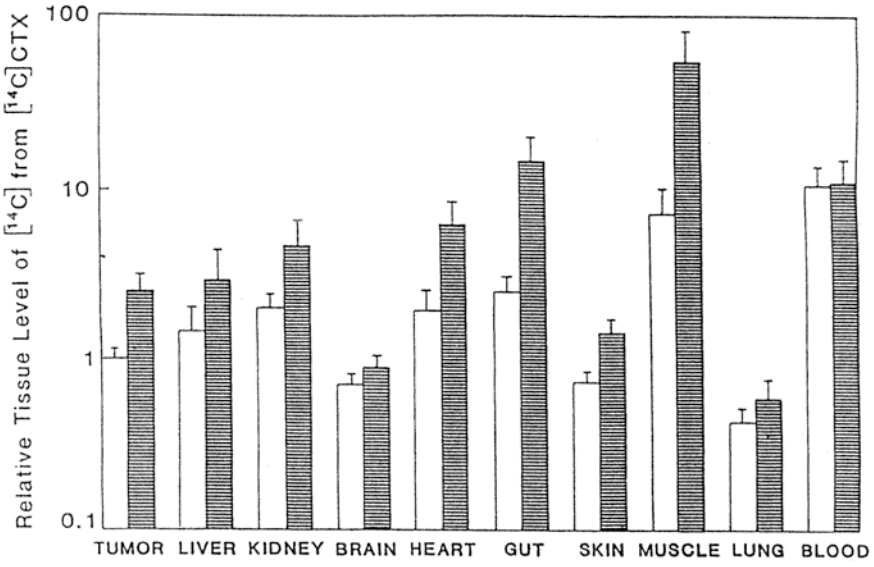

Fig. 4. Relative tissue levels of ^{14}C from [^{14}C]-CTX in C57BL mice bearing Lewis lung tumors sc in the hindleg 6 h after ip injection of 300 mg/kg of the drug alone on d 8 (open ❑) or after administration of the drug to animals treated with TNP-470 (30 mg/kg, sc) d 4, 6, 8, and minocycline (10 mg/kg, ip) daily d 4–8 after tumor cell implantation (■). Data are expressed relative to ^{14}C levels in the tumor in animals treated with the drug alone set equal to 1.0/g tissue.

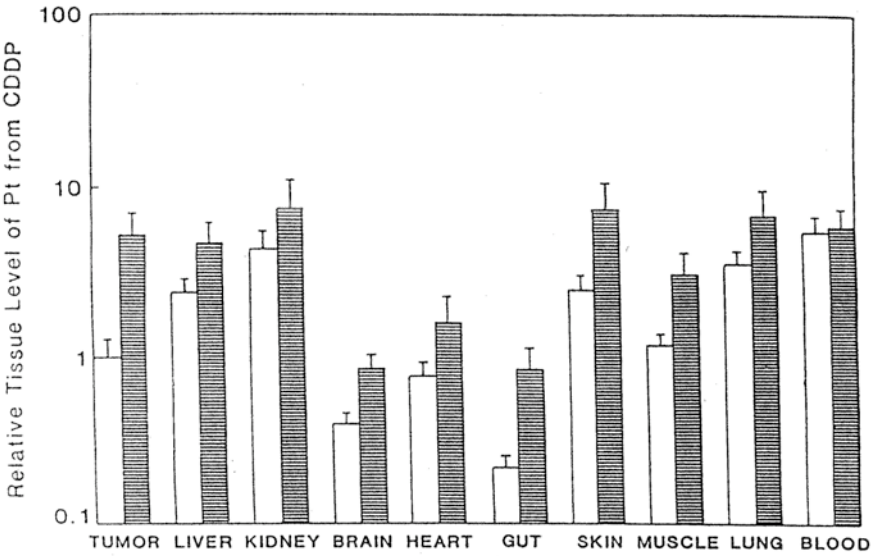

Fig. 5. Relative tissue levels of Pt from CDDP in C57BL mice bearing Lewis lung tumors sc in the hindleg 6 h after ip injection of 20 mg/kg of the drug alone on d 8 (open ❑) or after administration of TNP-470 (30 mg/kg, sc) d 4, 6, 8, and minocycline (10 mg/kg, ip) daily d 4–8 after tumor cell implantation (■). Data are expressed relative to Pt levels in the tumor in animals treated with the drug alone set equal to 1.0/g tissue.

Table 13
DNA Crosslinking Factors
from Lewis Lung Tumors by Alkaline Elution[a]

Treatment group	*DNA crosslinking factor[b]*
TNP-470–minocycline	1.2
Cyclophosphamide	
150 mg/kg	3.9
300 mg/kg	4.7
500 mg/kg	5.6
TNP-470–minocycline–cyclophosphamide (300 mg/kg)	6.2
CDDP	
10 mg/kg	1.7
20 mg/kg	2.0
30 mg/kg	2.8
TNP-470–minocycline–CDDP (20 mg/kg)	8.9

[a]For DNA alkaline elution studies, LLC-bearing animals were treated with TNP-470 (30 mg/kg) sc on d 4, 6, and 8, with minocycline (10 mg/kg) ip daily on d 4–8; and/or with cyclophosphamide (150, 300, or 500 mg/kg) ip or CDDP (10, 20, or 30 mg/kg) ip on d 8 after tumor cell implantation. [^{14}C]-thymidine was administered ip on d 7 and 8. The animals were sacrificed on d 9.

[b]A DNA crosslinking factor of 1.0 indicates no crosslinks.

Both cyclophosphamide and cisplatin are cytotoxic through formation of crosslinks in cellular DNA. DNA alkaline elution from tumors treated in vivo showed that there was increasing DNA crosslinking with increasing dose of cyclophosphamide (Table 13). Treatment with cyclophosphamide alone resulted in a crosslinking factor of 4.7; treatment with the same dose of cyclophosphamide in animals pretreated with TNP-470–minocycline resulted in a crosslinking factor of 6.2, which extrapolates to an equivalency of about 650 mg/kg of cyclophosphamide. Increased DNA crosslinking also was detected with an increasing dose of cisplatin. Treatment with cisplatin alone resulted in a crosslinking factor of 2.0; treatment with the same dose of cisplatin in animals pretreated with TNP-470–minocycline resulted in a crosslinking factor of 8.9, which extrapolates to an equivalency of about 85 mg/kg of cisplatin *(4)*.

[^{14}C]Paclitaxel was administered to LLC-bearing animals pretreated with TNP-470–minocycline, or not pretreated, on d 8 after tumor implantation, and tissues were collected over a 24-h time-course (Fig. 6; *39*). At early time-points (1 and 15 min) after iv administration of the [^{14}C]paclitaxel, there was a fivefold higher concentration of the drug in the tumors of animals that had been pretreated with TNP-470–minocycline. At the intermediate time-points, the [^{14}C]paclitaxel levels were similar in both the pretreated animals and those that had not been treated with TNP-470–minocycline; however, by 24 h, there was a twofold greater concentration of [^{14}C]paclitaxel in the tumors of the animals pretreated with TNP-470–minocycline, compared with those that had not received the antiangiogenic therapy. The pattern of [^{14}C]paclitaxel distribution into the other tissues was similar, with greater peak levels of [^{14}C]paclitaxel in the tissues of animals pretreated with TNP-470–minocycline. In the liver, however, there was a prolonged increased level of

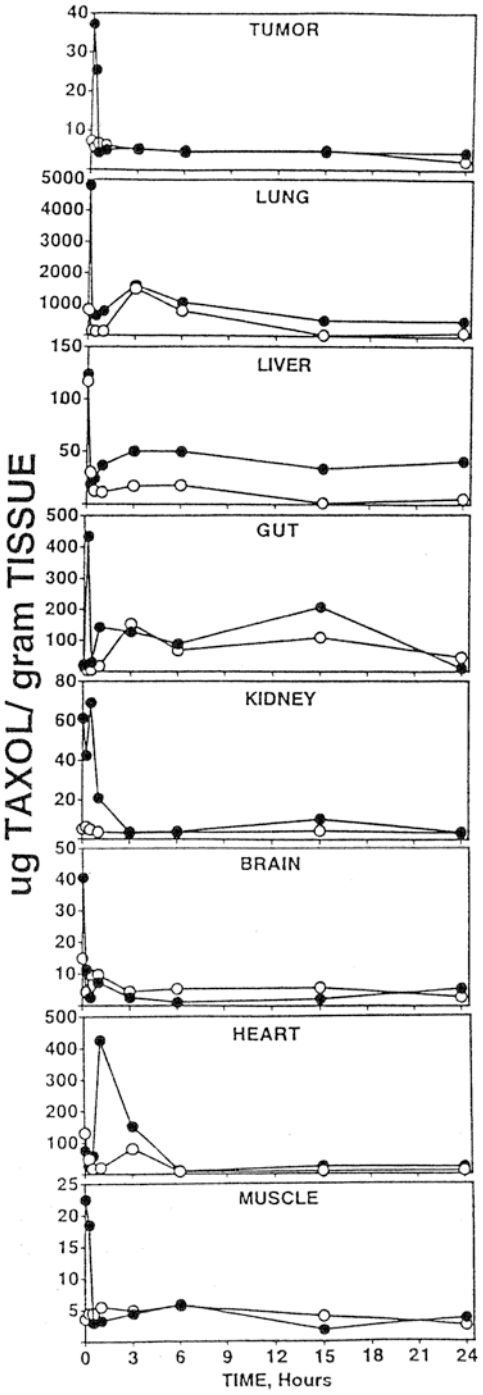

Fig. 6. Tissue levels of ^{14}C from [^{14}C]paclitaxel in C57BL mice bearing Lewis lung tumors sc in the hindleg over a time-course after iv injection of 36 mg/kg of the drug alone on d 8 (❍), or after administration of the drug to animals treated with TNP-470 (30 mg/kg, sc) d 4, 6, 8, and minocycline (10 mg/kg, ip) daily d 4–8 after tumor cell implantation (●).

[^{14}C]paclitaxel of the pretreated animals. By far, the highest levels of [^{14}C]paclitaxel were found in the lungs of the animals in which the peak level in the pretreated animals reached 4800 μg/g tissue. Other tissues with relatively high paclitaxel concentrations were gut and heart.

Concentrations of platinum from carboplatin were two- and threefold higher in the tumors of animals pretreated with TNP-470–minocycline at 15 and 30 min after drug administration than in the tumors of animals that had not received the antiangiogenic therapy, respectively (Fig. 7). Between 6 and 24 h after carboplatin administration, platinum levels in the tumors of pretreated animals remained about twofold greater than in animals that did not receive the antiangiogenic therapy. Overall the tissues of the animals pretreated with TNP-470–minocycline had higher platinum levels with the greatest differentials being in kidney, brain, muscle, and liver. The highest platinum levels overall were in kidney, gut, liver, and brain.

To determine whether pretreatment with TNP-470–minocycline might also alter the tissue distribution of large molecules into tumors and tissues, [^{14}C]albumin was administered to TNP-470–minocycline-pretreated and nonpretreated animals (Fig. 8). There was a two to threefold higher concentration of [^{14}C]albumin in the tumor over the first hour after protein injection, and a concentration differential, with higher concentrations in the tumors of the pretreated animals, persisted over the 24 h examined. A similar pattern pertained for the other tissues, with TNP-470–minocycline pretreated animals having higher tissue concentrations of [^{14}C]albumin than the tissues of nonpretreated animals. The highest peak levels of [^{14}C]albumin were in liver and lung.

The LLC growing subcutaneously in the hindleg of male C57BL mice is very hypoxic, having 92% of the pO_2 measurements ≤5 mmHg, as determined with a polarographic oxygen electrode *(52)*. Administration of a perflubron emulsion, along with carbogen breathing, increased the tumor oxygen level, so that 82% of the pO_2 readings were ≤5 mmHg. Treating tumor-bearing animals with TNP-470–minocycline daily, beginning on d 4 after tumor cell implantation, resulted in decreased hypoxia in the tumors on d 9, when pO_2 measurements were made. The percent of pO_2 readings ≤5 mmHg in the tumors of the TNP-470–minocycline-treated animals was 75%, which, upon administration of the perflubron emulsion, along with carbogen breathing, was reduced to 45%. Therapeutically, daily fractionated radiation (2, 3, or 4 gy × 5) was used as an oxygen-dependent cytotoxic modality. The radiation response of the tumors in TNP-470–minocycline-treated animals was greater than that in the untreated tumors (Fig. 9). The addition of carbogen breathing for 1 h prior to, and during, radiation delivery further increased the radiation response, so that overall there was a 2.2-fold increase in the tumor growth delay produced by the fractionated radiation in the animals treated with TNP-470–minocycline, compared with untreated animals. Administration of the perflubron emulsion, along with carbogen breathing prior to, and during, radiation delivery, resulted in a 3.4-fold increase in tumor growth delay by the fractionated radiation regimens in the TNP-470–minocycline-treated animals, compared with the tumor growth delay obtained with radiation alone. There was a linear relationship between decrease in the percent of pO_2 readings ≤5 mmHg and tumor growth delay at each radiation dose, indicating that the diminution in tumor hypoxia produced by these treatments may be directly responsible for the increase in the effectiveness of the radiation therapy (Fig. 10).

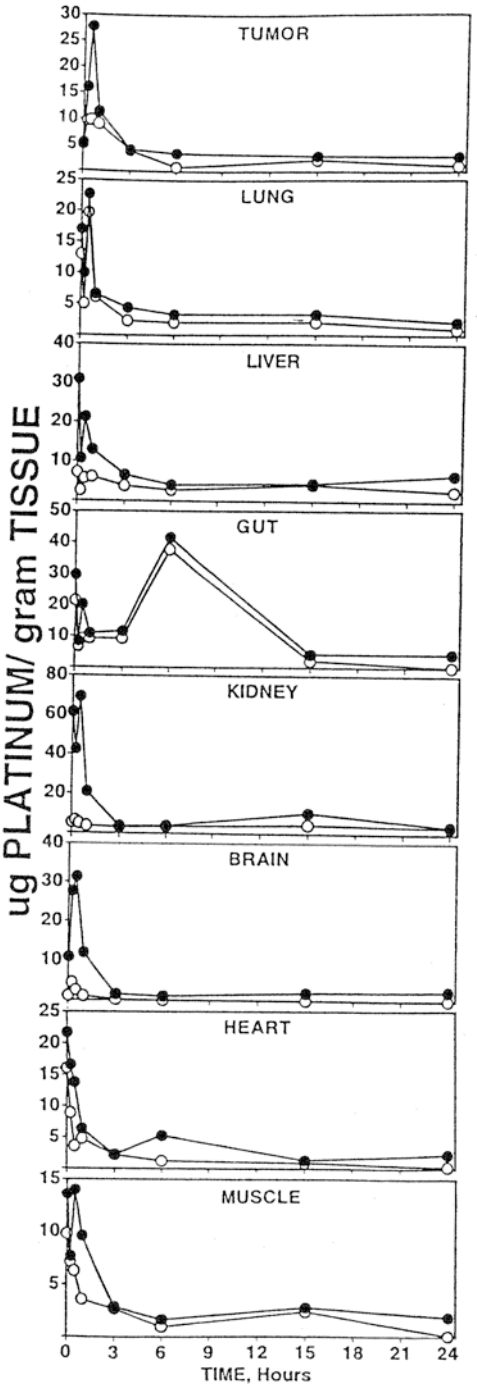

Fig. 7. Tissue levels of platinum (Pt) from carboplatin in C57BL mice bearing Lewis lung tumors sc in the hindleg over a time-course after iv injection of 300 mg/kg of the drug alone on d 8 (❍), or after administration of TNP-470 (30 mg/kg, sc) d 4, 6, 8, and minocycline (10 mg/kg, ip) daily d 4–8 after tumor cell implantation (●).

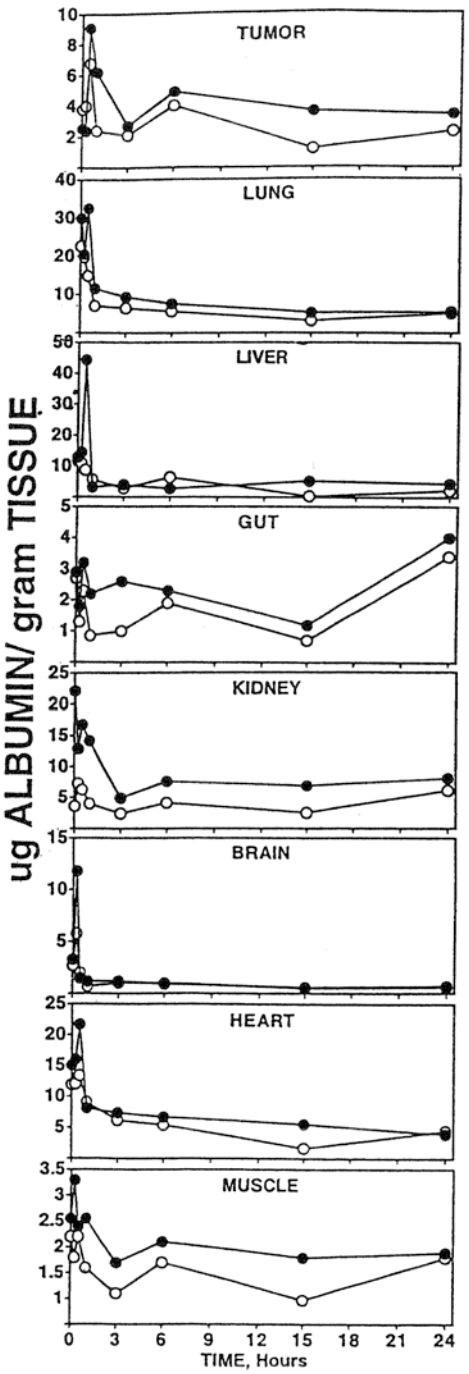

Fig. 8. Tissue levels of ^{14}C from [^{14}C]albumin in C57BL mice bearing Lewis lung tumors sc in the hindleg over a time-course after iv injection of 10 mg/kg of the protein alone on d 8 (❍), or after administration of the protein to animals treated with TNP-470 (30 mg/kg, sc) d 4, 6, 8, and minocycline (10 mg/kg, ip) daily d 4–8 after tumor cell implantation (●).

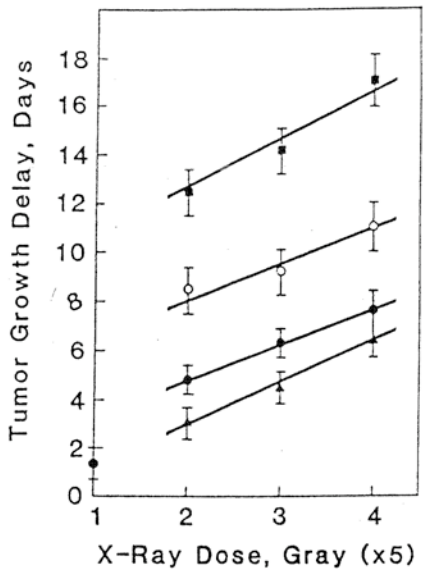

Fig. 9. Growth delay of the LLC produced by daily fractionated radiation delivered in fractions of 2, 3, or 4 gy locally to the tumor-bearing limb for 5 d on d 7–11 alone (▲), in animals treated with TNP-470 (30 mg/kg, sc) on alternate days and minocycline (10 mg/kg, ip) daily on d 4–18 (●), in animals treated with TNP-470–minocycline (as above), and allowed to breathe carbogen for 1 h prior to, and during, radiation delivery (○); and in animals treated with TNP-470–minocycline (as above), and injected intravenously with the perflubron emulsion (8 mL/kg), and then allowed to breathe carbogen for 1 h prior to, and during, radiation delivery (■). The points are the means of 15 animals, and the bars are the SEM.

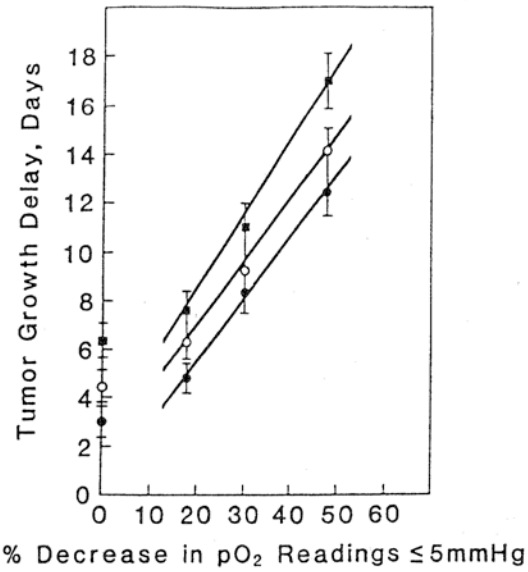

Fig. 10. Relationship between decrease in the percent of tumor pO_2 readings ≤5 mmHg and tumor growth delay at each radiation dose: 5 × 2 Gy (●), 5 × 3 Gy (○), and 5 × 4 Gy (■). Points are the means of 10 tumors. Bars = SEM.

4. PRECLINICAL THERAPEUTIC STUDIES: PROTEINS

4.1. Interleukin-12

In addition to small molecules, the search for antiangiogenic substances has led to the discovery of proteins that inhibit various steps in the breakdown of the basement membrane *(55,56)*. These include naturally occurring proteins, such as protamine *(57)*, interferon (IFN)-α *(58,59)*, IFN-γ *(60)*, platelet factor 4 *(57,61)*, tissue inhibitors of metalloproteinases (TIMPs; *62,63*), interleukin-12 (IL-12; *64–66*), angiostatin *(67)*, peptides derived from cartilages *(68,69)*, vitreous humor *(70)*, smooth muscle *(71)*, and aorta *(71)*, as well as synthetic peptides, such as synthetic laminin peptide (CDPG) YIGSR-NH_2 *(72)*, somatostatin analogs, such as somatoline *(73)*, and antibodies, such as MAb LM609 to human integrin $\alpha_v\beta_3$ *(74–76)*.

IL-12 is a naturally occurring cytokine that serves as a link between the innate and the cognate cellular immune systems *(66,77–79)*. IL-12 has the ability to act as a natural killer (NK) cell and a T-cell growth factor *(80–82)*, to enhance NK/LAK cell cytolytic activity *(82–84)*, to augment cytolytic T-cell responses *(83)*, and to induce secretion of cytokines, particularly IFN-γ, from T- and NK-cells *(85)*.

IL-12 has been shown to induce tumor regression and rejection in a variety of murine tumor models, when administered as a single agent *(86–90)*. This tumor regression results from activation of immune mechanisms that involve IFN-γ, $CD4^+$, and $CD8^+$ cells *(87,88)*. IL-12 has also been described as an antiangiogenic agent through the induction of IFN-γ *(64)*.

Both T- and NK-cells have been implicated as antitumor effector cells *(91)*, and IFN-γ has been shown to have antitumor activity in animals *(92,93)*. IL-12 has the potential to be used as an immunomodulatory cytokine in the therapy of malignancies *(92,94,95)*, as well as in gene therapy *(96,97)*. Brunda et al. *(87)* have shown that systemic administration of murine IL-12 can slow, and, in some cases, inhibit, the growth of both established sc tumors in mice and experimental pulmonary or hepatic metastases of B16F10 murine melanoma, M5076 reticulum cell sarcoma, or RenCa renal cell adenocarcinoma, and that local peritumoral injections of IL-12 can result in regression of established sc tumors. Based on results obtained using mice deficient in lymphocyte subsets, and an antibody depletion experiments, Brunda et al. *(87,98)* concluded that the antitumor efficacy of IL-12 is mediated primarily through $CD8^+$ T-cells.

Interleukin-12 (rmIL-12) was found to be an active antitumor agent against the LLC. The antitumor activity was dependent on rmIL-12 dose, duration of treatment, and the tumor burden at the initiation of treatment. The effect of the schedule of rmIL-12 administration alone, and along with a 1-wk regimen of fractionated radiation therapy (Table 14 and Fig. 11), or a 2-wk regimen of fractionated radiation therapy (Table 15 and Fig. 12), was examined. Beginning treatment with rmIL-12 on d 2 after tumor cell implantation, and treating for 5 d, resulted in about 5 d of tumor growth delay; beginning treatment with rmIL-12 on d 2, and treating for 10 d, and then for 5 d again, resulted in about 7.7 d of tumor growth delay (Table 14). Delaying the initiation of rmIL-12 treatment to d 7 after tumor cell implantation, and treating for 5 d, resulted in a tumor growth delay of about 2.7 d; extending that treatment to 10 injections increased the tumor growth delay to 5.4 d. Further delaying the initiation of rmIL-12 treatment until d 14 posttumor cell implantation, and treating for 10 injections, resulted in about 4.3 d of tumor growth delay. Finally, treating bulky disease beginning on day 21 posttumor cell implantation with rmIL-12 for 5 d did not alter tumor growth.

Table 14
Growth Delay of LLC, and Number and Percent of Large Lung Metastases on d 20 After Treatment with IL-12 and Fractionated Radiation Therapy Delivered Locally to Tumor-Bearing Limb

Treatment group	*Tumor growth delay, (d)*[a]	*Lung metastases (% large)*[b]
Control	–	25 (53)
IL-12 (45 μg/kg) ip[c]		
d 2–6	4.9 ± 0.4	15 (43)
d 7–11	2.7 ± 0.3	16 (47)
d 2–11; 14–18	7.7 ± 0.5	8.5 (29)
d 7–11; 14–18	5.4 ± 0.4	9 (31)
d 14–18; 21–25	4.3 ± 0.4	16 (50)
d 21–25	0.3 ± 0.3	
5 × 2 gy, d 7–11[d]	3.1 ± 0.4	17.5 (62)
5 × 3 gy, d 7–11	4.3 ± 0.4	17 (53)
5 × 4 gy, d 7–11	6.2 ± 0.6	17 (55)
IL-12 (45 μg/kg) ip, d 2–6		
+ 5 × 2 gy, d 7–11	6.8 ± 0.6	14 (27)
+ 5 × 3 gy, d 7–11	7.8 ± 0.9	13 (35)
+ 5 × 4 gy, d 7–11	10.0 ± 1.1	12 (39)
IL-12 (45 μg/kg) ip, d 2–11; 14–18		
+ 5 × 2 gy, d 7–11	8.7 ± 0.8	8 (40)
+ 5 × 3 gy, d 7–11	11.2 ± 1.3	7 (35)
+ 5 × 4 gy, d 7–11	16.1 ± 1.9	6 (33)
IL-12 (45 μg/kg) ip, d 7–11; 14–18		
+ 5 × 2 gy, d 7–11	6.9 ± 0.8	10 (24)
+ 5 × 3 gy, d 7–11	12.5 ± 1.6	6 (30)
+ 5 × 4 gy, d 7–11	19.8 ± 2.1	4 (27)
IL-12 (45 μg/kg) ip, d 14–18; 21–25		
+ 5 × 2 gy, d 7–11	4.2 ± 0.3	17 (39)
+ 5 × 3 gy, d 7–11	7.4 ± 0.5	15 (32)
+ 5 × 4 gy, d 7–11	10.6 ± 1.2	14 (23)
IL-12 (45 μg/kg) ip, d 21–25		
+ 5 × 2 gy, d 7–11	3.3 ± 0.3	19 (50)
+ 5 × 3 gy, d 7–11	6.6 ± 0.4	18 (61)
+ 5 × 4 gy, d 7–11	9.6 ± 1.0	16 (50)

[a]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in 12.5 ± 0.3 d. Mean ± SE of 18 animals.

[b]The number of external lung metastases on d 20 posttumor implant, as counted manually and scored as ≥ 3 mm in diameter. Data are the means from 6–12 pairs of lungs. Numbers in parentheses are number of large (vascularized) metastases.

[c]IL-12 was administered by ip injection.

[d]Radiation therapy was delivered in fractions of 2, 3, or 4 gy daily on d 7–11 locally to the tumor-bearing limb (100 rad/min; Gamma Cell 40 Nordion Inc., Ottawa, ON, Canada).

Fractionated radiation therapy in a 5-d regimen resulted in increasing tumor growth delay, with increasing radiation dose (Table 15). Administering rmIL-12 for 5 d prior to radiation therapy resulted in an additive effect of the two treatments, but did not increase the response of the tumor to the radiation therapy. Administration of rmIL-12 prior to, during, and after radiation therapy resulted in a highly effective therapy, which included

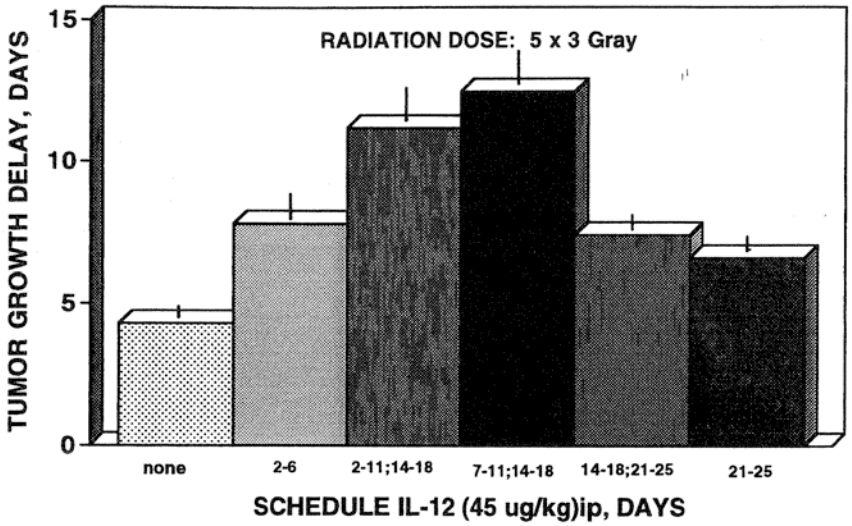

Fig. 11. Growth delay of the LLC after treatment of the tumor-bearing animals with fractionated radiation therapy (2, 3, or 4 gy) delivered locally to the tumor-bearing limb once per day on d 7–11 after tumor implantation alone, or along with rmIL-12 (45 μg/kg, ip) on d 2–6; d 2–11 and 14–18; d 7–11 and 14–18; d 14–18 and 21–25; or d 21–25. Data are the means of three experiments ± SEM.

an additive effect of the two therapies, and dose modification of the radiation therapy, with a dose-modifying factor of 2. Administering rmIL-12 during and after fractionated radiation also resulted in a highly effective therapeutic regimen, including an additive effect of the two therapies, and a radiation dose modifying factor of 3. Delaying administration of rmIL-12 until 2 d or 1 wk after completion of the radiation regimen resulted in less efficacious treatments than when the rmIL-12 was given concurrently and after the radiation therapy. Nevertheless, treatments in which the rmIL-12 was administered only after the radiation were as effective or more effective than radiation alone. Using 3 gy (×5) as a representative radiation dose, there was a maximal 2.8-fold increase in tumor growth delay when rmIL-12 was administered during and after fractionated radiation therapy (Fig. 11). The efficacy of these regimens against systemic disease, represented by lung metastases, paralleled the effects observed in the primary tumor (Table 14). When rmIL-12 was administered prior to, during, and after radiation therapy, or during and after radiation therapy, the number of lung metastases was decreased to 20–40% of the controls. The percent of large (vascularized) metastases was also decreased by treatment with rmIL-12.

When the fractionated radiation regimen was extended to 2 wk, the results shown in Table 15 were obtained. Administration of rmIL-12 for 5 d prior to radiation therapy resulted in an additive effect of the two therapies, but no dose modification of the radiation occurred. Concurrent administration of rmIL-12 just prior to each of the 10 radiation fractions formed a highly effective treatment regimen, resulting in additivity of the two therapies, and a radiation dose-modifying factor of 5. Administering rmIL-12 along with the second week of the fractionated radiation regimen, and then for 5 d the week after radiation therapy, also produced a highly effective treatment regimen, with an additive effect of the two therapies and a radiation dose-modifying factor of 2.7. When rmIL-12 administration was delayed until the completion of the radiation therapy, only a modest

Table 15
Growth Delay of LLC, and Number and Percent of Large Lung Metastases on d 20 after Treatment with IL-12 and 2 wk of Fractionated Radiation Therapy Delivered Locally to Tumor-Bearing Limb

Treatment group	*Tumor growth delay, (d)*[a]	*Lung metastases (% large)*[b]
Control	–	25 (53)
10 × 2 gy, d 7–11; 14–18[c]	4.1 ± 0.313 (32)	
10 × 3 gy, d 7–11; 14–18	5.1 ± 0.411 (38)	
10 × 4 gy, d 7–11; 14–18	8.9 ± 0.7	6 (27)
IL-12 (45 μg/kg) ip, d 2–6[d]		
+ 10 × 2 gy, d 7–11; 14–18	5.5 ± 0.4	7.5 (33)
+ 10 × 3 gy, d 7–11; 14–18	7.6 ± 0.7	6 (30)
+ 10 × 4 gy, d 7–11; 14–18	10.6 ± 0.9	5 (20)
IL-12 (45 μg/kg) ip, d 7–11; 14–18		
+ 10 × 2 gy, d 7–11; 14–18	7.2 ± 0.5	3.5 (43)
+ 10 × 3 gy, d 7–11; 14–18	16.6 ± 1.3	2.5 (40)
+ 10 × 4 gy, d 7–11; 14–18	28.9 ± 2.3	2 (50)
IL-12 (45 μg/kg) ip, d 14–18; 21–25		
+ 10 × 2 gy, d 7–11; 14–18	5.4 ± 0.5	10.5 (38)
+ 10 × 3 gy, d 7–11; 14–18	11.1 ± 0.9	6 (42)
+ 10 × 4 gy, d 7–11; 14–18	16.4 ± 1.1	5 (40)
IL-12 (45μg/kg) ip, d 21–25		
+ 10 × 2 gy, d 7–11; 14–18	5.6 ± 0.4	15 (32)
+ 10 × 3 gy, d 7–11; 14–18	7.7 ± 0.6	14 (52)
+ 10 × 4 gy, d 7–11; 14–18	11.8 ± 1.1	6 (38)

[a]Tumor growth delay is the difference in d for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in 12.5 ± 0.3 d. Mean ± SE of 15 animals.

[b]The number of external lung metastases on d 20 posttumor implant as counted manually and scored as ≥ 3 mm in diameter. Data are the means from 6–12 pairs of lungs. Numbers in parentheses are number of large (vascularized) metastases.

[c]Radiation therapy was delivered in fractions of 2, 3, or 4 gy daily on d 7–11 locally to the tumor-bearing limb (100 rad/min; Gamma Cell 40).

[d]IL-12 was administered by ip injection.

increase in tumor growth delay, compared with radiation therapy alone, was observed. Using the fractionated radiation regimen of 3 gy (×10) as a representative treatment, concurrent administration of rmIL-12 and radiation therapy resulted in a 3.3-fold increase in tumor growth delay, compared with radiation therapy alone (Fig. 12). A reduction in the number of lung metastases on d 20 was seen with each of the 2-wk radiation therapy regimens (Table 14). However, the treatment regimen, including concurrent administration of rmIL-12 and radiation therapy, which was most effective against the primary tumor, was also most effective in decreasing the number of lung metastases on d 20.

Because treatment of rmIL-12 markedly decreased lung metastases after radiation therapy locally to the sc tumor-bearing limb, the LLC was implanted into each hindleg of mice, and fractionated radiation therapy administered only to the right hindleg in the presence or absence of rmIL-12 treatment (Table 16). The presence of the second sc tumor in the animal did not alter the response of the treated tumor to the radiation therapy, or the number or size of lung metastases on d 20. Radiation therapy on d 7–11 to the tumor in the right hindleg resulted in a small but measurable tumor growth delay in the tumor

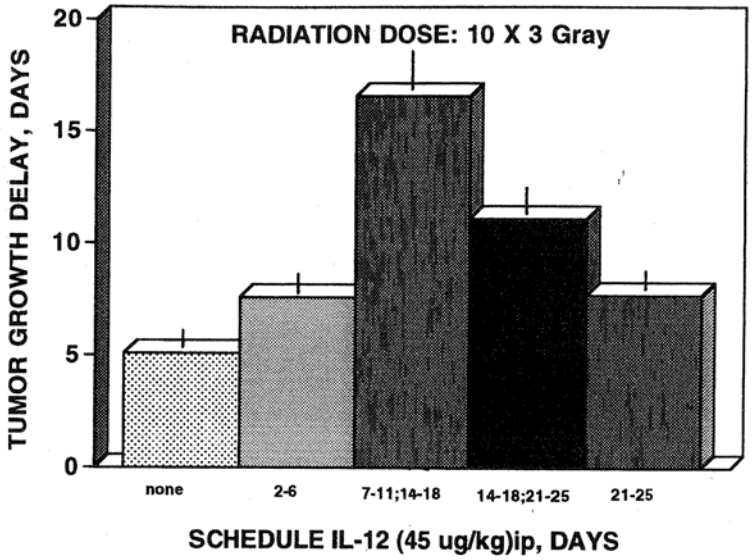

Fig. 12. Growth delay of the LLC after treatment of the tumor-bearing animals with fractionated radiation therapy (2, 3, or 4 gy) delivered locally to the tumor-bearing limb once per day on d 7–11 and 14–18 after tumor implantation alone, or along with rmIL-12 (45 μg/kg, ip) on d 2–6; d 7–11 and 14–18; d 14–18 and 21–25; or d 21–25. Data are the means of three experiments ± SEM.

in the left hindleg. Administration of rmIL-12 on d 7–11 and 14–18, along with fractionated radiation, resulted in increased tumor growth delay in the irradiated sc tumor, a response similar to rmIL-12 alone in the tumor in the contralateral limb, and a marked decrease in the number and percent of large lung metastases, compared with radiation to the right tumor only. Two other schedules of rmIL-12, also to the same total dose of 450 μg/kg, were tested. rmIL-12 (75 μg/kg) was administered on alternate d 7–18, or as two doses of 225 μg/kg on d 7–14 alone, or along with fractionated radiation therapy on d 7–11. Both of these schedules were less effective, as determined by each of the three experimental end points, than the rmIL-12 (45 μg/kg) on d 7–11 and 14–18 regimen.

rmIL-12 was also found to be an active antitumor agent in the MB-49 bladder carcinoma. The antitumor activity was dependent on IL-12 dose, duration of treatment, and the tumor burden at the initiation of treatment (Table 17). When IL-12 treatment was initiated on d 4 (tumor volume approx 30 mm^3), there was no statistically significant difference in the tumor growth delay produced by IL-12 (0.45 or 4.5 μg/kg) administered for 5 or 11 doses. However, when IL-12 (45 μg/kg) was administered to the animals for 11 doses, the tumor response was significantly greater than that obtained with the five-dose regimen. Delaying IL-12 treatment until d 10, when the tumors were approx 200 mm^3 in volume, resulted in decreased tumor growth delay, so that only the highest dose of IL-12 produced a significant tumor response. The number of lung metastases in these animals on d 20 was significantly decreased only at the highest dose of IL-12, and the percent of large (vascularized) lung metastases was not different from that seen in the controls.

In the design of treatment regimens, including systemic administration of IL-12 and chemotherapy, two major issues were the possible damage to the IL-12 target T-cells by

Table 16
Growth Delay of LLC in Animals Bearing Two sc Tumors, and Number and Percent Large Lung Metastases on d 20 After Treatment with Rmil-12 and Fractionated Therapy Delivered Locally to sc Tumor in Right Leg

	Tumor growth delay, d[a]		
Treatment group	*Right (X-rays)*	*Left*	*Lung metastases*[b] *(% large)*
Control	–	–	24.5 (56)
5 × 2 gy[c]	3.2 ± 0.4	–	17.5 (62)
5 × 3 gy	4.3 ± 0.4	–	17.0 (53)
5 × 4 gy	6.2 ± 0.6	–	17.0 (55)
5 × 2 gy	3.1 ± 0.4	1.2 ± 0.3	18.5 (53)
5 × 3 gy	4.0 ± 0.4	1.7 ± 0.3	14.0 (46)
5 × 4 gy	5.8 ± 0.5	2.5 ± 0.3	13.0 (50)
IL-12 (4.5 μg/kg)[d] 7–11; 14–18[d]	3.6 ± 0.4		15.0 (33)
above + 5 × 2 gy	5.9 ± 0.7	2.2 ± 0.3	5.0 (20)
above + 5 × 3 gy	10.6 ± 0.9	3.0 ± 0.4	5.0 (17)
above + 5 × 4 gy	15.6 ± 1.4	3.4 ± 0.4	1.5 (0)
IL-12 (45 μg/kg) d 7–11; 14–18	5.4 ± 0.4		7.0 (25)
above + 5 × 2 gy	6.9 ± 0.7	4.5 ± 0.4	16.0 (24)
above + 5 × 3 gy	14.3 ± 1.6	5.4 ± 0.4	5.0 (31)
above + 5 × 4 gy	22.7 ± 1.9	5.9 ± 0.5	3.0 (19)
IL-12 (75 μg/kg) d 7, 9, 11, 14, 16, 18	1.5 ± 0.3		14.0 (32)
above + 5 × 2 gy	6.0 ± 0.4	3.5 ± 0.3	95.0 (45)
above + 5 × 3 gy	12.4 ± 1.3	4.6 ± 0.4	9.0 (29)
above + 5 × 4 gy	17.6 ± 1.6	4.9 ± 0.4	8.0 (27)
IL-12 (225 μg/kg) d 7 and 14	3.0 ± 0.3		14.5 (29)
above + 5 × 2 gy	6.9 ± 0.7	2.9 ± 0.3	10.5 (30)
above + 5 × 3 gy	7.8 ± 0.8	3.4 ± 0.3	10.0 (26)
above + 5 × 4 gy	10.0 ± 1.0	3.6 ± 0.4	6.0 (24)

[a]Tumor growth is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 18 animals.

[b]Number of external lung metastases on d 20 posttumor implant as counted manually and scored as ≥ 3 mm in diameter. Data are the means from 6–12 pairs of lungs. Numbers in parentheses are number of large (vascularized) metastases.

[c]Radiation therapy was delivered in fractions of 2, 3 or 4 gy daily on d 7–11 locally to the tumor-bearing limb (100 rad/min; Gamma Cell 40).

[d]IL-12 was administered by ip injection.

the chemotherapy, thus ablating the IL-12 effect, and increased toxicity of the combination therapy. Therefore, IL-12 was studied over a dosage range with the chemotherapy, and on schedules prior to, after, and overlapping with the chemotherapy. Each of the chemotherapeutic agents studied (adriamycin, cyclophosphamide, and 5-fluorouracil) were active antitumor agents against the MB-49 bladder carcinoma (Table 18). IL-12 treatment did not increase the toxicity of the chemotherapy. There was increased anticancer activity when IL-12 administration was added to treatment with each chemotherapeutic agent. The increased tumor response was dependent on IL-12 dose and schedule, with overlapping therapy producing the greatest effect (Table 18). Adriamycin on d 7–11 produced 10.8 d of tumor growth delay. However, the greatest tumor growth delay was

Table 17
Tumor Growth Delay and Number and Size of Lung Metastases in Animals Bearing the MB-49 Bladder Carcinoma Treated with rmIL-12 on Different Schedules

Treatment group	*Total rmIL-12 dose, μg*	*Tumor growth delay, d*	*No. lung metastases (% large)*
Controls		–	22 (44%)
Daily d 4 → 8 posttumor implantation			
rmIL-12 (1 μg)	5	7.3 ± 1.2	12 (38%)
rmIL-12 (0.1 μg)	0.5	5.9 ± 0.9	17 (35%)
rmIL-12 (0.01 μg)	0.05	3.8 ± 0.6	18.5 (46%)
Daily d 4 → 14 posttumor implantation			
rmIL-12 (1 μg)	11	10.9 ± 1.5	8 (42%)
rmIL-12 (0.1 μg)	1.1	5.7 ± 0.9	14 (35%)
rmIL-12 (0.01 μg)	0.11	4.3 ± 0.6	18 (42%)
Daily d 7 → 11 and 14 → 18 posttumor implantation			
rmIL-12 (0.1 μg)	1	4.5 ± 0.7	—
Daily d 10 → 14 posttumor implantation			
rmIL-12 (1 μg)	5	3.2 ± 0.6	9 (43%)
rmIL-12 (0.1 μg)	0.5	1.6 ± 0.3	14 (45%)
rmIL-12 (0.01 μg)	0.05	1.5 ± 0.3	16 (47%)

[a]Tumor growth is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 18 animals.

obtained with extended IL-12 treatment, d 4–14, combined with adriamycin, which resulted in 23.4 d of tumor growth delay. Cyclophosphamide administered on d 7, 9, and 11 produced 8.0 d of tumor growth delay. When IL-12 was administered along with cyclophosphamide on the longer schedule, d 4–14, a tumor growth delay of 21.7 d was produced. 5-Fluorouracil administered on d 7–11 produced 6.2 d of tumor growth delay. IL-12 treatment extended to d 4–14, along with 5-fluorouracil, resulted in 16.5 d of tumor growth delay. Shorter-duration treatment with IL-12 before or after chemotherapy regimens was less effective in all treatment groups (Table 18). Administering IL-12 simultaneously with the chemotherapy, and then again on d 14–18, was not a very effective combination therapy (Table 18).

IL-12 included in the therapeutic regimen markedly increased the efficacy against metastatic disease (Table 18). The highest dose of IL-12, 45 was most effective against metastasis to the lungs. Unlike tumor growth delay, the combinations with adriamycin, cyclophosphamide, or 5-fluorouracil were similar at all schedules evaluated. In combination with adriamycin or 5-fluorouracil, there was little impact of IL-12 administration on the percent of lung metastases ≥3 mm in diameter, indicating that these treatments were not altering the growth pattern of the metastases. However, IL-12 in combination with cyclophosphamide did decrease the percent of large lung metastases, indicating that the growth rate of the metastases was slowed.

The B16 melanoma is a highly metastatic murine solid tumor that grows more slowly than the MB-49 bladder carcinoma, thus providing a convenient model in which to address the question of cycling IL-12 administration with cytotoxic therapy. Several schedules of rmIL-12 and cyclophosphamide were tested in which rmIL-12 administration was initiated after cyclophosphamide therapy, in a manner that overlapped the ter-

Table 18
Tumor Growth Delay and Number and Size of Lung Metastases in Animals Bearing the MB-49 Bladder Carcinoma Treated with rmIL-12 and an Anticancer Agent

Treatment group	*Tumor growth delay, d*[a]	*No. lung metastases*[b] *(% large)*
Controls	–	22 (44%)
Adriamycin (1.25 mg/kg) d 7 → 11		
Alone	10.8 ± 1.2	18 (42%)
+ rmIL-12 (45 μg/kg) d 4 → 8	17.1 ± 2.0	7.5 (33%)
+ rmIL-12 (4.5 μg/kg) d 4 → 8	15.0 ± 1.7	15 (37%)
+ rmIL-12 (0.45 μg/kg) d 4 → 8	14.6 ± 1.6	17 (38%)
+ rmIL-12 (45 μg/kg) d 4 → 14	23.4 ± 3.7	8 (38%)
+ rmIL-12 (4.5 μg/kg) d 4 → 14	15.1 ± 1.8	11.5 (38%)
+ rmIL-12 (0.45 μg/kg) d 4 → 14	13.8 ± 1.7	12 (38%)
+ rmIL-12 (45 μg/kg) d 7 → 11, 14 → 18	12.8 ± 1.7	–
+ rmIL-12 (45 μg/kg) d 10 → 14	14.8 ± 1.4	5 (42%)
+ rmIL-12 (4.5 μg/kg) d 10 → 14	13.6 ± 1.4	12 (46%)
+ rmIL-12 (0.45 μg/kg) d 10 → 14	13.0 ± 1.3	14 (41%)
Cyclophosphamide (100 mg/kg) d 7, 9, 11		
Alone	8.0 ± 0.8	12 (38%)
+ rmIL-12 (45 μg/kg) d 4 → 8	17.0 ± 1.7	3 (17%)
+ rmIL-12 (4.5 μg/kg) d 4 → 8	15.6 ± 1.6	4 (38%)
+ rmIL-12 (0.45 μg/kg) d 4 → 8	15.0 ± 1.3	3.5 (28%)
+ rmIL-12 (45 μg/kg) d 4 → 14	21.7 ± 3.3	4 (21%)
+ rmIL-12 (4.5 μg/kg) d 4 → 14	16.5 ± 1.8	5 (23%)
+ rmIL-12 (0.45 μg/kg) d 4 → 14	14.3 ± 1.5	6 (28%)
+ rmIL-12 (45 μg/kg) d 7 → 11, 14 → 18	12.8 ± 1.6	–
+ rmIL-12 (45 μg/kg) d 10 → 14	17.6 ± 1.9	1.5 (50%)
+ rmIL-12 (4.5 μg/kg) d 10 → 14	14.6 ± 1.7	5 (33%)
+ rmIL-12 (0.45 μg/kg) d 10 → 14	12.7 ± 1.2	8 (27%)
5-Fluorouracil (30 mg/kg) d 7 → 11		
Alone	6.2 ± 0.7	17 (42%)
+ rmIL-12 (45 μg/kg) d 4 → 8	12.3 ± 1.1	10 (25%)
+ rmIL-12 (4.5 μg/kg) d 4 → 8	11.9 ± 1.0	11.5 (39%)
+ rmIL-12 (0.45 μg/kg) d 4 → 8	10.2 ± 0.9	16 (38%)
+ rmIL-12 (45 μg/kg) d 4 → 14	16.5 ± 1.8	10 (27%)
+ rmIL-12 (4.5 μg/kg) d 4 → 14	11.0 ± 1.0	13.5 (35%)
+ rmIL-12 (0.45 μg/kg) d 4 → 14	8.5 ± 0.7	19 (39%)
+ rmIL-12 (45 μg/kg) d 7 → 11, 14 → 18	7.1 ± 0.6	–
+ rmIL-12 (45 μg/kg) d 10 → 14	11.2 ± 1.1	11 (43%)
+ rmIL-12 (4.5 μg/kg) d 10 → 14	9.2 ± 0.9	21 (44%)
+ rmIL-12 (0.45 μg/kg) d 10 → 14	9.1 ± 0.9	22 (41%)

[a]Tumor growth is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 18 animals.

[b]Number of external lung metastases on d 20 posttumor implant as counted manually and scored as ≥ 3 mm in diameter. Data are the means from 6–12 pairs of lungs. Numbers in parentheses are the number of large (vascularized) metastases.

minal portion of the chemotherapy regimen, or that started 2 d after the completion of the chemotherapy regimen and extended for 1–3 wk (Table 19). Cyclophosphamide was administered for one course (d 7, 9, and 11), or for two courses (d 7, 9, 11, 28, 30, and

Table 19
Growth Delay of B16 Melanoma, and Number and Size of Lung Metastases on d 30 Produced by Treatment with IL-12 and/or Cyclophosphamide

Treatment group	*Tumor growth delay, (d)*	*Lung metastases (% large)*
Controls		21 (49)
IL-12 (45 μg/kg), ip		
d 10–14	4.6 ± 0.4	8 (21)
d 14–18	5.1 ± 0.4	10 (23)
d 10–14; 18–22	6.4 ± 0.5	3 (25)
d 14–18; 21–25	6.3 ± 0.5	10 (23)
d 14–18; 21–25; 28–32	6.8 ± 0.5	8 (44)
IL-12 (4.5 μg/kg), ip		
d 10–14	2.1 ± 0.3	11 (24)
d 10–14; 18–22	3.2 ± 0.3	16 (27)
Cyclophosphamide (125 mg/kg), ip		
d 7, 9, 11	16.8 ± 1.4	3.5 (31)
d 7, 9, 11; 28, 30, 32	28.5 ± 2.1	3.5 (29)
CTX + IL-12 (45 μg/kg)		
CTX, d 7, 9 ,11 + IL-12, d 10–14	25.8 ± 2.7	2.5 (27)
CTX, d 7, 9, 11 + IL-12, d 14–18	19.0 ± 1.6	3.5 (43)
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25	30.9 ± 2.5	2 (0)
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25; 28–32	33.4 ± 2.1	1 (0)
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25 + CTX, d 28, 30, 32 + IL-12, d 35–39	40.0 ± 2.2	1 (0)
CTX + IL-12 (4.5 μg/kg)		
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25	23.2 ± 1.3	6 (17)
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25; 28–32	23.7 ± 1.7	2.5 (0)
CTX, d 7, 9, 11 + IL-12, d 14–18; 21–25 + CTX d 28,30,32 + IL-12, d 35–39	28.2 ± 1.9	2 (25)
Cyclophosphamide (62 mg/kg) ip		
d 7, 9, 11; 15, 17, 19	6.8 ± 0.5	6 (50)
CTX, d 7,9,11 + IL-12 (45 μg/kg) d 10–14 + CTX, d 15, 17, 19 + IL-12 (45 μg/kg) d18–22	11.6 ± 1.0	0.5 (8)
CTX, d 7, 9, 11 + IL-12 (4.5 μg/kg) d 10–14 + CTX, d 15, 17, 19 + IL-12 (4.5 μg/kg) d 18–22	9.1 ± 0.8	4.5 (22)

[a]Tumor growth is the difference in days for treated tumors to reach 500 mm^3, compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 d. Mean ± SE of 18 animals.

[b]Number of external lung metastases on d 20 posttumor implant as counted manually and scored as ≥3 mm in diameter. Data are the means from 6–12 pairs of lungs. Numbers in parentheses are number of large (vascularized) metastases.

32). Both rmIL-12 and cyclophosphamide were active antitumor agents against the B16 melanoma. The tumor growth delay produced by rmIL-12 was dependent on the dose and duration of treatment. Administration of 45 μg/kg of rmIL-12 was more effective than administration of 4.5 μg/kg of rmIL-12. Administration of rmIL-12 for 2 wk was more effective than administration of rmIL-12 for 1 wk. However, administration of rmIL-12 for 3 wk did not increase the tumor response further. Greater tumor growth delay resulted when the 5-d rmIL-12 regimen was administered overlapping with the terminal portion

of the chemotherapy treatment than if a 2-d break was allowed from completion of the cyclophosphamide treatment to initiation of the rmIL-12 administration. Extending the rmIL-12 administration to 2 wk (10 injections) resulted in a highly effective therapeutic regimen, with a tumor growth delay of about 31 d. Adding a third week of rmIL-12 administration to that regimen increased the tumor growth delay only 2.4 d more. When the dose of rmIL-12 was decreased to 4.5 μg/kg, the tumor growth delays observed with the combination regimens were decreased to 23–24 d, which was significantly greater than cyclophosphamide alone. Administration of two courses of cyclophosphamide on d 7, 9, and 11, and again on d 28, 30, and 32, produced a tumor growth delay of about 28.5 d. When rmIL-12 was administered between and after completion of the cyclophosphamide courses, a tumor growth delay of 40 d resulted, which was greater than expected for additivity for the two therapies. When the same treatment regimen was carried out with the lower dose of rmIL-12, the tumor growth delay observed was about 28 d. To explore the effect of cyclophosphamide dose and schedule, a total dose of 375 mg/kg of cyclophosphamide, administered as three injections of 125 mg/kg alone, was divided into six injections of 62 mg/kg administered over two courses (Table 19). Decreasing the dose intensity of the cyclophosphamide resulted in a decrease in the tumor growth delay from 16.8 d, for 3 × 125 mg/kg of cyclophosphamide, to 6.8 d, for 6 × 62 mg/kg of cyclophosphamide. Administering the rmIL-12 between and after the chemotherapy treatment resulted in additivity of the two therapies.

The efficacy of rmIL-12 and rmIL-12 and cyclophosphamide combinations against B16 melanoma metastatic to the lungs was dependent on the day of treatment initiation, dose of rmIL-12, and duration of treatment (Table 19). Beginning treatment with rmIL-12 on d 10, when the tumor burden was lower, was more effective in reducing the number and percent of large lung metastases than was beginning rmIL-12 treatment regimens on d 14, and the rmIL-12 treatment regimens most effective against metastatic disease were those including administration of rmIL-12 for 2 wk or more. In general, treatment regimens including two courses of cyclophosphamide were more effective. The rmIL-12 treatment regimens decreased not only the number of lung metastases on d 30, but also significantly decreased the percent of lung metastases that were large enough to be undergoing angiogenesis.

In all of the combination-treatment regimens of IL-12 with chemotherapy, there was a marked effect on disease metastatic to the lungs with each of the tumors studied. IL-12 has been described as an antiangiogenic agent *(64)*. The antiangiogenic activity of IL-12 appears to be the result of the induction of INF-γ by the cytokine *(64)*. Although the mechanism by which INF-γ exerts antiangiogenic effects remains unelucidated, several studies have shown that the INFs inhibit production of matrix metalloproteinases *(99–102)*.

Gohji et al. *(99)* found that incubation of human KG-2 renal cell carcinoma cells with INF-β or -γ suppressed transcription of the 72-kDa gelatinase gene and, hence, production of gelatinase activity. These inhibitory effects of INFs were independent of their antiproliferative effects. Treatment of KG-2 cells with INF-β or -γ significantly inhibited cell invasion through reconstituted basement membrane toward chemoattractants produced by kidney fibroblasts. The inhibitory activity of INFs was specific to the KG-2 cells, since gelatinase activity by various fibroblasts was unaffected. In human A2058 melanoma cells, Hujanen et al. *(100)* found that INF-β and -γ were potent regulators of both M_r 72,000 and M_r 92,000 type-IV collagenase/gelatinase-A and -B genes, showing biphasic and parallel effects on mRNA levels of both enzymes, depending on the treatment time, and that the M_r 72,000

metalloproteinase/gelatinase-A was the predominant basement-membrane-degrading type-IV collagenase in the A2058 human melanoma cell line. Norioka et al. *(101)* found that INF-γ alone and in combination with IL-1 inhibited the proliferation of human umbilical vein endothelial cells stimulated with basic fibroblast growth factor (bFGF) in culture. Local administration of INF-γ inhibited bFGF, and stimulated angiogenesis in mouse skin. INF-γ, especially in combination with IL-1, downregulated expression of bFGF receptor on the endothelial cells. On the other hand, Hiscox et al. *(102)* found that IL-12 directly inhibited the attachment of the human colon cancer cell lines HRT18, HT29, and HT115 to Matrigel. IL-12 did not affect the growth of these colon carcinoma cell lines. Flow cytometry, Western analysis, and immunohistochemistry showed an upregulation of E-cadherin cell-surface adhesion molecules. These direct effects of IL-12 on colon cancer cells suggest a potentially important role for IL-12 in metastasis. Therefore, administration of IL-12 may act as an antiangiogenic agent, directly and/or indirectly, by preventing invasion and extravasation of tumor cells through vasculature, and by preventing angiogenic activity in implanted metastatic tumor cells.

The immune basis of IL-12 activity would suggest that combination of IL-12 with other therapies that enhance immune response could potentiate the antitumor activity of IL-12. The combination of IL-12 with IL-2, a cytokine with a similar pharmacologic profile, was found to be no more effective than the optimal dose of IL-12 alone *(98,103)*. It was hypothesized that this outcome may have resulted from the substantially increased toxicity associated with IL-12–IL-2 combination therapy *(98)*; however, pulse IL-2, along with IL-12, was less toxic and more efficacious *(103)*. The combination of IL-12 with M-CSF, a macrophage activator and growth factor, was synergistic, especially with local fractionated radiation therapy *(104)*. These results concur with and extend those of Lu et al. *(105)* that M-CSF is effective in enhancing the response of the LLC to radiation therapy. Macrophages are present in tumors *(106)*, have a significant role in antigen presentation and lymphocyte activation, and have been identified as a primary source of endogenous IL-12 *(98,107)*. They produce a variety of other inflammatory cytokines, such as TNF-α, IL-1, and INF-α and β, as well as oxygen radicals and other cytostatic and cytolytic factors. M-CSF augments many of these antitumor functions *(108)*.

4.2. Angiostatin and Endostatin

Angiostatin, a proteolytic fragment of plasminogen composed of kringles 1–4, originally isolated from the urine of C57Bl mice bearing the LLC, was shown to be an inhibitor of angiogenesis in several assays, and to inhibit the growth of tumor metastases, by O'Reilly et al. *(109)*. Angiostatin can be generated in vitro by the limited proteolysis of human plasminogen by elastase *(110,111)*. The enzymatic mechanism by which angiostatin is generated in vivo remains unknown. Angiostatin was shown to inhibit the proliferation of endothelial cells in culture, and to induce tumor dormancy as defined by a balance of apoptosis and proliferation, resulting in no growth in lung metastases *(112)*. O'Reilly et al. *(112)* went on to show that systemic administration of angiostatin to SCID mice bearing human tumor xenografts could inhibit the growth of those tumors, including PC-3 prostate carcinoma, Clone A colon carcinoma, and MDA-MB breast carcinoma. Administration of human angiostatin also inhibited the growth of the murine LLC, the T214 fibrosarcoma, and the M5076 reticulum cell sarcoma. The angiostatin was administered at a dose of 50 mg/kg every 12 h. Gately et al. *(113)* reported that human prostate carcinoma cells in culture (PC-3, DU-145, and

Table 20
Number of Intratumoral Blood Vessels as Determined by Immunohistochemical Staining for CD31 of LLC Tumors After Treatment of Animals with Various Potential Antiangiogenic Agents

Treatment group	*Number of intratumoral vessels by CD31 positivity*
Controls	180 + 50
Angiostatin (10 mg/kg) sc, 2× per day, d 0–8	117 + 10
TNP-470 (30 mg/kg) sc, alternate d 0–8	92 + 30
Angiostatin + TNP-470	130 + 10
TNP-470 + minocycline (10 mg/kg) ip, d 0–8	116 + 9
Angiostatin + TNP-470 + minocycline	55 + 5

LNCaP cells) can cleave human plasminogen to angiostatin through the action of serine protease(s). The Folkman laboratory has investigated the structural requirements for the activity of angiostatin, and found that the activity of this complex protein is centered primarily in kringles 2 and 3 of the protein (114). Kringle 1 has some antiangiogenic activity, but kringle 4 is inactive.

The ability of angiostatin to inhibit blood vessel formation in the LLC was determined by immunohistochemical staining of tumors from animals that had been treated twice per day with recombinant murine angiostatin, and compared with animals treated with TNP-470, TNP-470 along with minocycline, angiostatin along with TNP-470, or with the three-drug combination of angiostastin–TNP-470–minocycline (Table 20). Angiostatin administered at a dose of 10 mg/kg twice per day, beginning the day of tumor implantation, decreased the number of stainable intratumoral vessels to 65% of the number found in the untreated control tumors. TNP-470 deceased the number of intratumoral vessels to 51% of the number found in the controls; angiostatin, along with TNP-470, and TNP-470, along with minocycline, were a bit less effective. The three-drug combination of angiostatin–TNP-470–minocycline decreased the number of stainable intratumoral vessels to 31% of the number found in the untreated control animals. Administration of angiostatin on this same schedule of 10 mg/kg, sc twice per day, beginning the day of tumor cell implantation, resulted in a twofold increase in the killing of EMT-6 murine mammary tumor cells from tumors by cyclophosphamide.

Recently, O'Reilly et al. *(115)* identified another antiangiogenic protein isolated from a hemangioendothelioma and called endostatin. Endostatin is a smaller protein than angiostatin, and is a 20-kDA C-terminal fragment of collagen XVIII. Endostatin has been shown to inhibit the growth of the LLC when administered at doses of 10 or 20 mg/kg daily by intraperitoneal injection. Similar results were found in animals bearing the T241 fibrosarcoma, the B16 melanoma, and the EOMA hemangioendothelioma.

5. CONCLUSION

The molecules described herein as antiangiogenic agents and antimetastatic agents represent a wide variety of molecular structures with a wide variety of biological effects

and targets. Most often, these agents have been generally classified as antiangiogenic or antimetastatic by their effects in an in vitro bioassay system. The diversity in this group of molecules gives strength to the potential of this approach in therapeutic applications. The biological and biochemical pathways involved in angiogenesis are numerous and redundant. It is likely that there are many angiogenic factors and many pathways of invasion; therefore, it is likely that blockade of more than one pathway related to angiogenesis and/or invasion will be necessary to have impact on the natural progress of a malignant disease.

The vasculature forms the first barrier to penetration of molecules into tumors. Although the antiangiogenic agent treatments administered in these studies did not inhibit angiogenesis in these tumors completely, the vasculature present in the treated tumors may be impaired, compared to control tumors. Overall, therefore, the best speculation is that the chief targets for the antiangiogenic agents are extracellular matrix processes and/or tumor endothelial cells and that inhibition and/or impairment of these nonmalignant functions can improve therapeutic responses when used in combination with cytotoxic therapies. The incorporation of antiangiogenic agents and/or antimetastatic agents into therapeutic regimens represents an important challenge. The successful treatment of cancer requires the eradication of all malignant cells, and therefore treatment with cytotoxic therapies. The compatibility of antiangiogenic therapy and/or antiinvasion agents with cytotoxic chemotherapeutic agents is not obvious *(116)*.

The goal of the addition of any noncytotoxic potentiator to a therapeutic regimen is to take a good therapy, and, without additional toxicity, push it to cure. Cyclophosphamide is a good drug against the LLC, although no long-term survivors of animals bearing LLC are achieved with cyclophosphamide treatment alone. Adding antiangiogenic agents to treatment of this tumor with cyclophosphamide produced a cure rate of 40–50%, meaning that both the primary and metastatic disease has been eradicated in these animals. Cures were achieved only when the antiangiogenic treatments extended from d 4–18 post-Lewis-lung-tumor implantation. The results obtained with the addition of antiangiogenic agents to cytotoxic anticancer therapies in in vivo models of established solid tumors have been very positive, and provide direction for future clinical trials, including these antiangiogenic agents. Two conclusions may be drawn. First, combinations of antiangiogenic and/or antimetastatic agents evoke a greater effect on tumor response to therapy than does treatment with single agents of these classes. Second, treatment with antiangiogenic agents and/or antimetastatic agents can interact in a positive way with cytotoxic therapies.

The early phases of the clinical testing (phase I and II) of antiangiogenic therapies should be performed with the highest degree of science possible by including measurements of markers for the biological activity of these agents. In addition to pharmacokinetic studies of the test agent, patient blood and urine levels of angiogenic markers and tumor markers should be monitored. Ideally, a noninvasive measurement of endothelial cells metabolic activity could be devised. In lieu of that, a noninvasive measurement of tumor metabolic activity, such as positron emission tomography of [^{18}F]fluorodeoxyglucose, may be useful in elucidating a biological effect of these new agents in established disease. In terms of traditional clinical response criteria, stable disease may be considered a response over the relatively short time frame of many phase I clinical protocols *(117)*.

The true strength of antiangiogenic therapies may be in their use in combination with traditional cytotoxic therapies, in which they will add a new dimension to the anticancer armamentarium.

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18 Prognostic Significance of Tumor Vascularity

Giampietro Gasparini and Adrian L. Harris

CONTENTS

1. INTRODUCTION

The significance of tumor angiogenesis has been recognized for many centuries. However, it was not until the 1970s, when Brem et al. *(1)* aroused interest in pathological angiogenesis, that the first attempt was made to generate a systematic method for quantifying angiogenesis. It has been only in the last 10 yr, with the advent of highly specific endothelial markers *(2,3)*, which can be used in histological archival tissues, that quantitation studies have been performed. The majority of studies on quantifying tumor angiogenesis have followed the method of Weidner et al. *(4)*, and used antiendothelial cell antibodies and a variety of immunohistochemical techniques to highlight the tumor vasculature. Briefly, the three areas containing the maximum number of discrete microvessels are identified by scanning the tumor at low power. Individual microvessels are then counted on high power objectives. Any immunoreactive endothelial cell(s) that is separate from adjacent microvessels is considered a countable vessel. Vascular lumina are not a requirement to be included in the count, and the vessels within any central sclerotic area of the tumor are not included. Most of these studies have shown that an increased microvessel density as a measure of angiogenesis is a powerful prognostic tool in many human tumor types (*see* Sections 4. and 5.). Nevertheless, despite the initial confirmatory publications, numerous reports are now appearing in the literature that fail to show a positive association between increasing tumor vascularity and reduced patient outcome, and caution as to the clinical utility of tumor angiogenesis is being urged *(5)*. However, many of these negative studies may result from significant differences in methodologies. There are several major considerations to take into account when quan-

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tifying tumor angiogenesis in histological sections using immunohistochemistry. These include the method used to identify the tumor vasculature, the representative tumor area measured, the vascular parameter and the counting technique selected, and the cutoff used in the statistical analysis to derive the method.

2. VARIABLES IN QUANTITATION OF TUMOR ANGIOGENESIS

2.1. Methods to Identify the Tumor Vasculature

Studies vary in their method used to highlight tumor capillaries. Early studies used tinctorial stains, such as Masson's Tricrome *(6)*, which highlights the basement membrane of the microvessels. Other studies have used nonspecific endothelial cell markers, such as alkaline phosphatase *(2)*, vimentin *(7)*, antilectin antibodies *(3)*, and type IV collagen *(8)*, which only pick up a small proportion of the total tumor vasculature. The newer endothelial cell markers include factor VIII-related antigen, CD31 (PECAM), and CD34. Specific antiendothelial antibodies show significant heterogeneity between endothelia of different tissues *(9–11)*. After assessing numerous endothelial cell markers, this laboratory *(12)* selected an anti-CD31 antibody, which, when used here, is most sensitive, and gives reliable microvessel immunostaining in routinely handled, formalin-fixed, paraffin-embedded tissues *(13–15)*.

However, different antibodies have been compared, and many have provided prognostic information *(12,16–19)*. Several groups have investigated the use of antibodies that do not highlight all endothelium, but selectively identify only the tumor-associated vasculature. This might not only more accurately measure tumor angiogenesis, but might be useful as a objective for antiangiogenesis and/or vascular-targeting strategies *(20)*. Several antibodies have been generated that identify epitopes that are induced on tumor-associated endothelium, and are mostly absent, or show highly restricted expression in normal tissues (Table 1). Thus, E-9, an antiendoglin antibody is reported to be upregulated on tumor-associated endothelium, anti-CD31, a more standard endothelial cell marker, is negative *(21)*. Similarly, other restricted epitopes, such as a fetal fibronectin splice variant, are highly expressed on tumor-associated vessels (in up to 78% of breast cancers), compared to normal adult tissues *(22)*. However, to date, no quantification studies assessing the prognostic utility of these markers have been performed.

2.2. Selection of the Representative Tumor Area

Studies examining different tumor areas have shown significant heterogeneity between different tumors regions. The edge of the tumor is the most active area of angiogenesis *(23)*, with little or no endothelial cell proliferation occurring within its body. This is reflected in the microvessel density, which is usually highest at the periphery. Thus, the particular tumor area that is measured will have profound effects on the data derived. However, it is still remarkable that assessment of only one representative section can give a useful measure of tumor angiogenesis, since it has been estimated that one tissue section accounts for only one-thousandth of the total tumor mass.

Many tumors demonstrate significant heterogeneity within the tumor periphery, with the presence of so-called vascular hot spots. Although studies have measured random fields *(6)* and average vascularity *(3)*, most have assessed these hot spots, acknowledging that these are the areas that are likely to be biologically important in tumor cell growth and metastases *(4,19)*. However, there is little agreement as to the optimal number of hot

Table 1
Endothelial Cell Markers

Constitutive	*Antigen*	*Distribution*
CD13	Aminopeptidase-n	Many cell types, brain endothelium
CD31	PECAM	Endothelium; plasma cells
CD34	Sialomucin	Endothelium; fibroblasts
CD36	Glycoprotein IV	Most endothelium
CD54	ICAM-1	Endothelium; leukocytes
CD63	Lysosomal protein	Many cell types
CD102	ICAM-2	Most endothelium; leukocytes
FVIII RA	Von Willebrand factor	Most endothelium; platelets
Inducible		
CD62 P	P-selectin	Endothelium; platelets
CD62 E	E-selectin	Activated endothelium
CD106	VCAM-1	Activated endothelium; leukocytes
Tumor-associated		
CD105	Endoglin	Tumor > normal vessels
FB5	Endosialin	Mostly tumor vessels
EN 7/44	p30.5 kDa	Stains tumors and inflammation
PAL-E	Unknown	Normal small vessels and tumor
4A11	Blood group	Preferential tumor and inflammation

spots to assess, which currently ranges from 1 to 5. This number also has important bearing on the efficacy of the method, since tumors have a limited number of identifiable hot spots. Counting a large number of tumor fields will tend to diminish the power of the hot spot technique, since there will be a tendency to reduce the average tumor vascularity as more fields are assessed.

The tumor field area, determined by the microscopic magnification and particular objectives used, will also significantly effect the vascular index. A higher magnification gives an increased resolution, which enables more microvessels to be identified, but to the detriment that all fields at too high magnification become an angiogenic hot spot *(12)*. Conversely, a low magnification, with its lower resolution, will identify a smaller number of vessels, and will dilute out the hot spot. The authors have demonstrated that measurements within magnification ×200–400, over a range of 0.12–0.74 mm^2, give comparable information *(24)*.

2.3. Vascular Parameter and Counting Technique

Most studies quantifying tumor angiogenesis have measured microvessel density. However, the vascular perimeter might be a better measure of angiogenesis than microvessel density, since it may reflect the endothelial surface area available for interaction with the tumor, thereby increasing the likelihood of dissemination and metastasis. Similarly, the vascular surface area might give an indication of blood volume from which the tumor can derive nutrients in exchange for its waste products. The authors have examined the vascular parameters, microvessel density, luminal area, and perimeter *(24)* and have observed significant correlations between all, suggesting that these indices are equivalent for quantifying tumor vascularity.

In addition to the conceptual difficulties of microvessel density, there are several practical considerations that make it unsuitable for a diagnostic pathology service.

Microvessel counting is time-consuming, particularly when the tumor is of high vascularity, and there is significant inter- and intraobserver variation, even when accounting for possible variations in microvessel criteria. In a confirmatory study, two pathologists, trained by Weidner, assessed microvessel density in 220 breast carcinoma patients, with an extended period of follow-up *(25)*. No correlation between microvessel density and clinicopathological variables including patients survival, was observed. These findings are probably the result of the experience of the observers. It has been demonstrated that experienced observers differ significantly from trainee observers in quantifying tumor angiogenesis in breast tumors *(26)*. Nevertheless, in the authors' experience, highly trained observers also occasionally disagree on counts, even with strict adherence to guidelines.

Because of the above problems, together with the additional conceptual difficulty of adjacent microvessels with significantly different vascular parameters assuming equal importance with the set criteria *(4,19)*, the authors and others have explored the use of a number of different techniques, with the aim of developing a rapid and objective method for quantifying tumor angiogenesis.

In an attempt to automate the procedure, improve reproducibility, and overcome the variations associated with manual counting, computer image analysis systems have been assessed *(3,7,8,24–27)*. Using similar endothelial markers and vascular parameters, these reports have confirmed the findings of the manual studies, and shown that quantitative angiogenesis gives independent prognostic information. However, in a manner similar to manual counting, these computer systems have their own general and specific problems, above their capital and running costs. An endothelial marker that will give a high signal-to-noise ratio is essential. This is important, since the software employed to analyze the staining, unlike the human eye, is unable to distinguish specific from nonspecific immunoreactivity. The systems are not usually automated, and require a high degree of operator interaction, and will reproduce what the operator observes. To date, no software is available for identifying hot spots, although partially automated systems with area and shape filters, using defined color tolerances, are available, which require reduced human control. In the authors' experience, a computer-image analysis system is more costly, time-consuming, unsuited to routine diagnostic practice, and no more accurate than a trained observer.

To overcome the time and observer variation associated with microvessel density, the authors have assessed a method based on a point-counting method. The tumor section is scanned at low power (×10–100) to identify the three most vascular fields, before a 25-point Chalkley point eyepiece graticule is placed at high power (×250) over each hot spot *(24)*. The eyepiece graticule is oriented so that the maximum number of points are on or within areas of highlighted vessels, thereby bypassing interpretation difficulties of microvessel counting, and reducing observer bias to the selection of the tumor's area. This technique gives independent prognostic information for breast *(15,24)* and bladder carcinomas.

To simplify the procedure further, and analogous to assessing tumor differentiation, the authors have explored the use of a vascular grading system, based on the subjective appraisal by trained observers over a conference microscope. This method is significantly correlated with both microvessel density and Chalkley count.

2.4. Statistical Analysis

There is considerable variation in cutoffs and types of analyses performed on angiogenesis data. This alone can determine whether a particular angiogneic index correlates

with other clinicopathological characteristics and/or will give prognostic information. Studies have used a variety of stratification indices, including the highest and/or average microvessel density, the mean, the mean count in node-negative patients with recurrence, or variable cutoffs given as a function of tumor area (or microscope magnification. The authors and others have used the median *(12,14,15,28)* or tertile groups *(24)*, since these stratifications avoid making any assumption regarding the relationship between tumor vascularity and other variables, including survival.

3. ADDITIONAL METHODS FOR MEASURING TUMOR ANGIOGENISIS ACTIVITY

Tumor angiogenesis is a complex pathway involving extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis, and many molecules are involved in executing these events. Alternative strategies, using these newly identified molecules as indices of tumor angiogenesis, are being pursued, and in due course these might complement or supercede vascular counts as angiogenic markers.

3.1. Angiogenic Factors and Receptors

In adult tissues, angiogenesis is actively suppressed, so that new vessel formation only occurs during the female reproductive cycle or wound healing. Tumor neovascularization results from the loss of this suppression by altering the balance of angiogenic stimulators to inhibitors. Upregulation of angiogenic factors and their receptors, such as VEGF-A, bFGF, and thymidine phosphorylase (TP), both at the mRNA and protein level, has been reported in a range of histological tumor types *(29)*. In lung *(30)* and breast tumors *(31)*, a significant relationship between microvessel density and VEGF has been observed. VEGF expression is significantly higher in tumors that relapse, compared to those that do not recur, and, in the latter tumor, VEGF gives independent prognostic information (29. Recently, high expression of (KDR), a VEGF receptor, has also been correlated with high vessel counts and advanced-stage colon carcinomas. Furthermore, TP expression in breast carcinomas has also been shown to correlate with tumor vascularity in breast *(32)* and colon cancers *(33,34)*, although not all studies in breast carcinomas have confirmed this observation *(35)*. TP is expressed not only in the neoplastic tumor epithelium, but is also present in stromal fibroblasts, macrophages, and endothelium *(35)*. Thus, a tumor unable to express this angiogenic factor in its neoplastic component might still be able to raise total tumor TP by upregulating this angiogenic factor in ancilliary cells, such as macophages and fibroblasts. Indeed, in colon cancers, in which TP expression rarely occurs in the neoplastic epithelium, the intensity of staining in tumor-infiltrating cells correlates with tumors vascularity *(33)*.

Angiogenic factors have been measured in cancer patients' serum *(36,37)*, urine *(38)*, cerebrospinal fluid *(39)*, and in the cytosol of primary tumors *(39a,39b)*.

3.2. Endothelial Cell Proliferation

The current methods for measuring endothelial cell (EC) proliferation are cumbersome *(23)*, but it is feasible that, in the future, a rapid and technically robust measure might give an index of the angiogenic activity of a tumor. This particular measurement might have more utility in identifying which patients might benefit from those

antiangiogenic agents that effect EC proliferation rather than inhibitors of other aspects of the angiogenic pathway (*see* Sections 3.3.–3.5.).

3.3. Proteolytic Enzymes

The proteolytic systems such as the plasminogen activators *(40)* and the matrix metalloproteinases *(41)*, which are important in tumor cell invasion, are also used for remodelling the existing vasculature during angiogenesis. Elevated levels of urokinase-like plasminagen activator (uPA) and plasminogen activator inhibitor (PAI)-1 are associated with a poor prognosis in several tumor types including breast *(42)* and colon *(43)* A significant correlation between microvessel density and both uPA and PAI-1 has been observed in breast cancer patients *(44)*. Thus, measurement of proteases, particularly the urokinase system, might give an indication of the angiogenic activity of a tumor. Currently, assays using homogenates of primary tumor have shown their potential, but measurement of the components of this system in the serum of cancer-bearing patients might also be a possibility.

3.4. Cell Adhesion Molecules

Endothelial cell adhesion molecules of the immunoglobulin, selectin *(45)* and integrin *(46)* superfamilies, play a major role in angiogenesis. In melanoma patients, upregulation of E- and P-selectins on the tumor endothelium is significantly associated with a shorter survival *(47)*. Furthermore, in addition to the use of CAMs as a prognostic marker, they might be also serve as a target for antiangiogenic or vascular-targeting therapies *(20)*.

3.5. Tumor Vascular Architecture

Newly formed vessels are abnormal in several respects: the capillaries are dilated, saccular, sinusoidal, and tortuous, and there are multiple bifurcations, loops, and blind ending sprouts. It has been suggested that the morphological assessment of particular vascular patterns might distinguish benign from malignant lesions, and act as a prognostic indicator (e.g., a closed back-to-back loop vascular pattern in ocular malignant melanomas) *(48)*.

4. PROGNOSTIC IMPORTANCE OF MICROVESSEL DENSITY IN BREAST CANCER

4.1. Prognosis

Breast carcinoma is the tumor type in which, at present, more information is available on the degree of vascularization and prognosis. The results of the 32 studies published up to now in peer-reviewed journals have recently reviewed been *(49,50)*. Most of these studies have been performed in series including patients with both node-negative and node-positive invasive disease. The conclusions of some other studies should be considered, with caution, however, because of the small number of cases studied, the relatively short period of follow-up, and the heterogeneous findings related to the clinicopathologic characteristics, the criteria of evaluation of the degree of microvessel density, and the different treatments administered (reviewed in ref. *50*). In addition, some studies suffered from a weak statistical approach *(50)*. Since the primary aim of a study assessing the prognostic value of a new biological marker is to verify whether its determination adds

Table 2
Intratumoral Vascularization and Prognosis

Authors	*Endothelial marker*	*Number of patients*	*Stage*	*Median follow-up (yr)*	*Multivariate analysis*		*Ref.*
					RFS	*OS*	
Weidner	fVIII–RA	165	I–II	4.0	<0.001	<0.001	*52*
Bosari	fVIII–RA	180	I–II	9.0	<0.03	<0.03	*53*
Visscher	Type IV collagenase	58	I–IV	5.1	NS	ND	*54*
Obermair	fVIII–RA	64	I–II	4.1	<0.01	ND	*55*
Ogawa	fVIII–RA	155	I–II	7.0	<0.002	<0.001	*56*
Fox	CD31	211	I–II	3.5	ND	0.05	*57*
Toi	fVIII–RA/ CD31	125	I–II	5.1	<0.01	ND	*58*
Toi	fVIII–RA	328	I–II	4.6	<0.0001	ND	*59*
Simpson	fVIII–RA	178	I–II	6	0.002	0.016	*60*
Gasparini	CD31	531	I–II	6.3	<0.001	<0.001	*61*
Bevilacqua	CD31	211	I	6.6	<0.0001	0.044	*62*
Obermair	fVIII–RA	230	I	4.6	ND	<0.001	*63*
Fox	CD31	109	I	2	0.04	0.01	*64*
Heimann	CD34	167	I	20	0.04	ND	*65*
Barbareschi	CD31	91	I	5.5	0.006	ND	*66*
Gasparini	CD31	191	II	5.5	<0.01	<0.01	*67*
Gasparini	CD31	178	II	5.2	<0.01	<0.01	*68*
Hall	fVIII–RA	87	I–II	9.5; 1.5	NS	ND	*69*
Axelsson	fVIII–RA	220	I–II	11.5	NS	NS	*70*
Van Hoef	fVIII–RA	93	I	13	NS	NS	*71*

Published positive studies. ND, not done; RFS, relapse-free survival; OS, overall survival; NS, not significant.

significant information over the conventional prognostic indicators *(51)*, only results of the studies performed with a multivariate analysis are here presented and commented on. Of the 32 studies published, only 20 met this criteria, and, as reported in Table 2, had a multivariate analysis performed at least on relapse-free survival or overall survival *(52–71)*. Only four of these 20 studies reported totally negative results; the majority of authors (80%) found that assessment of intratumoral microvessel density is a statistically significant and independent prognostic indicator for relapse-free survival (RFS), overall survival (OS) or both. Among the four series *(54,69–71)* in which the prognostic value of vascularization did not appear, only the study by Axelsson et al, *(70)* was conducted in a large cohort of patients; less than 100 cases were enrolled in each of the three other negative studies *(54,69,72)*. Furthermore, Visscher et al. *(54)* used an antibody to type IV collagenase to highlight vascular vessels, which is a suboptimal endothelial cell marker, compared to the panendothelial markers used by the other authors. Van Hoef et al. *(71)* used a method different from that developed and suggested by Weidner et al. *(52)*, to evaluate the degree of vascularization, and, in particular, microvessel count was performed on a very small area. Finally, in the studies by Hall et al. *(69)* and Axelsson et al. *(70)*, the patients were not consecutive *(69)*, and they were selected from a larger series, respectively *(70)*.

Table 3
Prognostic Value of Microvessel Density
in Breast Cancer Patients Treated with Adjuvant Therapy

Author	Number of patients	Stage	Median follow-up (yr)	Adjuvant treatment	Univariate analysis		Multivariate analysis		Ref.
					RFS	OS	RFS	OS	
Weidner	82	II	4.0	Heterogeneous	<0.001	<0.001	<0.001	<0.001	*52*
Toi	198	II	4.6	Heterogeneous	<0.001	<0.001	<0.001	<0.001	*59*
Gasparini	191	II	5.5	CD31	<0.001	<0.001	<0.001	<0.001	*67*
Macaulay	88	I–II	2.5	Tamoxifen	0.02	ND	ND	ND	*73*
Gasparini	178	II	5.2	CD31	<0.001	<0.001	<0.001	<0.001	*68*

RFS, relapse-free survival; OS, overall survival; ND, not done.

Therefore, taking into account the above criticisms, the probability that these four studies may report false negative results on the prognostic value of intratumoral microvessel density seems to be quite high. Some positive studies also present weaknesses, because they were conducted on too small a series *(55,67)*, or because the period of observation was relatively short *(57,64)*, their results are to be considered as preliminary.

From a methodological point of view, homogeneity of the clinicopathologic characteristics among the patients studied is of relevance for its clinical implications in breast cancer. Up to now, only five studies were conducted in node-negative patients only *(62–66)*, but only two series assessed more than 200 cases *(62,63)*.

Similarly, only two studies evaluated the prognostic value of vascularization by multivariate analysis in node-positive patients treated with a well-defined schedule of adjuvant therapy *(67,68)*. The overall picture emerging from the above reported meta-analysis, performed on retrospective studies, is that determination of intratumoral microvessel density is a promising prognostic indicator. However, prospective studies performed on more homogeneous groups of patients, with quality controls and standardization of the method *(72)*, are warranted prior to a possible wider application of angiogenesis in clinical practice.

4.2. Prediction

A useful marker for clinical decision should also be predictive of the efficacy of therapy. Up till now, only a few studies evaluated the clinical outcome of patients with operable breast cancer treated with adjuvant therapy in relation to the vascularization of the primary tumor. Some conclusion can be drawn from the results of five published studies *(52,59,67,68,73)*, which enrolled patients treated with heterogeneous adjuvant treatments *(52,59)*, chemotherapy *(67)*, or hormone therapy (tamoxifen), *(67,68,73)* (Table 3).

The most interesting general observation is that all these authors *(52,59,67,68,73)* found that the patients with highly vascularized primary cancers had poor outcome, even if they received a conventional adjuvant therapy. Because it has been shown that in breast cancer the degree of vascularization of the tumors is significantly correlated to high expression of angiogenic peptides *(74–77)*, it seems reasonable to hypothesize that the subgroup of breast cancer patients characterized by highly vascularized cancers are those

Table 4
Prognostic Value of Microvessel Density in Nonsmall-Cell Lung Cancers

Author	*Endothelial marker*	*No. pts.*	*Stage*	*Median follow-up (mo)*	*Prognostic value*	*Ref.*
Macchiarini	fVIII–RA	87	I	60	Yes	*79*
Yamazaki	fVIII–RA	42	I–IV	71	Yes	*80*
Fontanini	fVIII–RA	253	I–III	24	Yes	*81*
Giatromanolaki	CD31	107	I–II	36	Yes	*82*
Angeletti	fVIII–RA	96	III	24	Yes	*83*
Apolinario	CD31	116	I–III	60 (stage II only)	Yes	*84*
Fontanini	CD34	470	I–III	29	Yes	*85*
Fontanini	fVIII–RA	73	I–III	47	Yes	*86*

that are more likely to obtain benefit from inhibition of angiogenesis, which represents a promising new therapeutic strategy for treatment of breast cancer and other solid tumors. Once safe and active antiangiogenic drugs are available for adjuvant therapy, the identification of surrogate markers, related to the target molecules involved in the pharmacological modulation of angiogenesis, will be of utmost importance in rationalizing the identification of patients who have a higher likelihood to be responsive to compounds inhibitory to angiogenesis, and to monitor the duration of efficacy to such agents *(78)*.

5. PROGNOSTIC IMPORTANCE OF MICROVESSEL DENSITY IN OTHER CANCERS

5.1. Cancers of the Respiratory Tract

Macchiarini et al. *(79)* first assessed whether the degree of angiogenesis correlates with metastasis in nonsmall-cell lung cancer (NSCLC). They determined intratumoral microvessel density in a series of 87 patients with initial tumor stage T1N0M0 who underwent radical surgery. The 22 patients who developed recurrence during the follow-up period had tumors with a statistically higher vascularization, compared to those alive and disease-free. Seven subsequent studies *(80–86)* investigated the prognostic significance of vascularization in NSCLC, and, in all the series, the patients with radically resected, highly vascularized primary tumors had poorer prognosis, compared to those with low angiogenic tumors (Table 4). Of particular interest were the studies by Apolinario et al. *(84)*, suggesting that the prognostic value of neovascularization is more relevant in patients with stage II disease, and that the degree of angiogenesis was not related to the other biological (p53, bcl-2, and bax oncoproteins) or pathological markers studied.

Fontanini et al. *(85)* recently published results obtained in a very large series (470 patients) with stages I–III NSCLC. On multivariate analysis, intratumoral microvessel density, tumor size, and regional lymph node status all retained independent prognostic value for overall survival. In another study, Fontanini et al. *(86)* also found that highly vascularized tumors had a significantly more elevated nuclear expression of p53 protein, and more elevated levels of VEGF. Microvessel count retained statistically prognostic value in a multivariate statistical model, also including the other biological variables above.

Table 5
Prognostic Value of Microvessel Density in Genitourinary Cancers

Author	*Endothelial marker*	*No. pts.*	*Stage*	*Median follow-up (mo)*	*Prognostic value*	*Ref.*
Testicular germinal cell tumor						
Olivarez	fVIII–RA	65	A	>3	Yes	*89*
Prostatic cancer						
Wakui	Antivimentin	101	B–D	ND	Yes	*91*
Fregene	fVIII–RA	34	B–D	ND	Yes	*92*
Weidner	fVIII–RA	74	B–D	4.5	Yes	*93*
Vesalainen	Collagen IV	88	B	>11	Yes	*94*
Brawer	fVIII–RA	37	B–D	ND	Yes	*95*
Silberman	CD31	109	B–D	7	Yes	*96*
Barth	fVIII–RA	41	B–D	ND	Yes	*97*
Rogatsch	CD31	46	B-C	ND	Yes	*98*
Bladder Cancer						
Dickenson	CD31	45	Invasive	4.0	Yes	*99*
Bochner	CD34	164	Invasive	6.6	Yes	*100*
Grossfeld	CD34	163	Invasive	7.7	Yes	*101*

ND, not done.

However, a recent study by Pezzella et al. *(87)* identified a minority of invasive NSCLC whose growth does not seem to require angiogenesis, and that are characterized by a histologic architecture with an alveolar pattern. Finally, Kumar-Singh et al. *(88)* assessed microvessel count on 25 specimens of mesothelioma, and found that the patients with high angiogenic lesions had a significantly shorter survival than those with lower microvessel counts. In addition, in this malignancy, the degree of vascularization was not related to p53 expression.

5.2. Cancers of the Genitourinary Tract

Up till now, only the study by Olivarez et al. *(89)* evaluated the clinical significance of neovascularization in testicular germ cell tumors. They assessed vascularity of 65 primary tumors of patients with clinical stage A, using the factor VIII-RA.

After surgery, 43 patients had pathologic involvement of the retroperitoneal lymph nodes (histolgic stage B); the other 22 patients had no metastasis in the lymph nodes (pathologic stage A). Retrospectively, the authors found that quantitation of microvessel density was significantly predictive of occult metastatic disease on univariate analysis. However, in the logistic regression multivariate analysis, only absence of yolk-sac tumor elements retained significance in predicting pathological stage B.

Viglietto et al. *(90)* analyzed the expression of mRNA VEGF in human germ cell tumors, and found that more than 80% of the tumors were VEGF-positive, and that high levels of its expression are associated with high vascularization.

Much more information is available on the association of microvessel density with stage disease in prostatic cancer *(91–98)* (Table 5). Wakui et al. *(91)* first reported that the blood capillary density ratio, as assessed by a marker antivimentin, was predictive of bone marrow metastasis. All the other authors *(91–98)* who performed the subsequent

studies confirmed that the degree of vascularization of the primary tumor is predictive of advanced pathologic stage or metastasis. Regarding invasive bladder carcinoma, all the published studies evaluating the prognostic value of microvessel density found that it is a significant and independent prognostic indicator *(99–101)*. Grossfeld et al. *(101)* also assessed the expression of p53 protein and thrombospondin-1, and found that microvessel density counts were significantly associated with both the above biological markers.

Several authors also evaluated the expression of angiogenesis factors, such as VEGF, fibroblast growth factors (FGFs), TP, hepatocyte growth factor (or scatter factor), and pleiotrophin in human cancer of the bladder (reviewed in ref. *102*). The most important factors mediating angiogenesis in such a tumor seem to be acidic and basic FGFs, VEGF, and TP. A differential expression of VEGF and TP in superficial and invasive cancers suggests that different angiogenic pathways may occur at different stages of this neoplasm.

5.3. Cancers of the Digestive Tract

The prognostic value of microvessel density was little investigated in esophageal cancers. The study by Kumano et al. *(103)* suggested a significant association of high vascularization with the expansive and down-growth patterns of tumor growth patterns. Moreover, both carcinoma *in situ* and microinvasive cancers had higher microvessel counts, compared to the adjacent normal mucosa. Tanigawa et al. *(104)* stained intratumoral microvessels of 43 esophageal squamous cell carcinomas with anti-CD34 and fVIII-RA antibodies. Both the markers were found to be significant and independent prognostic markers. Regarding gastric carcinomas, the studies by Maeda et al. *(105)* and Tanigawa et al. *(106)* found that tumor angiogenesis was predictive of recurrence and time to progression and overall survival, respectively, in patients with invasive gastric carcinomas. Microvessel density was found to be significantly higher in VEGF-positive gastric carcinomas than in those VEGF-negative. Moreover, the patients with VEGF-positive tumors had a significantly poorer prognosis, with a particularly high frequency of hepatic metastases *(107)*.

At least 5 studies *(108–112)* assessed the prognostic value of microvessel density in colorectal cancer (Table 6). Tumor angiogenesis was found to be significantly associated with transmural penetration and early death in a series of 48 patients with heterogeneous stages of rectal carcinoma *(108)*. Subsequent studies by Tomisaki et al. *(109)* and Takebayashi et al. *(110)* suggested that the degree of angiogenesis of primary colorectal cancers is in prognostic value, and predictive of high probability of liver metastasis. Bossi et al. *(111)* observed that invasive carcinomas of the colorectum had a significantly higher vascularization of adenomas of normal mucosa. However, angiogenesis was not found to be of prognostic value in the series of 178 patients with heterogeneous stage of disease. Finally, the study by Lindmark et al. *(112)* compared three markers to highlight microvessels, and then assessed neovascularization of 212 consecutive patients with colorectal cancer using fVIII-RA. Contrary to the findings reported in all the other studies published up until now on angiogenesis and prognosis in solid tumors, the authors *(112)* observed that the patients with highly vascularized cancers had a significantly longer survival than those with low angiogenic tumors. However, these unexpected results may be impaired by some weaknesses in the methodology used *(113)*. Studies performed by Vermeulen et al. *(114)* suggest that anti-CD31 antibody is the more sensitive marker for assessing angiogenesis in colorectal cancer, that the endothelial cells have a lower pro-

Table 6
Prognostic Value of Microvessel Density in Esophageal and Gastrointestinal Tumors

Author	*Endothelial marker*	*No. pts.*	*Stage*	*Median follow-up (mo)*	*Prognostic value*	*Ref.*
Esophageal cancer						
Tanigawa	CD34/fVIII–RA	43	I–V	3.1	Yes	*103*
Gastric cancer						
Maeda	fVIII–RA	124	I–IV	>5	Yes	*105*
Tanigawa	CD34	181	I–IV	4.1	Yes	*106*
Colorectal cancer						
Saclarides	fVIII–RA	48	A–D (Aster-Coller)	4.4	Yes	*108*
Tomisaki	fVIII–RA	175	A-D (Dukes)	5.0	Yes	*109*
Takebayashi	fVIII–RA	166	A–C (Dukes)	6.3	Yes	*110*
Bossi	CD31	178	I–IV (TNM)	5.0	No	*111*
Lindmark	II/CD31	212	A–C (Dukes)	4.5	Yes[a]	*112*

[a]High vascularity associated with good prognosis

liferative rate than tumor cells, and that there is a significant positive correlation between vascularity with tumor cell proliferation rate *(115)* and p53 protein overexpression *(116)* in human invasive colorectal adenocarcinoma.

5.4. Cancers of Head and Neck

Albo et al. *(117)* studied angiogenesis in a small series of patients with squamous cell carcinomas of the head and neck, using fVIII-RA to stain microvessels. They found that the patients with highly vascularized primary tumors had a significantly higher frequency of recurrent or metastatic disease than those with low angiogenic cancers. A subsequent study by Williams et al. *(118)* found that angiogenesis is a significant prognostic indicator of recurrence in a series of 66 patients with oral cavity tumors.

Klijanienko et al. *(119)* assessed vascularization of 114 primary head and neck squamous cell carcinomas by morphologic criteria without a specific endothelial marker. In this series, tumor vascularization correlated with tumor mitotic index, nuclear grade, peritumoral vessel invasion, and lymph node involvement. The authors concluded that tumor vascularization is an important histologic feature associated with the biological aggressiveness of such type of tumors.

Gasparini et al. *(120)* determined several predictive and prognostic markers in 73 patients with head and neck squamous cell invasive carcinoma, who were treated with concurrent cisplatin or carboplatin and radiation therapy for stage II–IV disease. Vascularization was assessed by the anti-CD31 antibody, and it was significantly predictive of the probability of response to therapy, but not of prognosis. The patients with highly vascularized primary tumors had a significantly lower probability of obtaining a complete response with therapy, compared to those with low angiogenic cancers. In addition, the degree of microvessel density did not correlate with bcl-2 expression or PCNA labeling rate, but it was significantly associated with p53 expression. As found in another report by the same investigators *(121)*, both microvessel density and p53 protein expres-

Table 7
Prognostic Value of Microvessel Density in Head and Neck Cancer

Author	*Endothelial marker*	*No. pts.*	*Stage*	*Median follow-up (mo)*	*Prognostic value*	*Ref.*
Albo	fVIII–RA	10.0	III–IV	ND	Yes	*117*
Williams	fVIII–RA	66.0	I–III	>5	Yes	*118*
Klijanienko	HαE	114.0	I–III	ND	Yes	*119*
Gasparini	CD31	73.0	III–IV	1.4	No[a]	*120*
Roychowdhury	fVIII–RA	30.0	I–IV	3.6	Yes	*122*

[a]High vascularized tumors had a significant poor response to chemoradiation therapy.
ND, not done.

sion are predictive of metastasis in head and neck cancer. Finally, Roychowdhury et al. *(122)* determined tumor angiogenesis and c-erbB-2 expression in the primary cancer of 30 patients with nasopharyngeal squamous cell carcinoma. Both the markers were found to be of prognostic value, and capable of identifying the subgroup of patients at high risk of recurrence and death who may benefit from more intensive therapeutic strategies (Table 7).

5.5. Gynecological Cancers

Three studies reported on the prognostic significance of angiogenesis in advanced stage ovarian carcinoma *(123–125)*. All the series studied enrolled patients with FIGO stage III–IV disease, who treated with cisplatin-based combined chemotherapy. Both Hollingsworth et al. *(123)* and Gaparini et al. *(124)* reported that the patients with highly angiogenic tumors had poorer prognosis, compared to those with low-vascularized carcinomas. Gasparini et al. *(125)* also observed that mucinous carcinomas were more vascularized than the other tumor types, and that the degree of microvessel density and performance status were significant and independent predictors of pathologic response to therapy. Van Diest et al. *(125)* used a less sensitive marker to stain endothelium (ulex europeus I), and did not find microvessel count to be of prognostic value.

Recently, Paley et al. *(126)* evaluated the expression of VEGF in early-stage (FIGO I–II) ovarian cancer, and found that elevated expression of VEGF was associated with poor survival. Some studies (reviewed in ref. *127*) assessed the prognostic value of vascular density in cervical cancer, and the majority of the authors found that it is a valid prognostic marker of survival and response to specific treatments. A smaller number of studies assessed tumor angiogenesis in endometrial carcinoma. Abulafia et al. *(128)* determined microvessel density, using the fVIII-RA antibody in 19 subjects with benign endometrium, 24 patients with endometrial hyperplasias, and 34 with FIGO stage I carcinoma. Patients with hyperplasia had a significantly higher vascularization than those with benign endometrium. Moreover, the patients with myometrial invasive carcinomas had higher vascularization than those without invasive carcinoma. Very similar results were also observed by Morgan et al. *(129)*, and, recently, Kaku et al. *(130)* found that microvessel density is a significant and independent prognostic factor for relapse-free survival and overall survival in patients with FIGO stage I–II endometrial carcinoma.

Table 8
Prognostic Value of Microvessel Density in Malignant Melanoma

Author	*Endothelial marker*	*No. pts.*	*Thickness (mm)*	*Median follow-up (mo)*	*Prognostic value*	*Ref.*
Srivastava	Ulex	20	0.76-4.00	>5	Yes	*131*
Fallowfield	Ulex	64	0.48-18.5	ND	Yes	*132*
Carnochan	Ulex	107	0.85-1.25	>5	No	*133*
Busam	Ulex	120	Invasive	8.9	No	*134*
Graham	CD34/fVIII–RA	37	0.76	>10	Yes	*135*
Vlaykova	CD31	31	0.76	>3	Yes	*136*

5.6. Malignant Melanoma

The vascularity of 20 primary skin melanomas of intermediate thickness was first assessed histologically using the Ulex europaeus-I-agglutinin conjugated with peroxidase and image analysis in 1988, by Srivastava et al. *(131)*. The percentage of vascular area at the tumor base was significantly higher in the patients with recurrence, compared to those disease-free.

Using the same marker, Fallowfield and Cook *(132)* studied the degree of vascularization in 64 primary cutaneous melanomas, and they found a significant association between the percentage of vascular volume and the thickness of the lesion. However, the studies by Carnochan et al. *(133)* and Busam et al. *(134)*, performed on larger and more homogeneous series of patients, did not confirm that tumor vascularity was of prognostic value. More recently, both Graham et al. *(135)* and Vlaykova et al. *(136)* used more-sensitive panendothelial markers, and found that the extent of vascularization was a significant prognostic indicator. Therefore, although the results of these studies are contradictory, it is encouraging that, if a sensitive and specific marker of endothelium is used, assessment of microvessel density is of prognostic value (Table 8).

5.7. Hematological Malignancies

Vacca et al. *(137)* assessed microvessel area in bone marrow by staining endothelial cells with fVIII-RA and an image analyzer in 46 patients with multiple myeloma (28 with active and 18 with nonactive disease), and in 21 patients with monoclonal gammopathies. They found that microvessel area was significantly higher in patients with myeloma, compared with those with benign gammopathies. Furthermore, among myelomas, those active, relapsed, or in progression had higher percentage of microvessel area than those with nonactive disease. A high correlation was also observed between the extent of bone marrow angiogenesis and the proliferating rate of plasma cells. The same group *(138,139)* first evaluated angiogenesis in B-cell lymphoproliferative disease and non-Hodgkin lymphomas (NHL). Angiogenesis in involved lymph nodes correlated with the Working-Formulation grade of malignancy and tumor cell proliferative activity.

Immature vessels were more frequent in diffuse intermediate-grade and high-grade B-NHL. A diverse pattern of vascularization was identified between nodular (follicular) and diffuse histologic-types of NHL. More recently, Perez-Atayde et al. *(140)* evaluated the spectrum of angiogenesis in the bone marrow of 40 children with acute lymphoblastic

leukemia (ALL). The degree of angiogenesis was also evaluated in 22 of these patients after the completion of remission chemotherapy. The authors observed that the patients with ALL had a significantly higher angiogenesis than normal controls. No difference in microvessel density was observed by comparing bone marrow biopsies before and after chemotherapy. Similarly, also, the urinary levels of bFGF were higher in ALL, compared with controls.

5.8. Brain Cancers

Two reports described the pattern of angiogenesis and the prognostic significance of microvessel density in human brain tumors. The first study was conducted by Li et al. *(141)* in brain tumors of infancy. Microvessel density correlated with the levels of bFGF in cerebrospinal fluid, and with the probability of recurrence and death in a series of 26 children with heterogeneous histologic types of primary brain tumors. The second study *(142)* assessed microvessel density as a prognostic indicator in a series of 117 adult patients with astroglial brain tumors. The patients with highly vascularized primary tumors had a significantly shorter survival than those with low vascularized cancers. The prognostic value of angiogenesis was retained also in multivariate analysis.

6. CONCLUSIONS

6.1. Prognosis

Taking into account the potential biases of retrospective studies in evaluating the prognostic relevance of a new marker, the value of determination of intratumoral microvessel density seems to be different among the diverse tumor types. An attempt at classification is proposed, as follows:

1. Highly promising prognostic value: Assessment of vascularization seems to be a new relevant prognostic tool in early-stage invasive breast, NSCLC, and prostate cancers. In these three tumor types, the majority of retrospective studies found a significant correlation between vascularization of the primary tumor and clinical outcome of the patients. Therefore, prospective controlled studies to evaluate the clinical significance of angiogenesis are particularly recommended in such tumors.
2. Promising prognostic value: When a specific panendothelial marker and valid methodology and criteria of evaluation were adopted, several studies on microvessel density and prognosis gave positive results in patients with head and neck, gastric, bladder, ovarian, and endometrial cancers.

   Furthermore, the two studies on vascularity and prognosis conducted in children with brain tumors *(141)* or adults *(142)* found a significant correlation between microvessel count and clinical outcome. Similarly, the few studies performed until now on angiogenesis in bone marrow or lymph nodes of patients with hematologic malignancies were positive.

   Prior to suggesting prospective clinical trials, more information from well-designed and large retrospective studies are needed to better identify the tumor types and subgroups of patients in whom the prognostic value of angiogenesis is more promising.
3. Contradictory results: Retrospective studies on microvessel density in primary colorectal and malignant melanoma of the skin gave contradictory results on the prognostic significance of such a marker. Therefore, more homogeneous groups of patients need to be evaluated prior to drawing any definitive conclusions.

6.2. Prediction

6.2.1. Adjuvant Therapy

The data summarized in Table 3 suggest that the patients with high-risk, operable breast cancer, with highly vascularized tumors, do not benefit from conventional adjuvant hormone or chemotherapeutic treatments. However, a proper evaluation of the predictive capability of a marker needs a prospective controlled, randomized comparative study of therapy vs no therapy. Such a study design may also identify the subgroup of patients who do not benefit from conventional anticancer therapy, for whom novel therapeutic approaches, such as inhibition of angiogenesis, may be useful.

6.2.2. Therapy for Advanced Stages

The studies by Hollingsworth et al. *(123)* and Gasparini et al. *(124)*, on vascularization of patients with advanced stages of ovarian cancer suggest that highly vascularized ovarian cancers are poorly responsive to platinum-based combined chemotherapy. Similar findings were reported also for patients with highly vascularized squamous cell carcinomas of the head and neck treated with concurrent chemoradiation therapy *(120)*. In addition, Vacca et al. found that the patients with active or recurrent or progressive multiple myeloma treated with chemotherapy had a significantly higher degree of angiogenesis in the bone marrow, compared to those with nonactive or responsive disease.

Once antiangiogenic compounds become suitable for phase II–III clinical trials, the search for surrogate markers of responsiveness to the pharmacological modulation of angiogenesis will then be another area of translational research of clinical importance, allowing for a more rationale therapeutic approach.

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19 Endpoints for Determination of Efficacy of Antiangiogenic Agents in Clinical Trials

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CONTENTS

1. INTRODUCTION

The field of angiogenesis research has undergone dramatic growth in recent years, as evidenced by the massive number of citations appearing in the medical literature *(1)*. Until very recently, the focus of most work has been on defining the steps in the angiogenesis cascade, and the naturally occurring factors that both induce and inhibit the process. Over 20 years ago, Judah Folkman postulated that if tumor angiogenesis could be inhibited, then a potentially novel and effective treatment strategy could be developed for solid tumors *(2)*. That hope is now coming to fruition as novel agents that inhibit tumor angiogenesis are entering clinical trials. As outlined in previous chapters, many of the compounds that are known to inhibit angiogenesis behave in a cytostatic fashion. Unlike classic cytotoxic chemotherapy agents, inhibitors of tumor angiogenesis may not cause tumor shrinkage but instead maintain a stable tumor size. The challenge facing clinical investigators and pharmaceutical companies developing these drugs is to carefully and thoughtfully design clinical trials that take into consideration the cytostatic behavior of angiogenesis inhibitors (AI) *(3)*. Assessments of efficacy of AIs must avoid the trap of requiring tumor shrinkage

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Table 1
Original Fibonacci Number Series Used for Dose Escalation of a Drug in a Phase I Clinical Trial

Multiple of initial number	*% Increase over previous dose*
1n	–
2n	100
3n	50
5n	67
8n	60
13n	63
21n	62
34n	61

in order to continue clinical development. As a result, end points other than tumor regression must be incorporated into clinical trial design.

2. CLASSIC PHASE I CLINICAL TRIAL DESIGN

The primary objective of a traditional phase I clinical trial is the toxicity assessment of a new agent undergoing its first evaluation in patients, or a new combination of existing drugs. The primary end point of interest in a phase I trial is defining the maximum tolerated dose (MTD) of a particular drug or combination of drugs using a specific administration schedule *(4)*. Secondary objectives of phase I trials include the characterization of the clinical pharmacokinetics and pharmacodynamics of the new agent, as well as any observation of antitumor activity as suggested by physical examination or radiological evaluation *(5)*. Although the primary objective of a phase I clinical trial is identification of the MTD, cytotoxic agents that show no hint of antitumor activity in phase I trials are unlikely to be successful in later phases of development.

Cancer patients generally recruited to phase I clinical trials are those who have become refractory to standard therapies, or for whom no standard therapies exist. Although many of these patients have been heavily pretreated, most phase I trials require that patients have an excellent performance status and relatively normal laboratory parameters (i.e., hematology, renal, hepatic) *(6,7)*.

The starting dose of a drug selected for a phase I clinical trial is based on the results of toxicology data generated in animals. Typically, at least two species are tested with the new agent; one of these is the mouse. Usually, the starting dose in humans is 10% of the lethal dose in 10% of mice (LD10), unless unexpected or irreversible toxicity is observed in the second species receiving the agent *(4,8)*. Cohorts of three patients are assigned to each dose level and doses are escalated according to a standard or modified Fibonacci schema (Table 1; *9*). Toxicity is graded according to a standardized system, such as National Cancer Institute (NCI) toxicity criteria *(10)*. If one patient develops grade 3 or 4 toxicity at a particular dose level, an additional three patients are assigned to that dose level. The MTD is usually defined as the dose of drug at which dose-limiting toxicity (grade 3/4) occurs in at least 2 of 6 patients enrolled at that dose level. No further dose escalation would occur, and the drug dose recommended for further evaluation in phase II, disease-oriented, clinical trials (RPTD) is the dose level immediately preceding

the MTD. Additional patients may be enrolled at the RPTD in an effort to better characterize the pharmacokinetics and pharmacodynamics of the drug *(4)*.

As suggested above, the typical phase I clinical trial includes a very heterogeneous patient population in terms of prior treatment and underlying malignancies. Phase I clinical trials can be disease-directed, if significant preclinical activity is observed in tumor xenograft models. This type of disease-directed trial will optimally define the toxicity and pharmacology of a new drug(s) in a homogenous patient population, but, depending on the prevalence of the disease (i.e., breast or lung cancer vs cholangiocarcinoma), patient accrual to the trial may be slow, and, as a result, delay the identification of the MTD.

More recently, alternative phase I clinical trial designs have been considered, in an effort to more quickly identify the MTD, while simultaneously limiting the number of patients required at each dose level *(4)*. These alternative phase I trial designs include continual reassessment methods *(11)*, toxicity-based escalation schemes *(12)*, stochastic approximation methods *(13)*, model-guided dosing *(14)*, pharmacokinetically guided escalation *(15)*, up and down designs *(16)*, patient choice and race/gender assessment designs, and mechanism of action-based dose escalation *(17)*. The latter type of trial design has particular relevance to evaluation of compounds that may inhibit angiogenesis, and are thought to be primarily cytostatic in behavior. A pharmacodynamic end point may not be relevant. As an example, an assay that detects the degree of enzyme inhibition by the new agent at a particular dose level may be most relevant.

3. PHASE I TRIAL DESIGN CONSIDERATIONS FOR INHIBITORS OF ANGIOGENESIS

In the initial evaluation of a classic cytotoxic agent, phase I trials define the MTD, and characterize pharmacokinetics and pharmacodynamics. Generally, a dose–response relationship is observed in subsequent evaluation of the drug, so that dose levels significantly lower than the RPTD result in lower overall tumor-response rates. Tumor response rates have been the gold standard for judging the efficacy of cytotoxic drugs commonly used to treat solid tumors *(18)*. A complete response (CR) is defined as complete resolution of all evidence of cancer on physical exam, and normalization of any abnormal radiology tests (i.e., CT scans, X-rays). A partial response (PR) is defined as greater than 50% reduction in the bidimensional tumor measurements, lasting for at least 4 wk. Stable disease (SD) is defined by no change in bidimensional tumor measurements or <5% increase in bidimensional tumor measurements for at least 4 wk. The overall response rate (ORR) equals the addition of the CR and PR rate. Of interest and relevance to the discussion of cytostatic agents, such as AIs, is the relatively recent incorporation of SD status into reports of overall response rates (CR + PR + SD = ORR). Clinical trials of hormonal agents in metastatic breast cancer (i.e., tamoxifen, aromatase inhibitors, and so on) commonly report SD of 6 mo duration or longer as part of the ORR *(19)*. This new convention is appropriate for cytostatic agents, with which disease regression may not be observed clinically.

The design of phase I clinical trials of AIs may require consideration of alternative end points in addition to, or instead of, those of primary importance in the evaluation of classic cytotoxic chemotherapy agents. The first consideration is that MTD may be important to characterize toxicities associated with the AI, but the actual dose of drug required to attain

the desired biologic effect may be significantly lower than the MTD. Determining the MTD may remain relevant and important, because AI therapy may be most efficacious when administered chronically. An understanding of the complete toxicity profile of a given AI may be important in order to anticipate toxicities that may not be detected with short-term dosing. Since AIs as a class of compounds are viewed as noncytotoxic, it may be feasible and reasonable to conduct single-dose studies in normal volunteers in an effort to define the MTD, if there is one to a single dose of the drug. This information may provide insight into the toxicities that may be observed in the formal phase I trial in patients.

4. OPTIMAL BIOLOGIC DOSE

Defining the optimal biologic dose (OBD) of an AI will ultimately be of primary importance in AI drug development, but it will also be the most challenging aspect of the drug to define. The OBD of an AI may be very different than the MTD. If one relies entirely on the MTD to identify the appropriate dose of the AI for a phase II trial, patients may be subjected to a dose of drug associated with toxicity in excess of what might be expected using the OBD dose. More importantly, exceeding the OBD may negate the desired biologic effect (Fig. 1). The "more is better" credo may not necessarily apply to cytostatic agents, such as AIs.

The experience with cytostatic hormonal agents in the treatment of metastatic breast cancer is particularly instructive in this respect. Tamoxifen is the endocrine therapy of choice for all stages of breast cancer, and the most prescribed cancer medication in the world *(20)*. The antitumor effect of tamoxifen is mediated primarily through the estrogen receptor (ER). The tamoxifen–ER complex that is formed is incompletely converted to the activated form (e.g., estrogen–ER complex), and as a result the complex is only partially active in initiating the programmed series of events necessary to initiate gene activation required for cell growth and proliferation *(20)*. Early clinical trials of tamoxifen demonstrated that, although higher daily doses than the standard of 20 mg/d did not result in a significant increase in acute toxicity, there was no additional antitumor activity, defined as an increase in ORR (e.g., CR + PR + SD) *(20)*. It is now appreciated that chronic administration of tamoxifen at does higher than 20 mg/d may be associated with an increase risk of endometrial carcinoma and thromboembolism *(20)*.

Another example that demonstrates this concept is the use of the new aromatase inhibitor, anastrozole, for the treatment of metastatic breast cancer. Anastrozole, as opposed to its predecessor, aminoglutethimide, is a selective aromatase inhibitor that blocks the conversion of androstenedione to estrone. The drug is most effective in postmenopausal women with ER-positive tumors, in whom nonovarian sites of aromatase activity predominate (i.e., adipose tissue, liver, muscle) *(21)*. During the development of anastrozole, several doses of drug were evaluated to determine the dose that most effectively suppresses estradiol levels *(21)*. A daily dose of 1 mg anastrozole was optimal, and, even in the pivotal clinical trial, there was no advantage to higher daily doses of anastrozole in terms of ORR. These examples support the notion of seeking the OBD, rather than the MTD, to guide AI development.

5. SURROGATE END POINTS

The corneal assay is a powerful tool that provides a means to assess the in vivo biologic activity of a systemically administered AI *(22)*. Obviously, such an approach is not

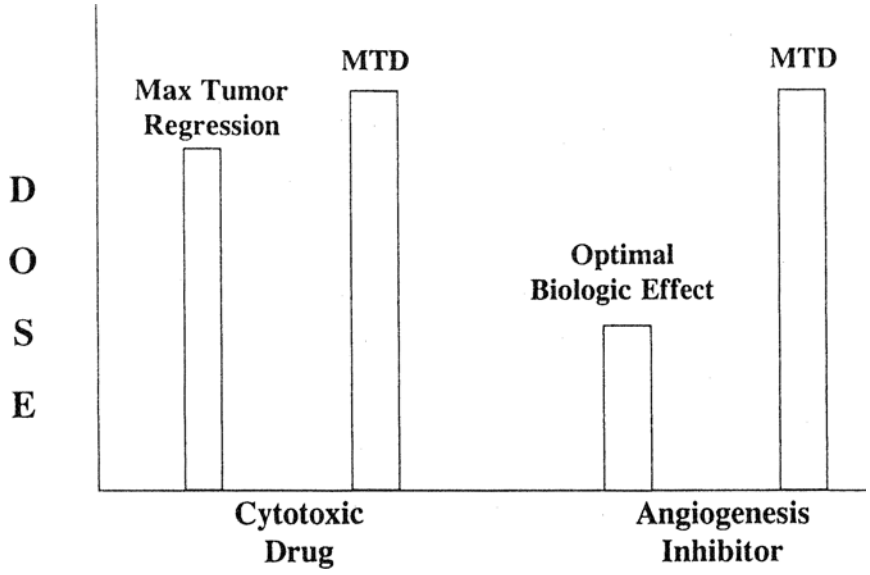


Fig. 1. Hypothetical difference between ideal dose of a cytotoxic drug and the OBD of an angiogenesis inhibitor, in relation to MTD.

appropriate in human subjects *(23)*. Surrogate end points need to be incorporated into clinical trial design of AIs, but surrogate end points that truly correlate with the biologic activity of the AI, and would be feasible to assess in patients, have proven difficult to identify.

If the presumed mechanism of action of a particular AI is the inhibition of an enzyme (i.e., matrix metalloproteinases [MMP]) that controls a step in the angiogenesis cascade, then a more relevant end point in a phase I trial may be to determine the dose of AI that will inhibit the activity of that enzyme to a certain degree. A corollary of that example is the presumption that preclinical data will help predetermine what degree of enzyme inhibition (i.e., 25 vs 50 vs 90% inhibition) is biologically relevant to achieve the desired effect. A more significant challenge will involve the identification of surrogate end points that correlate with the desired biologic effect at the target-tissue level.

Considering the example of matrix metalloproteinase inhibitors (MMPI), several issues need to be considered. MMPs are a family of structurally related zinc-containing enzymes involved in the degradation of extracellular matrix proteins during tumor growth and progression, as well as normal tissue remodeling, such as wound healing and bone fracture repair *(24)*. The MMP family includes collagenases, stromelysins, matrilysin, gelatinases, and metalloelastase *(24)*. The MMPI agents in development include those with broad inhibitory activity against several enzymes, as well as agents with more selective inhibitory activity. In addition, certain tumor types preferentially overexpress one or more MMPs *(25,26)*. This knowledge may influence trial design. One could determine if patients with tumors that strongly overexpress a certain MMP are more likely to benefit from treatment with a particular MMPI, compared to patients who weakly express that MMP. If sensitive assays are available, changes in the circulating serum concentration of the MMP target could be determined before therapy, and over the

course of treatment. Similarly, zymographic determination of intratumor ratios of the activated proenzymatic forms of MMPs, or urinary products of bone turnover, could be determined *(27)*. A recent report also suggests that circulating MMP-2:TIMP-2 ratios provide useful information regarding prognosis in patients with urothelial tumors after complete resection *(28)*. One could speculate that this analysis could be applied to patients while they undergo active therapy with an MMPI targeting MMP-2. This information may provide an insight into which patients are most likely to benefit from this form of therapy.

6. INTRATUMORAL MICROVASCULAR DENSITY

Intratumoral microvessel density (MVD) has been extensively studied in a variety of solid tumors and has been reported as a powerful independent prognostic factor in many tumor types *(29)*. Several clinical reports have suggested a strong correlation between intratumoral MVD and the development of metastases, primary tumor growth, and the expression of certain angiogenic peptides *(30)*. A variety of methods for assessing MVD have been reported *(31)*, but the most commonly used is that proposed by Weidner et al. *(32)*. Analysis of sections from paraffin blocks containing invasive tumor specimens are stained with hematoxylin and eosin. The sections are then stained with endothelial-cell-specific antibodies, such as anti-FVIII-Rag, anti-CD31, and anti-CD34. (The optimal endothelial cell antibody stain is also a subject of debate, since the sensitivity and specificity of individual antibodies is variable, as is their ability to stain intratumoral lymphatics *[31]*.) Vascular "hot spots" are identified by scanning the section under low-power magnification (×10–100). The rationale for identifying vascular hot spots is based on the notion that these areas of the tumor probably represent the location where angiogenic clones of tumor cells reside, and are most likely to enter the circulation and metastasize to distant sites. Once hot spots are identified, the magnification is increased to ×200–400, corresponding to a field size of approx 0.74 mm^2. Any highlighted endothelial cell or cluster of cells is counted as a distinct microvessel.

The relationship between intratumoral MVD and prognosis has been reported for several different tumor types, but the greatest number of reports have been in primary operable breast cancer. Gasparini *(33)* recently reviewed breast cancer reports accounting for 3200 cases of breast cancer. A statistically significant relationship between intratumoral MVD and relapse-free survival (RFS) and overall survival (OS) was seen in 69 and 80% of reports, respectively. Other ways of assessing MVD have included Chalkey point counting and multiparametric computerized image-analysis systems, which may have advantages over manual vessel counting, but are more time-consuming *(31)*.

Some investigators have suggested that assessing intratumoral MVD or other markers of endothelial cell proliferation, such as endoglin *(34)*, with serial tumor biopsies over time may offer an insight into the biological activity of a given AI. Unfortunately, the ability to obtain serial tumor biopsies in cancer patients has been difficult. Few tumor types manifest metastatic disease in easily accessible sites. Exceptions include malignant melanoma and Kaposi's sarcoma (KS), which often present with multiple skin lesions. Although there is much enthusiasm for evaluating AIs in patients with HIV-related KS, this disease entity may not behave in the same way as other, more typical, epithelial tumor types. The requirement for serial biopsies has been incorporated into other clinical trials, including those that attempt to determine the development of multidrug resistance (MDR) gene expression over time, as patients are treated with a particular cytotoxic chemo-

therapy agent. Many of these trials encountered difficulties obtaining serial biopsies, because patients refused, biopsy sites were either inaccessible or associated with significant biopsy-related morbidity, or patients were too ill to undergo the procedure.

An equally important issue, as it relates to the assessment of an AI with serial biopsies for intratumoral MVD, is whether changes in these indices actually reflect the biologic activity of the AI. If serial tumor biopsies could be easily obtained, a more informative measure of AI activity may be evaluating changes in markers for endothelial cell proliferation, apoptosis, or angiogenic peptide, or integrin expression within the tumor. A potential problem inherent in this strategy is sampling error from one biopsy to another. A clinical setting in which it may be feasible to assess these types of markers is a pilot study in which patients must undergo a biopsy to confirm the diagnosis of cancer, prior to definitive surgery. In the interval prior to definitive surgery, patients could be enrolled on a study to assess the short-term biologic effects of an AI. Such study designs have been utilized successfully, including a recent report describing the effects of a new pure antiestrogen, ICI 182780, in patients with primary breast cancer *(35)*. DeFriend et al. *(35)* assessed the pharmacokinetics, toxicity, and short-term biologic activity of ICI 182, 780 in 56 patients with primary breast cancer, who received daily intramuscular injections for 7 d. Following breast surgery, tumors were assessed for changes in the proliferation marker Ki67, PR, and ER expression. Patients with operable lung cancer, colorectal cancer, and sarcomas, as well as other tumor types, may be suitable candidates for this type of trial design, which may provide insight into the biologic activity of a particular AI.

7. CIRCULATING ANGIOGENIC FACTORS

Angiogenic factors can be detected in the serum and urine of cancer patients, and may offer an indirect means to assess the efficacy of an AI. Fujimota et al. *(36)* was the first to report that elevated levels of basic fibroblast growth factor (bFGF) could be detected in the serum of patients with renal cell carcinoma. Nyugen et al. *(37)* reported that bFGF levels could be detected in the urine of many patients with a variety of malignancies, and that frequently, these levels, particularly in patients with metastatic disease, were markedly higher than in controls without cancer. More recently, elevated serum levels of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) have been detected in the serum of recurrent breast cancer patients, at concentrations believed to be high enough to elicit a biologic effect on the endothelium *(38,39)*. Elevated serum VEGF levels have also been reported in patients with colorectal cancer *(40)*, brain tumors *(41)*, and other epithelial tumor types *(42)*. Other angiogenic factors, including TGF-α and IL-8, have also been reported as elevated in cancer patients *(43–45)*.

Some investigators have suggested that following changes in circulating angiogenic factors may be more useful than conventional tumor markers, such as CEA or CA15-3. Patients could be evaluated for their angiogenic profile prior to entry on a clinical trial of an AI. Changes in the concentration of circulating angiogenic peptides could be assessed as patients undergo treatment. It is important to recognize that changes in the concentration of circulating angiogenic peptides may not reflect a direct action of the AI, but rather represent an indirect effect of the AI on the angiogenesis cascade. Furthermore, even if an AI is able to decrease the circulating level of one angiogenic peptide, a tumor may be able to compensate by increasing the production of another. Therefore, an angio-

genic profile, rather than reliance on the measurement of a single angiogenic peptide, may be optimal. Finally, several reports note that not all cancer patients have elevated circulating levels of angiogenic peptides *(37,38)*.

8. TUMOR MARKERS

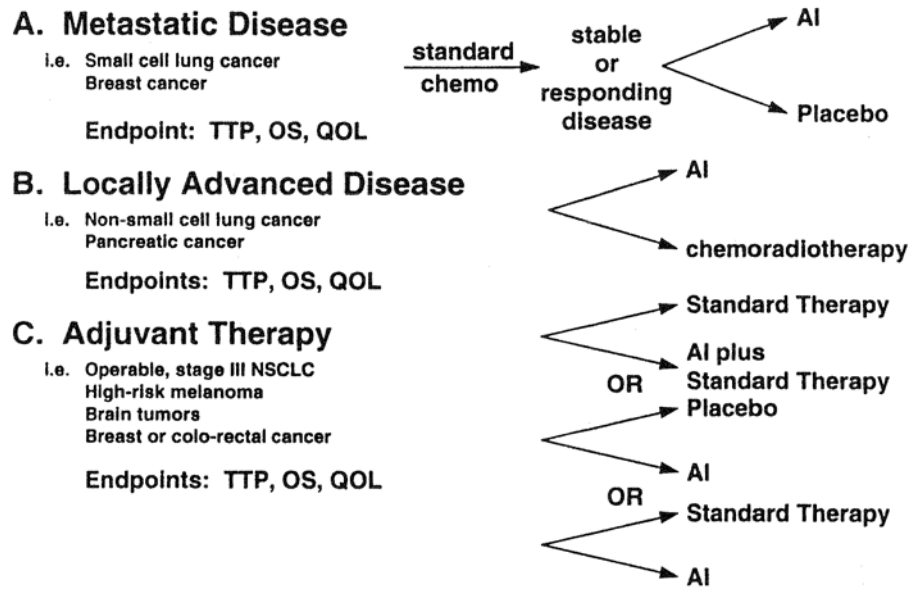
The development of the MMPI, Marimastat® by British Biotech (Annapolis, MD), has focused attention on the use of circulating tumor markers as a surrogate to assess biologic activity of the MMP. Patients with ovarian, pancreatic, colon, and prostatic cancers were eligible to participate in these early phase II trials, if they had a greater than 25% rise in circulating tumor markers (CEA, CA19-9, CA125, PSA) over a 4-wk period. During the screening phase of the trial, patients were followed without Marimastat therapy, to confirm that the tumor marker of interest was increasing. During the treatment phase with Marimastat, levels of tumor markers were followed and correlated with clinical response, as defined by radiological assessment. The biological effect was defined by the magnitude of change in the level of the circulating tumor marker after 1 mo of therapy with Marimastat. A complete biologic effect (CBE) was defined as no change or a decrease in the level of the tumor marker. A partial biologic effect (PBE) was defined as an elevation of the marker <25%. A negative biological effect was defined by an increase in the marker level of >25%. Patients were considered for long-term treatment beyond 4 wk if they experienced a reduced rate of antigen rise (<25%). In phase II trials with this design, patients with advanced colon, pancreatic, ovarian, and prostate cancer have been evaluated. Preliminary data from these trials suggest that a significant fraction of patients treated with Marimastat experience a biological response manifested by a reduced rate of rise in the tumor markers *(46,47)*.

Caution must be exercised in interpreting these interesting results, because the trials were not randomized. Within a population of patients with an advanced malignancy that expresses a tumor marker, there may be patients who spontaneously increase or decrease the rate of rise of a given tumor marker *(48,49)*. There are also other potential problems with tumor markers. There is intrapatient variability in the level of tumor markers, even in patients with no detectable tumor *(48,49)*. There can also be discordance between the pattern of tumor marker values and the clinical response observed in the patient. Even in tumor types in which tumor markers are generally expressed, not all patients have an elevation of the tumor marker *(48,49)*.

9. PHASE II TRIAL DESIGN

In phase II clinical trials of conventional cytotoxic chemotherapy drugs, the primary end points are ORR, survival, and further characterization of the toxicity profile associated with a new drug or combination of drugs. As already suggested, AIs may not produce tumor regression, so ORR may not be an appropriate end point for phase II trials. Rather, the fraction of patients attaining a stable disease status for a prolonged duration may be more appropriate to consider, and overall survival would remain important. Phase II trials would also be the appropriate setting to evaluate novel biological end points, and to attempt to correlate these findings with clinical outcome. Although these findings would not be definitive, they would provide hypotheses and questions that could be addressed in phase III randomized trials.

Table 2
Examples of Phase III Trial Design for Angiogenesis Inhibitors



10. PHASE III TRIAL DESIGN

Phase III trials typically compare the standard therapy for a particular disease to a new treatment strategy that has shown promise in phase II, disease-directed clinical trials. The new treatment strategy may simply represent a novel way of administering the standard therapy. One example of the latter is evaluating the efficacy of different ways of sequencing two different chemotherapy drugs, A and B (i.e., AB, then AB, then AB vs AAA, then BBB). Alternatively, a standard therapy can be compared to standard therapy plus a new agent, or a standard therapy can be compared to a new drug or combination of drugs. These examples represent only some of the possible design considerations for phase III randomized trials (Table 2).

The primary objective of phase III clinical trials is to define differences in clinical end points. For patients with metastatic disease, conventional chemotherapy trials typically focus on ORR, time to disease progression, and overall survival. More recently, instruments for assessing quality of life (i.e., Q-TWIST) are routinely incorporated into trial design, so that, even if clinical end points are identical between treatment arms, a treatment strategy associated with less toxicity or greater relief of symptoms may prove to be superior. In phase III clinical trials comparing different adjuvant therapies in patients deemed to be at high risk of disease recurrence, time to treatment failure, and overall survival, are the primary clinical end points. Quality of life assessments are particularly important for randomized adjuvant trials since patients are free of disease, and may participate in clinical trials assessing the efficacy of intense therapy (i.e., high-dose chemotherapy/peripheral blood stem cell transplantation), compared to standard chemotherapy or short-term therapy (i.e., mo) vs long-term therapy (i.e., yr).

Careful consideration must taken in the design of phase III clinical trials, particularly those that will incorporate AIs. The first consideration in designing the trial is the selection of the patient population. Patients with metastatic disease may offer advantages, but

there are also issues that make this patient population less than optimal. Patients with a specific type of metastatic cancer have a fairly well-defined survival available from natural history studies and prior clinical trial results. Because the survival of patients with metastatic disease is generally short, the duration of time necessary to complete the trial is also short. Another consideration is to design the trial with a comparison between a second-line therapy and a new therapy. Generally, patients and physicians are more willing to consider investigational trials if first-line therapies have failed, and they know that second-line therapies are generally less effective than first-line therapies. Alternatively, treatment with standard first-line therapy for a defined period, followed by randomization between placebo and the AI, could be considered. Similarly, the sample size (e.g., number of patients) required to detect a modest difference in outcome (i.e., survival) between the treatment arms is also generally small (usually <100–200 patients). If the clinical trial is conducted using patients with a disease that is common in the general population (i.e., lung, colon, or breast cancer), accrual to the trial can be very rapid and potentially provide results quickly.

The greatest problem with selecting a metastatic disease population to assess the efficacy of a new agent (AI or cytotoxic chemotherapy) is the biological diversity of the tumor at that point in its natural history. Metastatic tumors contain heterogeneous clones of tumor cells that not only express drug resistance to conventional cytotoxic chemotherapy, but, because of the large tumor burden, may not respond to any form of therapy. The tumor may be totally autonomous at that stage. Evaluating any new agent or treatment strategy in this patient population may falsely give the impression that the new agent is ineffective.

The adjuvant setting may ultimately be where AIs provide the greatest benefit; however, identifying the efficacy of the AI in this setting will require a commitment of patient resources, time, and money. The efficacy of adjuvant therapy, following complete surgical resection of a tumor, has been proven for breast cancer, colon cancer, and melanoma. The rationale for adjuvant therapy is to eradicate microscopic disease. In designing a clinical trial in the adjuvant setting for a particular disease, it is important to understand the natural history of patients rendered free of disease by surgery, radiation, and/or chemotherapy. As an example, response rates of 85–95%, including 50–60% CRs, can be achieved with combination chemotherapy in limited-stage, small-cell lung cancer *(50)*. Unfortunately, the median survival remains between 12–16 mo. Patients attaining a CR to standard cytotoxic chemotherapy could be considered for a trial randomizing patients between an AI or placebo. The number of events necessary to detect a difference in outcome based on treatment would be quickly observed, since the median overall survival of small-cell lung cancer is short. In contrast, the incorporation of an AI into an adjuvant therapy for breast cancer would be a larger undertaking, depending on the subset of patients selected for study. Two-thirds of all axillary node-negative breast cancer patients are cured following local therapy with mastectomy or lumpectomy and radiation therapy. Nevertheless, the standard of care dictates that the majority of such patients would benefit from adjuvant chemotherapy or hormonal therapy, or both. Since it may take several years for a recurrence to be detected, and in relatively few patients, adjuvant therapy trials in this subset of patients necessarily require large numbers of patients (1000–3000) and long follow-up, to detect differences in outcome between treatments. Clearly, many other subsets of cancer patients could be considered for adjuvant trials involving AIs including surgically resected brain tumors, nonsmall-cell lung cancer, high-risk, node-positive breast cancer, ovarian cancer, and others.

Table 3
Economic Implications of Clinical Trial Design

Choice of therapy
Sample size
Data collection
Duration of trial

The final issue that must be considered in the design of randomized phase III trials is cost, which can vary depending on the sample size, ancillary tests required, amount of data that must be collected, and the length of time patients must be followed (Table 3). Estimates of cost/patient enrolled on clinical trials have varied between $1000 and $10,000. The importance of clinical trial design involving AIs will be critical, so that resources can be used wisely, and the efficacy of AIs in patients can be defined.

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20 Retinoids and Interferons as Antiangiogenic Cancer Drugs

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Contents

1. MECHANISM OF RETINOID ACTION

Retinoids are a class of chemical compounds that include active metabolites of vitamin A (retinol) as well as a diverse array of synthetic derivatives. Vitamin A is required for normal embryonic development, epithelial homeostasis, maintainance of reproductive capacity, and functioning of the visual cycle *(1)*. Additionally, retinoids have been shown to modulate a wide variety of cellular processes, including proliferation, differentiation, homeostasis, and malignant transformation (for reviews *see* refs. *2–5*). Retinoids also act pharmacologically to restore regulation of differentiation and growth in certain premalignant and malignant cells in vitro and in vivo *(6,7)*. Consequently, retinoids are under study as therapeutic and chemopreventive agents for a variety of cancers (*see* refs. *8–10* for reviews). Retinoids are also potent drugs for the treatment of severe cystic acne, psoriasis, and several other dermatologic disorders *(11)*.

At least 24 different retinoid binding proteins have been identified to date. With the exception of specialized proteins in visual tissue, these proteins fall into three general classes:

1. The secreted retinoid binding proteins;
2. Cytosolic retinoid binding proteins; and
3. The nuclear retinoid receptors *(12)*.

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Early attempts at understanding the molecular basis of retinoid action were directed toward two families of cytosolic retinoid binding proteins, the cellular retinol-binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs). The cDNAs for these proteins have been cloned, and it has been shown that there are at least two CRABP proteins and two CRBP proteins, each encoded from a different gene *(13)*. These proteins have distinct spacial and temporal distributions in developing mouse and chick embryos *(14–17)*; however, double knockout mice lacking expression of both CRABPI and CRABPII develop normally and have almost no observable defects *(18)*. Although the lack of a knockout phenotype in these mice is difficult to explain, other evidence suggests that CRABP may either act as a sink for RA or enhance its metabolism, effectively reducing the amount of free RA in a cell *(19,20)*. Considered together, these findings suggest an important but auxilliary role for CRABPs and CRBPs in retinoid signaling.

Following the discovery and cloning of CRABPs and CRBPs, another class of retinoid binding proteins, the nuclear retinoid receptors, was discovered *(21,22)*. It is now believed that retinoids exert their effects primarily through these proteins. The nuclear retinoid receptors comprise two families of ligand-dependent, DNA-binding, transcriptional transactivators, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both members of the nuclear hormone receptor superfamily *(3,5,23,24)*. There are three members (types) of each family of retinoid receptors, designated, RARa, b, and g and RXRa, b, and g, each encoded by different genes. Each gene can generate multiple mRNA splice variants encoding receptor isoforms with unique amino-termini (*23, 25,* and references therein).

RARs are activated by all-trans retinoic acid (tRA) and its 9-cis isomer (9C-RA), whereas RXRs are only activated by 9C-RA. Retinoid receptors bind DNA and activate transcription primarily as RAR/RXR heterodimers (*23*; Fig. 1). Several in vitro studies demonstrated that ligand binding of both the RAR and RXR partner resulted in enhanced transcriptional activation compared to that caused by liganding of either partner alone *(26–28)*. This, along with evidence from studies of retinoid receptor knockout mice, strongly suggests that RAR/RXR heterodimers transduce the retinoid signal in vivo *(3,5,29)*.

The RAR and RXR proteins share a similar domain structure with other members of the steroid/thyroid hormone receptor superfamily, consisting of six regions designated A–F in the case of RARs and five regions A–E in the case of RXRs. The A/B domain contains a transcriptional activation function (AF-1) that acts in a promoter context- and receptor type-specific fashion and accounts for the constitutive transcriptional activity of RARs and RXRs *(30,31)*. The AF-1 of one of the RARs, RARα, can be phosphorylated by a component of the basal transcriptional machinery, resulting in enhanced transriptional transactivation *(32)*. The other transcriptional activation function (AF-2) is found within the highly conserved E region, which also contains the ligand-binding and dimerization domains (*30,31,33; see* refs. *3* and *24* for reviews). Ligand binding brings about conformational alterations in the receptors, of which one consequence is the exposure of an interacting surface contained within the AF-2 *(34,35)*. This surface contacts transcriptional intermediary factors (also called coactivators), which are thought to form a bridge between the RAR/RXR heterodimer and the basal transcriptional machinery and to establish contacts with proteins involved in chromatin remodeling *(36–41)*. Another consequence of ligand binding is the dissociation of the RAR partner with corepressor proteins, also called silencing mediators, which repress transcription when bound to unliganded RARs *(42–44)*.

The C region contains a zinc finger DNA-binding motif and is the most highly conserved domain between the different steroid receptor family members as well as between

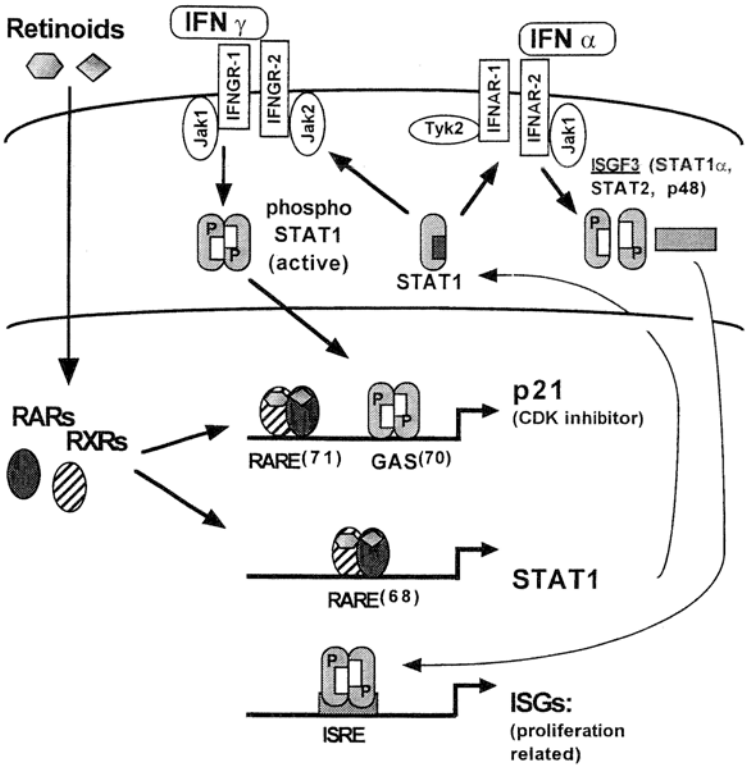


Fig. 1. Molecular mechanism of RA/IFN interaction. Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; IFN, interferon; IFNGR, interferon-γ receptor; IFNAR, interferon-α receptor; JAK, janus kinase; Tyk, tyrosine kinase; STAT, signal transducer and activator of transcription; ISGF3, interferon-stimulated gene factor 3; RARE, retinoic acid response element; GAS, interferon-γ activated sequence; ISRE, interferon-stimulated response element; ISG, interferon-stimulated gene. Numbers in parentheses are the references describing the isolation of the corresponding response elements.

members of the RAR and RXR classes. Cis-acting DNA to which the C domains bind are termed RA response elements (RARES), and they all share a paired repeat motif of the sequence PuG(G/T)TCA (Pu = purine) that can vary in spacing and orientation. There is a growing list of genes that contain RAREs, including both genes for which transcriptional modulation by RA is an immediate effect and genes for which it is delayed *(4)*.

The various RAR and RXR types and isoforms are highly conserved in evolution, and display distinct spatio-temporal expression patterns in developing organisms and in the adult, which suggests that each receptor exerts some distinct physiological functions (reviewed in ref. *5*). In addition to RARs, the RXRs can dimerize with several other steroid hormone receptor family members (*2,24,45–47*, and references therein). Upon heterodimerization, RXRs have been shown to modulate the DNA-binding and AFs of these receptors in vitro and in transfected cells. Recent studies provide evidence that ligand-binding of the RXR partner can also enhance the transcriptional activity of several of these receptors in a similar manner to RARs *(48,49)*. Thus, RXRs, and by extension 9C-RA, are likely to play a key role in the control of several signaling pathways.

2. MECHANISM OF INTERFERON SIGNALING

Interferons (IFNs) are cytokines, and, like retinoids, act as potent biological response modifiers. In humans, at least 26 IFN genes were originally classified into two groups according to the cell types in which they were found. Although the original classification has become obsolete, it is still in common use: IFN-α and IFN-β are type I and IFN-γ is type II *(50)*. A newer classification system designates IFN-α as a leukocyte IFN, IFN-β as fibroblast IFN, and IFN-γ as immune IFN *(51)*. IFNs were originally discovered as mediators of an antiviral state in virus-infected cells. It is now well established that IFNs also regulate proliferation, differentiation, and immune functions *(50)*. IFNs bind to transmembrane glycoprotein receptors belonging to a subclass (class II) of a large family of cytokine receptors (*52,* Fig. 1). IFN binding mediates oligomerization of receptor subunits, which results in their reciprocal tyrosine phosphorylation *(53)*. This is followed by a series of three phosphorylation events:

1. Phosphorylation and activation of a receptor-associated tyrosine kinases of the JAK (Janus kinase) family;
2. Phosphorylation of tyrosines on the cytoplasmic tail of the receptor and subsequent attachment of latent cytoplasmic transcription factors called signal transducing activators of transcription (STATs) to those phosphotyrosine sites; and
3. Phosphorylation of the STATs by the associated JAKs (*see* ref. *54* for review).

Phosphorylation of STATs results in their translocation to the nucleus, where they can modulate the transcription of a large number of genes (*54*; Fig. 1). In the case of IFN-α family members, binding to the interferon-α receptor-2 (IFNAR-2) subunit triggers dimerization with IFNAR-1 and phosphorylation of JAK1 and another JAK family member, Tyk2 (*55–57*; Fig. 1). JAK1 and Tyk2 subsequently phosphorylate and activate first STAT2 and then STAT1 *(58)*. After translocation to the nucleus, these proteins form a transcription factor complex with a third protein, p48. This complex is called IFN-stimulated gene factor 3 (ISGF-3) (*59,60* and references therein). ISGF-3 binds to consensus DNA sequences designated IFN-stimulated response elements (ISREs) found in the promoters of most IFN α/β responsive genes and activates their transcription (*59,60*; Fig. 1). IFN-β also binds IFNAR-2, which subsequently dimerizes with a distinct cell surface receptor and activates gene expression through the same JAKs and STATs as IFN-α *(50,54)*.

IFN-γ binds as a dimer to two IFN-γ receptor (IFNGR-1) molecules, which each dimerize with an IFNGR-2 subunit (*61*; Fig. 1). The IFN-γ signal is then transduced to STAT1 by JAK1 and JAK2, each bound to IFNGR-1 and IFNGR-2, respectively. The phosphorylated STAT1 enters the nucleus, but instead of forming the ISGF3 complex as in the case of IFN-α/β signal transduction, activates transcription as a STAT1 homodimer by binding to IFN-γ activated sites (GAS) (*50,61*; Fig. 1). GAS sequences are distinct from ISREs and are found in the promoters of a large number of genes *(50,61)*.

3. CROSSTALK BETWEEN RETINOID AND INTERFERON SIGNALING PATHWAYS

Retinoids and IFNs cooperate supra-additively or synergistically to inhibit proliferation and/or induce apoptosis of a large number of tumor cell lines, including cervical carcinoma, neuroblastoma, breast carcinoma, myeloid cell lines, head and neck squamous cell carcinomas (SCCs), and others *(62–65)*. Greatest success has been attained for

treatment of advanced head and neck premalignant lesions, renal cell carcinoma, and SCCs of the skin and cervix *(66)*. A likely mechanism for retinoid-IFN synergy is suggested by studies showing that RA can upregulate the expression of proteins involved in IFN signaling, including STAT1, STAT2, and p48 *(63,67–70)*. Recently it has been shown that RA can directly induce transcription of the STAT1 gene through a RARE in its promoter (*71*; Fig. 1). These results now provide a mechanistic rationale for the use of retinoid and IFN combinations in cancer therapy.

In addition to the induction of IFN signaling proteins, there are numerous examples in which retinoids directly induce the expression of ISGs *(63,69,71–74)*. Since IFNs function primarily to inhibit viral multiplication, it is not surprising that a number of the ISGs described to date are suppressors of viral DNA replication and protein synthesis. Many of these same proteins also suppress cellular DNA replication and protein synthesis and the upregulation of several ISGs by IFNs can be directly linked to their antiproliferative effects *(50)*. Examples of two such ISGs, IFN regulatory factor-1 (IRF-1) and 2'-5' oligoadenylate synthase (OAS), are induced by tRA in P19 embryonal carcinoma cells in the absence of added IFN *(71)*. The crossregulation of genes in this category suggests an overlap in the mechanism of proliferation control by retinoids and IFNs.

Although not as common, there are examples of the upregulation of retinoid signaling proteins, namely the RARs, by IFN. In breast cancer cell lines IFN-γ could upregulate RAR-γ expression, and in the NB4 acute promyelocytic leukemia cell line IFN-α,β and γ could upregulate RAR-α expression *(75,76)*. Thus, the possibility of a mutual enhancement of signaling between retinoid and IFN pathways could be exploited therapeutically.

Finally, RA and IFNs, as well as another hormone receptor ligand, vitamin D3 ($1,25(OH)_2D_3$), all directly regulate the transcription of the cell cycle regulator p21 through their respective upstream promoter regulatory elements, suggesting a common mechanism of cell cycle regulation for all of these agents (*77–79*; Fig. 1). p21 is one of a group of proteins that can interact with and inhibit cyclin-dependent kinases, causing cells to arrest in the G1 phase of the cell cycle *(80)*. Furthermore, the upregulation of the p21 gene is associated with differentiation induction in mouse primary keratinocytes *(81)*. The p21 protein therefore represents a potential intersecting point between the retinoid and IFN signaling pathways for the control of cellular proliferation and differentiation.

4. RETINOIC ACID-INTERFERON-α COMBINATION CANCER THERAPY

Retinoids and interferons (IFNs) show clinical promise in several carcinogenic settings. Retinoids have demonstrated significant clinical activity in randomized chemoprevention trials in the head and neck, skin, liver, and cervix *(82–90)*. Interest in retinoids for cancer therapy was greatly stimulated by the high complete response rate of acute promyelocytic leukemia (APL) to all-*trans*-retinoic acid (ATRA) *(91–93)*. Retinoids have shown activity in certain other hematologic malignancies as well *(10,94,95)*. In general, however, ATRA and other retinoids have disappointing single-agent results in established cancers. There are data indicating that 13cRA and ATRA may improve the antitumor efficacy of radiation, cisplatin, and IFN *(96–105)*.

Evidence that 13cRA and IFN-α interact favorably at the molecular level is provided by in vitro and in vivo preclinical data *(63–68,71,75,76,78,79,106–111)*. This combination has

achieved encouraging results in locally advanced untreated cervical cancer *(112–114)*, squamous cell carcinoma (SCC) of the skin *(115,116)*, and renal cell carcinoma *(117)*.

Combined 13cRA and IFN-α has achieved an overall major response rate of 45% in 55 patients with untreated locally advanced primary SCC of the cervix *(114–116)*. Based on promising pilot data on 13cRA/IFN-α integrated with radiotherapy (RT) *(117)*, a phase III study of 13cRA plus IFN-α plus RT vs RT alone was launched. This trial has produced promising preliminary data, including significantly improved survival for patients with locally advanced cervical cancer receiving the combined regimen *(118)*. Negative results with RA-IFN combinations have been reported by Hallum et al. (no response to 13cRA/IFN-α among 13 patients with heavily RT-plus-chemotherapy-pretreated recurrent SCC of the cervix) *(119)* and by Wadler et al. (no response among 26 patients with chemotherapy-naive recurrent cervical cancer to ATRA plus IFN-α-2A); *120*).

Results have been recently reported from a Southwest Oncology Group (SWOG)/NCI trial activated in 1992 *(121)*. This randomized phase II trial evaluated ATRA plus IFN-α and 13cRA plus IFN-α in 60 evaluable patients with recurrent chemotherapy-naive cervical SCC. Major response rates in the ATRA/IFN-α and 13cRA/IFN-α arms were 5 and 8%, respectively. All confirmed responses were partial, and one additional unconfirmed partial responses occurred in the 13cRA arm. Both regimens were generally well-tolerated, producing expected toxicities consistent with each agent's single-agent toxic effects. This trial represents the first clinical test of 13cRA plus IFN-α in chemotherapy-naive recurrent cervical SCC and the first randomized study of any IFN-α/retinoid combination in the setting of recurrent cervical SCC. There were nonsignificant differences between the two arms, including greater numbers, degrees, and durations of responses achieved by 13cRA/IFN-α. The basically promising clinical and laboratory data profile of 13cRA plus IFN-α supports further study of these combined agents plus concomitant radiation to establish the best role for these modalities in locally advanced, untreated cervical cancer.

The first phase II trial of a RA and IFN combination involved 28 evaluable patients receiving 13cRA (1 mg/kg/d) plus IFN-α2a (3 million units/d) for advanced SCC of the skin *(115)*. The overall major response rate was 68%. Other results of this trial included a significant inverse correlation between response and disease extent, significantly higher response rate in chemotherapy-naive patients, median response duration of approx 5 mo, and substantial toxicity (grade 3 or greater fatigue occurring in 12 patients, possibly because of study population age—median 67 yr).

A subsequent phase II trial of similar doses of 13cRA plus IFN in unresectable, recurrent, and/or metastatic SCC from many sites involved 32 evaluable patients *(116)*. There was a 50% major response rate in both head and neck as well as skin cancers, the two largest study subsets. Cancers of other sites with major responses included vulvar, esophageal, and penile cancers.

Combining cisplatin with the combination RA and IFN is a major thrust of current efforts with this combination. This direction is suggested by cisplatin's activity in advanced SCC of several sites and the ability of both RA and IFN-α to potentiate cisplatin antitumor activity in several in vitro and human tumor xenograft systems *(96,97)*.

The poor overall response rate of renal cell carcinoma (RCC) to cytotoxic and hormonal agents is <2%. The overall response rate for IFN-α in RCC is 12% (over 600 cases reported in the literature). Memorial Sloan Kettering Cancer Center conducted a phase II trial involving 44 evaluable RCC patients receiving 13cRA (1 mg/kg/d) and IFN-α

(3–9 MU/d) that achieved a major response rate of 30% (13 patients), including three complete responses *(117)*. Responses were durable (≥10 mo in 7 responding patients) and were observed in sites typically resistant to IFN. These investigators also found that results of in vitro studies of this combination in RCC suggested that activity is mediated by RAR-β. Prior Memorial Sloan Kettering clinical trials of IFN alone or combined with vinblastine or interleukin-2 achieved an overall response rate of 10% in 149 patients, which is threefold lower than the combined 13cRA-IFN-α rate.

Two German studies have involved RA plus IFN plus subcutaneous interleukin-2 plus 5-FU in 44 patients with progressive metastatic RCC, and RA plus IFN in 19 patients with metastatic disease refractory to IFN, interleukin-2, and chemotherapy *(122)*. The first study achieved a major response rate of 44% (20/44; 95% CI, 30–60%), including six complete responses. Responding cancers included lung, liver, and adrenal cancers; responses were durable in 19 of the 20 responding cases, and prolonged stabilization of disease occurred in 21 patients (47%). The second study achieved a major response rate of 21% (4/19).

Very recent, promising translational data on this combination come from a NCI-supported chemoprevention study in the head and neck *(122a)*. These data indicate that 13cRA plus IFN-α achieved striking clinical and molecular activity (e.g., effects on LOH and p53) in very high-grade premalignant lesions of the larynx.

5. ANTIANGIOGENIC ACTION OF RETINOIDS AND INTERFERONS

5.1. Suppression of Tumor Neovascularization

The importance of the aquisition of the angiogenic phenotype for the conversion of tumors from a benign to a malignant state will not be discussed here and is addressed in detail in Part I of this volume. We will instead comment on the specific role of retinoids and interferons in the suppression of tumor neovascularization. Several lines of evidence from in vitro and animal models indicate that at least part of the antitumor effect of retinoids is caused by inhibition of tumor vascularization *(123–127)*.

Early on in the use of retinoids for cancer chemotherapy it was hypothesized that they may be acting as antiangiogenic agents *(123)*. This was first verified by experiments in which tumors were implanted into the corneas of rabbits receiving either vitamin A or control injections. Vitamin A-treated rabbits showed a reduced vascular response in the adjacent limbic vessels (host tissue) as well as greatly reduced tumor vascularization and tumor size compared to controls *(123)*. Similar suppressive effects were observed for tumor-induced angiogenesis (TIA) of mouse tissue adjacent to the site of injection of transformed human keratinocyte cell lines *(125)*. Also, using a cervical SCC tumor xenograft model in nude mice, systemic tRA treatment suppressed vascularization within the tumors *(127)*.

A suppression of angiogenesis by other retinoids was observed in chick embryo chorioallantoic membranes (CAM) *(124)*. In this case, the normal vascularization that takes place by d 4.5 of chick embryo development was suppressed by implantation of retinoid-containing pellets directly onto the CAM *(124)*. This study and another showing the suppression of neovascularization in rat corneas by tRA *(128)* indicate that retinoid inhibition of angiogenesis can be attributed not only to direct effects on tumor cells, but also to suppression of the angiogenic activity of normal endothelial cells *(128)*.

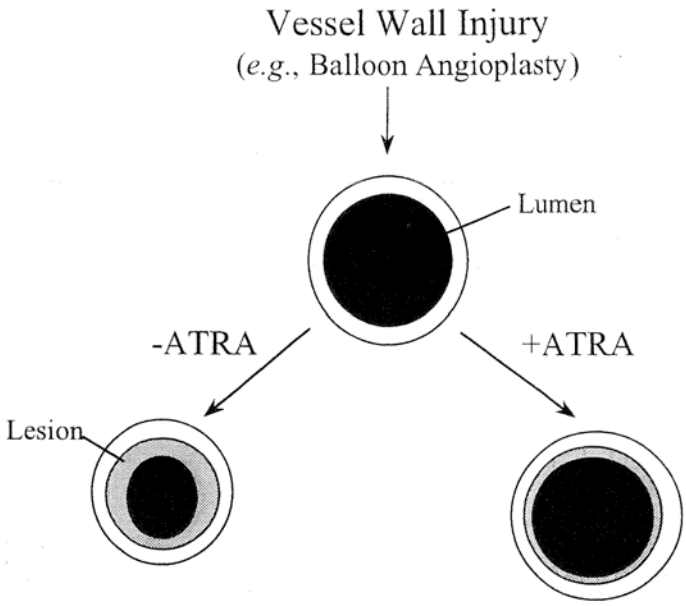
Analogous studies with IFNs have revealed an antitumor effect related to the inhibition of neovascularization, but not to an antiproliferative effect *(110)*. In these studies, TIA and lymphocyte-induced angiogenesis (LIA) were compared by counting blood vessels formed at the site of subcutaneous injection of tumor cells or allogeneic lymphocytes, respectively. Treatment of immunocompromised mice with human or mouse IFN-β caused a suppression of TIA from tumors of human or mouse origin, respectively *(110)*. This indicates that, unlike retinoids, the suppressive effect of IFN is directed primarily toward the tumors themselves, rather than on the adjacent host tissues. However, these same investigators also found that IFN-β inhibited LIA from both allogeneic lymphocytes and the mouse's own lymphocytes *(110)*. Thus, IFNs may inhibit the production of angiogenic factors from both tumors and host immune cells.

In the clinic IFN-α-2a was shown to be effective for the treatment of children with life-threatening hemangiomas, further confirming the antiangiogenic action of IFNs *(129)*. IFN-α was, in fact, the first angiogenesis inhibitor to be used in clinical trials. However, because of the occurrence of severe neurological side effects, IFN-α must be used with extreme caution for this disease *(130)*. Recently, another IFN-α isoform, IFN-α-2b, has been used successfully for the treatment of childhood hemangiomas and produced fewer side effects *(131)*. These investigators were also able to show a decrease in urinary levels of basic fibroblast growth factor (bFGF) that correlated with hemangioma involution *(131)*. bFGF is one of several inducers of angiogenesis and may provide an adequate biomarker for monitoring patient response (*131* and Chapter 7).

Because of the recent success of retinoid and IFN combination therapy for cancer (*see* ref. *66* for review), along with accumulating experimental evidence of a synergy between retinoids and IFN for the growth suppression of tumor cells *(62–65)*, it is logical to hypothesize that these agents may synergize to inhibit angiogenesis. Indeed, this has been shown using a mouse cutaneous TIA model *(111)*. Investigators employed the same assay system mentioned above *(125)*, and showed a synergistic inhibition of blood vessel formation at the site of injection of transformed keratinocytes or HeLa cells, by several retinoids in combination with IFN-α *(111,132)*. Further investigation into the precise mechanisms of inhibition of tumor vascularization (i.e., which inducers of angiogenesis are suppressed, or inhibitors of angiogenesis are upregulated) by both IFNs and retinoids will be necessary in order to better exploit their use in combination antiangiogenic therapy.

5.2. Effects of Retinoids and Interferons on Vascular Smooth Muscle Biology

The predominant cell type within the microvasculature of tumors is the endothelial cell whose migration, replication, and differentiation are central to angiogenesis. Higher caliber vessels, however, require circumferentially arranged vascular smooth muscle cells (VSMC) to perform the necessary function of contraction and the regulation of blood flow to the interior of the tumor. Interestingly, VSMC appear to play a dominant role in the neovascularization of several brain tumors (e.g., glioblastoma multiforme) *(133)*. Thus, any discussion of the effects of retinoids and interferons on angiogenesis should include their influence on VSMC function. Regrettably, there is virtually no literature with respect to these antiangiogenic agents on VSMC biology in the setting of cancer. Evidence for a role of retinoids and interferons in modulating VSMC proliferation and migration during tumor neovascularization is provided from the field of vascular pathobiology.


Vessel Wall Injury
(e.g., Balloon Angioplasty)
Lumen
-ATRA
+ATRA
Lesion

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21 Pentosan Polysulfate

A Polysaccharide That Inhibits Angiogenesis by Binding Growth Factors

William D. Figg, James M. Pluda, and Oliver Sartor

CONTENTS

1. INTRODUCTION

Sodium pentosan polysulfate (PPS; xylanopolyhydrogensulfate, Fig. 1) is a semisynthetic sulfated heparinoid polysaccharide that has been used in Europe as an anticoagulant for nearly 30 yr *(1–3)*. It is obtained from extract of beechwood shavings, and consists of a mixture of polymers with mol wt ranging from 1.8 to 9 kDa (mean 4.7 kDa) *(3)*. This glycosaminoglycan is a highly negatively charged compound that contains 1.9 sulfate groups per monosaccharide unit.

PPS's antitumor activity has been evaluated in several animal models (Fig. 2), and activity has been noted in vitro against several human tumor cell lines, including A204 (human rhabdomyosarcoma), SW-13 (human adrenal cortical carcinoma), and Ovar Cal (human ovarian carcinoma) cell lines, as well as nonsmall-cell lung cancer, prostate adenocarcinoma cell lines, and breast carcinoma cell lines *(6–9)*. The IC_{50}s for those cell lines evaluated were between 6 and 39 μg/mL *(10)*. In addition, PPS has been shown to inhibit basic fibroblast growth factor (bFGF) stimulation of angiogenesis in both bovine aortic endothelial cells and human umbilical vein endothelial cells. It is believed that the primary antitumor mechanism of action for PPS is through inhibition of bFGF-induced angiogenesis *(11)*. It has been shown to antagonize the binding of bFGF to its cell surface

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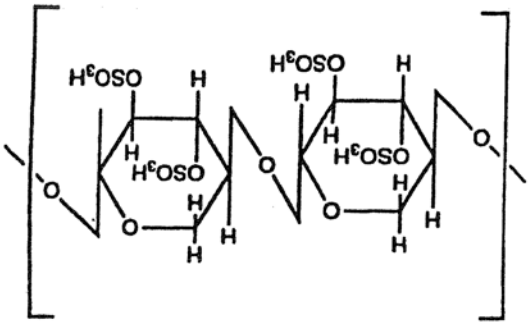


Fig. 1. Chemical structure of PPS.

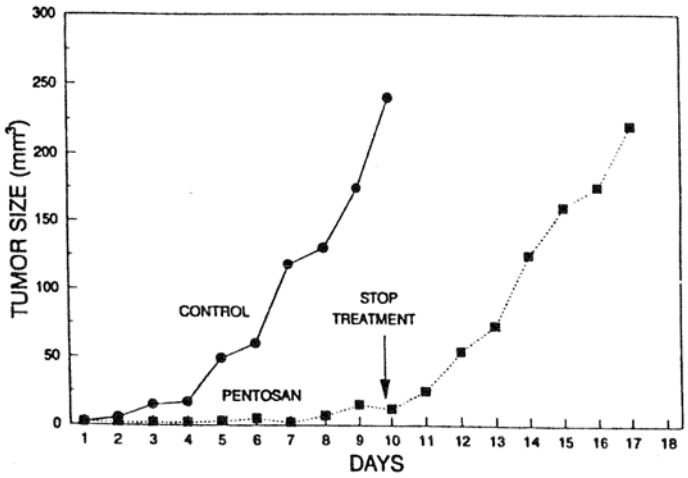


Fig. 2. The supression of in vivo prostate tumor growth by pentosan. 250,000 MAT-LyLu Dunning R3327 (MLL) cells were injected into the flank subcutaneously on d 0. Animals were treated with 150 mg/kg/d of pentosan orally in their drinking water starting on d 0. One animal was sacrificed per day. Pentosan-treated tumors were grossly white, without a surrounding blush by d 2, and were red in color by d 4. Reprinted with permission from ref. *6*.

receptor *(4,5)*. Furthermore, it has been shown to inhibit extracellular growth factor (EGF)-associated tyrosine kinase in lysed tumor cells.

The interaction between PPS and the coagulation and fibrinolytic systems is complex. Unlike heparin, PPS does not bind to antithrombin-II (AT-II). However, its most pronounced effect, like that of heparin, is to prolong the activated partial thromboplastin time (aPTT) *(12–14)*, which has been shown to arise primarily from pentosan's ability to potentiate thrombin inactivation by heparin cofactor II *(15)*. Normally, the small amounts of thrombin generated by activation of the clotting cascade plays a major role in converting zymogen factors VIII and V to their active forms, both of which are critical to the formation of the prothrombinase complex *(16,17)*. Thus, PPS, via its interaction with heparin cofactor II, circumvents this positive feedback loop *(18)*. PPS is essentially devoid of any in vitro anti-Xa activity; however, plasma obtained from patients receiving pentosan contains substantial anti-Xa activity, which is mostly (70–80%) attributed to enhancement by PPS of hepatic triglyceride lipase release *(19,20)*. Additionally, when

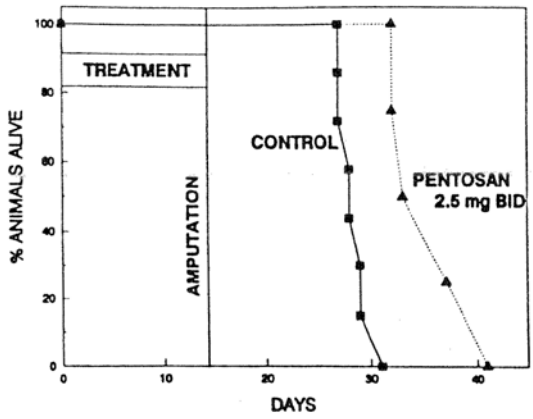


Fig. 3. Pentosan increased survival of animals injected with MLL cells. One million MML cells were injected into the hind limb of rats on d 0. Animals were treated with pentosan 2.5 mg/kg sc BID, the maximally tolerated dose, as demonstrated by earlier experiments. On d 14, treatment was stopped, and the tumor-bearing hind limbs were amputated. There was no significant difference in tumor size in treated vs control animals. Animals were then followed for survival. Control animals had a median survival of 28 d. Treated animals had a median survival of 35 d ($p < 0.05$). All animals died with large abdominal lymph node metastases and >100 lung metastases. Reprinted with permission from ref. *7*.

given as an injection subcutaneously, intramuscularly, or intravenously, or when administered orally, PPS has been shown to shorten the euglobulin lysis time. The effect may be mediated by the release of tissue plasminogen activator, and is also associated with activation of factor (f) XII and kallikrein *(21)*.

2. PHARMACOLOGY

The postulated anticoagulant mechanism of actions (as noted above) include inhibition of the activation and function of fVIII, possibly by complexing with fVIII *(22)*, and enhancement of fibrinolytic activities by activating kallikrein and fXII *(23)*. PPS appears to cause a release of hepatic triglyceride lipase, which interacts with lipoproteins and leads to weak anti-Xa activity. Fischer et al. *(21)* compared the in vitro and in vivo activity of heparin and PPS and found that PPS has relatively little effect on anti-fXa clotting assay following subcutaneous injection, and no effect as measured by the anti-fXa amidolytic assay. However, PPS is as effective as heparin in producing activation of lipoprotein lipase, shortening of the euglobulin clot lysis time and impairing the generation of fXa.

PPS inhibits cell growth of vascular smooth muscle cells *(24)*. At high concentrations, it inhibits bovine endothelial cell proliferation, and inhibits the metastatic potential of rat mammary carcinoma cells. PPS blocks bFGF-induced migration of endothelial cells. Pienta et al. *(6)* demonstrated that PPS could inhibit the in vivo growth of the MML prostate cancer cell line (Fig. 3). They speculated that the mechanism of action was through the blockage of cell motility. This group went on to show that PPS in combination with hydrocortisone, inhibited endothelial cell motility and tubule formation in vitro *(7)*. PPS inhibited capillary formation, using the chicken chorioallantoic membrane assay.

The authors have demonstrated that PPS can inhibit the formation and proliferation of endothelial tube formation, using a previously described in vitro model of angiogenesis *(25)*. This model uses rat aorta sections implanted in Matrigel and incubated for 5 d. Nicosia et al. *(26)* have demonstrated that, using this model, only 40% of nonpatent microvessels contain endothelial cells, but that all patent microvessels contain endothelial cells. Nonetheless, complete inhibition was noted with high concentrations (100 and 200 μg/mL), and significant inhibition was noted with 50 μg/mL (Fig. 4).

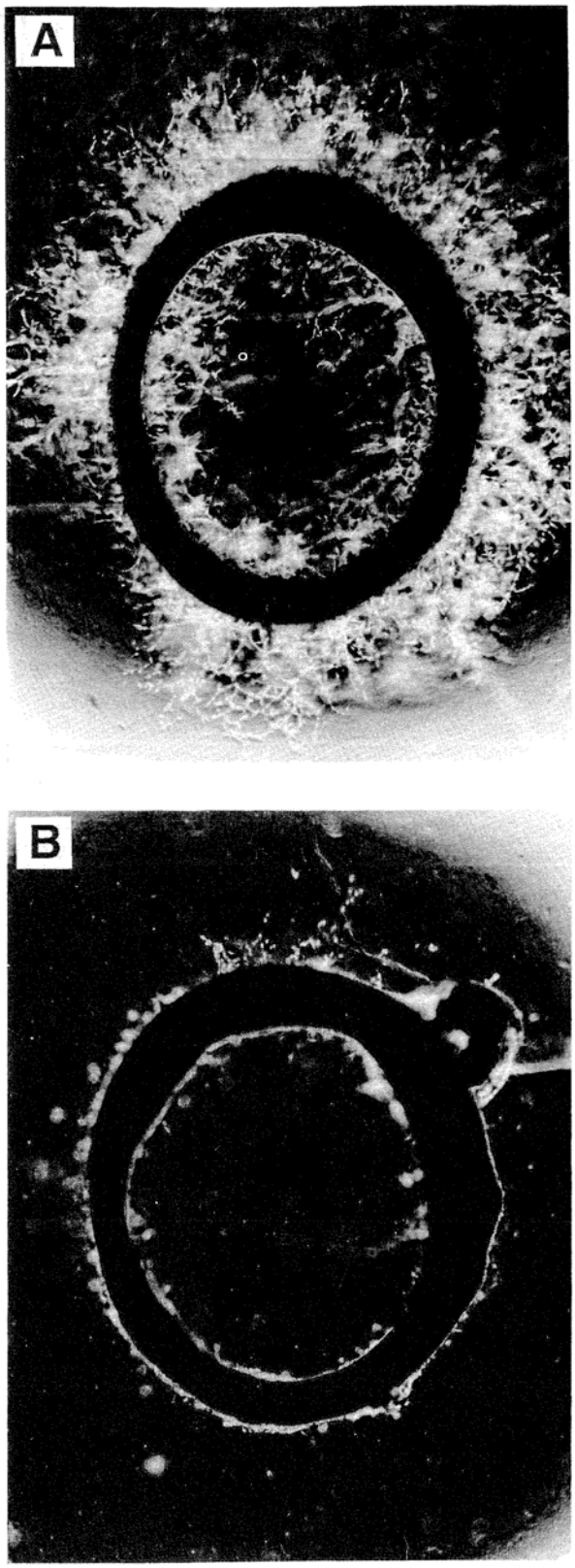
Wellstein et al. *(8)* developed a Kaposi's sarcoma (KS)-derived fibroblast growth factor (K-FGF)-secreting adrenal carcinoma cell line (SW-12/K-fgf). This transfected cell line develops colonies in soft agar, and produces large, highly vascularized tumors in athymic nude mice. PPS was a highly selective inhibitor of this cell line via blockade of both the autocrine and paracrine effects of K-FGF. Zugmaier et al. *(10)* evaluated the activity of PPS in seven different tumor cell lines (breast, lung, prostate, and epidermoid carcinomas, as well as rhabdomyosarcoma) that secreted a wide variety of heparin-binding growth factors. The majority of those cell lines were resistant in vitro to PPS in a soft-agar clonal assay. Despite the in vitro resistance, they showed that in vivo tumor growth of subcutaneously implant cells could be inhibited by the administration of PPS. McLeskey et al. *(27)* found that PPS inhibited in vivo tumor growth in mice inoculated with human breast carcinoma MCF-7 cells that had been transfected to express FGF-1 or FGF-4. However, PPS did not effect the in vitro growth of either transfected or parental MCF-7 cells.

Ca^{2+}–phospholipid-dependent protein kinase C (PKC) plays a pivotal role in both cell signal transduction and cellular proliferation. PKCs have been shown to be important in the signal transduction of platelet-derived growth factor, epidermal growth factor, FGF, the oncogenes $pp60_{src}$ and *ras* p21 protein *(28)*. PPS inhibits PKC activity via an interaction with the catalytic domain of PKC. PPS also showed strong inhibitory effect when tested on cAMP-dependent protein kinase and tyrosine protein kinase. Inhibition of PKC prevents phosphorylation of enzymes regulating proteins and DNA synthesis. Thus, part of the antitumor activity for pentosan may be through this mechanism.

3. CLINICAL PHARMACOLOGY

Because a reliable analytical method for quantitating PPS has not been developed, limited data is available on the disposition of pentosan in humans. Peters et al. *(29)* used a viral bioassay to estimate plasma concentrations in seven HIV infected patients. Following 100, 300, and 600 mg iv doses, peak concentrations ranged between 19 μg/mL for the low dose and 118 μg/mL for the high dose. Only with the 600-mg dose were plasma concentrations detected after 48 h. Cadroy et al. *(30)* used ^{125}I-labeled PPS to

Fig. 4. The thoracic aorta was removed from Sprague-Dawley rats. Using a dissecting microscope, 1-mm-long rings were cut. Each ring was rinsed 8 times with culture medium, embedded in Matrigel®, and incubated in growth factor-free endothelial cell basal medium with 10 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin at 37°C, 5% CO_2. Starting on d 2, the supernatant was replaced every 24 h with either 50 **(B)**, 100, or 200 μg/mL of PPS or control medium **(A)**. The aortic preparations were cultured for 6 d, and microvessel growth was assessed daily (figure is from d 6). 50 μg/mL of PPS resulted in significant inhibition in endothelium tube formations.


A
B

demonstrate that there was a significant correlation between dose and clearance in rabbits. They estimated the volume of distribution to be 246 ± 81 mL (2.1 times greater than the theoretical plasma volume for rabbits).

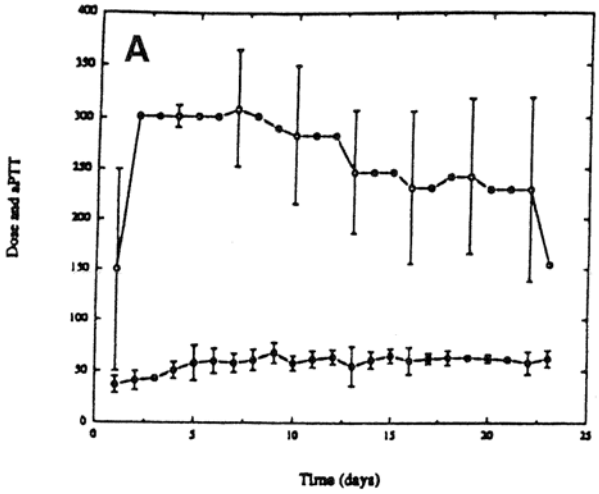
The pharmacokinetics of PPS have been reported to be similar to those of standard heparin. The clearance of PPS appears to be dose dependent *(29,30)*. When given to normal volunteers as an intravenous short-infusion of 1, 10, and 100 mg, the terminal elimination half-life was 7, 21, and 55 min, respectively. The bioavailability of PPS is poor, and only 4–8% is excreted unchanged in the urine. PPS is desulfated in the liver, and primarily excreted renally into the urine *(31)*. Lush et al. *(32)* reported an in vitro linear relationship between aPTT (25–60 s) and pentosan concentration (0–12 μg/mL), in an attempt to estimate in vivo plasma concentrations. As noted in Fig. 5A–C, they maintained clinical aPTTs of approx 50–60 s in 13 patients treated with continuous-infusion PPS (3, 5, and 8 wk of therapy). The plasma concentrations were estimated to be between 8.5 and 11 μg/mL. The IC_{50}s, as reported above for several cell lines, range between 6 and 39 μg/mL. Thus, it appears they were able to achieve plasma concentrations within the desired range for antitumor activity (albeit in the mid to lower portion of the range). Also noted from Fig. 5A–C, a reduction in dose was necessary throughout the duration of each exposure, to maintain the targeted aPTT (especially obvious in the 8-wk treatment cohort). In fact, such a modification in dose led them to speculate whether saturation of elimination was occurring. The terminal half-life had been estimated to be 55 h; thus, the time to steady state should be 11.4 d. However, looking at Fig. 5C suggests that steady state was not reached by 8 wk. This led them to question whether the half-life, of effect was different than the plasma half-life or if the terminal half-life was inaccurately estimated (i.e., a deep compartment that was not characterized). Nonetheless, continued dose reductions were necessary throughout the treatment course to maintain the aPTT in the desired range.

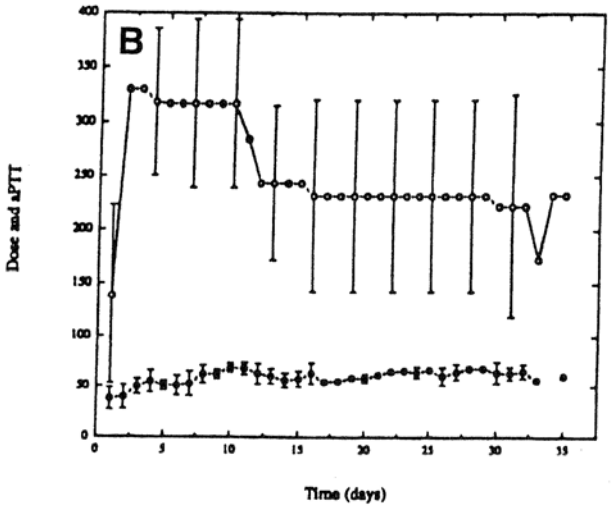
Parker et al. *(33)* suggest that the concentrations of PPS necessary to inhibit heparin-binding growth factors (HGFs) are substantially below those that alter the aPTT. A bioassay with SW-13 cells determined that aPTT changes were noted with concentrations above 1 μg/mL; however, HGF-dependent proliferation was inhibited at concentrations <0.1 μg/mL.

4. ANTICANCER CLINICAL TRIALS

As shown above, there is a relatively large amount of preclinical data regarding the antineoplastic activity of PPS, using both cell cultures and animal models. By contrast, the data from human clinical trials are relatively limited. Several types of phase I trials have been performed in patients with a variety of solid tumors, in an attempt to better define both the dose and duration of therapy. Phase II trials, exploring specific antitumor activity, have only been conducted in patients with human immunodeficiency virus

Fig. 5. Average dose (mg/kg/d) and aPTT (○) for patients treated with pentosan by continuous infusion. Data represents mean dose (mg/d) administered (○) and aPTT (s, ●) for patients treated for 5 wk **(A)**, 5 wk **(B)**, and 8 wk **(C)**. Bars represent standard deviation of mean dose and aPTT. Error bars presented for dose are only plotted for every third data point for clarity. PPS dose was adjusted to maintain an aPTT between 1.8 and 2.2 times baseline value. As shown in (A–C), the dose necessary to maintain that level of activity decreases with time. Reprinted with permission from ref. *32*.


A
Dose and aPTT
Time (days)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Dose and aPTT
Time (days)

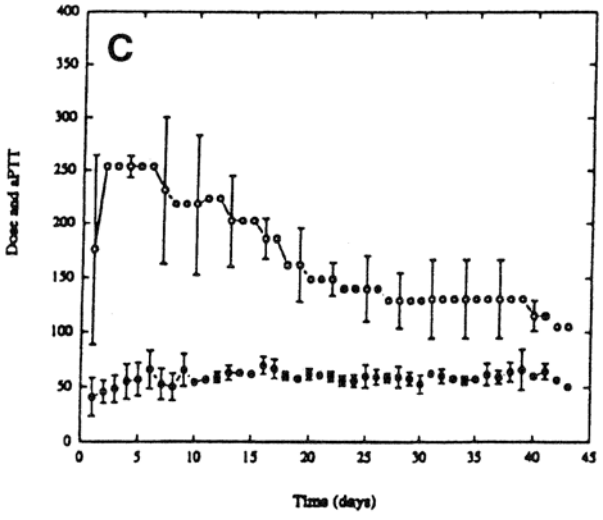
C
Dose and aPTT
Time (days)

Table 1
CTEP Grade II or Greater Toxicities Associated with Pentosan

Toxicity	*3 wk*	*5 wk*	*8 wk*
Hepatic			
T. bili	—	III	—
SGPT/SGOT	II	II,II,II	III
Alk Phos	—	—	—
Hematalogic			
Thrombocytopenia	—	II,II	II,II,IV
PT	n/a	n/a	n/a
aPTT	n/a	n/a	n/a
Anemia	—	III,III,III[a]	—
Catheter complications			
Infection	—	II,II[a]	—
Pneumothorax	—	—	II
Atrial fibrullation	—	II	—
Gastrointestinal bleeding	—	II	—

The roman number indicates the grade of toxicity, and the number of symbols indicate the frequency of the event.

[a]Same patient.

n/a, not applicable; all patients had prolongation of their aPTT/PT, but doses were adjusted to maintain the value within 1.8–2.2 of the baseline. Adapted with permission from ref. *32*.

(HIV)-associated KS. It is important to note that no trials have been reported in patients with early-stage cancers in an attempt to prevent the development of metastatic disease.

Three PPS phase I studies have been performed in patients with a variety of solid tumors. In 1996, Lush et al. *(32)* reported results of a small phase I study that was designed to administer continuous intravenous infusions for progressively longer durations (3, 5, or 8 wk of therapy). Participating patients had a variety of solid tumors. PPS was initially administered as a continuous iv infusion at dose of 4 mg/kg/d. Subsequent dosing was used to maintain an aPTT between 1.8 and 2.2× baseline. The coagulation parameters were initially determined on a daily basis, and dosages adjusted until the targeted range was achieved for three consecutive days. Subsequent dosage adjustments were performed less often. Three cohorts of patients were treated. Patients in each cohort received three, five, or eight consecutive weeks of PPS therapy. No objective responses were achieved using radiographic criteria or changes in tumor markers. Of the 13 total patients treated, 11 had therapy discontinued because of progressive disease. The median time to progression was 8 wk. Anticoagulation was regularly achieved in all patients. The dose necessary to achieve the targeted aPTT (1.8–2.2× normal) declined with time. The dose in all cohorts was approx 250–320 mg/d during the first 5 d. By wk 4 of infusion, however, the dose necessary to achieve the same degree of anticoagulation had fallen to approx 150 mg/d. This progressive decrease in dose administered was noted in all cohorts. Thus, continued dose reductions were necessary throughout the treatment course to maintain a constant aPTT. Grade II–IV thrombocytopenias (platelet counts between below $50 \times 10^3/\mu L$) were noted in five patients (Table 1). Of the four patients treated for 8 wk, three developed grade II or worse thrombocytopenia. Grade II–III elevations in liver function tests (between 2.6 and 20.0×) were also noted in five patients, but this toxicity did not appear

to be duration-dependent. The authors concluded that, when using this dosing schema, a 5-wk infusion duration would be appropriate for future studies.

A second phase I study, performed in various adult solid tumors, utilized PPS at three dose levels administered by intermittent sc injection every 6 h *(34)*. Dosages of PPS were 15, 22.5, or 30 mg/m^2/dose (60–120 mg/m^2/d). No objective radiographic or measurable responses were noted in the 19 patients enrolled on this study. Three patients were reported to have had stable disease lasting 16–76 wk. Dose limiting toxicities were achieved in the cohort receiving the 30 mg/m^2/dose. No clinical toxicities were observed. Laboratory abnormalities included both thrombocytopenias and liver function test abnormalities. These authors concluded that the recommended starting dose for phase II clinical trials should be 22.5 mg/m^2/dose (90 mg/m^2/d) administered subcutaneously every 6 h.

A third phase I PPS trial was conducted using an oral dosing schema *(35)*. To date, these data have been reported only in abstract form. PPS was administered 3 times daily (tid) at oral doses ranging from 180, 270, and 400 mg/m^2/dose. Treatment duration ranged from 4 to 40+ wk (median of 12 wk). No antitumor responses were noted. Toxicity was limited to increases in stool frequency and blood in the stool. The authors monitored inhibition of HGF activity in patient serum, but no evidence of this inhibition was detected. The authors concluded that PPS could be safely administered in tid oral doses up to 400 mg/m^2/dose, but stated that they found no evidence of systemic biological activity when using assays designed to assess inhibition of growth factor activity.

The rationale for evaluating PPS in HIV-associated KS is clear. FGF, as well as related molecules, are postulated to play an important role in the pathogenesis of this neoplasm, and PPS clearly inhibits FGF-dependent tumor grow under a variety of both in vitro and in vivo conditions. Pluda et al. *(11)* at the NCI reported treating 16 patients with HIV-associated KS on a phase I study with PPS. These investigators utilized a continuous iv infusion for 3–6 wk, followed by intermittent (3×/wk) sc doses. In this particular study, the continuous drug infusion was administered at several different dosages, including 2 mg/kg/d, 3 mg/kg/d, and 4 mg/kg/d. During the intermittent phase of treatment, a similar dose was administered by sc injection. Some patients also received direct intralesional injections as well. After 6 wk of PPS treatment, all patients were also administered AZT (zidovudine) at a dose of 100 mg by mouth every 4 h. In this trial, no patients had any evidence of antitumor response. This includes patients who received direct intralesional injections. Three patients were noted to have stable disease for periods up to 27 wk. Given the lack of controls in this study, it is difficult to conclude whether or not this simply reflects the natural history of the disease. No changes were noted in circulating immune (CD4) cells, or in viral antigen levels. Toxic effects were primarily related to anticoagulation, decreases in platelet counts, and hepatotoxicity. Each of these toxicities was reversible upon discontinuation of the drug. The authors concluded that the maximum tolerated continuous iv dose of PPS was 3 mg/kg/d.

A second clinical trial evaluated the activity of PPS in patients with HIV-related KS *(36,37)*. In this trial, PPS was administered at a dose of 25 mg/m^2/d every 6 h on d 1, followed by 25 mg/m^2 every 12 h by sc injections. All patients were homosexual males with histologically confirmed KS. A total of 16 patients were treated with PPS, and four patients also received antiretroviral agents (AZT ordideoxyinosine). Stable disease was noted in three patients, lasting up to 11 wk; one objective partial response (80% regression) was documented, which lasted for a total of 9 wk. Toxicity was restricted to mild

pain at the injection site, and low-grade fevers. No bleeding was observed, and no significant effects on anticoagulation parameters were noted when using this dosing schema. The authors concluded that a new trial was warranted at higher doses, given the lack of toxicity and the demonstration of a partial response in one patient.

When taken together, the clinical trial experience with PPS is quite limited. Furthermore, given the known biological activities of this agent, one could argue that the human clinical trials to date have neglected one of the most promising areas of potential clinical activity, i.e., trials designed to directly assess the antimetastatic activity of this compound.

5. OTHER INDICATIONS

PPS (Elmiron®, Baker Norton, Essex, UK) has recently been approved by the US Food and Drug Administration for oral treatment of interstitial cystitis *(38)*. The mechanism of action for the treatment of interstitial cystitis has not been fully elucidated, but it appears the anticoagulant and fibrinolytic effects of PPS causes changes in bladder surface mucin, which decreases bladder wall permeability *(39,40)*. A meta-analysis of four randomized placebo-controlled studies (n = 398) found that pentosan was more effective than placebo in the treatment of pain, urgency, and frequency *(41)*. One of those studies evaluated was conducted by Mulholland et al. *(42)* (n = 110 patients treated for 3 mo), who found that 28% of patients receiving PPS had improvement, but only 13% of the placebo group reported improvement ($p = 0.03$). Another trial noted that 38% ($n = 74$) of those patients treated with PPS reported moderate improvement in pelvic pain, compared with 18% in the placebo group ($n = 74$) *(40)*. Sixteen percent of those treated with PPS had an increase in mean urine volume. Finally, Baker Norton reports that retrospective nonblinded data find that 29% of patients (n = 2499) report symptomatic improvement after 6–12 wk of therapy *(38)*.

As noted at the beginning of the chapter, PPS has been used as anticoagulant for years. Bergqvist et al. *(18)* treated 20 patients with acute deep vein thrombosis (DVT) randomized to receive either heparin or PPS. No significant difference was noted in therapeutic effect. However, clinical trials have shown PPS to be effective in preventing DVT.

Calcium oxalate crystal growth and aggregation leads to the formation of renal calculi. Compared to pyrophosphate, heparin, and chondroitin-4-sulfate, PPS was a more potent inhibitor of calcium oxalate crystal growth. However, Fellstrom et al. *(43)* found no difference between the treatment and observation periods for the stone-formation episode rate and stone operation rate in 121 patients enrolled in an open-labeled trial. They did report a reduction in the size of the stone, and less pain associated with the passage.

6. SUMMARY

PPS, a sulfated polysaccharide used as an anticoagulant for many years, was the first agent to be evaluated for its abilities to inhibit angiogenesis. The agent has been shown to bind a host of tumor-secreted heparin-binding factors, including bFGF. In addition, pentosan inhibits in vitro endothelial cell proliferation and migration. Pentosan has also been shown to inhibit the in vivo growth of an adrenal cell carcinoma cell line transfected with a secretable form of bFGF, as well as the growth of a number of tumor cell lines that secrete HGFs in a paracrine manner, when implanted into athymic nude mice.

A number of clinical trials, administering PPS via continuous iv infusion, subcutaneous injection, and orally, have been reported. In general, no antitumor activity has been

noted. In addition, the administration of parenteral pentosan was associated with significant toxicity in the form of anticoagulation, thrombocytopenia, and abnormal hepatic function. Also, it would appear that pentosan is not adequately absorbed when administered orally as an antitumor agent. Thus, although pentosan inhibited in vitro endothelial cell functions consistent with the inhibition of angiogenesis, it did not appear to be a feasible antitumor agent because of its toxicity, lack of activity, and difficult administration. Perhaps analogs or other agents in this same class will be able to overcome the difficulties encountered with the administration of pentosan as an antitumor agent.

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22 TNP-470

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CONTENTS

1. INTRODUCTION

Proliferation of endothelial cells is critical to the formation of new blood vessels. These cells, which form the innermost layer of the vasculature, are normally in the G_0 phase of the cell cycle. They are able to respond to several different growth factors, and this response can result in neovascularization or angiogenesis. Angiogenesis is a normal physiologic component of growth, reproduction, and wound healing. It is also a pathogenic and critical component of tumor growth and metastasis, and it is this aspect of angiogenesis that the development of TNP-470 addresses.

O-(chloroacetyl carbamoyl) fumagillin, or TNP-470, is a synthetic analog of fumagillin, a compound secreted by the fungus *Aspergillus fumigatis fresenius.* The initial studies demonstrating its angioinhibitory effects were done by Ingber in the laboratory of Folkman, as a result of an observation of growth inhibition of an endothelial cell culture inadvertently contaminated by this fungus *(1)*. These studies demonstrated that fumagil-

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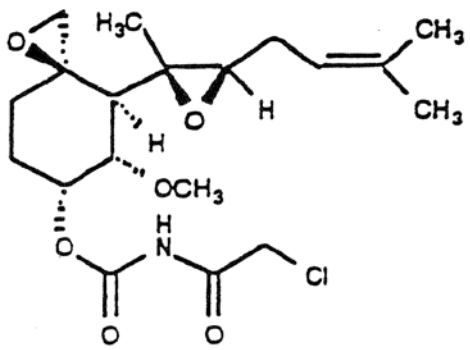

Fig. 1. Chemical structure of TNP-470. The chemical formula and mol wt are $C_{19}H_{28}ClNO_6$ and 401.89, respectively.

lin inhibited angiogenesis and tumor growth in vivo, as well as endothelial cell proliferation in vitro. Clinical development of this compound was not possible, because of the dramatic weight loss suffered by laboratory animals after prolonged exposure to the drug. A collaborative effort between scientists at Harvard and Takeda Chemical Industries resulted in the synthesis of a series of less toxic compounds, of which TNP-470 (previously known as AGM-1470) was among the most potent. Its chemical structure is shown in Fig. 1.

2. CELL BIOLOGY STUDIES

The effects of TNP-470 on proliferation are cell-specific. In general, TNP inhibits the mitogen (basic fibroblast growth factor [bFGF])-induced proliferation of endothelial cells (human umbilical vein [HUVEC], bovine capillary) with an IC_{50} in the pg/mL range; cytotoxic concentrations are orders of magnitude higher, i.e., ng-μg/mL. These concentrations correlate with those required to inhibit DNA synthesis and RNA and protein synthesis, respectively, in HUVECs *(1–4)*. The growth of transformed endothelial cells is not inhibited by TNP-470 *(5)*.

The effect of TNP on the proliferation of a wide variety of human and rodent transformed or tumor-derived cell lines has also been examined. In general, cytostatic and cytotoxic concentrations are comparable, and in the ng/mL–μg/mL range *(1–4,6,7)*. Exceptions have been noted, and include human embryonic lung fibroblasts and two of six tested human glioblastoma lines, which have IC_{50} values comparable to those determined for endothelial cells (i.e., pg/mL) *(8)*. It also appears that in the presence of normal T-cells, TNP is able to enhance the proliferation of murine *(9)* and normal human-, but not tumor-derived lymphocytes *(10)*.

TNP-470 inhibits mitogen-driven angiogenesis in a dose-dependent fashion in several model systems, including embryonic chick chorioalantoic membrane assay (CAM; *2*), rat and rabbit corneal micropocket assays *(6,7)* (and surgically implanted sponges *(2)*.

The effect of TNP on the rate of wound healing has been investigated. It was demonstrated that there is no effect if the drug is administered prior to wounding, but that healing is delayed if TNP is given immediately following injury *(7)*.

3. IN VITRO/IN VIVO TUMOR STUDIES

TNP-470 has been shown to be effective in decreasing the growth of primary tumors and the growth and incidence of pulmonary, hepatic, and lymph node metastases in a wide variety of rodent homograft and xenograft models, using a variety of dosing schedules and routes of administration. In most instances, antitumor effects were dose-dependent. Rodent tumor models included hemangioendothelioma, melanoma, osteosarcoma, reticulum cell sarcoma, glioma, hepatoma, fibrosarcoma, and mammary, lung, bladder, tongue, and pancreatic carcinomas. Human xenograft models included glioblastoma, neuroblastoma, medulloblastoma, meningioma, fibrosarcoma, neurofibrosarcoma, rhabdomyosarcoma, and gastric, colon, breast, prostate, and esophageal carcinomas. No antitumor activity was reported in these cases: a rat astrocytoma *(11)*, a human endometrioid carcinoma *(12)*, and a human gastric carcinoma *(12)*. With few exceptions, weight loss or failure to gain weight was observed in animals receiving high doses of TNP. There were two reports in which a decrease in splenic weight was noted *(13,14)*.

Routes of administration included subcutaneous (sc), intravenous (iv), intraperitoneal (ip), intra-arterial (ia), and instillation into the urinary bladder, with sc administration being the most frequently used. Dosing schedules ranged from 10–60 mg/kg every other day (qod) to 50–200 mg/kg once per week, with 30 mg/kg qod being the most common.

TNP, encapsulated into microspheres or dissolved in a lipid-based contrast medium or medium chain triglycerides, was delivered ia, and resulted in prolonged antitumor effects after even a single dose *(15,16)*. Instillation of 1 μg/wk into heterotopically implanted rat urinary bladders was sufficient to reduce the incidence of chemically induced tumors. The solution withdrawn from the bladders at the end of each week's treatment retained the ability to inhibit endothelial cell proliferation in vitro, indicating long-term stability of the drug's antiproliferative activity *(17)*.

In several studies, the antitumor effect was accompanied by an increase in tumor necrosis and an increase in mean survival *(11,14,18,19)*. A report documenting the effect of TNP against rat ascites hepatoma indicated that a 2-wk treatment interval was sufficient to increase the long-term survival of treated animals. In addition, although metastatic foci were present in all animals at the end of the 2-wk treatment period, only one small dormant metastatic focus was present in a single animal at 4 mo *(19)*. These data suggest a persistent effect of limited-study drug administration.

A direct antimetastatic effect of TNP was demonstrated in a rat fibrosarcoma model. Here, metastatic growth was inhibited, even when TNP administration was delayed until after resection of the primary tumor *(20)*.

4. COMBINATION THERAPY STUDIES

Potentiation of tumor response is observed in a number of rodent homograft and xenograft models when TNP-470 is co-administered with immunotherapy, hyperthermia, radiation, other antiangiogenic agents, and/or cytotoxic drugs. This is the case when TNP is used in combination with an anti-bFGF antibody in a murine squamous cell carcinoma model *(21)*, with tamoxifen *(22)* or 5'-deoxy-5-fluorouridine *(23)* in a rat mammary carcinoma model, with radiation against Lewis lung carcinoma (LLC) *(24)*, with mitomycinC or interferon (IAI)-α/β against murine melanoma and LLC *(25)*, and with 5-fluorouracil (5-FU) against LLC *(25)*. Xenograft models, in which combination therapy resulted in an increase in

efficacy, include breast *(26)*, prostate *(7)*, and esophageal and gastric carcinomas *(27)*, with tamoxifen, cisplatin, and hyperthermia, respectively.

Several studies included TNP in a regimen of multiple therapeutic agents. In one study, a 40% cure was effected in a LLC model using a combination of cyclophosphamide, TNP, and minocycline *(24)*. In a drug-resistant murine mammary carcinoma model, TNP, in combination with cisplatin, cyclophosphamide, or thiotepa, with or without minocycline, resulted in potentiation of cytotoxicity against the primary tumor, as well as a decrease in the incidence of metastatic disease, and an increase in long term survival *(28)*. In an exhaustive study using LLC as the model, combinations of several antiangiogenic agents were used, with or without traditional cytotoxic treatments. Regarding antiangiogenic treatment alone, the combination of TNP and INF-δ4 was the most effective. When TNP was used in conjunction with cisplatin, 5-FU, cyclophosphamide, BCNU, adriamycin, or radiation, an additional increase in efficacy was noted when minocycline or 14 (sulfated)-β-cyclodextrin–tetrahydrocortisol (14(SO_4)-β-CD–THC) was added to the treatment regimen. Inclusion of genistein improved the efficacy of TNP and cyclophosphamide or adriamycin. Addition of INF-δ4 to the regimen of TNP and cyclophosphamide, BCNU, adriamycin, or radiation also improved the response. Combinations that resulted in the best delay in primary tumor growth were not always the best combination when it came to inhibiting metastasis *(30)*.

Several experiments included attempts to elucidate the mechanism of these observed synergies. Adding TNP-470 and minocycline to cyclophosphamide in a breast carcinoma model resulted in an increase in the number of tumor cells killed by the treatment; an increase in bone marrow cell kill was also noted, but to a lesser degree *(24)*. Using a transgenic mouse model of progressive pancreatic cancer, a combination of TNP, minocycline, and INF-α/β resulted in growth inhibition of the primary tumor with a concomitant reduction in tumor capillary density, and an increase in the apoptotic index of tumor cells *(30)*. These observations suggest that, in addition to, or as a consequence of, its effect on the endothelium, TNP-470 is able to enhance the effectiveness of biologic or cytotoxic therapy by shifting the equilibrium between tumor cell proliferation and death.

A study using radiolabeled cyclophosphamide, or tracing the platinum moity of cisplatin, demonstrated an increase in DNA crosslinking, and an increase in both tissue and tumor uptake of cytotoxic drug, when TNP was included in the regimen *(31)*. This suggests that TNP, probably through its effect on the vasculature, increases the relative availability of therapeutic agents. In contrast, a study in a rat glioma model, which used a microdialysis probe to collect tumor interstitial fluid samples, indicated that TNP in combination with temozolamide (TMZ) resulted in a 25% decrease in interstitial TMZ levels *(32)*. This seems to conflict with what was found in the cyclophosphamide–cisplatin study. However, this combination of TNP and TMZ did result in a 59% decrease in tumor volume. In this study, neither the degree of DNA alkylation nor survivial time were determined.

A variety of dosing regimens were used in the combination therapy experiments. One report has indicated that the sequence in which the cytoxic agent and TNP are administered affects the degree of efficacy obtained *(33)*. Experiments are currently underway to investigate the schedule and sequence dependency of combination therapy.

5. MECHANISM OF ACTION STUDIES

Much effort has been directed toward elucidating the mechanism by which TNP-470 affects endothelial cell proliferation. Several laboratories have investigated how and when within the cell cycle TNP might block growth factor-induced proliferation. Time-course studies suggest that TNP affects events that occur late in G_1, but prior to the transition to S-phase *(5,34,34)*. The actual degree to which specific CDKs are inhibited is dependent on the source of endothelial cells, and the timing of exposure to TNP relative to that of mitogen. The effects of TNP within G_1 of HUVECs include inhibition or attenuation of several cyclins, attenuation of bFGF-induced activation of cyclin-dependent kinases cdc2 and cdk2, and inhibition of phosphorylation of the retinoblastoma (RB) protein *(35)*.

Maier et al. *(36)* report that TNP inhibits the activity of urokinase-type plasminogen activator (uPA) in endothelial cells. This same inhibition is observed when an antibody to endoglin (a component of the TGF-β receptor system) is used; there is no additive effect when the antibody is used in conjunction with TNP. The authors suggest that TNP and endoglin may have similar targets, or affect a similar pathway. It is difficult to draw conclusions from this report, because it is unclear whether cytostatic or cytotoxic concentrations of drug were used.

It has also been demonstrated that the addition of TNP to bovine aortic endothelial cells (BAE) leads to an increase in E-selectin mRNA and protein. That this increase was more pronounced in subconfluent than in confluent cells may suggest a mechanism by which TNP can inhibit tumor growth in vivo with no obvious effect on the normally quiescent endothelium *(37)*. It is counterintuitive that TNP would enhance the expression of E-selectin, which is expressed by dividing endothelial cells. This is further complicated by the fact that the concentration of TNP used in these experiments was in the cytotoxic range; it would be interesting to repeat these experiments using cytostatic concentrations of the drug.

There are several reports that indicate that TNP can also affect nonendothelial cells. As mentioned in Section 2., TNP enhances the proliferation of normal human, but not tumor-derived, B-lymphocytes; in vitro, this phenomenon requires the presence of normal T-cells *(9)*. Also in vitro, TNP stimulates the proliferation of murine B/T-lymphocytes in the presence of phytohemagglutinin *(13)*. Follow-up in vivo experiments showed an increase in the size of axillary or mesenteric nodes in a murine model; following treatment with TNP; this increase was not apparent in T-cell-deficient mice *(9)*. The mechanism by which TNP modulates the immune system warrants further investigation.

TNP inhibits PDGF- or IGF-induced proliferation of bovine aortic vascular smooth-muscle cells; this inhibition is associated with decreased cdk2 activity and mRNA levels induced by both mitogens, and with a partial inhibition of cdk4 mRNA induced by PDGF. At concentrations up to 100 ng/mL (cytotoxic), TNP could only partially inhibit the proliferation induced by fetal calf serum (FCS) *(38)*. This suggests that the effectiveness of TNP regarding cell proliferation depends not only on the cell type, but on the mechanism of proliferation induction.

There are also reports of TNP directly affecting tumor cells. A decreased secretion of bFGF has been reported for squamous cell carcinoma *(39)* and uterine endometrial carcinoma cells *(40)*. Kondo *(41)* has reported that TNP affects the cell cycle of colon 26 cells; however, the concentration of study drug used in these experiments was in the cytotoxic range.

It has recently been shown that a biotinylated derivative of fumagillol is able to bind to methioninaminopeptidase-2 (MetAP-2), a highly conserved, cobalt-dependent metalloproteinase expressed by endothelial and nonendothelial cells *(42)*. This same report demonstrated that fumagillin selectively inhibits the growth of yeast that are dependent on MetAP-2 for proliferation. MetAP-2, also known as p^{67}, a eukaryotic initiation factor 2 (eIF2)-associated protein, is able to interfere with the phosphorylation of eIF2 *(43,44)*, and is involved on posttranslational processing *(45,46)*. It may be that fumagillin–TNP is able to interfere with endothelial cell proliferation by affecting the activity or stability of proteins specifically involved in endothelial cell translation and/or cell-cycle signaling.

6. OVERVIEW OF CLINICAL TRIAL EXPERIENCE

Clinical trial experience with TNP-470 presently includes seven phase I and five phase II studies, in which more than 300 patients have been treated. All except one phase I study (pediatric malignancies) are for adult patient populations, including individual phase I studies in patients with hormone-refractory prostate cancer and cervical cancer, two in patients with solid tumors and two in AIDS-associated Kaposi's sarcoma (KS) patients. Three of these studies have concluded (prostate, cervical, and AIDS-associated KS), but the two solid tumor, one AIDS-associated KS and the pediatric study are ongoing. Phase II patient populations are limited to glioblastoma multiforme, locally advanced pancreatic cancer, and advanced breast, cervical, and renal cancer. Approximately two-thirds of the total number of patients exposed to TNP-470 derive from the phase I experience, and these are the data that will be discussed in this chapter. Findings in the pediatric phase I study are preliminary and will not be included, however. Nor will findings for the two phase I studies in AIDS-associated KS patients *(47)*, since these data reside with the institutional sponsors, and at present are not included in the TNP-470 database.

The dosing regimens employed in the phase I studies varied in both the duration and frequency of administration of TNP-470. Either a 1- or 4-h iv infusion was used. The latter was administered once weekly; the 1-h infusion was given once weekly or multiple times (either every other day [qod] or on a Monday, Wednesday, and Friday schedule [MWF]) per week. The final difference in dosing regimens is the inclusion of a rest period between treatment cycles. Maximum-tolerated doses (MTD) and dose-limiting toxicities (DLT) have been determined in all cases, with the exception of the 1-h once weekly schedule. The dosing regimen that has been taken into the initial phase II studies is 1-h, every MWF, and does not incorporate a rest period. The individual phase I study dosing regimens are summarized below:

Prostate cancer: 1-h, qod for 28 d, followed by a 14-d rest period
Cervical cancer: 1-h, qod for 28 d, followed by a 14-d rest period
Solid tumor: 1-hr, MWF, no rest period
Pediatric cancer: 1-h, MWF, no rest period
AIDS-associated KS: 1-h, qod, no rest period
AIDS-associated KS: 1-h, once weekly for 12 wk, followed by a 2-wk rest period
Solid tumor: 4-h, once weekly, no rest period

The data presented in the remainder of this chapter derive from four adult patient phase I studies (prostate, cervical, and two solid tumor), and represent findings for a total of 121 patients.

7. PATIENT DEMOGRAPHICS

The data presented in this overview of the phase I experience with TNP-470 are from 121 adult cancer patients, 56.2 and 43.8% of whom are male and female, respectively. Their mean age is 53.3 yr, with a range of 22–82 yr. The patients were predominantly Caucasian (86.8%); the percentages of Black, Hispanic, and Asian patients were 5.8, 6.6, and 0.8%, respectively.

Adenocarcinomas comprised the largest class of tumors tested in the four phase I studies (45.5%). Sarcomas represented 27.3%, with leiomyosarcomas accounting for more than half of these cases, and squamous cell carcinomas contributed 19.8%. The remaining 7.4% consisted of five cases of melanoma, and single cases of various tumor types.

8. DURATION OF TREATMENT AND OUTCOMES

The median duration of treatment ranged from 27 to 69 d, representing one treatment course in each of three studies (cervical cancer and two solid tumor) and two treatment courses in the prostate cancer study. Total duration of treatment ranged from 1 to 672 d. Four patients were treated for greater than 1 yr. These include two women with advanced cervical cancer (508 and 652 d), one patient with a fibrous histiocytoma (672 d), and one patient with advanced colon cancer (388 d).

The primary reason that patients were discontinued was disease progression, which occurred in 71.1% (86 of 120) of patients. Adverse events and intercurrent medical events each accounted for 9.1% (11 of 120) of patients' discontinuations. The incidence of all other reasons for termination did not exceed 3.3% for each category: death, personal reasons, lost to follow-up, and so on. Two deaths, both attributed to the patients' diseases, occurred within 10 d of the last dose of TNP-470. One cervical cancer patient discontinued treatment after experiencing and maintaining a complete response for more than 18 mo. Another cervical cancer patient with demonstrated stable disease of greater than 18 mo duration was transferred to an extended-use protocol, and continues treatment with TNP-470.

9. ADVERSE EVENT PROFILE OF TNP-470

Treatment-emergent medical events, which include adverse events (AE; events considered by the investigator to be possibly, probably, or definitely related to TNP-470 administration, or of unknown relationship) and complaints considered by the investigator to not be related to TNP-470, were reported for 100% of patients. The AEs most frequently (incidence in excess of 15%) reported by patients include asthenia (42.1%), nausea (32.2%), anorexia (18.2%), and dizziness (15.7%). It is important to note that the incidence of complaints of anorexia (18.2%) was not reflected in the incidence of weight loss greater than 10% (grade 2), which occurred in only six of 121 (5.0%) patients, and complaints of anorexia were recorded in only three of these cases. Overall, 99 of 121 (81.8%) patients experienced at least one AE, and a total of 408 AEs were recorded. Greater than 70% of AEs were categorized as mild or grade 1 (Common Toxicity Criteria). An additional 20% were moderate or grade 2 in severity, and only 7.6% were considered to be severe or meet grade 3 or 4 criteria. Included in this last group are complaints of abnormal gait (five of 408), asthenia, nausea, and dizziness (three each of

408); and anorexia and vomiting (two each of 408). The greatest incidence of severe or grade 3 or 4 AEs involved neurological function (16 of 110 AEs, or 14.6%). In addition to gait disturbances and dizziness, these neurologic AEs included single complaints of vision abnormalities (diplopia, amblyopia, and nystagmus), emotional lability, coordination difficulties, insomnia, nervousness, and vertigo. The incidence of AEs related to central nervous system and visual function increased in a dose-dependent manner, but this was not the case for AEs affecting other systems. Central nervous system AEs of grade 2 or higher severity (moderate or severe) increased in incidence at dose levels of 40 mg/m^2 and higher, when TNP-470 was administered as a 1-h infusion qod, or every MWF. The incidence of grade 2 or higher (moderate or severe) AEs did not differ between male and female patients.

10. DOSE-LIMITING TOXICITIES AND DETERMINATION OF MAXIMUM-TOLERATED DOSE

DLTs were reported for three of 32 prostate cancer patients, two of 21 cervical cancer patients, and five of 68 solid tumor patients. Excepting one report of hemoptysis (grade 1), which occurred in a patient with NSCLC with a history of similar episodes, all were neurological in nature. DLTs involving complaints of gait and coordination disturbances, memory impairment, increased anxiety, and emotional lability were observed in three prostate cancer patients at two dose levels, 70.6 (two patients) and 105.9 (one patient) mg/m^2. In two cases, the DLTs presented within the first 3 wk of treatment; in one case they were delayed and reported after the 2-wk rest period. Two cervical cancer patients experienced DLTs following treatment at 71 mg/m^2 for 4 wk; these consisted of gait disturbance as well as dizziness and nystagmus. In the solid-tumor study administering TNP-470 every MWF, a single DLT (grade 1 unsteadiness) was reported in a patient with melanoma after 102 d of treatment at 32.4 mg/m^2. The cohort was expanded, but no additional DLTs were reported at that dose level. However, at 76.5 mg/m^2, two patients experienced DLTs. In one case, a prostate cancer patient had complaints of grade 3 gait disturbance and nystagmus after 40 d of treatment. The second patient had complaints of grade 1 hemoptysis. Finally, in the solid-tumor study using a once weekly schedule, two patients experienced dizziness, grades 1 or 2, after 52, or 78 d of treatment at 235 mg/m^2, respectively.

Patients experiencing DLTs were evaluated utilizing radiographic and neuropsychologic assessments. Findings were negative for CT scans and MRIs, but psychometric testing showed effects on cognitive function and affect. In most cases, resolution of the DLTs occurred within several weeks, and, in all cases, the DLTs were reversible.

MTDs were determined in each of the four phase I studies. Two studies (prostate and cervical cancer) used the same dosing regimen, i.e., 1-h infusion qod for 28 d, followed by a 14-d rest period, and these MTDs were 47.1 and 60.0 mg/m^2, respectively. When TNP-470 was administered as a 1-h infusion every MWF in the absence of a rest period, the MTD was 57.4 mg/m^2. Extending the infusion to 4 h, and administering TNP-470 once weekly, resulted in an MTD of 177 mg/m^2.

11. OTHER SAFETY FINDINGS

Findings in preclinical toxicology studies suggested the need for careful monitoring of selected hematologic, coagulation, and serum chemistry parameters. In most cases,

decreases in hemoglobin of ≥2 mg/dL (incidence of 18 of 121 patients, or 14.9%) were not considered clinically significant, and, when clinically significant, were attributed to the patient's disease. With few exceptions, decreases in platelet (<100,000/μL; 9 of 121 patients, or 7.4%) and lymphocyte (<500/μL; 35 of 121 patients, or 28.9%) counts were either attributed to the patients' underlying disease or prior therapy, and not administration of TNP-470, or were considered to not be of clinical significance. Decreased lymphocyte counts were common in the patient population, and patients with low values at baseline were frequently enrolled. Increases in excess of 1.5× the upper limit of normal, in either prothrombin or partial thromboplastin times, were rare and consistently attributable to interventions with anticoagulants (administered systemically or used to maintain central line patency). SGPT values greater than 3× the upper limit of normal were observed in five cases, and were either related to the patients' disease (three cases) or judged to be not clinically significant (two cases). In all five cases, the elevations occurred sporadically, and resolution to baseline was documented.

An additional toxicology finding, the development of cataracts in dogs following 3 mo treatment with TNP-470, was monitored closely by the incorporation of serial ophthalmologic examinations into the study design. In two cases, a 69-yr-old prostate cancer patient and a 70-yr-old female patient with metastatic colon cancer, the development of cataracts was recorded. The former patient was treated with 47.2 mg/m^2 of TNP-470 for 126 d prior to cataract detection, and the latter received TNP-470 at 235 mg/m^2 for 85 d before mild cataract changes were noted. In both cases, earlier ophthalmologic examinations during treatment did not show these changes.

12. EFFICACY

The efficacy criteria that are commonly applied to oncology clinical trials may not be appropriate for antiangiogenesis agents such as TNP-470. This is because response criteria assume that the result of a chemotherapeutic intervention will be tumor regression. Under these conditions, positive outcomes are limited to either a partial (PR) or complete response (CR), and exclude stable disease. However, TNP-470 may be tumoristatic, not cytotoxic. Treatment resulting in a slowing of tumor growth could present as disease stabilization, which has traditionally been regarded as treatment failure.

The phase I experience with TNP-470, described in detail in Section 8., reflects this situation. Seven patients experienced disease stabilization of at least 5 mo duration, and one cervical cancer patient had a CR *(48–50)*. TNP-470 was administered under the same schedule (1-h infusion qod for 28 d, followed by a 14-d rest period) to prostate and cervical cancer patients. One prostate cancer patient with bone metastases was treated for 9 mo at 9.3 mg/m^2 prior to disease progression. Two cervical cancer patients were treated for 5 and 6.5 mo, respectively, before their disease progressed. These patients were permitted intrapatient dose escalations (14 → 47.5 and 31.5 → 47.5 → 71 mg/m^2, respectively). A third cervical cancer patient has been treated for more than 18 mo, and continues on treatment at 60 mg/m^2, with serial assessments of stable disease. When TNP-470 was administered as a 4-h infusion once weekly, three patients experienced disease stabilization. A patient with metastatic melanoma remained on treatment at 25 mg/m^2 for 6 mo prior to disease progression. At 50 mg/m^2, a patient experienced stabilization of a fibrous histiocytoma from May 1995 until March 1997, at which time he discontinued treatment, but had not progressed. Finally, a patient with metastatic colon cancer received

TNP-470, 235 → 177 mg/m^2, for 13 mo. At study discontinuation, the patient's disease remained stable.

The CR occurred in a patient with cervical cancer metastatic to the lungs (histologically documented), who was treated at 71 mg/m^2. Tumor regression (PR) was first noted after treatment for 3 mo, and this was followed by documentation of a CR. The patient was treated from February 1995 until January 1997. As of May 1997, the CR was maintained.

13. PHARMACOKINETICS

Determination of the pharmacokinetic profile of TNP-470 has been a challenging undertaking, chiefly because of to the rapid and extensive metabolism the drug undergoes, and its pH-dependent stability. Only two of the many observed metabolites have been characterized. These are AGM-1883 (M-IV), which results from an initial enzymatic degradation, and M-II, the hydrolysis product of M-IV. TNP-470 is most stable at a pH of 3.0–5.0. Thus, unless biological samples have their pH adjusted, TNP-470 rapidly degrades. These metabolic characteristics and stability concerns emphasize the importance of appropriate handling of biological samples containing TNP-470.

The current understanding of TNP-470 pharmacokinetics derives from the phase I study in which it was administered as a 4-h infusion once weekly. Data for three patients treated at 235 mg/m^2 suggested that the $t_{1/2}$ values for both TNP-470 and AGM-1883 were only 7–8 min, while the mean $t_{1/2}$ for M-II was 2.8 h. Mean $AUC_{0-\infty}$ values for TNP-470, AGM-1883, and M-II were 1610, 32, and 3280 ng · h/mL, respectively. M-II elimination appeared to follow a two-compartment model, with a terminal $t_{1/2}$ of about 3 h. Further studies are essential to confirm these preliminary findings.

14. DEVELOPMENT CHALLENGES

If the activity of antiangiogenesis agents results primarily in inhibition of tumor growth, and not tumor regression, the development of these compounds will truly be a challenge. The design paradigms for oncology clinical trials are deeply ingrained with expectations of response, when response is defined as a percent decrease in tumor mass, and, most important, disease stabilization is viewed as treatment failure. These expectations appear as early as phase I. Despite agreement that the primary end points of phase I clinical trials are determination of the MTD, description of DLTs, and generation of the pharmacokinetic profile, all trials include response evaluations and criteria. Phase II clinical trials are typically designed as single-arm studies, conducted in two stages, and requiring a minimum demonstration of response in the first stage, to be able to proceed to the second stage. Randomized, controlled clinical trials do not appear until phase III in oncology clinical research. This approach has worked well for agents that are cytotoxic. They do cause tumor regression, so the efficacy end points are appropriate. Their toxicities are not minimal, so establishment of an MTD and knowledge of DLTs is critical. However, for agents that may result in tumor dormancy *(51)*, the paradigm does not apply.

What are the appropriate end points for tumoristatic agents? Certainly time to progression/recurrence, disease-free survival, and, the ultimate assessment, survival. If tumoristatic agents provide benefit, it should be reflected in these parameters. Inherent difficulties include the extended duration of patient care and/or follow-up required to demonstrate benefit. In addition, elements of these data may not have been collected

systematically in the past, and may not be available for use as historical controls. Clinical trials with these end points may require concurrent controls, and increasing the numbers of patients required for study. Disease incidence and competition for appropriate patients thus become important factors.

Surrogate end points, once established, will facilitate the design of clinical trials of tumoristatic agents. The challenge resides in the establishment of surrogates, which requires validation with an accepted end point—survival being the most accepted. This is a critically important area of development, and should be a focus of activity.

What about measures of quality of life? Are these appropriate end points for tumoristatic agents? For cytotoxic agents? The answer is a resounding "yes," as affirmed by recent approvals by the FDA for treatment of pancreatic and prostate cancers. If the ability of a chemotherapeutic agent to induce a response does not correlate with a survival benefit, and, further, has an associated decrement in quality of life, is this the best approach to treatment of cancer? Is tumor dormancy an appropriate goal, assuming that the treatment to achieve this is accompanied by minimal negative impact on quality of life? These are among the difficult questions that the oncology community will find itself asking as the development of antiangiogenesis agents progresses. Focus on quality of life shifts the role of assessor from the clinician to the patient, requiring yet another transition from the way in which oncology clinical research has traditionally been conducted.

Paradigm shifts are not easy to achieve, and should not be undertaken lightly. However, in the face of a sufficient body of evidence that suggests that the present, accepted way of testing cancer agents may not be appropriate for the development of future treatment modalities, the direction becomes clear. Information transfer, from researchers presently addressing the development challenges, to the oncology community at large, will be a critical component of any transition, and books such as this should serve as vehicles for the process.

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23 Batimastat and Marimastat in Cancer
Summary of Early Clinical Data

Henrik S. Rasmussen

CONTENTS

1. INTRODUCTION

Batimastat® and Marimastat® are broad-spectrum matrix metalloproteinase inhibitors (MMPI) with potent activity against most of the major matrix metalloproteinases (MMPs). Batimastat is the prototype MMPI, and is highly active against interstitial collagenase (MMP-1) (IC_{50} = 3 n*M*), stomelysin-1 (MMP-3) (IC50 = 20 n*M*), gelatinase-A (MMP-2) (IC_{50} = 4 n*M*), gelatinase-B (MMP-9) (IC_{50} = 4 n*M*), and matrilysin (MMP-7) (IC_{50} = 6 n*M*) *(1)*. There is also some emerging evidence that Batimastat is a potent inhibitor of MT-MMP (MMP-14) (unpublished observations). The molecular structure of Batimastat is displayed in Fig. 1. Batimastat mimics the substrate of the MMPs, so that the drug works by competitive, potent, but reversible inhibition. Marimastat (Fig. 2) is another broad-spectrum MMPI, with an enzyme inhibitory spectrum very similar to batimastat, but improved pharmacokinetic properties. The only noticeable difference between Marimastat and Batimastat lies in the activity against stromelysin, because Marimastat is a weaker stromelysin inhibitor than Batimastat (IC_{50} of 230 n*M*, compared to 20 n*M* for Batimastat). Whether this weaker activity against stromelysin-1 (MMP-3) has any clinical significance is unclear; however, on theoretical grounds, this would be unlikely, because stromelysin-1 in itself has not been implicated as a significant factor in tumor progression or formation of metastases *(1)*. Batimastat and Marimastat are comparable in activity against all other major MMPs.

2. CLINICAL TRIALS

2.1. Design Considerations

Designing a clinical trials program for the development of MMPIs is fraught with difficulties. These drugs are not cytotoxic agents, and do not kill tumor cells; conse-

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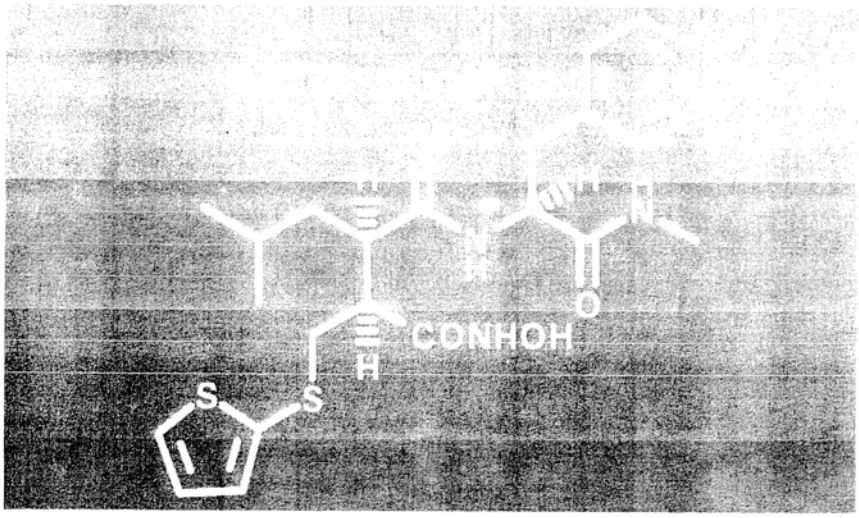

Fig. 1. Molecular structure of batimastat.

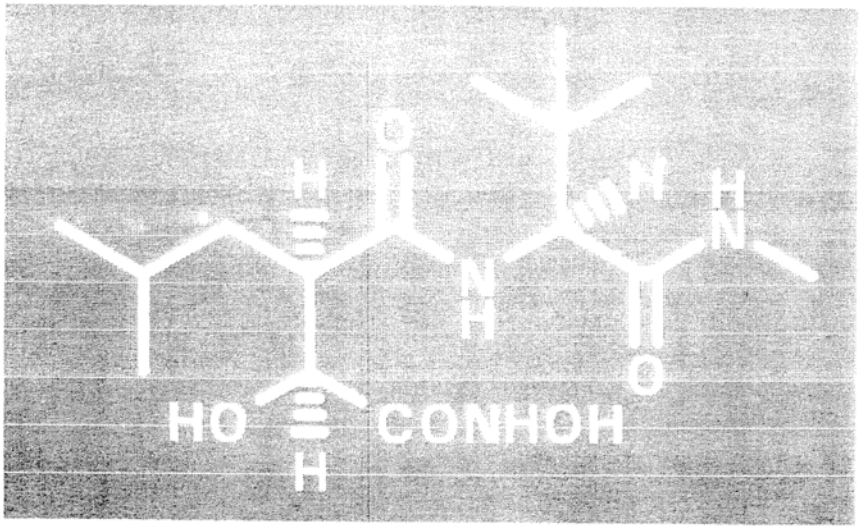

Fig. 2. Molecular structure of marimastat.

quently, a traditional cytotoxic development strategy would not only be inappropriate and time-wasting, but it would be potentially detrimental for the success of this class of agents. When one develops a new cytotoxic agent, the purpose of the phase I program is to define the maximum tolerated dose (MTD). This is a very appropriate strategy for a cytotoxic agent, which will be administered over a limited period of time on a cyclic basis; however, for a chronically administered enzyme inhibitor like Batimastat or Marimastat, which might have to be administered for years, this is not appropriate. Once complete enzyme inhibition has been achieved, no further activity would be expected, and it would be inappropriate to continue to increase the dose, simply to induce toxicity. Conse-

quently, the purpose of the phase I–II program with a drug of this class should be to identify the optimal biological dose (OBD) rather than the MTD. Also, in a traditional cytotoxic development program, tumor responses (that is, complete response, partial response, stable disease, and progressive disease) are the cornerstones of the early clinical development programs. However, MMPIs do not kill tumor cells; they are designed to work via prevention or reduction of further growth, or by prevention/reduction of metastases, not by killing tumor cells. Consequently, applying standard tumor-reductive criteria for efficacy evaluation in this class of agents would be inappropriate, and could lead to misleading conclusions of lack of activity.

The only way by which activity can be demonstrated for this, and similar, classes of agents is by randomized clinical trials, demonstrating increases in survival, progression-free survival and/or time to disease progression. However, this requires large studies, involving significant numbers of patients being treated for prolonged periods of time, and is, therefore, unsuitable for a traditional phase II program. Consequently, it is a major challenge for drug companies developing this class of agents to identify potential biological markers that could be used during the phase II program to get an early indication about the therapeutic potential of the drug, as well as being used to identify the OBD. Such an approach is described in Section 2.3.2., using cancer-specific antigens as surrogate markers of biologic activity, but undoubtedly a number of other strategies will be developed as more of these agents enter clinical trials.

2.2. Batimastat

2.2.1. Pharmacokinetics

Batimastat is poorly soluble, and, consequently, has a very poor bioavailability when administered orally, subcutaneously, intramuscularly, or intravenously. Thus, the only way the drug can be administered is by direct injection into various body cavities (peritoneal and pleural cavities). Intraperitoneal injection of Batimastat gave rise to high and sustained plasma concentrations (100–200 ng/mL) for up to 28 d after a single injection of Batimastat (150–1350 mg/m^2) *(1)*. The kinetics were linear within the dose range studied, with a dose of 1050 mg/m^2 producing a C_{max} at about 24 h of approx 1000 ng/mL. The slow dissolution rate of the Batimastat suspension within the peritoneum resulted in a half-life of 12–14 d, with blood levels still around 200 ng/mL at 4 wk *(1)*. Taking into account a protein binding of 98.5% and an IC_{50} for collagenase and 72 kDa gelatinase of 1–2 ng/mL, these blood levels are clearly within the pharmacological active range. Intrapleural administration results in significantly lower plasma concentrations, presumely because of the smaller surface area in the pleural cavity.

2.2.2. Intraperitoneal Administration

Batimastat has been tested in phase I studies in patients with malignant ascites and ovarian cancer, administered directly into the peritoneal cavity *(2)*. In these patients, Batimastat was generally well tolerated, although some local pain was experienced *(2)*. Also, there was an early indication that administration of Batimastat resulted in a reduced rate of reaccumulation of ascites *(2)*. As previously indicated, ip administration of batimastat gave rise to high and sustained systemic plasma concentrations well within the predicted therapeutic plasma concentration range; this observation led to a second phase I trial in patients with advanced lung cancer, without ascites *(3)*. Batimastat was administered at 600, 1200, and 1800 mg/m^2 to consecutive groups of three patients once every

4 wk. The drug was well absorbed from the peritoneal cavity, with maximum plasma concentrations reached within 24–48 h after administration. After the top dose of 1800 mg/m^2, total Batimastat levels remained well within the predicted therapeutic range 28 d after administration. Marimastat caused substantial local toxicity in this study in patients without ascites, including peritoneal irritation, severe abdominal pain requiring narcotic analgesia and vasovagal reactions. One patient developed bowel obstruction and adhesions that were felt to be caused by batimastat *(3)*. Systemic toxicities were minimal, but the local toxicities associated with drug administration were problematic.

2.2.3. Intrapleural Administration

The safety, toleration, and potential activity were assessed in a phase I study in patients with malignant pleural effusion *(4)*. Patients received single dosages of Batimastat (15–135 mg/m^2) given intrapleurally in 50 mL 5% dextrose, after aspiration of the existing effusion. Batimastat was well tolerated, and a significant reduction in the number of aspirations was observed, from an average of 2.33 aspirations in the month before treatment to 0.22 in the month after treatment ($p < 0.01$). As a consequence of these findings, two phase II studies have been initiated, in order to better assess the therapeutic potential of Batimastat in patients with malignant pleural effusion.

2.3. Marimastat

2.3.1. Pharmacokinetics

Because Marimastat is not a cytotoxic agent, the initial pharmacokinetic work was done in healthy volunteers *(5)*. A total of 31 healthy male volunteers were recruited into two studies—13 into a single-dose study, and 18 into a multiple-dose study. In the single-dose study, dosages from 25 to 800 mg were studied; in the multiple-dose study, dosages from 50 to 200 mg bd were explored. Up to a dose of 200 mg, a linear dose-plasma concentration relationship was found; at higher dosages, departure from linearity was apparent. The elimination half-life showed some variability, but was not dose-dependent, with a mean half-life of approx 8.5 h in the single-dose study and 10 h in the multiple-dose study. C_{max} was reached between 1.5 and 3 h *(5)*. The excretion was balanced, with 75% metabolized in the liver and the remaining 25% excreted unchanged via the kidney. Plasma concentrations at all dose levels studied were well in excess of IC_{90} concentrations, indicating that oral administration of the drug produces pharmacologically active dose levels *(5)*. Based on preclinical data, it is estimated that trough plasma concentrations of approx 30–40 ng/mL will result in approx 90% enzyme inhibition, when the protein binding has been taken into consideration. However, early pharmacokinetic data from patients with advanced cancer suggest that these patients have significantly higher (2–4×) plasma levels than normal healthy volunteers (data on file, British Biotech). The reason for this is unclear, but probably multifactorial. Patients receive a standard dose, irrespective of body mass or surface area, and this parameter varies greatly between a healthy population and advanced cancer patients. Second, impaired hepatic metabolism, as well as reduced renal clearance, resulted in a decrease in elimination. Third, protein binding is altered, which could also contribute.

2.3.2. Cancer Antigen Studies

Marimastat has been tested in 371 patients in studies with pancreatic *(6,7)*, ovarian *(8,9)*, colorectal *(10,11)*, and prostatic *(12)* cancer. All patients had advanced, mostly

Table 1
Demography in 371 Patients Enrolled in Cancer Antigen Studies with Marimastat

Cancer type	*N*	*Age (median and range)*	*Stage III/IV*	*Courses of chemo (%)*
Colorectal	131	63 (37–87)	85	2 (0–6)
Ovarian	124	60 (31–83)	95	2 (0–7)
Pancreatic	55	64 (43–84)	93	0 (0–2)
Prostatic	71	71 (45–88)	100	0 (0–4)

metastatic cancer, and had failed conventional treatment (Table 1). In all of these studies, cancer-specific antigens (CSAs) were used as surrogate markers for biological activity (CA 19/9 in pancreatic cancer, CA 125 in ovarian cancer, CEA in colorectal cancer, and PSA in prostatic cancer) *(12)*. In all of these studies, only patients who exhibited a more than 25% increase in their CSAs in the 4 wk prior to study entry were eligible for the studies. The rate of CSA rise during this drug-free screening period was then compared with an equivalent time interval (4 wk) on study drug. A significant reduction in rate of CSA rise in these patients with advanced, rapidly progressive disease would then be regarded as an indication that Marimastat was slowing down the growth of the cancer, and would be used to identify the optimal dose to take into randomized phase III studies of hard clinical end points *(12)*. All these studies were dose-ranging studies, in which dosages from 5 to 75 mg bd were explored. Meta-analysis of these studies *(13)*, 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. Dosages of 10 mg bd and above showed maximal activity, without any significant difference between 10, 25, and 50 mg bd; at all of these doses, a significant difference between rate of rise was detected before and after treatment (p-values at 0.0001, 0.0001, and 0.005 for 10, 25, and 50 mg bd, respectively). Twice-a-day dosing was more active than once-a-day dosing for comparison between equivalent total daily doses ($p < 0.005$ for a comparison between 10 mg bd and 25 mg qd). It is noteworthy that these results were achieved in patients with advanced, rapidly progressive, treatment-refractory cancer, a group which traditionally is very difficult to treat. Consequently, it is conceivable that better results will be achieved in patients with earlier-stage disease; theoretically, at least, the optimal setting for an antimetastatic, antigrowth agent like marimastat would be in early-state disease, and Marimastat is currently being tested in a number of studies in patients with earlier-stage disease.

2.3.3. Histology Studies

Another component of the Marimastat phase II program were the histology studies, in which patients with advanced malignancies and lesions, accessible for biopsies before and during treatment, were enrolled. Two such studies have reported so far *(14,15)*. In both studies, an increase in peritumoral, as well as intratumoral, fibrosis, consistent with the expected drug effect, was noted *(14,15)*. In the melanoma study, of the 16 patients evaluable for response, there was one partial response (PR), three patients with stable disease (SD), and 12 patients with progressive disease *(15)*. In the gastric cancer study *(14)*, 14 patients completed a 28-d study period. Six received a dose of 50 mg bd, the remaining eight a dose of 25 mg qd. Seven patients (2/6 at the 50 mg bd dose, 5/8 at the 25 mg qd dose) showed no evidence of disease progression over the 28-d period, based

on endoscopic examination, and continued in the extension protocol. One patient showed very significant changes in tumor appearance, with a decrease in tumor cellularity, and an increase in stromal tissue up to 7 mo of treatment. Macroscopic changes consistent with stromal formation have also been observed in the tumors of three other patients. The investigators concluded that there are early indications that Marimastat may slow the rate of progression of gastric cancer *(14)*; however, the study was uncontrolled, and any such conclusions are preliminary.

2.3.4. Safety and Tolerance in Clinical Trials

Marimastat has now been tested in more than 400 patients with a number of different solid tumors. By and large, the drug has been well tolerated *(13)*. The only clear-cut, drug-related toxicity identified so far is quite a characteristic musculoskeletal syndrome, consisting of joint pain, stiffness, edema, discoloration, and reduced mobility. The symptoms very often start in the small joints in the hands, spreading to the arms and the shoulder girdle, often on the dominant side. If dosing continues unchanged, the symptoms tend to spread to include other joints as well *(13)*. The symptoms respond poorly to nonsteroid anti-inflammatory drugs *(13)*. The symptomatology of the toxicity is very similar to what was seen in toxicological studies in the marmoset, and appears to be mostly tendinitis where the tendon is attached to the joint (unpublished data). The musculoskeletal side effects are dose-related, with the incidence, rate, and severity of onset increasing with higher doses of Marimastat. After 3–5 mo of treatment at a dose of 10 mg bd, approx 30% of the patients require dose reduction. Implementing a short dosing holiday, followed by dose reduction, has so far allowed the majority of patients to continue on treatment for prolonged periods of time.

No other drug-related toxicity has so far been identified. However, in the absence of randomized studies, it is difficult to draw firm conclusions.

3. CONCLUSIONS AND RECOMMENDATIONS

Based on the phase I-II program described above, the following conclusions can be drawn:

1. Marimastat has a favorable pharmacokinetic profile, with high systemic bioavailability, linear dose-plasma relationship, balanced excretion (75% hepatic, 25% renal), and an elimination half-life compatible with twice-daily dosing.
2. Musculoskeletal symptoms (by and large reversible upon drug discontinuation) have been identified as the primary dose-limiting toxicity. No other major toxicity has emerged.
3. Studies in patients with prostate, pancreatic, ovarian, and colorectal cancer, using changes in CSAs as a surrogate marker for drug activity, as well as histology studies in patients with lesions accessible for biopsies, have indicated that Marimastat is biologically active and have helped identify an appropriate dose range.

These encouraging phase I–II data have provided the foundation for a major pivotal phase III program, and major clinical trials have been initiated in patients with advanced pancreatic cancer, malignant glioblastoma, small-cell lung cancer, nonsmall-cell lung cancer, gastric cancer, ovarian cancer, and breast cancer. All of these trials are looking at hard clinical end points, such as survival and disease progression, and, when completed, should provide the first step toward identifying the potential clinical use of this class of agents.

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24 Thalidomide
A Prodrug That Inhibits Angiogenesis

William D. Figg, Eddie Reed, Shawn Green, and James M. Pluda

CONTENTS

1. INTRODUCTION

Thalidomide (*N*-phthalidoglutamide, C13 O4 N2 H9, mol wt = 258.23) (Fig. 1) is a glutamic acid derivative [S(-) and R(+) racemate] *(1)* that was first described in 1953 by the Swiss pharmaceutical company Ciba. Ciba discontinued research on the compound and a German company Chemie Grunenthal undertook development in 1954. It was later marketed as a sleeping pill, and was subsequently blamed for nearly 12,000 birth defects between the late 1950s and early 1960s *(2)*.

The maximal solubility of racemic thalidomide in water is approx 2×10^4 mol/L (45 to 60 mg/L) *(3)*. The ultraviolet spectrum of thalidomide is characterized by an absorbance maximum at 300 nm that is dependent on an intact phthalimide moiety. Four amide bonds present in the molecule are susceptible to hydrolytic cleavage in vitro at pH values higher than 6.0 *(3)* (Fig. 2). Only 20% of the parent compound is detectable 24 h after spiking a whole blood sample (pH 7.2) with thalidomide *(4)*. Nonenzymatic cleavage of one or more of the amide bonds produces hydrolysis products that contain at least one carboxyl group *(3)*. These products are thus more polar and can be expected to cross

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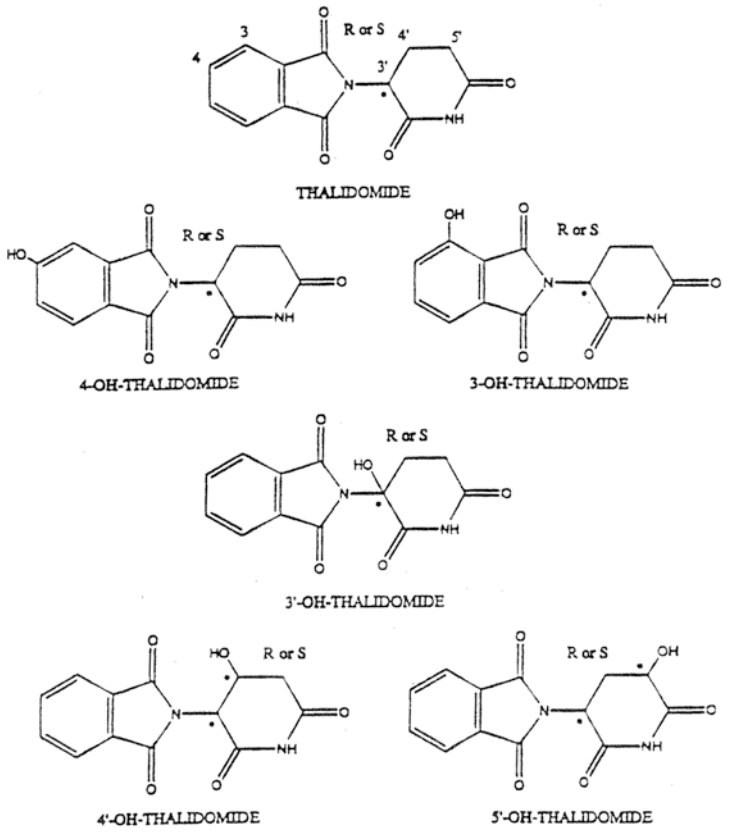

Fig. 1. Structures of thalidomide and the five known metabolites.

biological membranes less efficiently than the parent compound. Theoretically, >12 different degradation products can be formed from thalidomide by hydrolysis and five metabolic metabolites by other pathways (phenolic metabolites) *(5)* (Fig. 1). Each metabolite is also susceptible to hydrolysis *(5)*. Nonetheless, the main transformation of thalidomide in the body is by spontaneous chemical processes that do not involve an enzymatic reaction. Considering the possible combinations of hydrolysis, hydroxylation, and optical activity, there are more than 50 theoretical metabolites, or degradation products, of thalidomide in vivo.

Thalidomide is a potent teratogen that causes dysmelia (stunted limb growth) in humans. It was marketed as a sedative, but was withdrawn from the European market 30 years ago because of its teratogenic effects. The compound was later discovered to be extremely effective in lepromatous leprosy and has been used routinely for that indication around the world. In addition, trials have recently been initiated in a variety of diseases with an autoimmune character, including recurrent aphthosis of nonviral and nonfungal origin in human immunodeficiency patients, graft-vs-host disease (GVHD), lupus erythematosus, tuberculosis, wasting syndrome associated with HIV infection, and rheumatoid arthritis. Renewed interest in thalidomide has been generated following the publication of in vitro data suggesting it has antiangiogenic properties *(6)*. Based on those data, four solid tumor trials were initiated by the NCI *(7)*. Currently, more than 1000 patients in the United

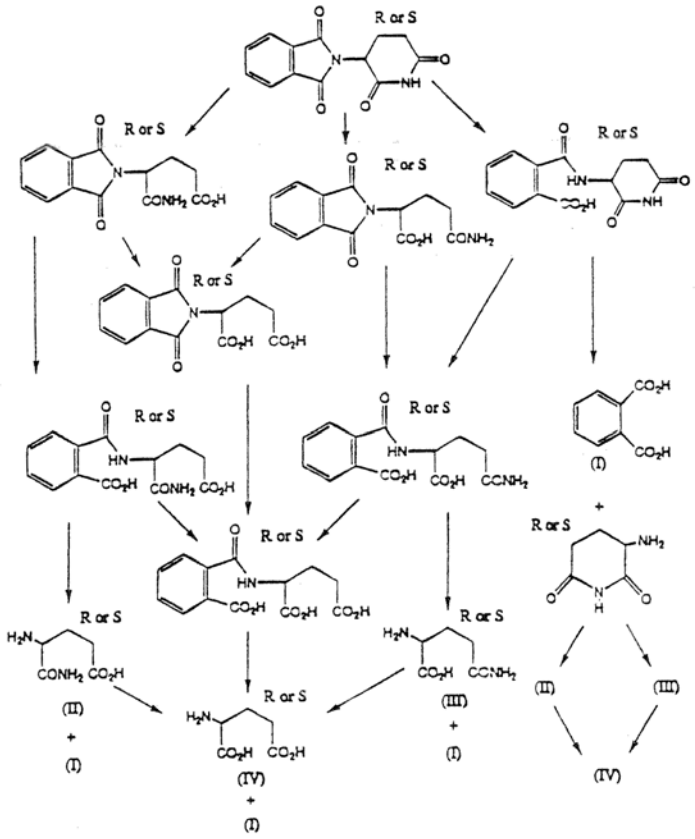

Fig. 2. Structure of thalidomide and the hydrolysis pathway for degradation.

States are receiving thalidomide either on a compassionate use basis or as part of a clinical trial.

2. HISTORICAL PERSPECTIVE

A new drug application (NDA 12–611) was originally submitted for thalidomide in September 1960 to the then Bureau of Medicine (a precursor to the FDA) *(8)*. It was being indicated for use in the treatment of somnolence and as a mild hypnotic. At that time thalidomide was widely used in Europe and was considered a safe and effective sedative. The application was found to have significant shortcomings by three reviewers and additional data was requested. In February 1961 reports emerged of peripheral neuritis occuring in individuals receiving long-term treatment with thalidomide, a further delay in the application, which caused review. In addition, concerns were starting to be raised about possible adverse effects to the fetus if administered during pregnancy. In November 1961 the application was put on hold and subsequently withdrawn because of data from Germany suggesting an association between phocomelia (a rare birth defect) and thalidomide. In 1965 efficacy was reported in the treatment of leprosy; this was later verified in large clinical trials. In addition, immunosuppression activity was detected in a skin model as well as efficacy in preventing GVHD. More recently, the FDA has approved the use of thalidomide for the treatment of skin lesions and fever-associated erythema nodusum leprosum in patients with leprosy.

3. TERATOGENIC EFFECTS

In 1961, McBride described an association between limb defects in babies and maternal thalidomide usage *(9)*. Although humans and rabbits are exquisitely sensitive to the teratogenic effects of thalidomide, the compound was shown to exert teratogenic effects in a number of animal species, including monkeys, rats, mice, and chickens. However, there are significant differences among the various species. For example, rodents are less sensitive than rabbits to the teratogenic effects of thalidomide *(10b,11,12)*. Interestingly, differences among strains of the same species also exist. Such differences are reported among hamsters and primates. The Galago, a prosimian primate, is relatively resistant to the teratogenic activity of thalidomide, whereas several other primates, including the Macaca sp developed limb abnormalities as a result of thalidomide *(10a)*. Over the past 30 years the mechanism of thalidomide's teratogenicity has been extensively studied, but has remained unsolved *(13)*.

4. PHARMACOLOGY

In addition to its chemical instability, the reported differences in biological activity add further complexity in understanding the fate of thalidomide and related metabolites. In the case of mice, thalidomide is a relatively poor teratogen *(10b)*; however, it effectively protects mice from endotoxin shock *(14)* and blocks angiogenesis induced in the mouse cornea by bFGF or VEGF *(15)*. These differences suggest that thalidomide targets multiple biochemical and molecular pathways. Hence, the differences in biological activity depend on both the species, as well as the biological processes under evaluation. Further understanding of both species-dependent biotransformation and the effects of thalidomide in angiogenic-dependent and angiogenic-independent processes will provide insight for the use of thalidomide in disease management and analog design.

The biochemical mechanism of the nonsedative effects of thalidomide is unclear. In addition, very little work has been done to understand the neurotoxic action or immunomodulatory effect of the compound on a molecular basis. Considering the possible combinations of hydrolysis, hydroxylation, and optical activity, there may be more than 50 metabolites of thalidomide in vivo. Naturally, it has been difficult to correlate in vivo observation with molecular evidence.

5. CLINICAL PHARMACOLOGY

The main pathway of degradation for thalidomide appears to be nonenzymatic hydrolytic cleavage *(3,16)*. Minor amounts of hydroxylated products have been detected in the urine of some species *(3)*. Hepatic metabolism probably involving the cytochrome P450 enzyme system, occurs to some degree *(17,18)*. However, only the parent compound is enzymatically modified to five known metabolites. Each of those products is susceptible to hydrolysis *(3,17)*. Thalidomide itself does not cause enzyme induction *(19)*, but possibly interferes with enzyme induction caused by other compounds *(19)*.

Piscitelli and colleagues evaluated the pharmacokinetics of a single dose of thalidomide (100 and 300 mg) in patients with HIV *(20)*. They reported a mean peak concentration of 1.17 ± 0.21 and 3.47 ± 1.14 μg/mL, respectively, and an elimination half-life of 6 h. Figg et al. characterized the pharmacokinetics of thalidomide in 24 males with prostate cancer *(21)*. Using a one-compartment model, they reported an oral clearance of

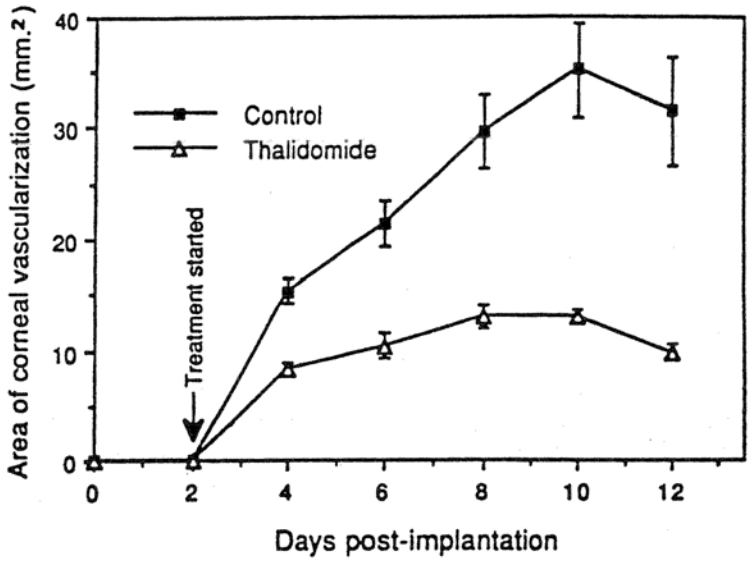

Fig. 3. Time course of inhibition of neovascularization with thalidomide. Mean areas of corneal neovascularization with standard error bars are presented from one experiment that is representative of the three experiments performed with thalidomide on nonirradiated animals. Data presented from the first time-point after administration of drug through the completion of the study are statistically different (n = 10 eyes; $p < 0.005$ for all time-points, one-way ANOVA with ranked data).

7.41 L/h and an apparent volume of distribution of 66.9 L in a group of patients receiving a single dose of 200 mg. A high-dose group (800 mg oral dose) had a oral clearance of 7.2 L/h and an apparent volume of distribution of 165.8 L. The elimination half-lives for the low and high dose were 7.08 and 16.19 h, respectively.

Drug interactions with thalidomide have not been systematically studied. Thalidomide enhances the sedative activity of barbiturates, alcohol, chlorpromazine, and reserpine, whereas its sedative action is antagonized by methyl amphetamine and methylphenidate.

6. ANGIOGENESIS

It has been postulated that limb defects seen with thalidomide are secondary to an inhibition of blood vessel growth in the developing fetal limb bud *(22)*. The limb bud is unique in requiring a complex interaction of a number of processes including angiogenesis. Since angiogenesis is the formation of new blood vessels from sprouts of preexisting vessels, the limb bud would be a particularly vulnerable target to a teratogen that inhibited vascular endothelial growth.

Thalidomide has recently been shown to inhibit angiogenesis (30–51% reduction in vascularization) induced by FGF in the rabbit cornea micropocket model *(22)* (Fig. 3). It appears that the antiangiogenic activity in this model may be the result of an epoxide active metabolite; thalidomide did not have activity in the chicken chorioallantoic membrane assay (a topical assay compared with the orally administered animal model). Bauer et al. evaluated thalidomide in a rat aorta model (as well as with human aorta endothelial cells) and noted antiangiogenic activity when thalidomide was coincubated with human liver microsomes *(5)* (Figs. 4 and 5). Furthermore, they failed to observe activity when thalido-

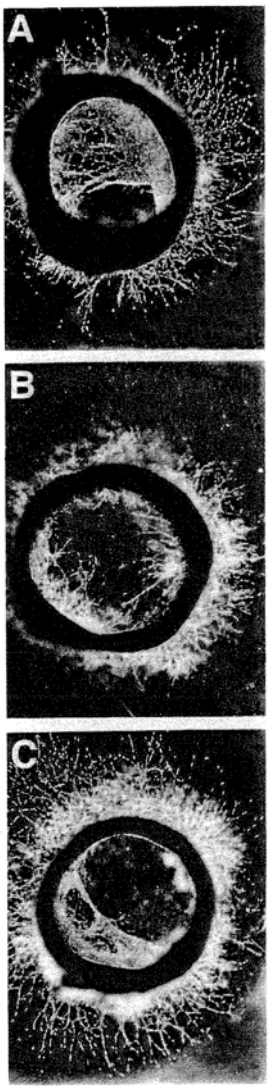

Fig. 4. Rat aortic sections treated with EBM and **(A)** DMSO vehicle, **(B)** human microsomes (0.2 mg/mL), and **(C)** thalidomide (8.0 μg/mL).

mide was coincubated with rat liver microsomes, but did note inhibition of growth with rabbit liver microsomes. These experiments confirm that neither the parent compound nor a hydrolysis degradation product is the active moiety, but rather is one of the enzymatic metabolites or an intermediate product.

Gordon et al. reported that rats were resistant to the teratogenic effects of thalidomide, but rabbits (as well as higher primates) were sensitive *(23)*. They speculated that differences were caused by alterations in biotransformation between the species. Furthermore, they noted that after thalidomide treatment, 4- and 5-hydroxylated metabolites of thalidomide were recovered from the urine of rabbits but not rats. The presence of phenolic derivatives of thalidomide suggests that the drug might undergo oxidative metabolism via an arene oxide intermediate. Arene oxides have been implicated as mutagens, cytotoxins, and terato-

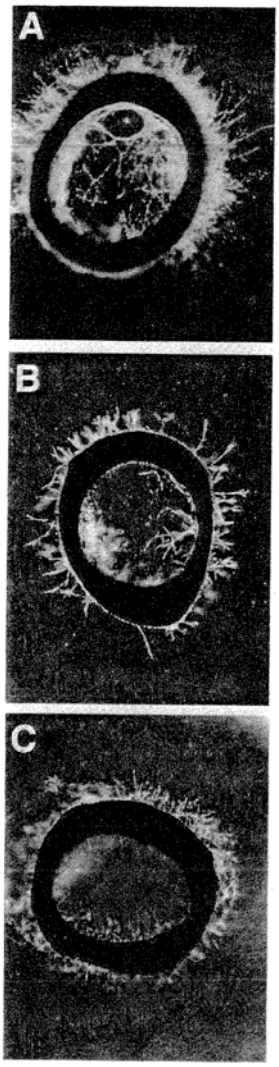

Fig. 5. Rat aortic sections treated with EBM and **(A)** thalidomide (8.0 μg/mL) and rat microsomes (0.2 mg/mL), **(B)** thalidomide (8.0 μg/mL) and human microsomes (0.2 mg/mL) and **(C)** thalidomide (8.0 μg/mL) and rabbit microsomes (mg/mL).

gens. The intermediate metabolite is also most likely responsible for the antiangiogenic activity. Furthermore, Gordon et al. showed that by inhibiting epoxide, hydrolase results in enhanced teratogenicity *(23)*. Likewise, D'Amato and colleagues noted that inhibition of epoxide hydrolase results in enhanced antiangiogenic properties *(22)*.

Based on these observations, thalidomide has recently entered clinical trials to assess its antiangiogenic activity in patients with solid tumors (i.e., breast cancer, prostate cancer, Kaposi's sarcoma, and glioblastoma) *(7)*. Preliminary results have been reported from the prostate cancer trial in which 12 patients with androgen-independent cancer have been enrolled (6 patients in the high dose group [1200 mg/d] and 6 patients in the low dose group [200 mg/d]) *(10c)*. Complications, which have been relatively minimal,

and have included: neuromotor dysfunction, constipation, dizziness, drowsiness, xerostomia, neurosensory dysfunction, and rash. Four patients *(30%)* have had prostate-specific antigen declines, with the longest maintained for 84 d.

Eisen and colleagues studied 48 patients with advanced solid tumors, with a thalidomide dose of 100 mg qHS *(24)*. This cohort included 17 individuals with ovarian cancer, 16 with melanoma, 8 with renal cell carcinoma, and 7 with breast cancer. Three patients responded to therapy, and an additional 10 patients had disease stabilization for up to 25 wk. Of particular interest was that patients with progressive disease developed rising serum and urinary VEGF levels, whereas patients with stable disease showed stable or falling VEGF levels. In addition, four patients had measurable serum bFGF, and all had rapidly progressive disease.

Politi and colleagues assessed thalidomide in AIDS-related Kaposi's sarcoma in a phase I study *(25)*. Twelve affected males were treated with one of the following doses; 200, 300, 400, or 600 mg/d. Antiretroviral therapy was continued while patients took thalidomide. There were two objective responses to therapy, and seven patients had disease stabilization. Somnolence was the dose-limiting toxicity. Thalidomide has been incorporated into a bone marrow transplantation approach for the treatment of metastatic breast cancer *(26)*. Although toxicities from this approach have been reported in a summary fashion, efficacy has yet to be detailed. The thalidomide dose was 200 mg BID, and again, somnolence was a major toxicity.

In 1965 Grabstald et al. reported their experience with thalidomide in patients with cancer *(27)*. They treated 71 patients with various types of cancer and at multiple doses (300 mg to 2 mg/d). No objective antitumor activity was noted, with the exception of one patient with metastatic renal cell carcinoma who had regression of pulmonary lesions following nephrectomy and the initiation of thalidomide. This patient was maintained on thalidomide for 7 mo until he developed peripheral neuritis. Within 1 mo after discontinuing therapy he developed brain metastases and subsequently died 4 mo later. The authors were unwilling to completely credit thalidomide with the antitumor activity noted in this case, but proposed spontaneous regression of renal carcinoma as an alternative hypothesis. Olsen and colleagues treated 21 patients with various types of cancer using thalidomide (duration of therapy ranged between 1 and 34 wk, and total dose administered ranged between 4.2 and 354 g) *(28)*. None of the patients experienced objective clinical benefit secondary to thalidomide. Two patients (multiple myeloma and fibrosarcoma) may have had a slowing of their tumor growth while on thalidomide. Seven additional patients reported a subjective benefit associated with thalidomide administration (i.e., increased sense of well-being, improved appetite, better sedation). This group also cited investigators at Merrell Co. that used thalidomide in the treatment of over 100 individuals with cancer. No objective evidence of antitumor activity was noted in their experience, but subjective improvement was apparently common.

7. ANALOGS

Despite its potential therapeutic value in the treatment of angiogenic-based diseases, such as cancer and macular degeneration, thalidomide undergoes rapid hydrolysis at neutral pH, and hence, is unstable. Its instability and a lack of understanding of the cellular and molecular targets of thalidomide has limited analog design and development. However, recent studies have focused on whether structural stability enhances biological activity. Whereas thalidomide rapidly decomposes into numerous metabolites, 2-phthalimidino-

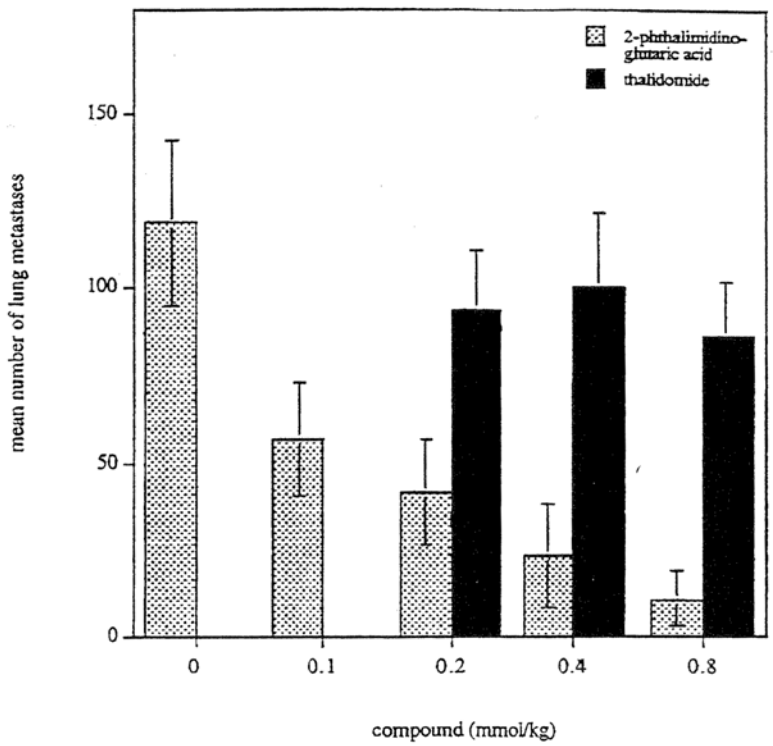

Fig. 6. Dose response of ip-administered thalidomide and 2-phthalimidino-glutaric acid in the B16-BL6 model. B16-BL6 melanoma cells (5×10^4) were iv injected into the tail vein of C57BI/6 mice. Three days later, mice were treated ip with increasing doses of thalidomide or 2-phthalimidino-glutaric acid on alternate days. Fourteen days after tumor cell inoculation, lungs were removed and surface pulmonary metastases were counted. The values shown are the mean of at least five mice per group; bars, SD.

glutaric acid, a partially reduced and relatively stable analog of thalidomide, is more resistant to hydrolysis with fewer than four hydrolysis products being detected under physiological conditions.

Recent studies have demonstrated the effects of thalidomide and 2-phthalimidino-glutaric acid on lung tumor colony formation following iv injection of B16-BL6 melanoma cells in C57/B16 mice *(29)*. Dose-response studies demonstrated that both oral (Fig. 6) and intraperitoneal (Fig. 7) administration of 2-phthalimidino-glutaric acid effectively inhibited the number of lung metastases, whereas thalidomide was significantly less effective at reducing the number of tumors at comparable concentrations.

2-Phthalimidino-glutaric acid was found to have potent antitumor activity as demonstrated by a single administration experiment *(68)*. Tumor-bearing mice given 0.8 μmol/kg of 2-phthalimidino-glutaric acid orally on d 3 showed a 50% reduction in the number of metastases when compared to a control group *(29)*. This experiment went on to suggest that 2-phthalimidino-glutaric acid is most effective at inhibiting metastatic disease when tumor cells are breaking through the subendothelial matrix and seeding the extracellular matrix. This process requires cells to migrate through the basement membrane. To support the growth of these newly established tumors, angiogenesis is required: Endothelial cells from postcapillary venules follow the same migratory process as tumor cells in

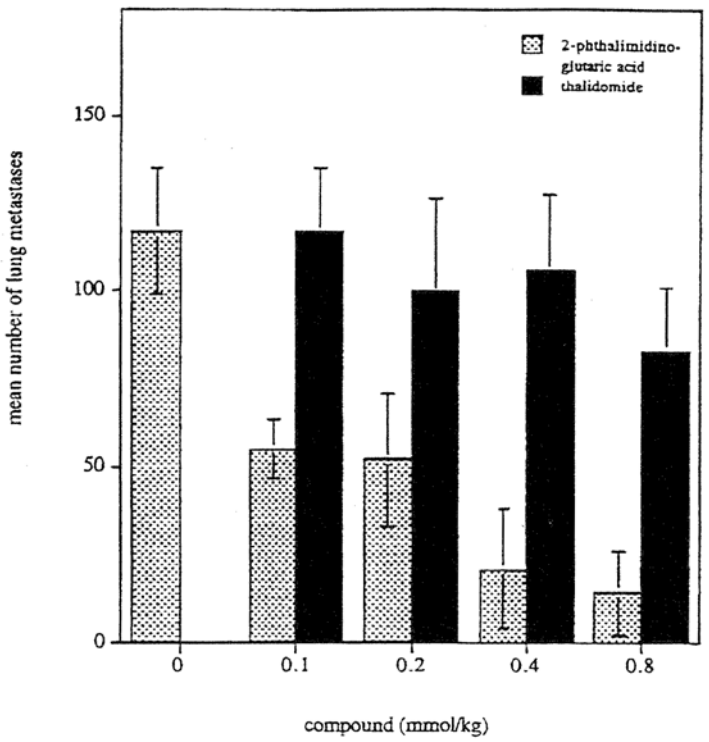

Fig. 7. Dose response of orally administered thalidomide and 2-phthalimidon-glutaric acid in the B16-BL6 model. B16-BL6 melanoma cells (5×10^4) were iv injected into the tail vein of C57BI/6 mice. Three days later, mice were treated orally with increasing doses of thalidomide or 2-phthalimido-glutaric acid on alternate days. Fourteen days after tumor cell inoculation, lungs were removed and surface pulmonary metastases were counted. The values shown are the mean of at least five mice per group; bars, SD.

response to the angiogenic signal provided by the advancing tumors. Both β3 and β3 integrins are required for this migratory process. Agents that block the interaction between αυβ3 and extracellular matrix proteins with an RGD motif inhibit cell migration and angiogenesis *(30)*. It is possible that thalidomide may affect αυβ3, since it has been found to downregulate β3 in the fetus of marmosets *(31)*.

These preliminary studies infer that 2-phthalimidino-glutaric acid may be working at the cell–cell and/or cell–matrix adhesion level. 2-Phthalimidino-glutaric acid, and to a lesser extent, thalidomide, appear to diminish HUVEC adhesion to various basement membrane components, but have no effects on their ability to adhere to plastic or effect proliferation. Furthermore, these compounds appear to cause thinning of HUVEC tubes on matrigel.

Such observations suggest that thalidomide and related analogs may disrupt end-stage events, such as tube formation of a developing vessel, e.g., block tight junction formation, resulting in leaky vessels. This hypothesis is consistent with past observation showing that thalidomide causes the thinning of endothelial cell processes during embryogenesis and fenestration of developing vessels in the cornea micropocket assay; fenestration has been reported to be specific to regressing corneal blood vessels after removal of proangiogenic agents *(32)*.

One may argue that "thalidomide-sensitive vessels" would facilitate the delivery of chemotherapy and promote oxygenation at the site where neovascularization is taking place. Thalidomide administration reportedly increases Lewis lung tumor sensitivity to combined treatment with radiation and bioreductive cytotoxin triapazamine *(33)*. These findings suggest that thalidomide may elevate hypoxia in the tumor, presumably via an antiangiogenic mechanism. The observations that thalidomide together with the antitumor agent dimethylxantheone-4-acetic acid regressed tumors in mice as compared with partial regression in mice receiving the antitumor agent alone is consistent with this concept *(34)*.

8. OTHER INDICATIONS

Thalidomide was first reported to suppress erythema nodosum leprosum (ENL) in 1965 *(35)*. Its beneficial effect was subsequently established in double-blind trials in which activity was noted in about 90% of patients *(35–37)*. The use of several thalidomide analogs in patients with ENL has led to contradictory results *(38–40)*. The mechanism of its action is not understood; however, an antibacterial effect has been excluded *(41)*. In addition, thalidomide does not inhibit the growth of *M. tuberculosis* in vitro *(42)*. These observations suggest that the compound exerts its beneficial effects through a direct action on the immune or inflammatory system. This view is strongly supported by the suppression of GVHD observed with thalidomide in animal experiments *(43–50)* and in humans *(51)*.

GVHD may develop in immunocompromised individuals who receive transplants containing immunocompetent donor cells *(52–54)*. Donor T cells are believed to induce an immune reaction against the recipient's issues, either immediately (acute GVHD) or with delayed onset (chronic GVHD). However, subpopulations other than cytotoxic T lymphocytes may be the mediators of the disease. Suppressor T cell numbers are also reduced *(55)*. Anatomic locations most prominently involved are skin and the gastrointestinal mucosa, but other structures may be affected. Mortality may be as high as 60% of affected individuals, despite immunosuppressive therapy. Administration of thalidomide improved the clinical condition of about 50% of patients with chronic GVHD after bone marrow transplantation in whom other therapeutic regimens had failed, but improvement was noted in only 1 of 7 patients with acute GVHD *(56)*. The dosing regimen explored in some of the anticancer trials is based on the pharmacokinetically guided trial conducted by Vogelsang and colleagues at Johns Hopkins University in patients with GVHD *(57,58)*. In that study the initial dose of thalidomide was 200 mg given four times a day to adult patients with GVHD. Seven of the 44 patients required larger doses (1200–1600 mg/d) to maintain a targeted concentration of 5 μg/mL 2 h postdose. The median duration of thalidomide therapy was 240 d (range, 2–700 d). Sedation was observed in virtually all patients; 91% reported sleepiness, especially during the first few weeks of therapy. Twenty-two patients (50%) had at least a single episode of constipation. Four patients had numbness and tingling of their hands or feet that was consistent with a peripheral neuropathy (4/44 = 9%). Their medication was discontinued and their symptoms resolved over the following days or weeks.

Thalidomide was an effective treatment for aphthous ulcerations in at least 19 patients *(59–64)*. Daily doses of 50–200 mg were used during the acute phase and complete remission was achieved within 3–14 d. Recurrence of ulcers was not observed during maintenance therapy (50 mg twice weekly or 100 mg every 5 d) in 6 cases *(59,64)*. Recurrence of ulcers after complete discontinuation of thalidomide was not observed in

9 patients *(62,63)*, and occurred only after several months in others. In a single patient, thalidomide did not affect the healing of an ulceration that ultimately responded to amphotericin-B *(42)*, suggesting that thalidomide has no antimycotic activity.

9. SIDE EFFECTS

As discussed above, thalidomide has been used for the treatment of leprosy, graft versus host disease, rheumatoid arthritis, aphthous ulcers associated with AIDS, and various dermatologic disorders. The side effects of thalidomide have been well documented and they include: drowsiness, constipation, peripheral sensory neuropathy, swelling of the limbs, erythema of the limbs, hair loss, fever, rash, and amenorrhea. Dizziness and mood changes occurred in 33–100% of all patients. Other frequent adverse effects are xerostomia, increased appetite, loss of libido, nausea, pruritus, and menstruation abnormalities have been occasionally observed. The most serious of these side effects is peripheral neuropathy. Its incidence has been estimated to be approx 1% in patients treated for lepra reactions *(81)*, 12% in rheumatoid arthritis *(65)*, 22% in prurigo nodularis *(66)*, and 25% in patients with discoid lupus erythematosus *(67)*. Based on electrophysiologic studies the incidence was estimated at 21% *(68)*. Peripheral neuropathy consists clinically of a symmetrical distal paresthesia with sensory loss in distal limbs, predominately the toes. Motor involvement is rare but can occur later, especially if the drug is continued despite development of sensory involvement. While some studies noted a dose dependent pattern with neuropathies occurring only after cumulative doses of greater than 40 grams *(69,70)*, other studies have not demonstrated a correlation between the incidence of the neuropathy and the dose or duration of thalidomide treatment *(68,71)*. Paresthesias are often noted early in the course of the neuropathy, before other neurologic signs develop *(68)*. A higher incidence of neuropathy has been reported in females and the elderly *(72)*. The neuropathy does not appear to progress after discontinuation of the drug *(71)*. In many cases the neuropathy appears to be reversible if treatment is discontinued once it is detected *(73,74)*.

A group from Columbia reported treating 17 patients with arthritis with a dose of 400–600 mg/d of thalidomide (mean duration of therapy, 24.8 mo, range 7–65 wk) *(75)*. They found that two patients developed symptoms of peripheral sensory neuropathy that was reversed on discontinuation of therapy. Crawford reported that 25% of patients ($n = 60$) receiving thalidomide for the management of chronic discoid lupus erythematosus developed neuropathies, and all patients ($n = 8$) receiving it for nodular prurigo and aphthous stomatitis were found to have neuropathies *(76)*. Sheehan reported the development of neurological complications in 2 of 5 patients receiving thalidomide for prurigo nodularis *(77)*. Polyneuropathy persisted in one of those patients for >12 mo after the discontinuation of therapy. Aronson et al. reported the development of sensory peripheral neuropathies (onset 2–12 mo into therapy) in 3 of 4 patients with prurigo nodularis that were receiving thalidomide (100–300 mg/d) *(78)*. Their in vitro work went on to show that thalidomide induced primary neuronal degeneration. Schroder et al. reported that there was a reduction in myelin thickness of sural nerve fibers (reduction in sheath thickness) and a decrease in conduction velocity in thalidomide-treated New Zealand white rabbits as compared to controls *(79)*. Patients with pre-existing neuropathies may be particularly sensitive to the development of thalidomide polyneuritis *(75,80)*. On the other hand, peripheral nerve abnormalities are affected in lepromatous leprosy. Neuritis is present

during ENL episodes, resulting in neuralgia and decreased motor conduction velocity. Thalidomide treatment improves these symptoms because of suppression of the inflammatory process *(36,81)*. Thalidomide neuropathy was not observed after repeated ENL episodes treated with thalidomide *(81)*, but it may be difficult to differentiate from the neuropathological changes caused by the underlying disease.

Effects of thalidomide on the endocrine system have been consistently observed in both clinical trials and animal experiments. These actions may be the result of an effect of the drug on the hypothalamus *(82)*. In humans, a tendency to normalize hyperthyroid states has been noted. Iodine uptake by the thyroid gland was slightly decreased, and myxedema was occasionally observed. Increased urinary secretion of 17-hydroxycorticosteroids associated with hypoglycemia has been reported. Thalidomide was found to antagonize the action of histamine, serotonin, acetylcholine, and prostaglandins in organ bath experiments, but had no influence on the uterine reaction to oxytocin, vasopressin, and histamine *(82)*.

10. CONCLUSION

Based on the preclinical data suggesting that thalidomide has antiangiogenic activity in rabbit cornea model and the rat aorta model when coincubated with microsomes, studies were initiated to evaluate thalidomide in patients with solid tumors (breast cancer, prostate cancer, Kaposi's sarcoma, and glioblastoma) *(8)*. Since the discovery of the beneficial effects of thalidomide in leprosy, the compound has been used in an increasing number of clinical conditions; thus, the side-effect profile and dosing information have been fully characterized. The teratogenic action of thalidomide necessitates contraceptive measures in all women of childbearing potential receiving the drug. However, its immunomodulatory action, its peripheral neurotoxicity, and its lesser known effects on the CNS and other adverse reactions may constitute special problems in other patients, especially those with preexisting neuropathies. To date, only preliminary data is available on the clinical antitumor activity of the compound.

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25 Ribozyme Targeting of Angiogenic Molecules

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CONTENTS

1. INTRODUCTION

The importance of blood vessel formation (angiogenesis) for the local growth of solid tumors and their metastatic spread is well established *(1,2)*. A large series of correlative clinical studies published by different groups over the past seven years showed that the number of blood vessels detected in a primary tumor is an independent prognostic indicator of the outcome of the disease and is directly related to the rate of metastasis of tumors of different origin, such as breast cancer *(3–8)*, nonsmall-cell lung cancer *(9)*, prostate cancer *(10)*, squamous cell carcinoma of the head and neck *(11)*, and melanoma *(12,13)*. In general, these studies showed that the numbers of blood vessels in a given primary tumor specimen is indicative of the rate of metastasis of the respective tumor and gives an independent measure of the outcome of the disease. Of additional significance are reports that in breast cancer patients with estrogen receptor (ER)-positive tumors, and hence apparently good prognosis, high microvessel density in the primary tumors seems to predict poor clinical outcome *(8)*.

Since angiogenesis is such an important feature in the progression of clinical cancer, the driving factors need to be understood to exploit this process therapeutically (reviewed in refs. *1,14,15*). It is conceivable that all angiogenic factors present in a given tumor

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contribute to and are required for the angiogenic and metastatic phenotype of the particular tumor. On the other hand, it is also possible that very few crucial gene products will emerge as rate-limiting factors and that metastasis of a given tumor is ultimately dependent on the production of a single major angiogenic growth factor. Unfortunately, most tumor cells express a multitude of candidate angiogenesis factors and additional growth factors are contributed by the tumor stroma (*see* e.g., ref. *14*). As far as functional studies go, very few specific and efficacious inhibitors have been generated to assess in vivo which of the gene products found in a tumor are only innocent bystanders and which contribute significantly to angiogenesis and metastasis. One of those few examples are recent studies with blocking antibodies to vascular endothelial growth factor/vascular permeability factor VEGF/VPF *(16,17)*. VEGF, is expressed in tumors and blockade of the pathway is rate-limiting for local growth of some tumors *(18–20)*. Furthermore, anti-VEGF antibodies blocked seeding of fibrosarcoma cells into the lungs after intravenous injection *(21)* and metastasis into the liver of intrasplenically injected colon carcinoma cells *(22)*.

Here we discuss a molecular targeting approach of mRNAs coding for defined angiogenic molecules with enzymatically active RNA molecules (ribozymes; for details *see* Section 2.2.). This approach allows the depletion from tumor cells of virtually any gene product and subsequently the study of the resulting phenotype. Ultimately, results from such studies should demonstrate whether a given factor plays any role in tumor growth, invasion, angiogenesis, and metastasis and allow a direct assessment of the functional redundancy of the various growth factors produced by tumor cells *(23)*. Furthermore, depletions to different residual levels of the gene product of interest permits studies on the "gene dose" needed for a particular phenotype. We will present studies in which we targeted two angiogenesis modulators, the secreted growth factor pleiotrophin (PTN), and a secreted FGF-binding protein (FGF-BP) and discuss the respective implications.

2. WHAT ARE RIBOZYMES?

2.1. Background

RNA was traditionally viewed as a set of molecules that provide structure or transmit information. The discoveries by Altman and Cech of enzymatic activity of RNA molecules changed this traditional view of the function of RNA. It is now obvious that RNA can act as an enzyme (*ribozyme* from *ribo*nucleic acid-derived en*zyme*) capable of catalyzing reactions previously associated only with protein enzymes. These findings not only lend support to a major role of RNA in the early stages of the development of life on our planet in an "RNA world" but also prompted attempts to develop specific ribozyme molecules as potential therapeutic entities for the treatment of human disease (*see* refs. *24–33*).

2.2. Principle of Action

Once the principle of action of naturally occurring ribozymes that act in cis (on themselves) was recognized, it became obvious that they can also be made to function in trans (on exogenous substrates) and thus can be designed to target virtually any RNA. This was initially shown for the group I intron and for the hammerhead ribozymes *(28,29)*. The most frequently used hammerhead ribozyme is derived from the original Haseloff-Gerlach ribozyme *(29)* and modified and minimized to a 22-nucleotide catalytic center (stem II in Fig. 1; *34*) based on the mutational analysis that had shown the essential

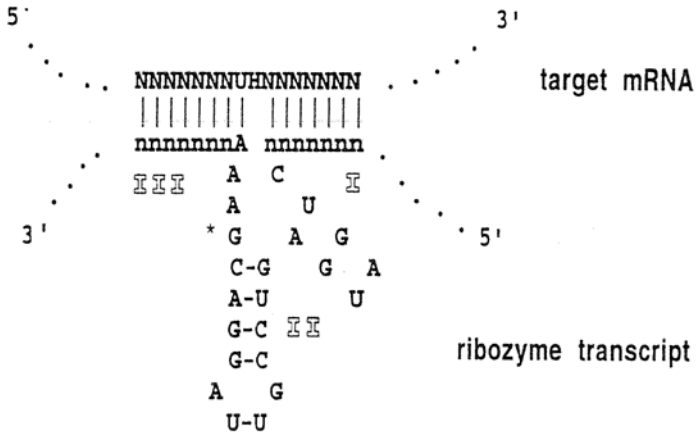

Fig. 1. General structure of a hammerhead ribozyme RNA hybridized to the target site. Stems I and III are antisense sequences that will hybridize to the targeted mRNA. Stem II is the catalytically active center of the ribozyme. The asterix indicates a mutation-sensitive site that will render the ribozyme enzymatically inactive when mutated.

elements for a catalytically active RNA *(35)*. Figure 1 illustrates the predicted structure of this type of ribozyme and its interaction with the target substrate. This hammerhead ribozyme requires only the sequence UH immediately 5' to the cleavage site in its target sequence (where H = A, C, or U but not G). The minimized enzymatically active core (stem II in Fig. 1) and two targeting arms of 4–9 nucleotides (stems I and III) in length results in a ribozyme of 30–40 nucleotides. The flanking sequences of stem I and III hybridize as antisense sequences with the targeted sense RNA by Watson-Crick basepairing (reviewed in ref. *31*). This requirement for substrate binding and the position of a cleavage consensus sequence 3' of a UH site contributes to the high specificity of hammerhead ribozymes.

2.3. Specificity

A general question is whether the ribozyme effects could be caused by degradation of RNA species related in sequence that is fortuitously cleaved by the particular ribozyme constructs. For a particular set of ribozymes several essential pieces of experimental evidence are required to rule this out. First, different constructs targeted against different regions (at least two) in the target gene should be effective. Second, ribozyme constructs should show similar efficacy against the endogenous gene product as well as against a cotransfected target cDNA in different cell systems.Third, the ribozymes should lack an effect against a closely related gene. Finally, ribozyme-transfected cells that do not express the targeted gene should stay viable and not change their phenotype.

2.4. Antisense Activity

Antisense sequences that flank the ribozyme core and target its activity to a specific stretch of nucleotides by basepairing can also block translation of the targeted mRNA and thus act as an antisense molecule. In our hands point-mutant constructs (*see* Fig. 1), which contain the same flanking sequences as wild-type ribozyme, were ineffective in

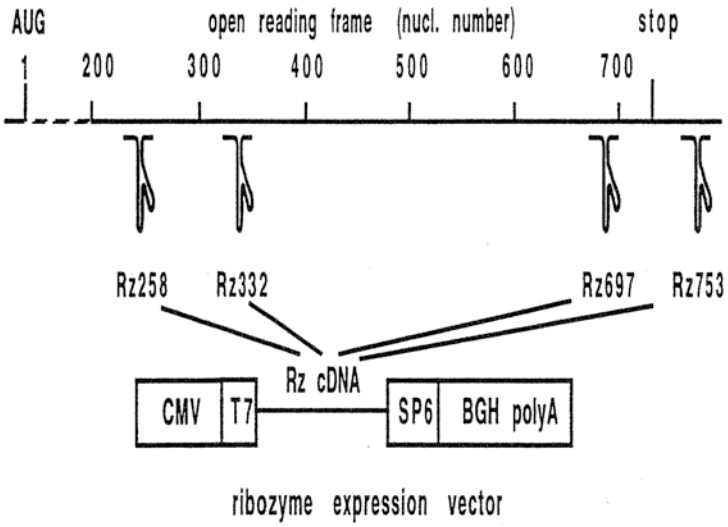

Fig. 2. FGF-BP and ribozyme target site and the structure of the functional elements in the ribozyme expression vector. The ribozymes are numbered according to their target cleavage sites relative to the translation start site in the FGF-BP mRNA. Ribozyme expression is under the control of a CMV promoter *(37)*.

downmodulating the targeted gene products. This suggests that antisense effects appear to be of minor importance in ribozyme targeting.

2.5. Selection of Target Sites

Because of the complex secondary and tertiary structure of mRNAs and their association with proteins, the predictive value of the currently available computer model is still low. Consequently, a random selection of ribozyme targets may work as well. Fortunately, the majority of predicted target sites yields satisfactory results, so that in our hands an initial number of three to five different ribozymes was always sufficient to select one with excellent in vitro and in vivo efficacy *(33,34,36–40)*.

2.6. Selection of Expression Vector

Delivery of ribozymes to the target cells can be accomplished either through exogenous delivery of synthetic ribozymes or through gene therapy approaches by intracellular expression of ribozyme transcripts from plasmid or viral expression constructs. Each approach raises different issues for ribozyme delivery, stability, activity, and potential toxicity. Our experimental design required the generation of stable cell lines expressing the PTN mRNA at differently reduced levels. For that reason we chose a standard eukaryotic expression plasmid to stably express our Hh-Rz constructs. We used the commercially available pRc/cytomegalovirus (CMV) plasmid (or the newer version pcDNA3; *see* Fig. 2) from Invitrogen (San Diego, CA) for the following reasons:

1. Most importantly, the CMV promoter provides high transcription levels in the cell lines used in this study;
2. The BGH polyadenylation (poly(A)) signal facilitates transcriptional termination and addition of a poly(A) tail to increase RNA stability;

3. T7 and SP6 promoter binding sites flanking the multiple cloning side (MCS) enable the generation of in vitro runoff transcripts of Hh-Rz inserts; and
4. A eukaryotic transcription unit for G418 resistance allows selection of stably transfected cells.

In addition to the standard constitutively active CMV expression vector we successfully used a tetracycline (Tc)-regulatable promoter system, which is reviewed in detail in ref. *33*.

2.7. Cloning of Ribozymes

We inserted the oligonucleotides coding for ribozymes targeted to different sites or different gene products into the multiple cloning site of the pRc/CMV plasmid (Fig. 2; *see also* ref. 33). This expression vector contains the CMV promoter for eukaryotic cells as well as a polyadenylation signal to enable transcription termination and the addition of a poly(A) tail (Fig. 2). These elements should increase RNA stability and transport to the cytoplasm if required *(31,41)*. Ribozyme expression cassettes were generated by synthesizing the respective sense and antisense DNA-oligonucleotides coding for the binding regions in the targeted mRNA, the catalytic core sequence of the ribozyme, and additional sequences that will generate appropriate restriction enzyme overhangs for ligation into the expression plasmid. We used 5'-*Hin*dIII and 3'-*Not*I overhangs for a directional cloning although other restriction sites worked as well. It is important to obtain high-quality high performance liquid chromatography (HPLC) or gel-purified oligos to make sure that the majority of the oligos has the full length. The flanking regions of the ribozyme are connected by a center portion that confers catalytic activity and was modified from the original design of Haseloff and Gerlach *(29)* taking into account more recent mutational analyses *(35)*. The asterix indicates the position of a single basepair substitution from G to U to generate a mutant ribozyme that is predicted to lack catalytic activity *(35,42)*.

2.8. Generation of Stable Cell Lines

Cell lines are stably transfected with 10 μg of each ribozyme plasmid using Lipofectamine (Life-Technologies; Gaithersburg, MD) at 7 μL/μg DNA as a transfection reagent. Stable, pooled cell lines are selected for 4–8 wk with G418 at 500–1500 μg/mL. Individual clonal cell lines were generated by limited dilution technique from pooled cell lines *(33)*.

3. RIBOZYMES TARGETED TO A SECRETED FGF-BINDING PROTEIN

3.1. Background

Polypeptide growth factors of the FGF family regulate important developmental processes, such as mesoderm induction, show neurotrophic activities, and stimulate the growth of new blood vessels in healing wounds or during tumor growth (reviewed in refs. *43–45*). High concentrations of biologically active aFGF and bFGF (also called FGF-1 and FGF-2; ref. *43*) are found in extracts of normal embryonic and adult tissues *(46–49)* as well as in tumor tissues of different origin *(50–52)*. However, the biological activities of both FGFs are quenched by tight binding to heparansulfate proteoglycans present in the extracellular matrix *(53–57)*. It is only partly understood how these FGFs become solubilized and thus activated in embryonic or in tumor tissues that require angiogenesis

for their growth. One established mechanism that can solubilize bFGF from this storage site is the digestion of the glycosaminoglycan portion of the cell attachment molecule by heparanases *(58–61)*. An alternate mode of delivering active bFGF from the storage site to its receptor can be via noncovalent binding to a secreted carrier protein. Such a secreted binding protein for FGFs was described by D. Sato's laboratory in 1991 *(62)*. The purified protein protected bFGF from acid inactivation and bFGF bound to it retained its mitogenic activity *(62)*.

3.2. Function and Expression of FGF-BP

The originally described characteristics of FGF-BP *(62)* make this protein an excellent extracellular carrier molecule for immobilized and hence inactivated FGFs. In studies addressing this hypothesis, we found that expression of FGF-BP in cell lines that express bFGF leads to a tumorigenic and angiogenic phenotype of these cells *(63)*. We also found that FGF-BP-transfected cells release the protein into their media together with bFGF in a noncovalently bound form. This released bFGF is biologically active. Based on these observations, we speculated that spontaneously expressed FGF-BP in human cancer may play a role for the tumorigenic phenotype through its activating effect on locally stored and ubiquitously present bFGF or lesser abundant aFGF. Indeed, we found FGF-BP expressed in SCC cell lines from different organs and at very high levels in almost all SCC tumor tissue specimens from patients *(63)* as well as in some colon cancer samples and cell lines (unpublished data). In contrast, normal adult tissues did not appear to express FGF-BP mRNA as evidenced by Northern blotting *(63)*. Another striking observation was that FGF-BP expression is upregulated transcriptionally by the phorbolester TPA in cultured cells and increased dramatically in the initial stages of carcinogen-induced skin tumors in mice *(64)* as well as in carcinogen-treated human skin grafted onto SCID mice (unpublished data).

3.3. Ribozyme Targeting Reveals a Rate-Limiting Function of FGF-BP for Tumor Growth and Angiogenesis

In a recent study *(37)*, we explored the significance of endogenously produced FGF-BP for the tumorigenic and angiogenic phenotype of human carcinoma. We used the genetic approach of ribozyme targeting to downregulate spontaneous expression of FGF-BP mRNA in model cell lines for human squamous cell cancer (SCC; ME-180) and colon cancer (LS174T). Both cell lines express and store bFGF in addition to FGF-BP. In vitro we found that depletion of FGF-BP increases the cell-bound bFGF and at the same time quenches the release of biologically active bFGF into the media of cells (not shown; *see* ref. *37*). As a consequence in vivo, tumor growth and angiogenesis in animals were reduced *(37)*. Data using the ME-180 cells are shown in Fig. 3. Most surprisingly, an as little as 20% reduction of FGF-BP mRNA already caused a delay in tumor growth (Fig. 3).

In conclusion, we demonstrate that spontaneous FGF-BP expression is a rate-limiting factor for the tumorigenic phenotype of FGF-BP-positive human SCC and colon cancer cells, most likely because of a reduction of the release of biologically active bFGF from its extracellular storage. We propose that FGF-BP should be a worthwhile therapeutic target in FGF-BP-positive cancers because of its otherwise limited expression in the healthy adult.

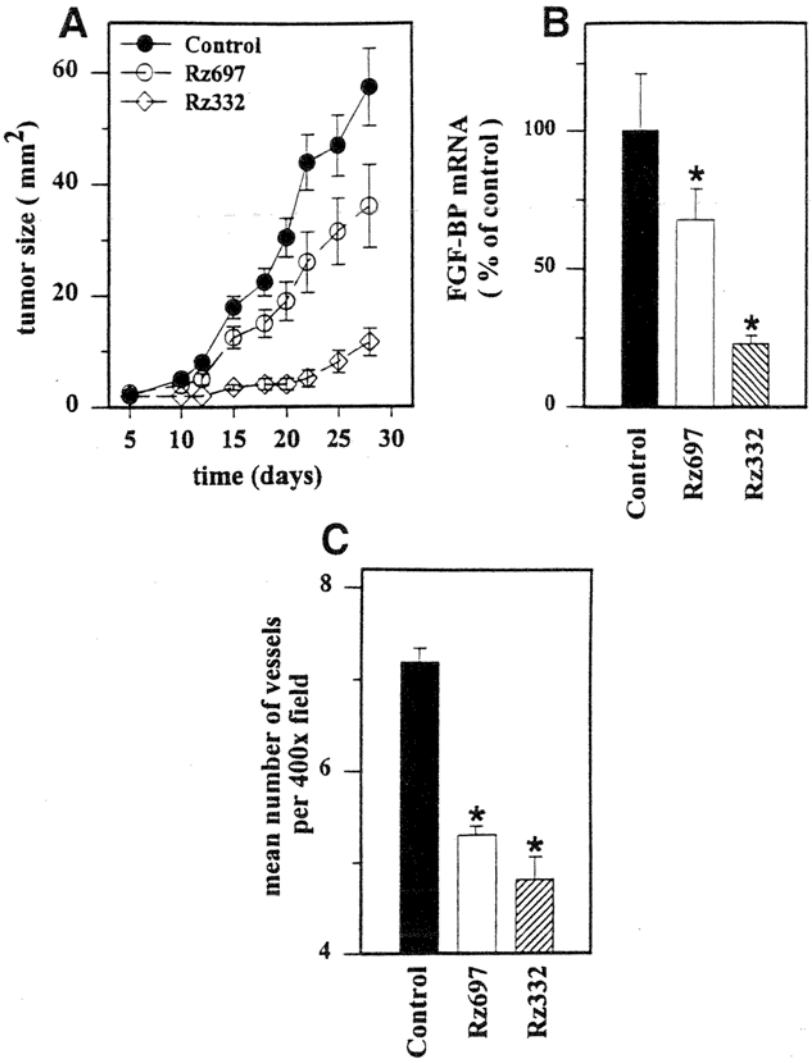

Fig. 3. ME-180 human squamous cell carcinoma cells stably transfected with FGF-BP-targeted ribozymes. **(A)** Growth as xenografts in athymic nude mice. **(B)** FGF-BP mRNA levels in the tumors. **(C)** Angiogenesis in the tumors. One million each of the different ribozyme-transfected ME-180 cell lines in 0.1 mL of serum-free media were injected subcutaneously into female athymic nude mice (NCr nu/nu; $n = 10$ tumors per group) and the tumor size measured as described *(40)*. The tumors were surgically removed when they reached a size of 50–70 mm^2 and subsequently analyzed for FGF-BP mRNA levels by Northern analysis (B; mean ± SEM, $n = 6$) and for the number of microvessels by staining for PECAM *(40)* (C; mean number of microvessels per × 400 field ± SEM of three tumors per group scored by three independent, blinded investigators). *Significantly different from control ($p \leq 0.001$). *See* ref. *37* for more details.

4. RIBOZYMES TARGETED TO PLEIOTROPHIN

4.1. Background

PTN was originally purified and cloned from developing brain, uterus, and placental tissue *(65–70)*. Recent *in situ* hybridization studies in rodents *(71,72)* demonstrated that

the gene is expressed at the highest levels in the neuroepithelium and ependyma during the second half of intrauterine development. During the same developmental phase PTN mRNA was also detected in the mesenchyme of the lung, reproductive tract, gut, kidney, and bone. With progressing organ differentiation PTN gene expression was increasingly restricted to a few sites and dropped sharply around birth. Finally, in the adult, only a few structures, such as the hippocampal region and cerebral cortex, showed detectable levels of gene expression. The developmental regulation of the PTN protein appears to parallel the expression profile of the mRNA and the highest protein levels in the growing brain coincide with periods of rapid sprouting of nerve cells *(67)*. This very distinct pattern of gene expression suggests an important role for PTN during brain development and organogenesis (for a recent review *see* ref. *73*).

4.2. Expression and Function of PTN in Tumors

4.2.1. Expression

We found the highest levels of PTN expression in tumors originating from neuroectodermal cell lineages, i.e., in human melanoma and glioblastoma multiforme tissues and in cell lines derived from these malignant lesions *(74)*. This finding supports the notion that developmentally regulated genes may be activated in a preferential fashion during malignant transformation *(75)*.

4.2.2. Function

PTN stimulates neurite outgrowth *(66,70,76)*, is mitogenic for fibroblasts *(66,69,74)* and for endothelial cells *(74,77),* and can thus serve as an angiogenesis factor for tumors. A potential role of PTN during tumor growth is suggested by studies in which overexpression of PTN in SW-13 cells *(74)* and in 3T3-fibroblasts *(78)* supported tumor growth of these nontumorigenic cells in athymic nude mice. However, a wide variety of growth factors is expressed at any given time in human tumor tissues as well as in cultured cell lines *(75,79)* and it is very difficult to decipher which are essential for tumor growth and are ultimately promising targets for specific therapeutic agents. In particular, melanoma cells that express high levels of PTN *(74)* also express fibroblast growth factors (FGFs) *(79)* that have biologic activities similar to those of PTN. Much like PTN, FGFs stimulate neurites, fibroblasts, and endothelial cells and can also serve as tumor angiogenesis factors *(43,80)*. To directly assess whether the expression of PTN in tumor cells, such as melanoma, plays a significant role for their malignant growth, we decided to inactivate the PTN gene product by ribozyme targeting and to study the resulting phenotype of the cells. For our studies we chose 1205LU human melanoma cells generated by the laboratory of Meenhard Herlyn (Wistar Institute, Philadelphia, PA) as a model. These cells express high levels of PTN and metastasize to the lungs after subcutaneous inoculation in athymic nude mice. We used ribozyme targeting of PTN in these tumor cells to generate a panel of cell lines expressing different levels of the growth factor and then subjected these cell lines to further in vitro and in vivo studies.

4.3. Ribozyme Targeting of Endogenous PTN mRNA in 1205LU Human Melanoma Cells

The 1205LU human melanoma cell line expresses easily by Northern–blot detectable amounts of PTN mRNA and was one of the highest expressors among all of the cell lines

studied by us (*see also* refs. *34,40,74*). Ribozyme targeting of PTN in the 1205LU cells was applied to generate a panel of derivative cell lines that express different extents of residual PTN. Expression vectors for hammerhead ribozymes that are targeted against two separate sites in the PTN transcript were used *(34)*. The efficacy and specificity of the PTN-targeted ribozymes transcribed from these vectors was shown earlier against spontaneously expressed PTN in WM852 melanoma cells and in cotransfection studies with a PTN expression vector in PTN-negative SW-13 cells *(34)*. After transfection of 1205LU melanoma cells with the ribozyme expression constructs, we used G-418 drug selection to generate stable mass-transfected and clonal derivative cell lines. We assessed the amount of residual PTN mRNA in different cell lines by Northern blotting. The quantitative results from the seven cell lines used in the further studies is depicted in Fig. 4A. We chose cell lines that express high, medium, and low levels of PTN mRNA and the data presented below are organized accordingly. We wish to emphasize that mass-transfected and clonal cell lines as well as cell lines transfected with either of the ribozyme constructs (Rz66 and Rz261) are represented in this set (details are given in ref. *40*).

4.4. Characterization of the 1205LU Melanoma Cells Expressing Different Residual Levels of PTN

4.4.1. In Vitro Growth

Previous studies with SW-13 cells *(74)* and NIH3T3 fibroblasts *(78)* showed that PTN is an autocrine-acting growth factor for these cells. In parallel with this, we demonstrated recently that the reduction of PTN in WM852 human melanoma cells by ribozyme targeting reduces their colony formation in vitro and coincidently tumor growth in nude mice *(34)*. Obviously, in the WM852 melanoma cells endogenously expressed PTN also serves as an autocrine growth factor. In contrast with these previous studies, PTN does not appear to be a rate-limiting factor for the in vitro growth of 1205LU melanoma cells. The reduction of PTN did not affect spontaneous colony formation in soft agar of the different 1205LU cell lines (Fig. 4B) or their proliferation on plastic surfaces (not shown).

4.4.2. Growth Factor Activity in the Cell Supernatants

Since PTN is a secreted growth factor *(81)*, we also assessed residual growth factor activity in the supernatants of the different 1205LU cell lines using soft agar colony formation of SW-13 cells as a bioassay *(81)* (Fig. 4C). In this semiquantitative assay an average reduction of growth stimulatory activity by 40–75% in the "medium PTN" and by 70–90% in the "low PTN" group was found. In endothelial cell assays a similar reduction of growth factor activity was observed as with the SW-13 cell assay. This result suggests that PTN is the predominant growth factor activity among a number of growth factors that can stimulate these two indicator cell types and are commonly found expressed in melanoma *(23)*.

4.5. Tumor Growth of 1205LU Melanoma Cell Lines Expressing Different Levels of PTN

When injected into nude mice, subcutaneous tumor growth of the melanoma cells producing different residual levels of PTN was reduced in parallel with the reduced levels of growth factor mRNA levels (compare Figs. 4A and 5A,B). Much to our surprise, even an only 40% reduction of endogenous PTN mRNA resulted in a dramatic reduction by 65% of the growth rate of the subcutaneous tumors ("medium PTN" group of cell lines;

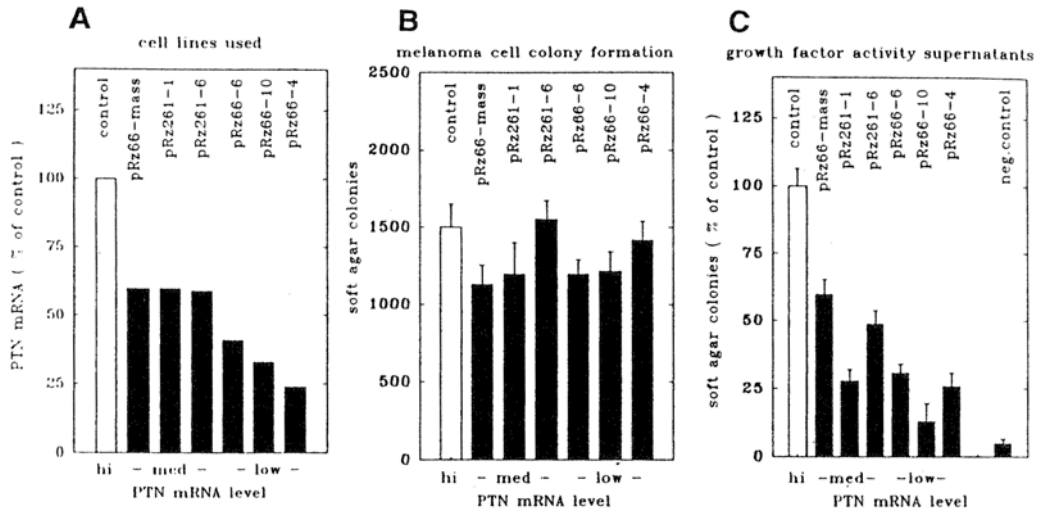

Fig. 4. In vitro characteristics of human melanoma cells (1205LU) after stable transfection with PTN-targeted ribozymes (40). **(A)** Relative PTN mRNA levels of all cell lines used in the studies. After transfection of 1205LU cells with different ribozyme constructs, PTN mRNA levels of G418-resistant clonal or mass-transfected cell lines were analyzed by Northern blotting with β-actin as a loading control. Values are expressed relative to control. Clonal cell lines (pRz66-4, pRz66-6, pRz66-10, pRz261-1, pRz261-6) and a pool of G-418 resistant cells (pRz66-mass) were used. The cell lines are grouped according to their PTN mRNA levels into high (100%), medium (>50 %), and low (<50%). **(B)** Soft agar colony growth of melanoma cells. The number of colonies (>60 μm) grown after 10 d of incubation is shown. **(C)** Secreted growth factor activity. Medium conditioned by the different 1205LU cell lines was passed over heparin-Sepharose columns and fractions were eluted with a NaCl-gradient. The fractions containing the highest PTN immunoreactivity levels (0.9 *M* NaCl) were tested for their bioactivity using colony formation of an indicator cell line in soft agar (SW-13; *See* ref. *81* for more details).

compare Figs. 4A and 5B). A further reduction of PTN in the tumor cells ("low PTN" group) resulted in a further reduction of the growth rate of the primary tumors (*see* Fig. 5A,B). Tumors in this group took almost three months to grow to a size reached by control tumors in 3 wk. These data suggest to us that PTN is rate-limiting for the growth of these tumors in vivo. Furthermore, the effect of the PTN reduction on tumor growth in animals, in contrast to the lack of an effect on cell growth in vitro (Fig. 4B), supports the notion that PTN acts as a paracrine growth factor toward the tumor stroma. This feature distinguishes the role of PTN for 1205LU melanoma cells from its role in WM852 melanoma cells, where it was also an autocrine growth factor for the tumor cells *(34)*.

4.6. Tumor Growth of Mixtures of High and Low PTN 1205LU Melanoma

Further analysis of the data presented above shows that tumor growth of heterogeneous, mass-transfected cells was no different from tumor growth of clonal, homogeneous cell lines that produced comparable levels of PTN (compare pRz66-mass vs pRz261-1 and pRz261-6; Figs. 4A and 5B). From this we hypothesized that higher or lower amounts of PTN produced by different subpopulations of tumor cells is averaged out in the tumor bed and that tumor growth is dependent on the overall paracrine stimulus generated by the tumor cell mass as a whole. Consequently, individual tumor cells or subpopulations

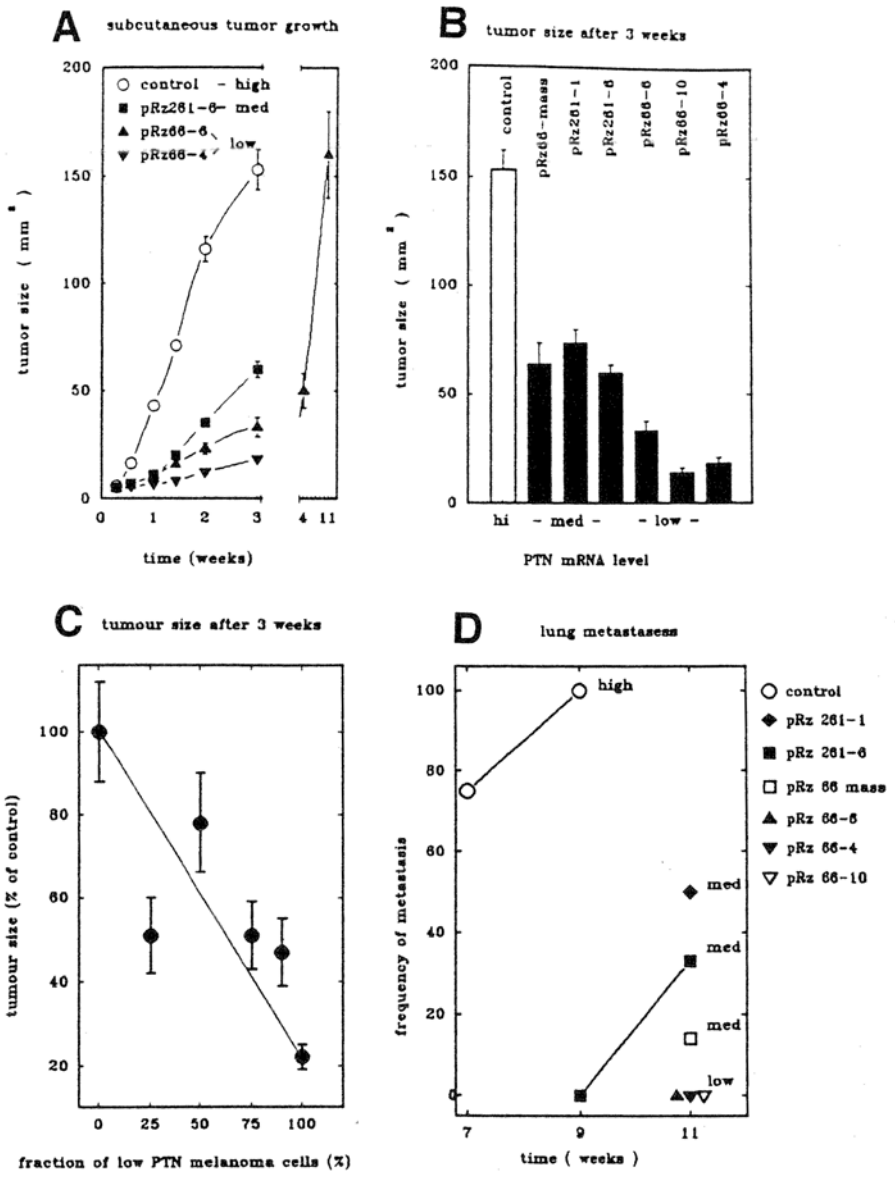

Fig. 5. Subcutaneous tumor growth and lung metastasis in nude mice of 1205LU melanoma cells producing different levels of PTN mRNA *(40).* **(A,B)** Complete growth curves of subcutaneous tumors from representative cell lines (A) and tumor sizes of all cell lines after 3 wk (B) are shown. The cell lines producing different levels of PTN mRNA (*see* Fig. 4A) were injected subcutaneously into nude mice ($n = 5–12$ animals per group) at 1,000,000 cells per injection site and two sites per animal. **(C)** Tumor growth in nude mice of mixtures of different ratios of melanoma cells producing high and low levels of PTN mRNA. Control cells producing high levels and ribozyme-transfected cells producing low levels of PTN were mixed at different ratios and injected subcutaneously into two sites per mouse ($n = 5$ animals per group). Tumor sizes after 3 wk of growth are shown relative to control. **(D)** Quantitation of lung metastases. Subcutaneous tumors were resected after 3 wk (controls) or 11 wk (pRz66-6) or when they reached at least 50 mm^2. Lungs were examined macroscopically or after hematoxylin and eosin staining. Animals with any detectable metastases were scored positive and the relative incidence of animals with lung metastases is shown.

would not gain or lose a selective growth advantage from a higher or lower production of PTN. In support of this hypothesis we found no in vivo selection for high PTN producer subpopulations in Northern blots with tumors harvested at the end of the animal study (not shown).

To test this hypothesis more rigorously, we mixed cells from the "high PTN" and the "low PTN" group at different ratios and inoculated a fixed total number of cells into nude mice (Fig. 5C). Indeed, after 3 wk the size of the subcutaneous tumors grown from these cell mixtures mimicked the results obtained with individual cell lines expressing different levels of PTN and tumor growth was reduced in parallel with a decreasing portion of "high PTN" cells (Fig. 5C). Furthermore, PTN levels in these tumors assessed by Northern blots followed the levels predicted from the different ratios of cell mixtures (not shown). From this set of data it is tempting to speculate that therapeutic targeting of PTN should have an impact even if only a fraction of malignant cells can be reached in a solid tumor by the respective targeting method.

4.7. Angiogenesis in 1205LU Tumors and PTN Levels

To establish whether PTN acts as a mediator of tumor angiogenesis in vivo, we quantitated the number of microvessels in subcutaneous tumors using an endothelial cell marker (platelet-endothelial cell adhesion molecule [PECAM], CD31; ref *4*). For this analysis we selected tumor samples from each of the groups with different levels of residual PTN. We took care that tumors of equal size were used in the assessment. A significant reduction of the number of microvessels per field by approx 70% ($p < 0.01$ vs control) was found in the "medium PTN" group and by 85% ($p < 0.002$ vs control) in the "low PTN" group (Fig. 6A). We conclude from this result that PTN acts as a tumor angiogenesis factor in vivo. Furthermore, we propose that the reduced blood supply of the tumors caused by the reduced levels of PTN results in the slower rate of tumor growth observed.

4.8. Apoptosis in 1205LU Tumors and PTN Levels

A recent study describing the targeting of integrins to inhibit angiogenesis reported that the extent of apoptosis in adjacent tumor tissues was increased, and may cause tumor regression in a clinical setting *(82)*. In our study, a significant increase in apoptotic DNA fragmentation (detected as DNA/histone complexes *[83]*) was found in tumors from the "low PTN" group (149 ± 25% of control; $p < 0.05$; Fig. 6B), and a small but insignificant increase of this parameter was observed in the "medium PTN" group (115 ± 23% of control). The increase in apoptosis was corroborated by staining of the tumor tissues for apoptotic nuclei using the TdT-mediated dUTP nick-end labeling method (TUNEL) *(84)* (not shown). This finding suggests to us that the decreased angiogenesis in the tumors resulting from decreased levels of PTN causes a shift of more tumor cells into apoptosis.

4.9. Melanoma Cell Metastasis and PTN Levels

The ultimate goal of our studies was to assess the metastatic spread of the 1205LU melanoma cells expressing different levels of PTN. In most of the groups we resected the subcutaneous tumors when they reached at least 50 mm^2 and followed the development of lung metastases for up to 11 wk (Fig. 5D). One set of subcutaneous tumors growing from a "low PTN" cell line (pRz66-6) was left to grow for the complete study period to reach (or surpass) the size of tumors in the control group (*see* Fig. 5A).

Animals carrying tumors from the control group ("high PTN") showed metastases to their lungs 7–9 wk after tumor cell inoculation (Fig. 5D) and the majority of these

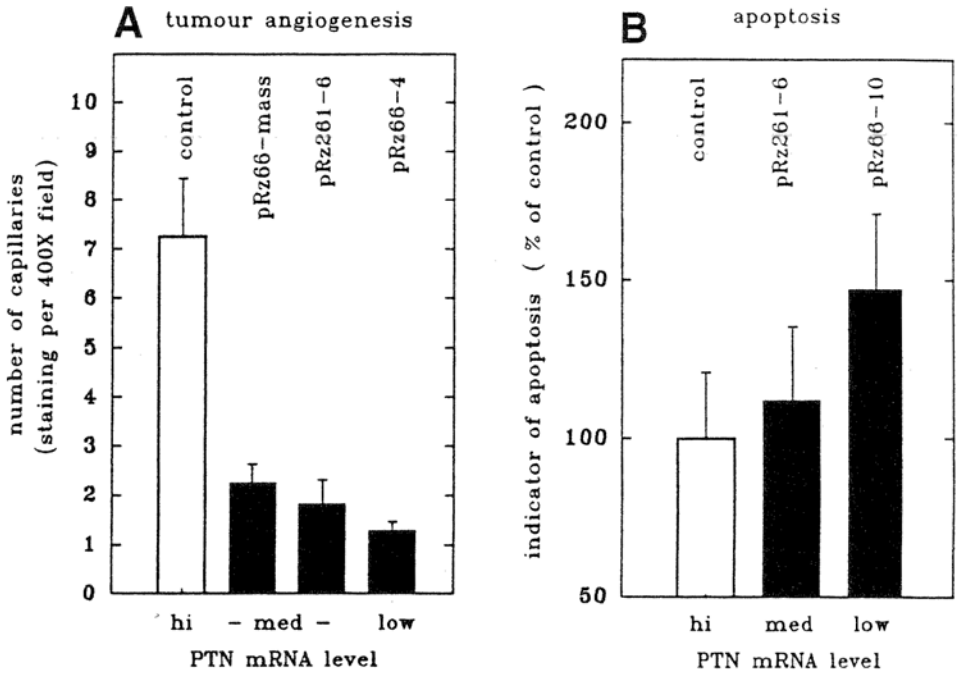

Fig. 6. Reduction of PTN mRNA levels leads to a decrease of tumor angiogenesis **(A)** and an increase of apoptosis **(B)** in the tumor tissues *(40)*. (A) Subcutaneous tumors grown from representative groups of the melanoma cells expressing different levels of PTN mRNA were analyzed. Capillaries in tumors were highlighted by staining for PECAM (CD31; *4*). In three representative tumors from each group the average number of capillaries in eight high power fields (×400) was counted. The number of capillaries/field ± standard error of the counts of three blinded investigators are shown. (B) The extent of apoptosis was assessed in tumor tissues from three representative groups by an enzyme-linked immunosorbent assay ("cell death detection;" Boehringer Mannheim) that quantitates apoptotic DNA fragmentation by detecting DNA/histone complexes *(83)*. From each of the groups three tumors were selected and three separate biopsies from each of the tumors were used for the assay.

metastases was visible by macroscopic inspection of the lungs (*see* ref. *40*). In contrast with this, none of the tumor cell lines in which PTN was reduced generated macroscopic metastases. Upon further microscopic inspection of the lungs, no micrometastases were found in any of the "low PTN" group even after 11 wk ($p < 0.002$ vs control; *40*). This was independent from the continuous presence (pRz66-6) or surgical resection of the primary tumors (pRz66-10, pRz66-4). In the "medium PTN" group we found one or more micrometastases in one-third of the animals at this time point ($p < 0.01$ vs control; Fig. 5D). Obviously, the reduction of PTN mRNA to different levels is reflected in a gradual reduction of the incidence of metastasis. This result support the thesis that the reduction of angiogenesis in the primary tumors is the cause for this effect on metastasis.

4.10. Choriocarcinoma Growth, Angiogenesis, Invasion, and PTN

The trophoblast (placenta) is one of the most vascular and invasive tissues and choriocarcinomas derived from the trophoblast are also characterized by this aggressive growth.

In the course of our studies on the regulation of PTN, we found a germline retroviral insertion into the PTN gene that occurred some 25 million years ago and generated a novel promoter with trophoblast-specific activity. As a consequence of this promoter activity, primate trophoblast, trophoblast cells in culture, and human choriocarcinoma express high levels of PTN *(39)*. To assess whether the high levels of PTN expression contributes to the invasive and angiogenic phenotype of choriocarcinoma (and by extension of the human trophoblast), we depleted choriocarcinoma cells of their PTN by ribozyme targeting *(39)*. By stable transfection of JEG-3 human choriocarcinoma cells we reduced their PTN levels by >80%. Very similar to the findings with the 1205LU melanoma cells (*see above*), this reduction of PTN did not affect the proliferation of JEG-3 cells in culture. Upon implantation of the choriocarcinoma cells subcutaneously or intraperitoneally into immune-compromised animals, these cells form highly vascular and invasive tumors and thus mimic the human disease. Much to our surprise, tumor growth, angiogenesis, and invasion into the surrounding tissues was almost completely abolished in the PTN-reduced JEG-3 cell xenografts. This was observed for both orthotopic (i.e., intraperitoneal) and heterotopic (i.e., subcutaneous) inoculation of tumor cells *(39)*.

4.11. Conclusion

Our results with PTN demonstrate that specific and dose-dependent reduction of a single factor that mediates angiogenesis can have a significant inhibitory effect on tumor angiogenesis, invasion, and metastasis. Furthermore, our data suggest that ribozyme targeting has the potential to be of therapeutic significance in the prevention of metastatic malignancies.

5. RIBOZYMES AS DESIGNER DRUGS

5.1. Background

In principle, ribozymes can be designed to specifically cleave a particular RNA species and not affect the background expression and function of cellular RNA. This cleavage event renders the respective mRNA untranslatable and thus abrogates protein expression. In general, the mRNA coding for any protein associated with a disease may be cleaved selectively. To function as therapeutic agents, ribozymes must be delivered to the target cells and destroy the target RNA. Delivery of ribozymes to the target cells can be accomplished either through exogenous gene therapy or through drug delivery approaches, with each approach raising different issues for ribozyme delivery, activity, and potential toxicity. In our studies the effects on tumor growth, angiogenesis, and metastasis followed the extent of the reduction of PTN or of FGF-BP mRNA in the tumor cells by ribozyme targeting. Most surprising for us, a relatively small reduction of PTN mRNA by less than one-half already showed a significant effect in vivo. This is further supported by the experiment with mixtures of different ratios of tumor cell populations producing high and low levels of PTN (*see* Fig. 4C). From this data it is tempting to speculate that therapeutic targeting of PTN using ribozymes would only require partial efficacy to achieve a significant therapeutic effect on tumors.

5.2. Gene Therapy

In more recent studies we applied adenoviral vectors expressing different ribozymes to assess whether this approach would be feasible for gene targeting. Interestingly, the

efficacy of the system was very high in cell culture with tumor cells expressing the targeted mRNAs, i.e., the oncogene HER-2 as well as pleiotrophin *(38)*. The duration of a downmodulation of the targeted proteins was several days, suggesting that this approach could indeed have therapeutic benefits. Animal studies with in vivo delivery are currently planned.

5.3. Synthetic Ribozymes

The small size of the hammerhead motif and its minimal substrate requirements also make the hammerhead ribozyme very amenable to chemical synthesis and ultimately the delivery as a small molecule designer drug. For the purpose of use in vivo, these ribozymes have to be stabilized by chemical modifications and a large body of work comparing stability and activity has been published in the last few years addressing this problem (*see* ref. *24*). In preliminary, unpublished animal studies we used synthetic ribozymes that were designed to target pleiotrophin (i.e., the ribozymes Rz66 and Rz 261 mentioned above). These preliminary studies showed that ribozyme treatment of animals with 1205LU melanoma xenograft tumors inhibited growth of the tumors. Furthermore, when we monitored the serum levels of PTN in the animals, we found that ribozyme treatment prevented the serum levels from increasing along with the tumor growth. The treatments were via intraperitoneal injection of the ribozyme drugs every other day or twice a week (Malerczyk et al. unpublished data).

6. OUTLOOK

The growth and metastatic spread of cancer is directly related to tumor angiogenesis *(2)* and the driving factors need to be understood to exploit this process therapeutically *(14,15)*. However, tumor cells and their normal stroma express a multitude of candidate angiogenic factors *(14)* and very few specific inhibitors have been generated to assess which of these gene products are only innocent bystanders and which contribute significantly to tumor angiogenesis *(16,39,40)* and metastasis *(21,22,40)*. In our experience, ribozyme targeting is a very elegant approach that allows an assessment of the relative contribution of suspected angiogenic modulators expressed by tumor cells. One of the most surprisingly findings was that tumor angiogenesis and the resulting tumor growth, invasion, and metastasis are exquisitely sensitive even to small reductions of the driving factors. Since the inhibitory effects on tumor angiogenesis were strictly "gene dose"-dependent, inhibition of specific growth factor pathways with ribozymes or other pharmacologic means has the potential to be therapeutically efficacious. Ribozyme targeting definitely is advantageous when trying to sort out which factor to pick under which circumstances.

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