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ANGIOGENESIS AND THE CLINICAL IMPLICATIONS OF SELECTED ANGIOGENESIS INHIBITORS

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ANGIOGENESIS AND THE CLINICAL IMPLICATIONS OF SELECTED ANGIOGENESIS INHIBITORS

By

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ABSTRACT

ANGIOGENESIS AND THE CLINICAL IMPLICATIONS OF SELECTED ANGIOGENESIS INHIBITORS

By
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Angiogenesis is the induction of blood vessels from preexisting blood vessels. It is an essential process for cancer growth and serves as a conduit for micrometastases to travel to distant sites. There are numerous endogenous factors that are released by different types of cells, including tumor and endothelial cells that are involved in the induction and inhibition of angiogenesis. Often some of the angiogenic inducers such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and matrix metalloproteases (MMPs) work in synergism and have overlapping function, while other factors such as tissue inhibitors of metalloproteases (TIMPs) and angiopoietin-2 function in inhibiting the actions of these inducers. Part I of this thesis is a review of the process of angiogenesis and many of the factors that help to induce and inhibit it. Part II of this thesis proposes an experiment that could be conducted to test the efficacy of a particular combination of antiangiogenic therapy, using angiostatin, endostatin, and radiation therapy on a highly vascularized tumor model, rat C6 glioma cells. The purpose of this proposed experiment is to study the effectiveness of combining antiangiogenic therapy on vascularized glioma in a murine model.

Dedicated to my parents, Jayanta and Gopa, my sister Sumana,
and Nathan Venno for their unwavering support in my academic career.

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(Note: Images in this thesis are presented in color.)

KEY TO ABBREVIATIONS

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
CBP	creb binding protein
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GRB-2	growth factor receptor bound protein-2
HIF-1	hypoxia inducible factor-1
MAP	mitogen activated protein (kinase)
MEK	mitogen activated protein kinase kinase
MLCK	myosin light chain kinase
MMP	matrix metalloprotease
N-cadherin	neural-cadherin
PAI	plasminogen activator inhibitor
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3 kinase
PLC-γ	phospholipase C-γ
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloprotease
tPA	tissue plasminogen activator
TSP	thrombospondin
uPA	urokinase plasminogen activator
VE-cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor

Part I:

Angiogenesis Overview

Angiogenesis

Angiogenesis is the formation of new vasculature from preexisting blood vessels. Though angiogenesis and vasculogenesis literally mean the same thing (the *genesis* of new blood vessels), the two processes differ in their context and process. Angiogenesis is the sprouting of new blood vessels from preexisting vessels, whereas vasculogenesis is the formation of blood vessels *de novo.* Vasculogenesis occurs primarily during embryogenesis and is driven by the recruitment of undifferentiated mesodermal cells to the endothelial lineage, followed by the assembly of such cells into blood vessels (Drake, 2003). In contrast, angiogenesis is characterized by endothelial cell proliferation, vascular discontinuity, migration, and the maturation of new vasculature. Angiogenesis occurs in normal conditions in the female reproductive system as well as in wound healing and embryogenesis. However, this process can also pathologically occur in conditions such as ocular neovascularization in diabetics and rheumatoid arthritis.

Tumor cells need angiogenesis to occur for growth and delivery of oxygen and nutrients. Normally, tumor cells can receive the required nutrients and oxygen by diffusion from the nearest microvessel. However, as the tumor mass increases in size, and distance from the tumor cells to the nearest blood vessel increases, diffusion alone cannot maintain the tumor's growth and viability. If the tumor does not promote nearby vascular growth, the lack of gas exchange and nutrient supply will starve the tumor and result in the surrounding area to become hypoxic. The formation of new vasculature around the tumor also serves as a conduit for micrometastases from the primary tumor to

travel to distal sites. Therefore, angiogenesis is needed for tumor growth and to increase the tumor's metastatic potential.

Judah Folkman hypothesized in 1971 that targeting the vasculature instead of tumor cells may prove to be effective therapy in inhibiting tumor growth. The notion of targeting endothelial cells instead of tumor cells was a novel idea at the time, as most of the anticancer drugs studied and developed at the time were targeted to the tumor cells.

Tumor cells are inherently genetically heterogeneous, unstable (i.e. more likely to acquire mutations), and susceptible to acquiring resistance to pharmaceutical inhibitors as seen in a study conducted by Fernandez et al, 2001. This study exhibited that TNP-470, an angiogenesis inhibitor, administration led to the overexpression of the antiapoptotic protein, Bcl2, that allowed prostate cancer cells to become resistant to this angiogenesis inhibitor's actions (Fernandez et al, 2001). In contrast to the tumor cells' genetic instability, endothelial cells are a relatively genetically homogenous group of cells that are more stable and less likely to accumulate mutations that would make them gain resistance to therapeutic agents. Therefore, many current antitumor studies are focusing on inhibiting endothelial cell growth in cancer.

There are numerous growth factors that have been identified that are involved in normal and pathological development of vasculature such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), basic and acidic fibroblast growth factor (bFGF and aFGF), and platelet derived growth factor (PDGF). These inducers are expressed in all stages of angiogenesis and their binding to their respective growth factor receptors is often targets for antiangiogenic therapy.

Though it may seem counterintuitive, administration of angiogenic inhibitors does not inhibit vasculature formation to the extent that there is a decrease in the delivery of cytotoxic chemotherapeutic agents to the tumor cells. It has been demonstrated that immediately after angiogenic inhibitor administration, there is increased blood flow and oxygen delivery to the tumor, and this is thought to occur as a result of decreased leakage of plasma proteins from the tumor vessels that results in a decreased intratumoral pressure (Jain, 1989). The increased blood flow also results in an increased delivery of chemotherapeutic agents to the tumor site. Therefore, antiangiogenic therapy can have synergistic effects with chemotherapy in fighting tumor growth.

Process of Angiogenesis

The process of angiogenesis needs the induction of vascular discontinuity, endothelial cell proliferation and migration towards the angiogenic stimulus, and structural reorganization of the new vasculature (Figure 1). The induction of vascular discontinuity occurs by the local degradation of the basement membrane by activated endothelial cells using molecules such as plasminogen activators and matrix metalloproteases (MMPs). The activation of endothelial cells result from the release of chemotactic growth factors from the tumor such as VEGF and FGF (Philip, 2000). The network of existing vessels expands by sprouting or intussusception. During the structural reorganization of the vasculature, the individual capillary or small venule sprouts and loops merge to form a vascular lumen, followed by the establishment of blood flow (Figure 2). The stabilization of the new vasculature requires the recruitment

and differentiation of precursor cells into smooth muscle cells that are mediated by adhesion molecules such as cadherins.

It has been suggested that angiogenesis is made of two general phases: phase of activation and phase of resolution. The phase of activation includes increased vascular permeability and extravascular fibrin deposition, vessel wall disassembly, basement membrane degradation, endothelial cell proliferation and migration, and capillary lumen formation (Pepper, 2001). The phase of resolution is characterized by decreased endothelial cell proliferation, termination of cell migration, basement membrane stabilization, and the establishment of blood flow follows vessel wall assembly (Pepper, 2001).

Pericytes surround endothelial cells and provide structure and integrity to the vasculature. Pericytes also help to maintain a state of endothelial cell non-proliferation via cell-cell contacts. Normal blood vessels are made of endothelial cells that line the lumen of the vessel. The vessel walls are also made of smooth muscle cells and extracellular matrix (ECM) proteins. Angiogenesis requires endothelial cell morphology to change from tubular (parent venule) to flat and elongated (sprout growth) and back to tubular (established capillary blood vessel) (Folkman et al, 1992). Tumor vessels have distinct structural features that distinguish them from normal vasculature. Tumor blood vessels are not quiescent, and instead, are in a state of proliferation, and characterized by increased vessel branching and chaotic blood flow. They have open interendothelial junctions, and are associated with a discontinuous basement membrane. Furthermore, tumor vessels have loosely associated pericytes and the thin vessels have irregular shapes, and are leaky and dilated (Figure 3) (Bergers, 2003). It has been demonstrated

that some tumor vessels in xenografted and spontaneous human colon carcinomas are mosaic in nature, that is, the vessels are made of both endothelial and cancer cells, although it is unknown as to whether these cells invade the vessel wall, are mimicking endothelial cells (vasculogenic mimicry), or are exposed when the overlying endothelial cells undergo apoptosis (Chang et al, in press).

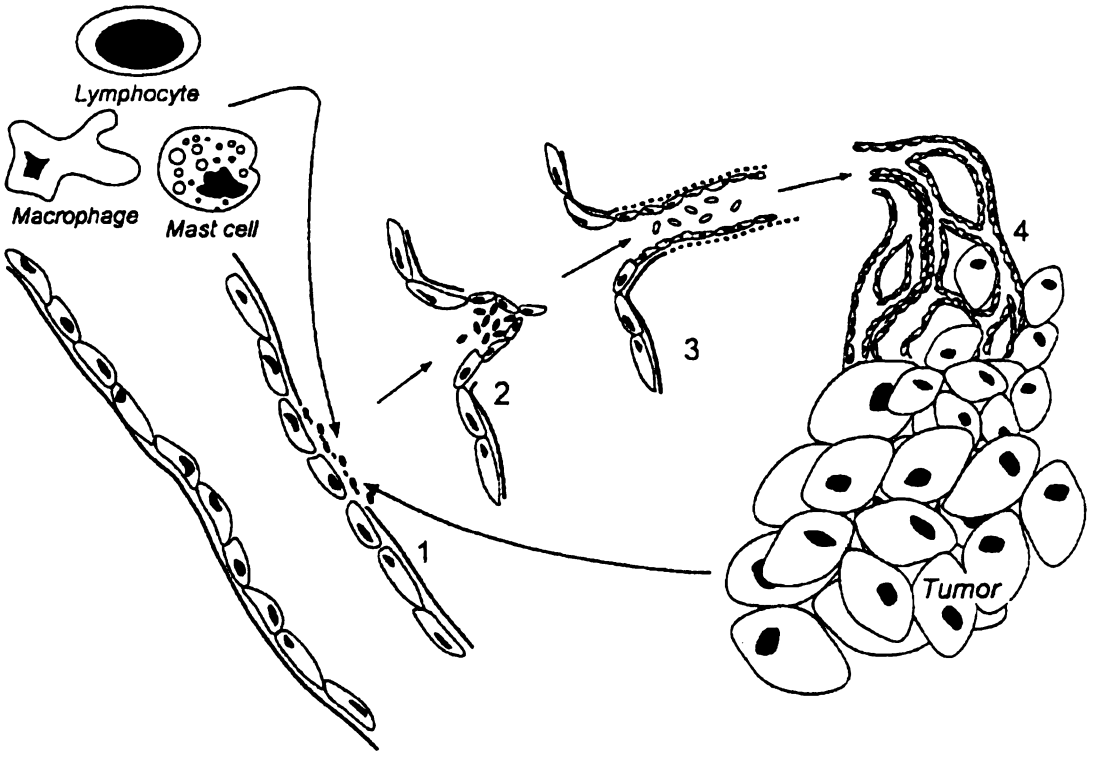

Figure 1: Process of Angiogenesis. 1 induction of vascular discontinuity; 2 endothelial cell proliferation; 3 endothelial cell migration; 4 structural reorganization of new vasculature. (Reijneveld JC, Voest EE, Taphoorn MJB. (2000) Angiogenesis in malignant primary and metastatic brain tumors. *J Neurol.* 247:597-608.)

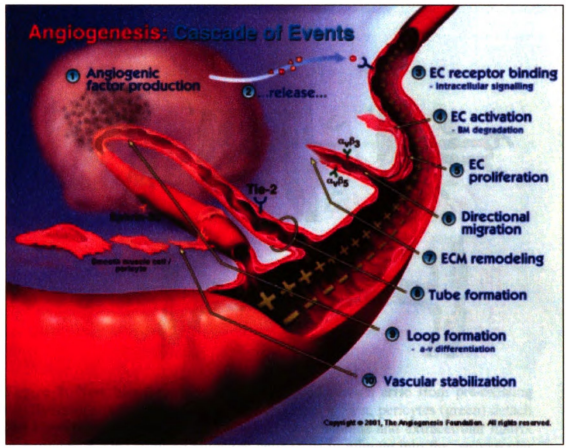

Figure 2: Angiogenesis: the formation of new blood vessels. (The Angiogenesis Foundation, Inc., 2001)

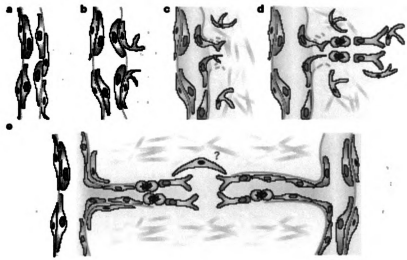

Figure 3: New blood vessel formation. Blood vessels arise from pre-existing capillaries or post-capillary venules in tumours **a**. **b** First, pericytes (green) detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded. **c** This allows endothelial cells (red) to migrate into the perivascular space towards angiogenic stimuli produced by the tumour cells or host cells. **d** Endothelial cells proliferate, loosely following each other, and are presumably guided by pericytes. **e** Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte attachment. Finally, blood-vessel sprouts will fuse with other sprouts to build new circulatory systems. Little is known about this fusion mechanism. (Bergers G, Benjamin LE. (2003) Tumorigenesis and the Angiogenic Switch. *Nature Reviews.* 3:401-410).

Angiogenic Switch

Not all tumors become angiogenic and the transformation from nonangiogenic to angiogenic occurs with an imbalance between tumor cell proliferation and apoptosis that could lead to angiogenesis (Figure 4). The angiogenic switch in which the rate of tumor cell proliferation is greater than tumor cell apoptosis can be activated by an imbalance in positive and negative angiogenic factors. An angiogenic switch can be triggered by metabolic stress (such as hypoxia, low pH, hypoglycemia), mechanical stress (such as pressure generated by proliferating cells), immune/inflammatory response (immune cells infiltrating the tissue), and genetic mutations (such as tumor-suppressor gene deletion and activation of oncogenes) (Carmeliet et al, 2000). Furthermore, the angiogenic switch is potentiated by hypoxic conditions in which mutated, apoptosis-resistant tumor cells upregulate factors such as VEGF and FGF to induce angiogenesis.

Activation of oncogenes and the role of tumor suppressors have been demonstrated in angiogenesis. Oncogenic activation results in increased expression of angiogenic inducers and growth factors such as vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (aFGF and bFGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). For example, the wildtype p53 gene normally inhibits angiogenesis by downregulating hypoxia inducible factor (HIF)-1 that increases VEGF expression during tumor hypoxia (Ravi et al, 2000 and Zhang et al, 2000). However, the mutated tumor suppressor p53 gene makes tumor cells resistant to apoptosis in hypoxic environments and may be involved in the angiogenic switch.

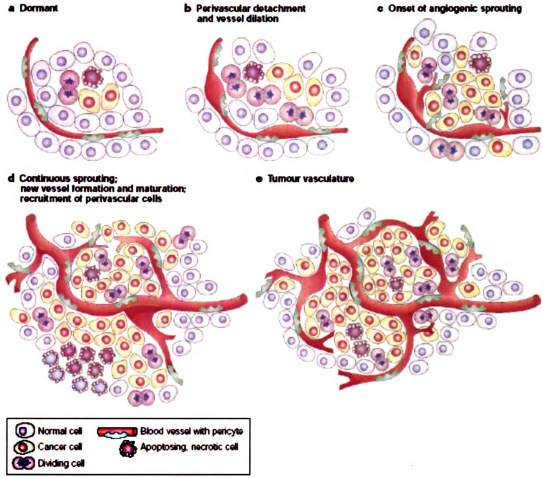

Figure 4: The classical angiogenic switch. The angiogenic switch is a discrete step in tumour development that can occur at different stages in the tumour-progression pathway, depending on the nature of the tumour and its microenvironment. Most tumours start growing as avascular nodules (dormant) (**a**) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis, or the "angiogenic switch," has to occur to ensure exponential tumour growth. The switch begins with perivascular detachment and vessel dilation (**b**), followed by angiogenic sprouting (**c**), new vessel formation and maturation, and the recruitment of perivascular cells (**d**). Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (**e**). (Bergers G, Benjamin LE. (2003) Tumorigenesis and the Angiogenic Switch. *Nature Reviews.* 3:401-410)

Hypoxia

Tumor size is limited by the extent of the surrounding vasculature. If no new vascularization occurs, tumor cells can become hypoxic. Angiograms can reveal tumors with dark ischemic areas whose perimeter is lined by a rim of vascularized tumor cells. There is a distinct border between the viable tumor cells that are within diffusion distance for oxygen from a nearby microvessel and the dead tumor cells that are a few microns too far to receive the diffused oxygen (Figure 5) (Kerbel et al, 2002). In vascularized tumors, there usually lies a viable cuff of tumor cells around a microvessel, and outside this radius of nutrient supply, tumor cell necrosis can be observed. Tumor cells that have higher metabolic needs (high rates of oxygen or nutrient consumption), such as in glioblastomas, have small cuffs that are only two to three cells deep (Hlatky et al, 2002). Therefore, a tumor cell mass's metabolic needs and cuff size surrounding a microvessel is inversely proportional. Interestingly, results obtained by Bergers et al, 1999, showed that tumor cells that make up the cuff surrounding the microvessel were apoptotic, even though they were closer to the vessel. This phenomenon known as the periendothelial apoptotic pattern suggests that tumor cell death in the cuff may not necessarily by hypoxia induced programmed cell death (Bergers et al, 1999).

It has been demonstrated that during hypoxia, endothelial mitogens such as PDGF and FGF are induced by macrophages (Kuwabara et al, 1995). Furthermore, hypoxia can induce the expression of hypoxia inducible factor (HIF)-1. HIF-1 is a heterodimeric transcription factor, and is made of HIF-1α and HIF-1β (HIF-1β is a nuclear translocator that is not oxygen responsive, but is constitutively bound to HIF-1α). HIF-1 binds to hypoxia-response elements and induces the expression of angiogenesis inducer genes,

such as VEGF and PDGF (Carmeliet et al, 1998). Carmeliet and colleagues demonstrated that HIF-1α affects tumor vascularization not only by upregulating VEGF expression (via HIF-1 binding to a consensus sequence on the VEGF gene), but also that HIF-$1\alpha^{+/+}$ embryonic stem cells undergo apoptosis in response to hypoxia, whereas HIF-$1\alpha^{-/-}$ embryonic stem cells do not. The authors also suggested that the p53, Bcl-2, and HIF-1α pathways may interact with each other, suggesting that programmed cell death is an interplay of various pathways (Carmeliet et al, 1998). Furthermore, work conducted by Giordano and colleagues demonstrated the essential role that HIF-1 plays in development as their studies revealed that HIF-$1\alpha^{-/-}$ embryos die prenatally (Giordano et al, 2001). Studies conducted by An and colleagues have demonstrated that though hypoxia is a potent inducer of the wild-type p53 gene, p53's own induction is dependent on hypoxia induced HIF-1α (An et al, 1998).

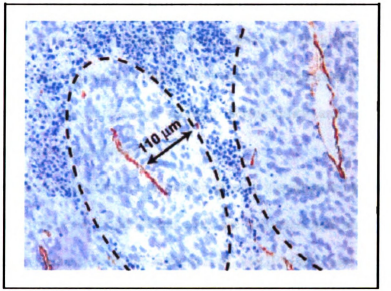

Figure 5: Supported tumor cells forming cuffs (areas of viable tumor cells surrounding functional vessels—indicated by black dashed ovals) are shown for a Dunning rat prostate carcinoma xenograft. Cuff size is roughly indicative of the metabolic burden of the carcinoma cells. Tumor cells within approximately 110 µm of the vasculature are viable; beyond this radius of oxygen and nutrient support, an abrupt shift to necrosis is observed. Section was stained with hematoxylin for DNA, highlighting areas of necrosis, and with an antibody to CD31, showing the endothelium. (Hlatky L, Hahnfeldt P, Folkman J. (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *Journal National Cancer Institute.* 94:883-893.)

Angiogenic Inducers

Angiogenic inducers such as serine proteases that include urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), matrix metalloproteases (MMPs), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin-1 play important roles in angiogenesis. These roles include the formation of new vasculature with differing roles in the breakdown of the extracellular matrix and basement membrane, directly promoting vascular permeability and endothelial cell growth, and new vasculature remodeling.

These factors are involved in the formation of new vasculature by functioning in different stages of angiogenesis. Endothelial cells are activated and transform from a quiescent to proliferative phase with the induction of VEGF, FGF, and PDGF. These growth factors prepare endothelial cells for detachment, migration, and attachment by inducing vessel hyperpermeability that results in extravasation. Protease activators that are released primarily by tumor cells induce endothelial cell detachment from the surrounding matrix and basement membrane. These protease activators aid in the degradation of the surrounding extracellular matrix (ECM) and are involved in plasma protein deposition that forms a temporary matrix that is utilized during the process of endothelial cell migration. This temporary matrix is composed of fibrin that provides a scaffold for the migrating endothelial cells, which, at the end of the angiogenic process, is replaced by a mature collagenous matrix (Pepper, 2001). Adhesion molecules such as integrins and cadherins are involved in endothelial detachment from the ECM and reattachment to the surrounding ECM in the newly formed vasculature. During matrix degradation and endothelial cell migration, integrin receptors $\alpha v\beta_3$ and $\alpha_v\beta_5$ on

endothelial cells bind to extracellular matrix proteins such as fibronectin and vitronectin and aid in endothelial cell detachment and migration. Endothelial cells then assemble themselves to form a vessel lumen, and recruit pericytes and smooth muscle cells to stabilize the tumor vasculature.

Serine Protease Activators

Serine protease activators play a role in the breakdown of the ECM and allow for endothelial cell migration that is needed for angiogenesis. Different types of cells including tumor cells function in degrading the matrix and the basement membrane of the surrounding vasculature and release these protease activators. Some of the cells that synthesize these protease activators are smooth muscle cells, endothelial cells, monocytes and macrophages, epithelial cells, and fibroblasts.

These protease activators include tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) both playing a role in angiogenesis, and catalyzing the conversion of inactive plasminogen to active plasmin. Plasmin, a protease, hydrolyzes ECM proteins such as fibrin that allow for endothelial cells to migrate. It is thought that the protease activator tPA is synthesized when fibrolysis is needed, and uPA is synthesized for cell migration (Panchenko et al, 1999, and Vassalli et al, 1991). It has been shown *in vitro* that tPA is involved in capillary-like tube formation on matrices made of type I collagen (Sato et al, 1993). The protease activator, uPA binds to its receptor, uPAR. There is colocalization of uPA, uPAR, and plasminogen that occurs on the plasma membrane, which ensures the activation of plasminogen to plasmin. The protease uPA stimulates endothelial proliferation, chemotaxis, and invasion paralleled by

glucose-dependent DAG synthesis, suggesting that uPA affects the protein kinase C (PKC) pathway.

The levels of the endothelial cell membrane-associated activator uPA, and its receptor uPAR, are increased in migrating endothelial cells at cell-substrate and cell-cell contact sites, resulting in localized plasmin production that causes the proteolysis of the surrounding ECM. Furthermore, it has been demonstrated that the expression of uPA, uPAR, and PAI-1 can be upregulated by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Mignatti et al, 1996). This suggests that conditions such as hypoxia that upregulate these growth factors also increase the expression of matrix proteases (and their inhibitors) to facilitate the formation of new vasculature. Both uPA and tPA are repressed by factors known as plasminogen activator inhibitors, PAI-1 and PAI-2. It has been demonstrated that plasmin inhibitors can suppress cell migration both *in vitro* and *in vivo* (Jackson et al, 1992, and Okada et al, 1996).

Matrix metalloproteases

There are two metalloprotease family members that are involved in matrix degradation so that endothelial cells can migrate: matrix metalloproteases (MMPs) and metalloprotease-disintegrins (ADAMs). This review will focus on MMPs. MMPs include a family of over 20 soluble and membrane bound enzymes that are Zn-dependent and degrade the extracellular matrix. MMPs are released by the tumor and degrade extracellular matrix proteins such as gelatin and collagen. MMPs can be secreted in the form of inactive proenzymes (zymogens) or can exist as membrane bound MMPs.

Secreted proMMPs are activated in the ECM and membrane type-MMPs (MT-MMPs) are activated intracellularly. MT-MMPs are then associated with the plasma membrane and therefore degrade ECM proteins such as fibrin, fibronectin, and vitronectin close to the surface of the cell (Murphy et al, 1999, Pepper, 2001, and Seiki, 1999). MT-MMP-1, the best-characterized MT-MMP, has been shown to activate proMMP-2, one of the metalloproteases that is involved in angiogenesis. MT-MMP has been shown to degrade a fibrin gel that helps endothelial cells to migrate through the ECM. MMPs have been shown to exhibit angiogenic and antiangiogenic roles: by releasing matrix bound factors such as the angiogenic molecule TGF-β, and cleaving the extracellular matrix protein components, respectively (Chang et al, 2001).

Inhibitors of MMPs are secreted tissue inhibitors of metalloproteases (TIMPs). TIMPs bind MMPs and inhibit their degradative activity. Members of the family of TIMPs show antiangiogenic activity, by blocking extracellular matrix degradation and inhibiting endothelial cell proliferation. Levels of both MMPs and TIMPs are increased in endothelial cells during the process of angiogenesis in wound healing, embryogenesis, the female reproductive cycle, and tumor growth. The coexpression of TIMPs and MMPs is thought to function in preventing excessive degradation of the ECM. MMPs can affect other angiogenic inducers, such as cleaving and activating latent TGF-β, through a process known as ectodomain shedding. Furthermore, MMPs's proteolytic effects can release matrix-bound growth factors from ECM stores such as bFGF.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) also known as vascular permeability factor, is approximately a 40 kD glycosylated protein that is secreted as a dimeric protein by the tumor and acts specifically on endothelial cells. VEGF is a mitogen and chemotactic factor for endothelial cells, and promotes endothelial cell survival. VEGF also acts as a permeability factor to enhance the permeability of blood vessels to macromolecular solutes, without causing mast cell degranulation, endothelial cell damage, or significant inflammatory response (Machein et al, 2000). VEGF mRNA expression is upregulated in hypoxic conditions in normal cells; in cancerous cells, VEGF expression is dramatically increased. It is hypothesized that VEGF increases vascular permeability by increasing the expression of vesicular/vacuolar organelles in endothelial cells and loosening adherens junctions in endothelial cell-cell contacts. Furthermore, VEGF binding to its receptor has been shown to induce nitric oxide production that may mediate vasodilation and increased blood flow that may precede angiogenesis (Dulak et al, 2003). It has also been shown that VEGF induces the production of proteases such uPA, PAI-1, and interstitial collagenase by endothelial cells (Pepper et al, 1991, and Pepper, 1997, and Unemori et al, 1992).

The VEGF family is comprised of six members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor. In humans, there are four VEGF isoforms, VEGF-121, VEGF-165, VEGF-189, and VEGF-206 (Machein et al, 2000). VEGF homodimers binds to its receptor primarily by hydrophobic interactions and act through a family of protein tyrosine kinase receptors (Kliche et al, 2001). The binding of the tyrosine kinase receptors lead to the activation of endothelial cells that, in turn, lead to

increased endothelial cell proliferation, cell adhesion molecule expression, protease secretion, migration, and invasion (Brooks, 1996). The VEGF receptors, flt (fms like tyrosine)-1/VEGFR-1, flk (fetal liver kinase)-1/VEGFR-2, and VEGFR-3 are expressed on endothelial cells. VEGF receptors are comprised of 7 extracellular immunoglobulin-like domains, one membrane-spanning segment, and a conserved intracellular tyrosine kinase domain (Quinn et al, 2000).

VEGFR-1 has the highest affinity for VEGF, is expressed on endothelial cells in adult and embryonic mice, and plays a role in wound healing. VEGFR-2 has a lower affinity for VEGF, and has a role in endothelial cell proliferation. VEGFR-2 is expressed on endothelium in embryonic vasculature, but its expression is decreased in quiescent adult vasculature (Millauer et al, 1996). VEGFR-3 is primarily expressed on lymphatic endothelium and may play a role in lymphangiogenesis. In the brain, VEGF may be responsible for the peritumoral edema that is often associated with brain tumors.

In *in vitro* experiments, VEGF expression can be regulated by factors such as TGF-β, PDGF, and oncogenes such as Ras, Raf, and Src (Machein et al, 2000). Mice with the VEGF receptor knocked out exhibit deficient blood vessel formation during embryogenesis (Shalaby et al, 1995). A correlation has been demonstrated between high concentrations of plasma VEGF and poor prognosis in solid tumors (Poon et al, 2001). In contrast, Bergers and colleagues demonstrated that in a mouse model of pancreatic beta cell carcinogenesis there is not net increase in VEGF expression; instead, VEGF is mobilized from matrix stores (Bergers et al, 2000). Therefore, it is unclear whether measuring VEGF transcription is a good measure of the level of induction of angiogenesis.

Mice null for the VEGFR-1 and VEGFR-2 exhibit vascular defects that resulted in lethality. VEGFR-2 lacking mice embryos exhibit lethality around embryonic day 9 (Shalaby et al, 1995). Furthermore, targeted homozygous disruption of the VEGFR-1 resulted in mice embryos without organized vasculature (Fong et al, 1995). Experimental approaches have been developed that target the VEGF/VEGF-R pathway to inhibit angiogenesis. These methods include utilizing anti-VEGF antibodies, soluble VEGF receptors, dominant-negative VEGFR-2, and antisense VEGF. Studies using an anti-VEGF antibody have proven to give results such as decreased tumor vascularity and permeability, and decreased tumor metastases (Margolin et al, 2001). For example, administering the VEGFR-2/flt-1 antagonist, SU5416, which inhibits the kinase activity of the receptor, has shown to have an antiproliferative effect on endothelial cells, thereby decreasing tumor size (Rosen, 2002).

Fibroblast growth factors

Fibroblast growth factors (acidic and basic) are mitogenic for many types of cells including endothelial cells, tumor cells, smooth muscle cells, fibroblasts, and epithelial cells. Both acidic and basic FGF (aFGF and bFGF, respectively) are ubiquitously expressed as 18-25 kDa polypeptides and play a role in mitogenesis, differentiation, chemotaxis, angiogenesis, tissue integrity and repair, and wound healing. Specifically, acidic (aFGF/FGF-1) and basic (bFGF/FGF-2) FGFs play a role in angiogenesis by inducing endothelial cell proliferation, migration, and tube formation (Dunn et al, 2000). FGFs are involved in angiogenesis by regulating VEGF expression in tumor cells and

stimulating endothelial cell proliferation and activity so that these cells can migrate into the extracellular space.

The factors aFGF and bFGF lack a signal sequence and are not secreted proteins. Instead, FGFs are cytoplasmic or bound to the ECM through an affinity to heparin. Heparin functions in activating aFGF and bFGF. It is thought that FGFs are released upon cell injury and play a role in localized angiogenic repair. It has been shown that FGFs induce sprouting of existing vasculature *in vivo* in the cornea (Folkman et al, 1983). However, aFGF and bFGF may not be essential in vascular formation as mice deficient in aFGF and bFGF exhibit normal vasculature development *in vivo*, but do exhibit deficient wound healing (Miller et al, 2000).

The growth factor FGF binds to its receptor, and receptor dimerization occurs, initiating protein tyrosine kinase activity. Receptor activation and transphosphorylation of the intrinsic tyrosine kinases followed by signaling events through Ras and Raf ultimately leads to modulation of gene transcription. The degradation of the ECM that occurs is due in part to FGF enhancing the expression of molecules such as the urokinase-type plasminogen activator (uPA) that activates the ECM protease plasmin. The factor bFGF has been shown to upregulate the expression of uPA on endothelial cells, as well as to induce the expression of the uPAR (Mignatti et al, 1991, and Moscatelli et al, 1986).

Platelet-derived Growth Factor

Platelet-derived growth factor (PDGF) is a 45-kDa molecule that exists as a homodimer (PDGF-AA) or heterodimer (PDGF-AB) and is a mitogen that targets

endothelial cells, vascular smooth muscle cells, osteoblasts, glia, and neurons. Both the homodimer and heterodimer are expressed by most cells and bind to PDGF receptors that homo- and heterodimerically consist of α- and β- subtypes. Though PDGF was originally purified from platelets, it has since been found in other types of cells such as fibroblasts, myoblasts, astrocytes, epithelial cells, and macrophages (Heldin et al, 1999).

PDGF plays a role in embryonic and CNS development, vasculature development, and wound healing. Furthermore, PDGF has an angiogenic effect, though is less potent than VEGF and FGF. It binds to protein tyrosine kinase receptors and induces receptor dimerization followed by transautophosphorylation, signal transduction events, and finally gene transcription. Increased PDGF expression may stimulate VEGF expression in endothelial cells and in tumor cells leading to endothelial cell proliferation and migration.

PDGF's role in angiogenesis has been shown to be essential in *in vivo* experiments conducted by researchers where it was demonstrated that mice deficient in the PDGF-β receptor exhibited vasculature that had edema, dilated vessel lumen, and died perinatally from hemorrhage (Leveen et al, 1994, and Soriano, 1994). Furthermore, Lindahl and colleagues showed that PDGF-B null mice embryos had vasculature defects such as a lack of pericytes, and had ruptured capillary microaneurysms (Lindahl et al, 1997). Furthermore, Hellstrom and colleagues demonstrated that pericytes are recruited to the microvasculature independently of PDGF. The pericytes proliferate and migrate along the angiogenic sprouts upon PDGF's action, thereby establishing a role for PDGF in vessel wall stabilization (Hellstrom et al, 1999).

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a 25 kDa disulfide-linked homodimer that plays a role in cell proliferation, differentiation, motility, adhesion, and apoptosis. An inactive form of TGF-β is secreted by the cell and can only bind the TGF- β receptor once it is activated by proteases such as plasmin, or acidic conditions (Lawrence et al, 1985 and Lyons et al, 1988). TGF- β and its receptor are expressed on endothelial cells and pericytes. TGF-β has been shown to stimulate and inhibit cell proliferation; furthermore, it is involved in cell adhesion by regulating the synthesis of the ECM, protease inhibitors, and integrins (Messague, 1990). TGF-β has been shown to have both angiogenic and antiangiogenic effects. *In vivo* studies have shown TGF-β to have a positive role in angiogenesis, but *in vitro* assays have shown for TGF-β to inhibit endothelial cell proliferation (Fajardo et al, 1996, and Press et al, 1989). The switch from TGF-β's inhibitory to proliferative effects may be due to selective resistance of TGF-β binding to its' receptor, or due to a downregulation of the receptors (Yamada et al, 1995). There have been reports that TGF-β's stimulatory and inhibitory effects depend on TGF-β dosage, i.e. low doses (≤0.5 ng/ml) stimulate, and high doses (1-5 ng/ml) inhibit endothelial tube formation (Myoken et al, 1990).

TGF-β's signaling cascade differs from FGF and PDGF signaling. TGF-β binds to two different serine/threonine kinase receptors, known as type I and II. TGF-β binds as a dimer to the type II receptor that leads to a phosphorylation of type I receptor and results in an heterotetrameric receptor. Upon activation of this receptor tetramer, TGF-β activates numerous signaling pathways including extracellular signal regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase pathways (Takekawa et al,

2002). Smad proteins are activated downstream of receptor dimerization and activation, and TGF-β results in Smad translocation from the plasma membrane to the nucleus where they modulate gene transcription by interacting with transcriptional factors such as Runx, and p300/creb binding protein (CBP) (Miyazono et al, 2003).

TGF-β antiangiogenic role includes modulating the activity of uPA, PAI levels, inhibiting proteases, and stimulating protease inhibitor production to prevent matrix degradation. TGF-β has been to have proangiogenic effects *in vivo* (in chick embryo and rabbit cornea) when in the presence of inflammatory mediators such as monocytes and fibroblasts. This suggests that TGF-β dependent angiogenesis may be mediated by angiogenic factors produced by these inflammatory cells (Papetti et al, 2002). *In vitro* studies have shown that mice deficient in TGF-β seem to have normal differentiation of mesodermal precursor cells into endothelial cells, but is followed by embryonic lethality as these mice have deficient yolk sac vasculature, and frail blood vessel walls because of defective cell-cell contacts (Dickson et al, 1995 and Oshima et al, 1996).

TGF-β's role in angiogenesis and invasiveness has led scientists to believe that inhibiting its actions may have therapeutic applications in fighting tumor growth. Therapeutic methods such as utilizing antisense TGF-β, receptor antagonists like decorin, and drugs that reduce TGF-β expression such as Tranilast have resulted in tumor growth inhibition in experimental models, including rat glioma cells (Platten et al, 2000, and Stander et al, 1998).

Angiopoietin-1

Angiopoietin-1 and its Tie2 receptor does not play a role in the initial phase of vasculogenesis, they play roles in angiogenic outgrowth, vessel remodeling, and maturation (figure 6 and 7) (Sato et al, 1995). Angiopoietin-1 binds to the Tie2 receptor tyrosine kinase on the endothelial cell, activates the receptor, and recruits periendothelial cells that will surround it, and these periendothelial cells then become the pericytes or smooth muscle cells of the blood vessel.

Knockout mice for angiopoietin-1 have defects in vascular organization that is characterized by decreased vessel branching, remodeling, and homogenous sized vessels. Mice deficient in the Tie2 receptor show similar results to mice null for angiopoietin-1, both resulting in embryonic lethality (Suri et al, 1996). Though adding angiopoietin-1 does not induce *in vitro* tube formation, angiopoietin-$1^{-/-}$ mice embryo vasculature lack periendothelial cells, is scattered with collagen-like fibers, and have rounded endothelial cells instead of their normal flattened morphology (Figure 8) (Suri et al, 1996). Furthermore, the angiopoietin-$1^{-/-}$ cells lack the necessary contacts with the surrounding mesenchymal cells, thereby making the vasculature unstable. Though angiopoietin-1 and its Tie2 receptor have effects on the vasculature similar to VEGF's effects, angiopoietin-1 or Tie2 receptor null embryos live longer than mice homozygous for a null mutation in the VEGF gene, or mice lacking or VEGF receptor (Flk-1) lacking embryos, suggesting that angiopoietin-1 plays a later role in vasculogenesis in developing embryos.

Angiopoietin-2 is a receptor tyrosine kinase antagonist for the Tie2 receptor. Upon binding the Tie2 receptor, angiopoietin-2 inhibits angiopoietin-1 binding and receptor activation. To elucidate the specific role of angiopoietin-2, adult human tissues were examined using Northern blot and the presence of angiopoietin-1 and angiopoietin-

2 was studied. It was found that angiopoietin-1 was expressed in many tissues such as skeletal muscle, small intestine, prostate, ovary, uterus, and placenta; whereas angiopoietin-2 was expressed only in tissues such as in the ovary, placenta, and uterus. These results suggest that angiopoietin-2 plays a role in adult tissues where physiologic angiogenesis and vascular remodeling is needed (Maisonpierre et al, 1997). When a Tie2 receptor antagonist was used, destabilized vasculature followed by endothelial cell apoptosis and vessel regression occurred (Brat et al, 2001). In gliomas, angiopoietin-2 mRNA is expressed in hyperplastic and nonhyperplastic vessels, suggesting that angiopoietin-2's inhibitory effects on vessel outgrowth may play a role, even before the angiogenic switch occurs (Zagzag et al, 2000).

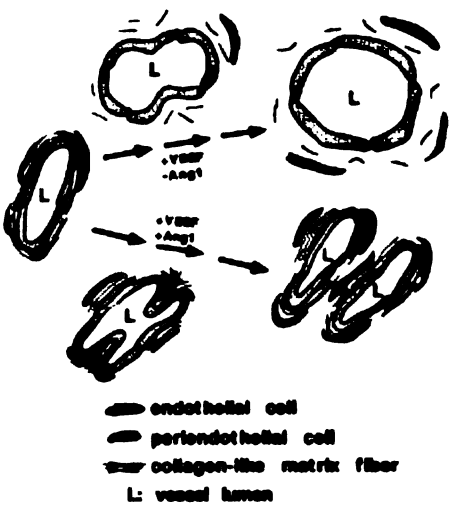

Figure 6: Schematic Depiction of Defective Vascular Development in the Absence of Angiopoietin-1. Shown are deficient tissue folds and decreased branching resulting in dilated vessels in the absence of Angiopoietin-1. (Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. (1996) Requisite Role of Angiopoietin-1, a Ligand for the TIE2 Receptor, during Embryonic Angiogenesis. *Cell.*87:1171-1180.)

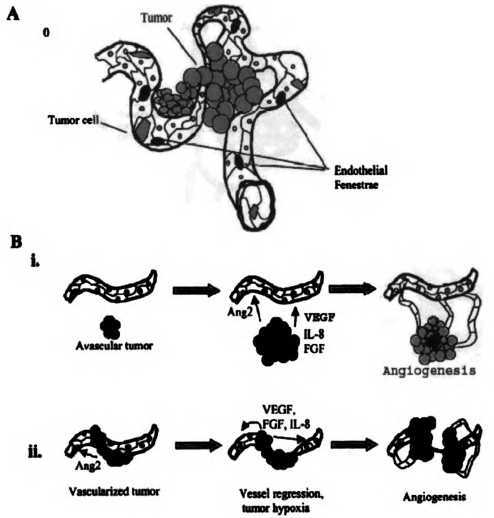

Figure 7: Mechanism of tumor angiogenesis. *A:* schematic of tumor blood vessel (green, normal tumor cells; black, necrotic tumor cells). Notice the thin walls, tortuous shape, absence of pericytes and variations in diameter. Numerous gaps or fenestrae are found between endothelial cells. The vessel wall is mosaic and can consist of both tumor cells as well as endothelial cells. *B:* model of tumor-induced neovascularization. In *i,*an initially avascular tumor grows until inner regions become hypoxic and upregulate production of angiogenic factors such as VEGF, FGF, and interleukin (IL)-8. In *ii*, a tumor grows on an existing blood vessel. Soon the tumor induces Ang2 expression in the preexisting vessel, and it regresses due to endothelial cell apoptosis. The tumor is now avascular, and by upregulating angiogenic factors as in *i,* it induces the production of a new blood supply. (Papetti M, Herman IM. (2002) Mechanisms of normal and tumor derived angiogenesis. *Am J Physiol Cell Physiol.* 282:C947-C970.)

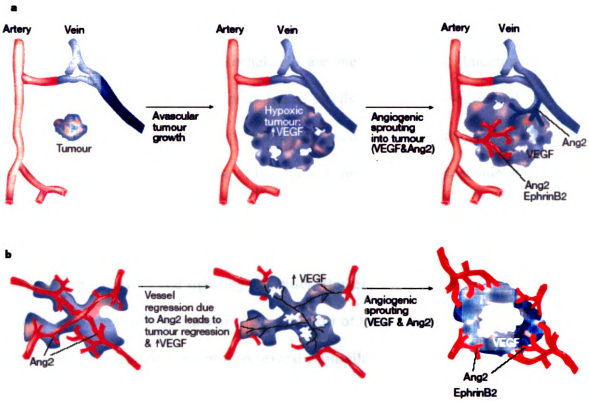

Figure 8: Models of tumour angiogenesis. a, Model of avascular tumour initiation contrasted with **b,** tumour initiation involving host vessel co-option. An attempt is made to assign the indicated vascular growth factors to roles in the various indicated steps in tumour development, and to indicate their expression patterns. (Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. (2000) Vascular-specific growth factors and blood vessel formation. *Nature.* 407:242-248)

Role of Adhesion Molecules

Integrins

Most of the ECM receptors on endothelial cells are integrin receptors. Integrins are adhesion proteins found on the surface of endothelial cells and are involved in cell cycle regulation, cell attachment, proliferation, migration, differentiation, and programmed cell death. Integrins are made of two non-covalently associated chains, α and β, and often cluster in the cell membrane. There are 18 α and 8 β subunits that yield at least 24 integrin heterodimers; each of the unique alpha and beta chain configurations result in unique ligand specificity and signaling properties (Brakebusch et al, 2002).

Integrins share a common αv subunit, and all or most of these subunits recognize a RGD (arginine-glycine-aspartate) sequence on several extracellular matrix proteins that they bind, such as fibronectin, laminin, collagen, and vitronectin (Brooks, 1996, and Giancotti et al, 1999). The intracellular domains of the integrin molecules can link to the cytoskeleton and interact with components such as α-actinin and play a role in numerous signaling pathways between the ECM and the cytoskeleton.

Integrins are primarily responsible for the adhesion of endothelial cells to the ECM and basement membrane. Integrins play a role in angiogenesis by facilitating cell adhesion, migration, proliferation, survival, and ECM degradation. Migration involves the movement of polarized cells. Integrins are found in the polarized protruding ends of the migrating endothelial cells, known as the leading edge. The rear end of the cell dissociates from the substratum by releasing the integrin contacts and releasing degradative ECM proteases. They have been shown to stimulate cell contraction and

movement by stimulating ECM degrading enzymes that facilitate tumor cell migration (Brakebusch et al, 2002).

The αv integrins have been shown to play roles in implantation and placentation, bone remodeling, and angiogenesis. To study the role of αv integrins, Bader and colleagues generated a strain of mice with a null mutation in the αv integrin gene. In the study some of the mice died prenatally and exhibited features such as smaller heads, pericardial edema, and enlarged hearts (Bader et al, 1998). To the scientists' surprise, contrary to results obtained using blocking agents against αv integrins, Bader and colleagues found that in αv-null mice, developmental angiogenesis that takes place in the CNS vasculature exhibited sprouting, invasion, and vessel branching (Bader et al, 1998). The scientists hypothesized that in the CNS, αv integrin-deficient endothelial cells may not associate normally with the surrounding accessory cells such as pericytes or glial cells resulting in dilation of brain capillaries (Bader et al, 1998).

The β_1 subunit of the integrin molecule has been shown to play a role in angiogenesis. *In vitro* studies using β_1 antagonists resulted in inhibition of endothelial cord formation (Davis et al, 1993). Furthermore, *in vivo* studies using antagonists of β_1 integrins, showed that when these antagonists were injected into quail embryos, there was a disruption in vascular development (Drake et al, 1991). These *in vivo* and *in vitro* results demonstrate the role of β_1 integrin in angiogenesis.

In another study conducted by Brooks and colleagues, it was demonstrated that integrin αvβ3, the vitronectin receptor, is expressed on endothelial cells in increased levels during angiogenesis, and negligibly expressed in quiescent blood vessels (Brooks et al, 1994). It is not clear which ECM molecules interact with αvβ3 in angiogenesis

(Brooks, 1996). In an earlier study, Brooks and colleagues showed that the integrin αvβ3 expression is increased in human and chick vasculature upon stimulation with solid human tumors; furthermore, in another study conducted by the investigators, it was shown that upon using integrin αvβ3 antagonists, tumor growth induced by bFGF and other growth factors was inhibited, and in fact, tumor regression occurred (Brooks et al, 1994 and Brooks et al, 1994). However, in a study conducted by Friedlander and colleagues, it was demonstrated that VEGF-induced angiogenesis was not inhibited by using integrin αvβ3 antagonists (Friedlander et al, 1995). However, VEGF-angiogenesis was inhibited using antagonists to the related vitronectin receptor, αvβ5. These results demonstrate that inhibiting tumor growth may depend on what specific integrin subtype is targeted by antagonists.

Integrin signaling comprises numerous pathways and is involved in cytoskeletal modifications, cell contraction, gene transcription, cell invasion, and cell migration. The binding of the integrin subunits to the ECM leads to the recruitment and activation of intracellular focal adhesion kinase (FAK) that binds and activates numerous adaptor proteins such as growth factor receptor bound protein 2 (GRB2) that activates the small G protein, Ras (Schlaepfer et al, 1997). Activated Ras then leads to activation of phophatidylinositol 3 kinase (PI3K). Fak activation also leads to Src-dependent Shc phosphorylation and Grb2 recruitment (Schlaepfer et al, 1997). This leads to Ras activation and Raf recruitment to the plasma membrane where it can be phosphorylated by kinases such as Src, leading to mitogen-activated protein kinase kinase (Mek) followed by extracellular signal regulated kinase (ERK) activation. Erk activation can lead to the activation of myosin light chain

kinase (MLCK). Ultimately, these signaling pathways alter gene transcription and modulate the cytoskeleton so that cell migration can occur.

Cadherins and Catenins

Cadherins are involved in endothelial cell adhesion using homophilic interactions. Cadherins help to maintain vascular integrity, and may play a role in angiogenesis. Cadherins are calcium-dependent cell adhesion molecules that include two subtypes: neural (N)- and vascular endothelial (VE)-cadherin. N-cadherins are present on the entire surface of endothelial cells, and VE-cadherins are expressed on endothelial cells in intercellular junctions known as adherens junctions. Cadherins are transmembrane glycoproteins involved in cell adhesion. Their cytoplasmic domains interact with α- and β- catenin, which links these transmembrane proteins to the microfilaments via α-actinin (Blaschuk et al, 2000). Normal blood vasculature has endothelial cells surrounded by pericytes embedded in the basement membrane. These pericytes function as stabilizers for the blood vessels. Tumor blood vessels, however, often have no pericytes, and therefore no organized basement membrane. Unlike the tightly fit adherens junctions in normal endothelial cells, these tumor blood vessels have open interendothelial junctions, due to decreased endothelial cell adhesion that is thought to be due to VEGF (Figure 9) (Blaschuk et al, 2000). Furthermore, the VE-cadherin/β-catenin complex is associated with the VEGF-receptor 2 and PI3K signaling pathway. It has been demonstrated from *in vitro* studies that VE-cadherin dimers, β-catenin dimers, and the VEGF-R2 associate with PI3K and exists as a supramolecular complex in endothelial cells (Carmeliet et al, 1999). The scientists hypothesize that the effect of VEGF-R2 on endothelial cells may depend on whether it is bound by the complex.

Treating cells with antibodies for N-cadherin and VE-cadherin caused these endothelial cells to show a loss of adhesion and organization (Gulino et al, 1988). In a separate experiment, mice embryos null for VE-cadherin died prenatally due to severe cardiovasculature defects. These embryos exhibited defects in vasculature expansion and vascular remodeling (Carmeliet et al, 1999). When the catenin binding region was mutated in endothelial cells, the lethal phenotype was identical to the VE-cadherin null mutation, suggesting that catenins are essential in cadherin-mediated cell adhesion activities (Carmeliet et al, 1999). N-cadherin null mice exhibit embryonic lethality at embryonic day 10 (E10) and have defective heart tube and yolk sac vasculature (Radice et al, 1997). Gerhardt and colleagues demonstrated that N-cadherins are needed for endothelial cell and pericyte interaction in the process of brain angiogenesis during chick embryo development (Gerhardt et al, 2000). Furthermore, Saffell and colleagues have demonstrated that N-cadherin binds directly to the FGF-R and led to FGF-R dimerization and activation, suggesting N-cadherin's role in angiogenesis (Saffell et al, 1997).

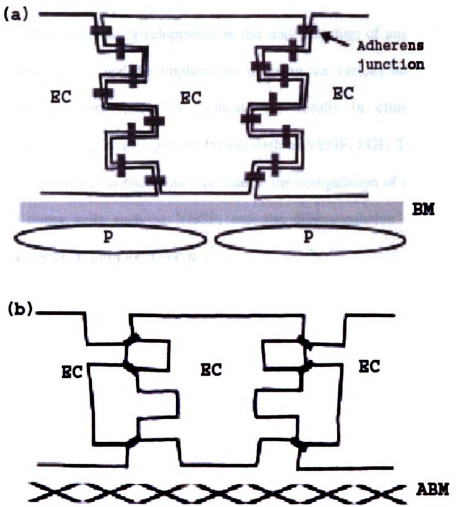

Figure 9: Diagrammatic representations of normal (a) and tumor (b) blood vessels. Normal blood vessels are composed of endothelial cells (EC) surrounded by pericytes (P). Both of these cell types contribute to the deposition of the basement membrane (BM). Pericytes are frequently absent from tumor blood vessels. Consequently, the basement membranes (ABM) of these blood vessels are abnormal. (Blaschuk O, Rowlands T. (2000) Cadherin as modulators of angiogenesis and the structural integrity of blood vessels. *Cancer Metastasis Rev.* 19:1-5)

Angiogenesis Inhibitors

There have been numerous developments in the understanding of angiogenesis, the inhibition of angiogenesis, and the implications of using the various inhibitors in experimental models, and attempting to duplicate the results in clinical trials. Angiogenesis is induced by angiogenic growth factors such as VEGF, FGF, TGF-β, and PDGF. Furthermore, mutations in oncogenes can lead to the upregulation of angiogenic growth factors by tumor cells such as VEGF, and the downregulation of natural antiangiogenic factors such as thrombospondin-1.

To combat these activities, there are numerous exogenous and endogenous antiangiogenesis factors that have been identified since Judah Folkman's group discovered the body's natural antiangiogenesis factors, angiostatin and endostatin years ago (O'Reilly et al, 1994, and O'Reilly et al, 1997). Many of these antiangiogenic factors' mechanisms of action have been elucidated, but some are still unknown.

In animal studies, angiogenesis inhibitors have successfully stopped the formation of new blood vessels, causing the tumor to regress. However, it is not known yet whether these angiogenesis inhibitors will be effective against all human cancers. Numerous angiogenesis inhibitors are currently in clinical trials in various trial phases. These inhibitors include drugs that block matrix breakdown, inhibit endothelial cells directly, block angiogenesis activators, and inhibit endothelial-specific integrin signaling. As tumors grow, they begin to produce a variety of angiogenic inducers. Thus, if investigators inhibit the actions of only one inducer such as VEGF, the tumor can then express other angiogenic inducers such as FGF. Therefore, the investigators should use a cocktail of inhibitors in the clinical trials to suppress angiogenesis in the patients.

Furthermore, if a mixture of antibodies/inhibitors is used, care must be taken to ensure that normal cells that express similar markers are left unharmed to minimize tissue toxicity and death.

Angiostatin and Endostatin

Angiostatin and endostatin are endogenous inhibitors of angiogenesis. Angiostatin and endostatin are released by the primary tumor, have long half-lives, and are thought to function in keeping micrometastases dormant and in a nonproliferative state. Thus, removal of the primary tumor can result in the loss of inhibition of growth of these metastatic foci, and the metastases can proliferate and induce angiogenesis in distal sites. For example, angiostatin has been shown to keep lung metastases in a dormant state by inducing a state of insufficient vascularization and normal proliferation, but increased apoptosis (Cao et al, 1998).

Angiostatin is an internal 38 kDa fragment of plasminogen, and was initially purified from Lewis lung carcinoma bearing mice by O'Reilly and colleagues (O'Reilly et al, 1994). Angiostatin inhibits endothelial cell proliferation, induces endothelial cell apoptosis, and inhibits chemotaxis (Eriksson et al, 2003). Furthermore, angiostatin was shown to inhibit endothelial cell proliferation, and endothelial cell tube formation *in vitro* (Gately et al, 1996, Gately et al, 1997, and O'Reilly et al, 1994). Administration of angiostatin to endothelial cells in culture resulted in endothelial cell apoptosis in a dose-dependent manner (25 mg vs. 50 mg angiostatin/kg/day) (Claesson-Welsh et al, 1998). The inhibition of endothelial cell proliferation may be due to angiostatin's actions on the α/β ATP synthase on the surface of the endothelial cell through unknown signaling

pathways (Fig 11) (Moser et al, 1999). Troyanovsky and colleagues suggested that a binding protein called angiomotin was needed to bind angiostatin in order for angiostatin to inhibit endothelial cell chemotaxis (Troyanovsky et al, 2001). Stack and colleagues showed that angiostatin also inhibited ECM-stimulated plasminogen activation that resulted in decreased endothelial invasiveness (Stack et al, 1999). Angiostatin has been shown to inhibit tPA-catalyzed plasminogen activation in endothelial cells by binding tPA and preventing it from binding plasminogen and ECM cofactors, thereby inhibiting endothelial cell migration (Stack et al, 1999). Furthermore, angiostatin has been shown to inhibit the activation of mitogen-activated protein kinase (MAPK) pathway by activating a tyrosine phosphatase (Redlitz et al, 1999). However, when another study used bovine capillary endothelial cells and angiostatin, angiostatin did not inhibit FGF-induced MAPK activation, suggesting that angiostatin's actions may depend on the type of endothelial cell that is used (Claesson-Welsh et al, 1998).

Mauceri and colleagues demonstrated that combining angiostatin and radiation treatment targeting endothelial cells proved to be more effective than radiation therapy alone, i.e. the number of tumor vessels decreased with combination therapy (as seen under the microscope) (Mauceri et al, 1998). Continuous administration of angiostatin, as compared to a bolus injection of angiostatin, resulted in increased inhibition of angiogenesis (Drixler et al, 1999). Angiostatin administration to human prostate carcinoma to immunodeficient mice resulted in prolonged tumor dormancy, even after the conclusion of the experiment (O'Reilly et al, 1995). Angiostatin concentrations that inhibited ERK activation also inhibited bFGF stimulated invasion of collagen gel and subsequent cord/tube formation by endothelial cells indicating that angiostatin-induced

dephosphorylation of ERKs may play a role inhibiting the invasion of endothelial cells (Redlitz et al, 1999).

Endostatin was also discovered by O'Reilly's group and was shown to inhibit growth factor-induced proliferation and migration of endothelial cells, as well as to induce endothelial cell apoptosis, although its mechanism of action is unclear (O'Reilly et al, 1997). Endostatin is a 20 kDa fragment of the C-terminal section of collagen XVIII, a component of the walls of blood vessels, and has been shown to mediate its antiangiogenic properties by specifically inhibiting proliferation, migration, and tube formation of endothelial cells (Eriksson et al, 2003 and O'Reilly et al, 1996).

Upon endostatin administration, tumor regression was observed, and when endostatin therapy was discontinued, tumor growth began again (O'Reilly et al, 1997). It was demonstrated in one study that endothelial cell apoptosis occurred with endostatin treatment where endothelial cell apoptosis was measured using annexin V and the TUNEL assay (Dhanabal et al, 1999). Furthermore, it was shown that endostatin treatment did not result in the endothelial cells acquiring drug resistance, a problem often encountered in tumor cells (Boehm et al, 1997). Repeated endostatin treatment to Lewis lung carcinoma bearing mice as well as to mice bearing T241 fibrosarcoma unexpectedly resulted in tumor dormancy that remained indefinitely after the conclusion of the therapy (Boehm et al, 1997). With the continuous administration of endostatin, investigators found through positron-emission tomography (PET) scans, that there was a dose dependent decrease of tumor blood flow (Herbst et al, 2001). Endostatin can bind to various ECM components such as heparin sulfate proteoglycans. It was hypothesized that endostatin's antiangiogenic activity may be mediated by competitively binding

bFGF's binding site on heparin. However, mutating endostatin's heparin binding site did not inhibit endostatin's antiangiogenic activity (i.e. the inhibition of VEGF induced endothelial cell migration was not affected) (Yamaguchi et al, 1999). It is hypothesized that endostatin's antiangiogenic mode of action involves its binding to these ECM components, perhaps through its inhibition of FGF binding to specific ECM components (Sasaki et al, 1999). Endostatin treatment resulted in tumor regression in a murine hemangioendothelioma model, however, when endostatin treatment was discontinued, tumor regrowth was observed (O'Reilly et al, 1997). Endostatin administration to tumor bearing mice led to a reduction of Bcl-2, the anti-apoptotic protein (VEGF has been shown to augment Bcl-2 levels in endothelial cells) and resulted in an increased apoptotic index of the tumor cells and a decreased proliferation rate (O'Reilly et al, 1997).

Endostatin can bind α5- and αv-integrins to prevent endothelial cell migration (Figure 10). Integrins are associated with focal adhesion kinases (FAKs) that lead to tyrosine kinase activation. To understand endostatin's actions on FAKs and the downstream signaling events, investigators administration of endostatin to porcine aortic endothelial cells (PAEs), used anti-FAK immunoprecipation and found that there was induction of FAK activity (Claesson-Welsh et al, 1998). These results were duplicated when angiostatin was administered to murine pancreas endothelial cells and resulted in an increase in FAK induction in the immunoprecipates. However, with angiostatin administration to Swiss 3T3 fibroblasts, there was no FAK induction, suggesting FAK induction may depend on the type of endothelial cell (Claesson-Welsh et al, 1998).

Although, angiostatin and endostatin have been identified as angiogenesis inhibitors, their mechanisms of action are not clearly understood. It has been shown that

angiostatin and endostatin's inhibition of VEGF and FGF-induced endothelial cell migration is not due to any actions on signaling pathways including phospholipase C-γ (PLC-γ) (involved in FGF-mediated cytoskeletal reorganization), Akt/PKB (regulates cell survival), p44/42 mitogen-activated protein kinase (MAPK) (regulates mitogenicity), p38 MAPK (FGF-mediated differentiation and VEGF-mediated migration), p21-activated kinase (PAK) activity, and Rac activity. The investigators demonstrated that upon VEGF and FGF induction, there was no change in the intracellular phosphorylation status of these factors with the angiogenesis inhibitor administration (Eriksson et al, 2003). Furthermore, FGF was shown to be responsible for inducing mitogenicity of human dermal microvascular endothelial cells (HDMECs), however, with administration of angiostatin or endostatin, there was no change in FGF and VEGF-induced endothelial cell proliferation that was measured by ^{3}[H]thymidine incorporation (Eriksson et al, 2003). These results were confirmed by studies conducted by Wajih and colleagues in which it was found that angiostatin does not affect VEGF and FGF-mediated mitogenicity in human umbilical vein cells (Wajih et al, 2003). The joint administration of angiostatin and endostatin to mice with Lewis lung carcinoma resulted in increased tumor regression (Boehm et al, 1997). Hajitou and colleagues have demonstrated that some of the antiangiogenic effects of angiostatin and endostatin are through their effects on the VEGF expression by tumor cells (Hajitou et al, 2002). Although, it has been shown by Eriksson and colleagues that neither angiostatin nor endostatin affects the signaling pathway that is involved in endothelial cell proliferation and migration, specifically the GTPase Rac, PI3K, and p21 activated kinase (PAK), signaling pathway (Eriksson et al, 2003).

It has been demonstrated that angiostatin administration and radiation therapy used in combination to inhibit rat C6 glioma xenograft growth resulted in increased tumor growth inhibition, as compared to angiostatin or radiation treatment alone. Furthermore, when murine angiostatin and endostatin (each fused to the Fc fragment of the murine immunoglobulin heavy chain) was administered together to a mouse model of pancreatic islet cell carcinogenesis, there was a reduction in tumor size that did not result from individual therapy administration (Bergers et al, 1999).

In the second part of this study, an experiment will be proposed that will use rat C6 glioma cells that will be administered angiostatin, endostatin, and radiation therapy. The rat C6 glioma cell is highly angiogenic and is an established glioma model that is often used to study angiogenesis. Rat C6 glioma cells have been shown to form intracranial tumors that are highly invasive and are known to express the growth factors that are involved in angiogenesis such as FGF, PDGF, and VEGF (Okumura et al, 1989 and Hamel et al, 2000). In a study conducted by Kubiatowski and colleagues, it was shown that the PI3K pathway in the rat C6 glioma cells were involved in invasion (Kubiatowski et al, 2001). When the investigators inhibited PI3K activity, the cells exhibited decreased invasiveness (Kubiatowski et al, 2001). These and other studies indicate that the rat C6 glioma cell line is an appropriate model to study angiogenesis and tumor invasion. The purpose of proposing this experiment is to study the efficacy of combining antiangiogenic therapy in a rat C6 glioma murine model.

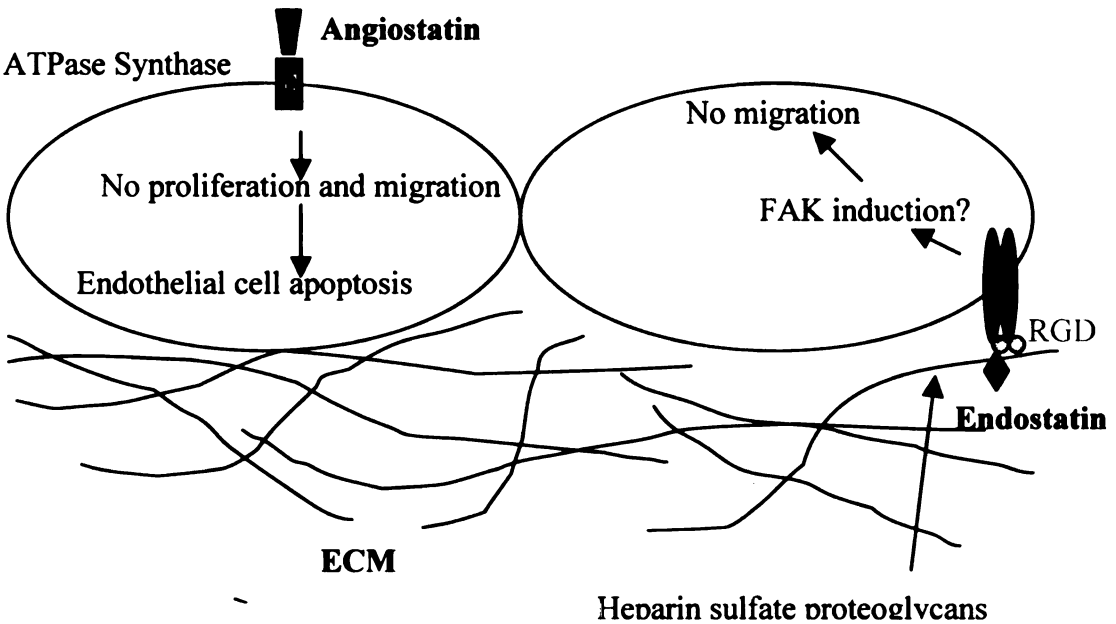

Figure 10: Angiostatin and endostatin effects on endothelial cells. Angiostatin binds to the α/β subunits of ATPase Synthase and results in the inhibition of endothelial cell proliferation and migration through unknown signaling mechanisms. Endostatin binds to integrin α_5 and αv subunits and to heparin sulfate proteoglycans in the ECM. Angiostatin and endostatin administration has been shown to lead to endothelial cell apoptosis. The combination of angiostatin and endostatin administration to tumor cells is proposed in the study in Part II to study the additive effects, if any, of the two inhibitors. Angiostatin and endostatin have different binding sites, and therefore presumably affect different signaling pathways. It is hypothesized that the multiple signaling pathways that will be inhibited by angiostatin and endostatin will result in better antitumor efficacy.

Part II:

Experimental Proposal

Background Information about Initial Clinical Trial Design

Numerous *in vitro* and *in vivo* experiments using angiogenesis inhibitors in various animal models have given results in which there was tumor growth inhibition, and in some cases, tumor regression. However, when scientists tried to duplicate these results in clinical trials, the results were not as promising. Results from these initial clinical trials using various angiogenesis inhibitors have not shown the desired and expected antitumor effects that were expected following the preclinical studies. However, it has been proposed that the reason that these initial clinical trials did not result in many successful antiangiogenic therapies may be due to the limitations of the trial design.

Initial clinical trial studies using endostatin that began in 1999 revealed that endostatin administration did not result in any acquired resistance and decreased formation of new blood vessels. Phase I clinical trials using an angiostatin cocktail and recombinant angiostatin, as well as angiostatin in combination with radiation therapy began in 2000. Scientists and physicians studying angiostatin as an effective antiangiogenic agent in human trials have observed no detrimental side effects thus far, and are currently designing the next phase of the study.

The clinical trial designs have been studied and new parameters have been suggested to obtain better results. Changing parameters such as dosage, the type of angiogenesis inhibitor used, the type of endpoint measured, and using inhibitor(s) in conjunction with chemotherapy and/or radiation therapy may improve overall therapeutic efficacy.

It has been shown that when an angiogenesis inhibitory drug is administered, there is often an increase in tumor vasculature and blood flow. After obtaining such undesirable results, the trial was often discontinued. Instead, investigators have proposed that in these situations, adding a second inhibitor to the regimen may increase the antiangiogenic effectiveness, though some initial increase in tumor size and vasculature may still occur. The increase in tumor size and blood flow may be due to a decreased intratumoral pressure that results from the antiangiogenic treatment. However, with the increased delivery of oxygen and nutrients, any chemotherapeutic agents that are being administered will also be delivered at increased rates, suggesting that synergism may occur between antiangiogenic agents and chemotherapy.

In the initial clinical trials, when researchers recruited patients, only patients with highly vascularized tumors were allowed to enter the trials. The investigators believed that highly vascularized tumors respond better to antiangiogenic therapy, with the established fact in mind that cytotoxic therapy responds better to highly vascularized, rapidly growing tumors. However, it has been demonstrated by Beecken and colleagues that slow growing, poorly vascularized tumors respond as well as highly vascularized, rapidly growing tumors to antiangiogenic therapy (Beecken et al, 2001). Therefore, patients with slowly growing tumors and decreased levels of vascularization will now be allowed to participate in antiangiogenic clinical trials.

Angiogenic inhibitors were initially administered in bolus maximally tolerated doses, a therapeutic measure that is effective for the administration of cytotoxic chemotherapeutic agents. However, it has been found that angiogenic inhibitors produce

maximal results when administered in doses that maintain a constant concentration of the drug in circulation, with no off-time in the course of the therapy.

To measure the efficacy of the angiogenesis inhibitors on tumor growth and angiogenesis, certain clinical trial endpoints are used. In the initial clinical trials using angiogenesis inhibitors, tumor regression was used as an endpoint. Currently, investigators suggest that instead of tumor regression, retardation of tumor growth should be used as an endpoint because in rapidly growing tumors, such as high grade gliomas, tumor regression may not occur.

Initial trials only tested the efficacy of an angiogenic inhibitor administered alone. Current trial design is incorporating the concurrent administration of an angiogenesis inhibitor with chemotherapy and/or radiation therapy to maximize the antitumor effects. The conduct of clinical trials with antiangiogenic inhibitors used either alone or in combination with other standard therapies has been modified substantially, often incorporating magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning, to assess tumor vessel density and tumor blood flow. Mauceri and colleagues have demonstrated that in a Lewis lung carcinoma model, the administration of angiostatin in conjunction with normal-dose radiation therapy resulted in increased tumor vasculature toxicity, but no tumor cell toxicity as compared to results obtained with sole treatment of angiostatin that produced modest tumor growth inhibition (Mauceri et al, 1998). Furthermore, it was demonstrated by te Velde and colleagues that when a model of early colorectal liver metastasis was treated with angiostatin or endostatin and adjuvant chemotherapy, there was decreased tumor size, decreased

metastatic lesions in the immediate surroundings of the tumor, and overall increased antitumor efficacy (te Velde et al, 2002).

The complexity of factors involved in angiogenesis has proven to be great. There are numerous endogenous factors that are released by cells such as tumor, endothelial, fibroblasts and macrophages, and the ECM that induce and/or inhibit the process of tumor angiogenesis. Multiple pro- and antiangiogenic molecules are released at different stages of angiogenesis. Further study must be undertaken to understand the temporal sequence of the release of angiogenic inducers and inhibitors during the process of vascular growth and maturation to design suitable drugs to fight tumor growth at various stages of angiogenesis.

Tumor regression by antiangiogenic therapy is slow and can take more than a year, in contrast to the relative rapid tumor regression that can be obtained by chemotherapy. Therefore, in the clinical trials, the patients' tumor vascularization, blood vessel density, tumor blood flow, endothelial cell apoptotic rate, and tumor size must be monitored for a longer period of time than trials using chemotherapeutic agents.

Finally, many initial clinical trials used patients with end-stage cancer, heavy tumor burden, and little life expectancy (te Velde et al, 2002). It is suggested that clinical trials should try to recruit more patients that have less tumor burden and fewer metastatic lesions for adjuvant antiangiogenesis therapy.

In this proposed study, the endogenous angiogenesis inhibitors, angiostatin and endostatin will be transfected into an established glioma model, rat C6 glioma cells, as established by Peroulis et al, 2002. The rat C6 glioma cells are characterized as being highly angiogenic and is a model for malignant gliomas. The mice implanted with

transfected angiostatin and endostatin rat C6 glioma cells mice will also be given radiation therapy to observe if any additive effects occur on tumor growth inhibition.

Hypothesis

Administering combination antiangiogenic therapy (administering angiostatin and endostatin) in conjunction with radiation therapy will result in tumor regression and decreased tumor vessel density in rat C6 glioma tumors in a murine model.

Proposed Experiment

In this study, a rat C6 glioma model will be used to study angiogenesis inhibitors on glioma cells. The rat C6 cells are cultured in RPMI 1640/5% newborn calf serum (NCS, heat inactivated) in a humidified atmosphere of 5% CO_2 (Peroulis et al, 2002). The study uses 6 groups of male nude (*nu/nu)* athymic mice that are 7-8 weeks old. In order to ensure that there is a sustained supply of angiostatin and endostatin in circulation, cells transfected with angiostatin and/or endostatin are implanted into mice brains. The cells are transfected using an adenoviral vector that is stably expressed. The expression of the adenovirus vector uses the human cytomegalovirus immediate-early promoter as described in Griscelli et al, 2000.

Group 1 consists of control mice that are transfected with an empty viral vector, group 2 is transfected with sense angiostatin, group 3 is transfected with sense endostatin, group 4 is treated with radiation therapy alone, group 5 is transfected with sense angiostatin and sense endostatin, and group 6 is transfected with sense angiostatin and

sense endostatin and is irradiated (30 Gray/day). As a general measure of toxicity, body weights are determined daily on all mice (n=15 in each group).

A Western blot analysis, using rabbit polyclonal antibodies against angiostatin and endostatin (Abcam Ltd, Cambridge, UK), is performed after the rat C6 glioma cell transfection to verify the presence of angiostatin and/or endostatin in the tumor cells. The mice are then anesthetized by intraperitoneal injection of pentobarbital (50mg/kg of body weight) and their heads are placed in a stereotactic head frame (Gossmann et al, 2002). The transfected C6 glioma tumor cells are inoculated using the protocol established by Peroulis et al, 2002. The C6 cells are implanted into the brains of the mice through the coronal suture left of the midline to a depth of 5 mm as described in Peroulis et al, 2002. After 30 days, all 6 groups of mice are euthanized by carbon dioxide inhalation, and the tumors are excised. Following tumor excision, tumors are fixed in cold acetone, dried, and stored at -80° Celsius for the quantification of blood vessels as described in Teicher et al, 2001. Tumor volume is measured by calipers (determining width, height, and depth of tumor). An antibody against CD31 is used to immunohistochemically stain 5 tumor sections that are 5 mm thick. Endothelial cell apoptosis is measured by an Annexin V assay. Annexin V is a calcium dependent phospholipid binding protein with a high affinity for phosphatidylserine that is used to detect early stage apoptosis. To measure blood vessel density, regions of high vascularity are observed using 10 low power (x100) microscopy fields, and regions of low vascularity are observed using 10 high power (x200) microscopy fields. As described in Teicher et al, 2001, the data that will be analyzed is a mean ± SE for the 10 high and low power fields.

Possible Outcomes

The proposed experiments will study rat C6 glioma cells *in vivo* in a murine model, and will be administered with the two angiogenesis inhibitors, angiostatin and endostatin, and concurrently with radiation therapy. According to previously mentioned results, jointly administering angiostatin and endostatin in combination with radiation therapy will most likely result in increased tumor regression and decreased tumor vessel density. The combination therapy is predicted to augment the antitumoral effects of administering angiostatin, endostatin, and radiation therapy alone.

The administration of angiostatin and endostatin is predicted to have synergistic effects in antiangiogenic therapy due to their differing binding sites. Because angiostatin binds to ATP Synthase on the endothelial cell membrane and endostatin is thought to bind to heparin sulfate proteoglycans, the resulting intracellular signaling pathways that are induced are most likely to be different. In order for a cell to become angiogenic, numerous signaling pathways are induced by different factors that all result in the activation of the endothelial cell. Therefore, inhibiting one pathway may not be enough to prevent the angiogenic switch from occuring. However, if more than one pathway is inhibited, the endothelial cell may be inhibited from becoming angiogenic. The purpose in jointly administering angiostatin and endostatin is to inhibit multiple signaling paths in the endothelial cell that result in angiogenesis (figure 10). Furthermore, radiation is added to the therapy because it is has been demonstrated that radiation, through mechanisms that are unknown, resulted in improved tumor eradication when combined with angiostatin (Mauceri et al, 1998).

It is expected that the control mice (group 1) will exhibit heavy tumor burden, a high tumor vessel density, and increased mortality rates. Group 2 and 3 (implanted with transfected sense angiostatin and sense endostatin rat C6 glioma cells) are predicted to exhibit less tumor burden, and decreased tumor vessel density. Group 4 (treated with radiation alone) is likely to show similar tumor burden and blood vessel density as in group 2 and 3. Group 5 (implanted with transfected sense angiostatin and sense endostatin cells) is expected to show a further decrease in tumor burden and tumor blood vessel density. Finally, group 6 (implanted with sense angiostatin and sense endostatin in conjunction with radiation therapy) is likely to exhibit the lowest tumor burden and tumor vessel density.

In the different groups of mice, levels of VEGF, FGF, PDGF, and TGF-β expression can be measured to quantify the antiangiogenic effects of angiostatin, endostatin, and radiation therapy. Furthermore, vessel stability can be studied by observing the presence of periendothelial cells such as pericytes and smooth muscle cells in the vessel wall. Antibodies against adhesion molecules such as integrins, cadherins and catenins can be used to measure endothelial cell-cell interactions.

Conclusion

To interpret the results of this proposed study, one has to take into consideration the numerous variables in the experiment. Though it has been demonstrated that the joint administration of angiostatin and endostatin does not result in normal cell toxicity, adding radiation therapy to the regimen may lead to normal cell toxicity and undesirable side effects. These side effects may not have resulted when treating with one inhibitor and

radiation therapy or treating with two inhibitors alone, though O'Reilly and coworkers demonstrated that angiostatin can be administered up to 100 mg/kg without observing any toxicity (O'Reilly et al, 1997). The proposed study can be modified by adding a cytotoxic chemotherapeutic agent to the antitumor therapy. The function of adding a cytotoxic chemotherapeutic agent is to target the tumor cells while concurrent antiangiogenic therapy targets the endothelium.

Though it is expected that a decrease in vessel density, an increase in endothelial cell apoptosis, and a decrease in tumor size will occur following the joint angiogenesis inhibitor administration along with the concurrent radiation therapy, the results may prove that this regimen is not so effective. Though in previous studies rat C6 cells implanted subcutaneously into mice have yielded positive results in tumor size regression, the murine model suggested in this study may not give the same results. The reason for this may be due to the different type and the combination of inhibiting agents used, as well as the different location of tumor cell implantation.

Similar to some results obtained, an increase in vascularization and tumor size may occur upon the two-inhibitor and radiation therapy treatment. It has been suggested that the increase in tumor size may be due to increased blood flow to the treated tumor because of a decrease in intratumoral pressure. Although this proposed study quantifies tumor vessel density as a measure of the angiogenesis inhibitor efficacy, it has been shown by Hlatky et al, 2002, that measuring microvessel density is a good prognostic tool, but may not be a good indicator of antitumor efficacy. However, in the proposed study, tumor size is also measured to study the efficacy of the inhibitors and radiation.

Further study needs to be conducted to understand the effects of combining three antiangiogenic therapeutic methods in treating glial tumors in a murine model. Studies that incorporate new experimental parameters should also look at tumor burden and histological characteristics such as blood vessel density and apoptotic index when studying the efficacy of antiangiogenic therapy in cell culture, animal models, and human clinical trials (Bergers et al, 1999).

Discussion

The process of angiogenesis is essential for cancer growth. The viability of tumor cells depends on the induction of new blood vessels from the surrounding vasculature. The induction of angiogenesis is often due to hypoxia and the hypoxia inducible factor (HIF)-1 that has been shown to induce the expression of endothelial mitogens VEGF and PDGF (Carmeliet et al, 1998). During the induction and the subsequent angiogenic process, inducers such as VEGF, PDGF, bFGF, and TGF-β, are expressed by numerous cells such as tumor cells and endothelial cells.

These inducers help to activate endothelial cells from a quiescent, nonproliferative state to a proliferative state. Furthermore, VEGF helps to enhance the permeability of the blood vessels that results in edema and endothelial cell injury. VEGF also induces the production of serine proteases by the tumor that aids in endothelial cell migration. PDGF is released by macrophages and fibroblasts among other cells, and targets endothelial cells and smooth muscle cells in the vessel wall to provide vessel structural integrity. Acidic and basic FGF are mitogenic for endothelial and tumor cells and play a role in differentiation, chemotaxis, endothelial cell migration, and tube

formation. TGF-β's inhibitory and stimulatory effect on endothelial cell proliferation, differentiation, motility, adhesion, and apoptosis depends on the dosage. Angiopoietin-1 has a role in vessel outgrowth, remodeling, and maturation. The presence of angiopoietin-1 has been shown to be essential in providing vessel integrity because of its interaction with the surrounding pericytes. These growth factors are involved in numerous signaling pathways that modulate downstream gene transcription (Figure 11).

Antiangiogenic factors such as the protease activators such as uPA and tPA and the MMPs function to inhibit the process of angiogenesis. The inhibitors of these factors such as PAI and TIMPS are often coexpressed with angiogenic inhibitors and help to prevent the excessive degradation of the ECM so that endothelial cell migration can occur.

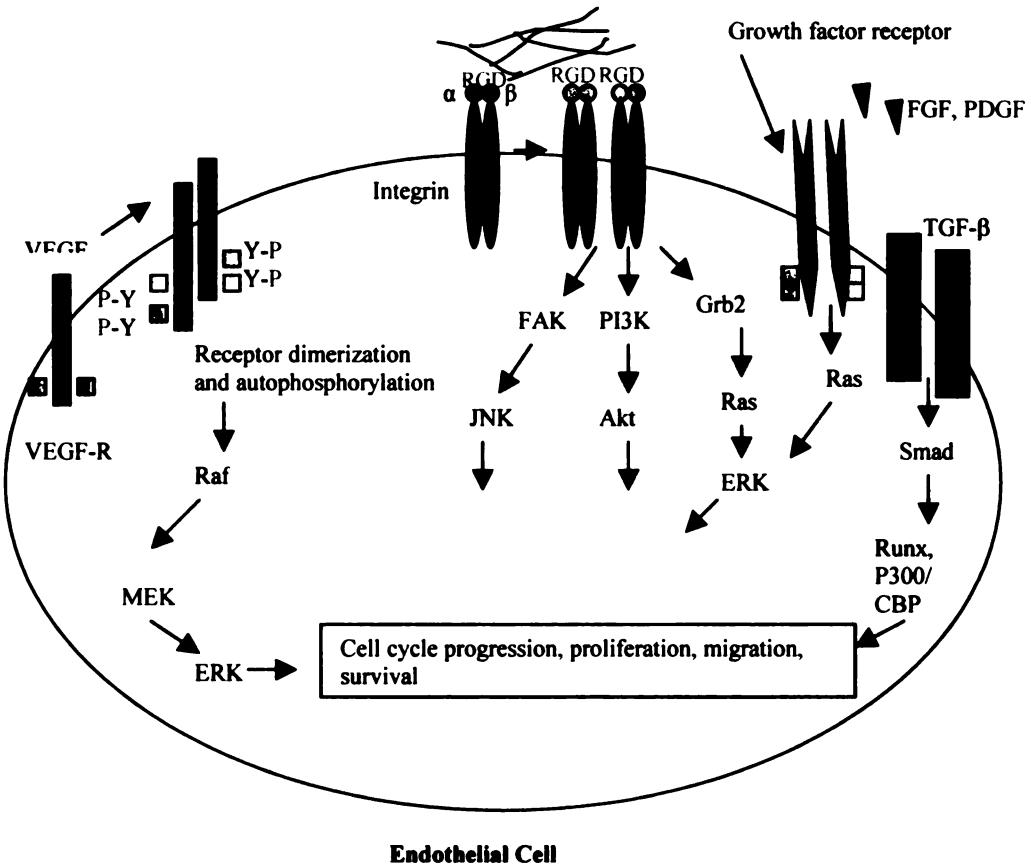

Figure 11: The angiogenic endothelial cell. Angiogenic inducers such as VEGF, FGF, TGF-β, and PDGF are promote angiogenesis by modulating gene transcription through their effects on numerous intracellular signaling pathways. These pathways affect endothelial cell cycle progression, proliferation, migration, and survival.

Adhesion molecules such as integrins, cadherins and catenins play an essential role in endothelial cell, ECM, and periendothelial cell attachment, proliferation, and migration. Integrins recognize a RGD sequence on the ECM components that they bind and help to link the ECM to the intracellular signaling pathways involving cytoskeletal elements that aid in endothelial cell migration. Cadherins and catenins are involved in cell adhesion using homophilic interactions and help to maintain vascular integrity by linking the cell-cell contacts with intracellular signaling pathways to affect cell adhesion and migration.

There are numerous antiangiogenic factors that are endogenous and are released by the tumor to inhibit angiogenesis. Factors such as angiostatin and endostatin have been shown to function in inhibiting distant tumor metastatic foci to proliferate. The presence of these angiogenic factors results in metastatic cells to remain dormant and unable to induce angiogenesis. Angiostatin and endostatin were discovered by O'Reilly and colleagues, and were shown to inhibit endothelial cell proliferation and chemotaxis, and induces endothelial cell apoptosis (Eriksson et al, 2003). The mechanisms of action by which these antiangiogenic factors exhibit their antiproliferative and apoptotic effects are unclear. It is known that angiostatin binds to the α and β subunits of the ATPase synthase on the endothelial cell membrane. Endostatin is thought to bind integrin α subunit and heparin sulfate proteoglycans in the ECM, thereby inhibiting endothelial cell migration. Angiostatin and endostatin have been shown to inhibit angiogenesis in experimental *in vitro* and *in vivo* models where they have resulted in the inhibition of endothelial cell migration, tube formation, and in inducing endothelial cell apoptosis.

Angiostatin and endostatin are currently in clinical trials using new trial designs to improve the efficacy of the antiangiogenic inhibitors in human trials. Changing parameters such as dosage (bolus versus inhibitor in continuous circulation), the type of angiogenesis inhibitor used, type of endpoint measured (tumor regression vs. reduced tumor growth rate) and using angiogenesis inhibitors in conjunction with chemotherapy and/or radiation. The purpose of proposing the experiment in this thesis was to assess the efficacy of administering angiostatin and endostatin in conjunction with radiation therapy to a highly angiogenic model, rat C6 glioma cells. In order for antiangiogenic therapy to be efficacious, further study needs to be undertaken to understand the temporal sequence of events and the overlapping functions of many of the angiogenic inducers and inhibitors in order to target specific factors that are involved in different stages of angiogenesis.

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