Commentary

Glomeruloid Microvascular Proliferation Orchestrated by VPF/VEGF

A New World of Angiogenesis Research

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One can hardly imagine a more comprehensive investigative assault than that occurring in the field of tumor angiogenesis. Scores of basic and clinical researchers in academics and industry are entrenched in a search for underlying mechanisms and a means for antagonizing the proliferation of new blood vessels in neoplastic diseases with the common goal of selectively inhibiting angiogenesis as an effective therapy for all sorts of cancer. To this end, angiogenesis is being approached from a variety of research angles: genetic, biochemical, molecular biological, transgenic and knockout mouse models, and large scale clinical trials involving anti-angiogenesis regimens in cancer patients.^{1,2} In this period of heightened research activity, enthusiasm, and even media coverage, it seems inconceivable that a time existed when angiogenesis research either did not exist or was not fashionable. In fact, the concept of angiogenesis as a key regulator of neoplasia is relatively new, having emerged in the early 1970s.³ Even after its conceptual birth, meaningful results in angiogenesis research were limited by the experimental model systems available for investigation. Years passed before researchers studying angiogenesis developed model systems that could adequately address their fundamental biological questions. Some have argued that appropriate models are still lacking.^{1,4}

Angiogenesis: Forming New Vessels from Old

To the uninitiated, the biological steps involved in new vessel formation may seem straightforward. In reality, nothing could be further from the truth. Angiogenic events are complex and tightly regulated by multiple independent pathways that ensure vessels are formed only in the appropriate developmental, physiological, or

pathological context. Although angiogenesis occurs at high levels in embryonic and fetal development, in adulthood it is largely restricted to uterine and ovarian menstrual cycle events and to pathological states including wound healing, inflammatory conditions, and neoplasms.

Investigations of angiogenesis in experimental animals, both in disease states and in response to pro-angiogenic factors such as vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), have demonstrated a well-defined pattern of events resulting in the formation of new vessels.^{5,6} Initially, mature vessels in a region of VPF/VEGF expression show increased permeability, leading to extravasation of plasma, plasma proteins, and deposition of pro-angiogenic matrix proteins. Basement membranes and extracellular matrices must be modified by a regulated, limited proteolytic digestion and remodeling to accommodate new vessel formation from the old. Dilated, pericyte-poor vessels, called mother vessels, emerge and give rise to daughter vessels through a complex series of endothelial rearrangements. In response to the mitogenic effects of VPF/VEGF and other pro-angiogenic cytokines, endothelial cells and fibroblasts undergo cell division and begin to migrate along a chemotactic gradient leading to the extracellular matrix previously modified for their entrance. Once established in their new matrix, endothelial cells assemble themselves to form a central lumen, elaborate a new basement membrane, and eventually recruit pericytes and smooth muscle cells to surround the mature vessels. Clearly, angiogenesis involves a multitude of tightly controlled signaling cascades and structural changes, that occur in a defined order and continue, until a new vasculature has stabilized.

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Table 1. Endogenous Promoters of Angiogenesis

Vascular endothelial growth factors (VEGF-A, -B, -C, -D)
Angiopoietins
Angiogenin
Basic fibroblast growth factor (bFGF)
Ephrins
Hepatocyte growth factor (HGF; scatter factor)
Interleukin-8 (IL-8)
Platelet-derived growth factor (PDGF)
Transforming growth factor- β (TGF- β)
Tumor necrosis factor- α (TNF- α)

Under developmental and reparative conditions, there is an obvious need for a tight balance between angiogenesis and its inhibition. Unchecked regional or global vascular growth could easily become deleterious in developmental, physiological, or pathological states if blood flow to aberrantly formed vessels deprived other regions of nutrient and oxygen supply. A balance of pro-angiogenic (Table 1) and anti-angiogenic (Table 2) forces are also at work in neoplasms, and a shift toward angiogenesis favors new vessel growth.^{7,8} Manipulation of this balance in favor of anti-angiogenic factors is a natural target for therapies.^{9,10}

A New Model of Angiogenesis

It is in the context of animal models for angiogenesis that the article by Sundberg et al in the current issue of *The American Journal of Pathology* is so exciting.¹¹ The authors describe the formation of a specific type of vascular proliferation, glomeruloid bodies, in a mouse model after local injection of an adenovirus vector directing the expression of VPF/VEGF. Vascular permeability, microvessel enlargement, and finally glomeruloid bodies—a focal proliferative budding of endothelial cells that resembles a renal glomerulus—appeared in direct relationship to the level of VPF/VEGF expression by infected host cells. The finding of glomeruloid body formation in this experimental setting is significant in its own right, as it represents the first detailed description of this type of angiogenesis in an animal model. Previous attempts to overexpress VPF/VEGF in a variety of mouse and rat tissues resulted in a high degree of microvascular permeability, edema, and formation of mother vessels. Beyond these findings, vascular changes were highly variable and tissue-dependent.¹² In the current investigation, adenoviral VPF/VEGF delivery to mouse ears was followed by a tightly regulated sequence of events that have been meticulously described by the authors and include glomeruloid body

Table 2. Endogenous Inhibitors of Angiogenesis

Angiostatin
Brain angiogenesis inhibitor-1 (BAI1)
Endostatin
Interferons
Platelet factor-4 cleavage products
Prolactin fragment (16 kd)
Thrombospondin-1
Vascular endothelial growth inhibitor (VEGI)
Vasostatin

development, stabilization, and finally regression. Considering the temporal and morphological complexity of angiogenesis, as well as the large number of cytokine pathways thought to be involved, perhaps the most striking finding in the article by Sundberg et al is the ability of a single secreted factor, VPF/VEGF, to orchestrate these events and result in a semistable vascular structure.

This most recent investigation comes from the laboratory of one of the early pioneers of angiogenesis research, Harold Dvorak, whose group first described the functional activity of vascular permeability factor (VPF), a tumor-associated soluble compound that made blood vessels leaky.^{13,14} This glycoprotein was later purified, cloned, and further characterized for its mitogenic activity on endothelial cells and became more widely known as vascular endothelial growth factor (VEGF, VEGF-A, or VPF/VEGF).^{15,16} The VEGF family of secreted proteins has recently expanded with the discovery of other endothelial-specific growth factors including VEGF-B, VEGF-C, and VEGF-D.^{17,18} These factors share significant sequence homology with VPF/VEGF, but receptor binding properties, tissue specificity, and biological properties differ within the family. Adding to the complexity, each of these factors appears to have a variable number of splice forms. Alternative splicing of the human VPF/VEGF gene yields isoforms of 121, 145, 165, 189, and 206 amino acids, and at least in some experimental systems, these isoforms have differential activity in tumor neovascularization.^{19,20}

Central Importance of VPF/VEGF in the Angiogenic Response

The glomeruloid bodies that develop in the presence of high levels of VPF/VEGF are structural units whose function is not well understood. The normal sequence of angiogenesis alluded to above is interrupted by an atypical proliferative process. The animal model introduced by Sundberg et al does not, at this point, suggest whether these bodies are an immature, dysfunctional, or abortive form of angiogenesis. It is not clear what types of modifying influences other angiogenic and anti-angiogenic cytokines might have in the formation of glomeruloid bodies, or in the potential transition of glomeruloid bodies to a more mature vascular phenotype. Numerous regulating factors besides VPF/VEGF are necessary for normal new vessels, and the balance of such factors may be significant in determining the type and/or success of angiogenesis (Cavallaro U, Tenan M, Castelli V, Perilli A, Maggiano N, Van Meir EG, Montesano R, Soria MR, and Pepper MS, submitted).

On the pro-angiogenic side, the angiopoietins (most importantly Ang-1) working through specific tyrosine kinase receptors that are selectively expressed on endothelial cells (Tie2 and, perhaps to some extent, Tie1), have roles that are permissive but nonetheless requisite for the development of stable and functional vessels from their immature precursors. Ang-1, acting through Tie2, is thought to enhance cohesiveness between endothelial cells themselves, and between endothelial cells and the

surrounding matrix, thereby stabilizing vessels and reducing permeability.²¹ At a slightly later point, ephrin-B2 and its tyrosine kinase receptor, EphB4, assist in shaping the vessel identity in the direction of either arterial or venous differentiation.^{22,23} A host of other angiogenic factors have been identified that are beyond the scope of this Commentary, but are certainly critical in the regulation of new vessel formation (Table 1).

Although other angiogenesis factors should not be ignored, the article by Sundberg et al highlights the central role that VPF/VEGF plays in the initial events of angiogenesis. Its role in tumoral angiogenesis is believed to be just as vital. VPF/VEGF is thought to be a principle component of the angiogenic switch that tips the balance in favor of new vessel formation in multiple forms of neoplasia.⁸ In addition to gliomas of the central nervous system, VPF/VEGF is expressed in solid malignancies including breast, lung, colon, gastric, bladder, kidney, thyroid, cervical, and ovarian carcinomas.²⁴ Germ cell tumors and some sarcomas also show increased expression. Somewhat surprisingly, even malignant hematopoietic cells overexpress VPF/VEGF.²⁵ At least in some gastrointestinal, genitourinary, and bronchial tumors, pre-neoplastic lesions (ie, dysplasia and carcinoma *in situ*) show up-regulation of VPF/VEGF, suggesting early involvement of this cytokine in tumor angiogenesis, perhaps even before mass formation.^{26–28}

Some of the strongest evidence for the central significance of VPF/VEGF in angiogenesis of all forms comes from the genetic manipulation of mice. Mice with a targeted loss of a single VPF/VEGF allele have an embryonic lethal phenotype in which embryos die between days 11 and 12.^{29,30} In these heterozygous embryos, blood vessels develop but are highly abnormal. This severe phenotype in the setting of inactivation of a single allele is uncommon and points to the developmental importance and critical dosage of VPF/VEGF in embryonic angiogenesis. Mice lacking the high affinity tyrosine kinase receptors for VPF/VEGF (VEGFR-1 and VEGFR-2) have dramatic phenotypes as well. Homozygous targeted disruption of VEGFR-2 (Flk-1) causes death of embryos at 8.5 to 9.5 days.³¹ Endothelial cells fail to differentiate in these embryos, and thus the vascular system is never initiated developmentally. Mouse embryos with targeted mutations to both VEGFR-1 (Flt-1) alleles develop endothelial cells, but these cells do not organize properly into normal vascular channels.³² Mice engineered to lack other angiogenic cytokines and cytokine receptors, such as the Ang-1 protein and Tie2 receptor, have also been studied.^{33,34} The absence of these receptors is also devastating to the developing vascular system, but does not compare in magnitude to the changes elicited by a loss of the VPF/VEGF signaling pathway.

Although components of the angiogenesis machinery continue to be defined and reassessed, it remains clear that VPF/VEGF, at least in some instances, is sufficient to orchestrate the formation and regression of glomeruloid bodies. Sundberg et al found that primitive glomeruloid bodies formed 3 to 4 days after injection of VPF/VEGF adenovirus in tissues expressing high levels of VPF/VEGF

mRNA. These bodies matured at days 7 to 10 and finally regressed at day 14, a time when tissue expression of VPF/VEGF was declining. The formation and regression of these structures in direct relation to VPF/VEGF expression confirms the importance of this cytokine to both the initiation and sustaining of endothelial structures. VPF/VEGF is known to participate in the proliferative and migratory responses of endothelial cells that begin the process of angiogenesis as well as its maintenance through anti-apoptotic activity. Additional triggers for individual events within the cascade leading to glomeruloid body formation and regression have yet to be defined. Though it appears likely that these structures originate from pre-existing mother vessel endothelium, it cannot be excluded that VPF/VEGF might attract hematopoietic cell precursors. Furthermore, the role of pericytes and macrophages that appear during glomeruloid body maturation is unclear at this stage. Nonetheless, this animal model for formation of glomeruloid bodies by Sundberg et al carries great potential for future investigations of biological mechanisms as well as pharmacological manipulation.

Mechanisms of Regression

It is becoming evident that the decreased growth and even regression of blood vessels due to anti-angiogenic factors is not passive, but rather represents a sequence of receptor-mediated events that result in vascular destabilization and apoptosis.^{1,2,34,35} This phenomenon has been studied most extensively in animal models of metastases. New metastatic foci are thought to gain access to a vascular supply by co-opting existing mature vessels. To counteract this co-option, vessels undergo a form of regression that appears to be mediated by specific cytokines including Ang-2, a cytokine that is massively up-regulated by co-opted vascular endothelial cells. Ang-2 is believed to act in an autocrine fashion, most likely acting as a Tie2 receptor antagonist. In the absence of vascular growth factors, such as VPF/VEGF, Tie2 receptor blockage leads to vascular destabilization and, eventually, apoptosis and regression. Only those metastases that can induce angiogenesis in this setting will remain viable and grow.

Events mediating vascular regression are complex, but the potential for therapeutic intervention within this cascade begs for a better understanding. The mouse model of glomeruloid body formation described by Sundberg et al offers a unique opportunity to study vascular regressive events. Glomeruloid bodies regressed 14 days after adenoviral delivery of VPF/VEGF, corresponded to a loss of tissue expression of VPF/VEGF, and was characterized by significant levels of apoptosis in vascular endothelial cells. After regression, a more typical-appearing microvasculature remained. This active regression of vascular structures is an excellent model to better study signaling pathways and molecular mechanisms that potentially could be targeted therapeutically.

Figure 1. Glomeruloid vascular proliferation in a glioblastoma multiforme (arrow). Newly sprouted vessels arranged in tufted aggregates resemble renal glomeruli. Adjacent vessels demonstrate other morphological forms of microvascular hyperplasia in glioblastoma.

Glomeruloid Vascular Proliferation in Glial Neoplasms

Glomeruloid bodies that formed after adenoviral expression of VPF/VEGF at least superficially resembled glomeruloid vascular proliferations that occur in a number of human neoplasms. Whether these bodies represent an accelerated form of angiogenesis or a dysfunctional, perhaps abortive, type of proliferation remains an open question. Glomeruloid bodies are best known in high grade glial neoplasms (Figure 1), where they are one of the diagnostic histopathological features of glioblastoma multiforme (GBM).^{36,37} Ultrastructural analysis has demonstrated that they are tufted collections of newly formed vascular channels lined by hyperplastic endothelial cells and surrounded by basal lamina and an incomplete layer of pericytes.^{38,39}

Angiogenesis differs substantially from tumor to tumor, in both quantitative and qualitative terms, depending on the organ system involved and is thought to be dependent on differential expression of cytokines by organ-specific stroma.^{40–42} The brain is unique in this regard, since its “stroma” consists of a rich network of oligodendrocytes, astrocytes, and numerous intertwining neuronal processes. Perhaps in part because of the distinctive stromal component and the constellation of cytokines expressed,^{43–45} vascular proliferation in GBM takes the form of glomeruloid bodies more frequently than in tumors occurring in other organ systems.⁴⁶

Glioblastoma multiforme is the most malignant form of astrocytic neoplasm (World Health Organization (WHO) grade IV) and has a highly irregular vasculature. To better comprehend the sequence of events that lead to this atypical vascular phenotype, it is useful to examine the vascular structure of lower grade astrocytomas, because GBMs can evolve from them (Figure 2). Low grade, infiltrating astrocytomas (WHO grade II) have a vessel density that is only slightly greater than non-neoplastic brain, and its vessels have a normal structure. As an astrocytoma progresses to become more cellular and atypical (anaplastic astrocytoma, WHO grade III), vessel density increases noticeably. In the transition from anaplastic astrocytoma to glioblastoma multiforme (WHO grade IV), two histopathological features emerge: microvascular hyperplasia, including glomeruloid vascular proliferation, and necrosis with pseudopalisading. These two features are closely related spatially and temporally, but the exact sequence of events in their evolution has not been completely defined. In the setting of a high grade infiltrating astrocytoma, either the presence of microvascular proliferation or pseudopalisading necrosis fulfills criteria for glioblastoma multiforme.

Aside from its place as a defining histopathological feature of GBM, vascular proliferation is believed to significantly influence the biological behavior of malignant astrocytomas and to signal the beginning of an aggressive growth phase. Survival of patients with GBMs is approximately 1 year after diagnosis, despite state-of-

Figure 2. Schematic diagram showing the histopathological progression of infiltrating astrocytoma (WHO grade II, left) to glioblastoma multiforme (WHO grade IV, right). In grade II infiltrating astrocytomas, individual tumor cells are scattered within the central nervous system neuropil. Blood vessel architecture and density are similar to normal brain white matter (far left). Neoplastic cells become more numerous and atypical, and show occasional mitotic figures in anaplastic astrocytoma (WHO grade III). In glioblastoma multiforme, microvascular hyperplasia, including glomeruloid vascular proliferation (**open arrow**), and necrosis with pseudopalisading (**black arrows**) are seen. Glomeruloid vascular proliferation is often noted in zones surrounding pseudopalisading necrosis. Illustration by Bill Winn.

the-art neurosurgical resection and postoperative radiation therapy. Patients with anaplastic astrocytomas, tumors that lack vascular proliferation and pseudopalisading necrosis, typically survive twice as long. Thus, the presence of vascular proliferation and pseudopalisading necrosis in a high grade astrocytoma heralds the onset of rapid tumor growth and clinical progression. Most researchers would agree that accelerated angiogenesis and glomeruloid vascular proliferation are either key factors responsible for this accelerated growth or a side effect of chaotic and excessive signaling events that initiate glioblastoma development.

The intimate relationship between necrosis and glomeruloid vascular proliferation in glial neoplasms cannot be a coincidence. The most likely explanation is that hypoxic conditions associated with necrotic zones induce an angiogenic response. In fact, dense collections of neoplastic astrocytoma cells seen palisading around necrotic foci in glioblastoma show some of the highest expression of hypoxia-inducible regulators of angiogenesis.^{44,45,47,48} Hypoxia is one of the most potent stimulators of VPF/VEGF expression, and acts through a hypoxia-responsive element (HRE) within the VPF/VEGF promoter to increase VPF/VEGF transcription.⁴⁹ This response is mediated by transcription factors of the hypoxia-inducible factor (HIF) family, and inhibition of this pathway has been shown to prevent tumor growth.^{50,51} Through these mechanisms VPF/VEGF, a potent stimulant of angiogenesis in gliomas, is up-regulated in hypoxic tumor cells surrounding necrotic foci at both the mRNA and protein levels. In the cystic, partially necrotic regions of a GBM, concentrations of secreted VPF/VEGF protein are 200- to 300-fold higher than concentrations in

serum.⁵² VPF/VEGF binds to its high affinity tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (Flt-1 and Flk-1, respectively) which are expressed on endothelial cells of high grade gliomas, but not on endothelial cells of normal brain.⁴⁸ Binding of VPF/VEGF to its receptors, in turn, initiates a cascade of signaling events that eventually results in new vessel formation in surrounding hypoxic regions. Thus, the combination of increased expression of VPF/VEGF by tumor cells in hypoxic zones and the specific up-regulation of VPF/VEGF receptors on endothelial cells within the neoplasm are thought to be critical in the development of a hypoxia-induced angiogenic response in glioblastoma.

Collectively, these new findings by Sundberg et al, emphasizing the significance of VPF/VEGF in glomeruloid bodies, combined with structural observations in glioblastoma and the recognized role of Ang-2 in the collapse of co-opted vessels, suggest a model for the evolution of vascular changes during astrocytoma progression (Figure 3). In this model, low grade infiltrating astrocytoma cells receive oxygen and nutrients by co-opting existing small capillaries.^{35,53,54} As neoplastic cells proliferate to form dense aggregates around normal capillaries, endothelial cells resist co-option by releasing Ang-2, which acts in an autocrine fashion via Tie2 receptors on endothelial cells to cause apoptosis and vascular regression in the absence of VPF/VEGF. Those astrocytoma cells in the immediate vicinity of degenerated, co-opted vessels begin to die, forming initial foci of necrosis. These foci become tightly surrounded by tumor cells which will form the pseudopalisade by mechanisms nascent which are not yet understood (cell migration, cell

Figure 3. Proposed model for the evolution of vascular changes during astrocytoma progression to glioblastoma multiforme. In early stages of tumorigenesis (**left**), low grade infiltrating astrocytoma cells co-opt existing small capillaries. As neoplastic cells proliferate to form a more cellular neoplasm (**middle**), endothelial cells resist co-option by releasing Ang-2, which acts in an autocrine fashion to cause endothelial apoptosis and vascular regression in the absence of VPF/VEGF. Astrocytoma cells in the immediate vicinity of degenerated, co-opted vessels begin to die, initiating the well-recognized pattern of pseudopalisading necrosis (**right**). Hypoxic astrocytoma cells in zones surrounding central necrosis up-regulate the expression and secretion of VPF/VEGF, which acts on nearby vessels to cause vascular hyperplasia, including glomeruloid vascular proliferation.

proliferation, or reduced cell death). Surrounding hypoxic tumor cells up-regulate the expression and secretion of VPF/VEGF and causes vascular hyperplasia, including glomeruloid vascular proliferation. This proposed model explains the finding of hypercellular zones that surround foci of necrosis in glioblastoma, as well as the close temporal and spatial relation between glomeruloid vascular proliferation and pseudopalisading necrosis. Already, investigations have shown that Ang-2 mRNA is expressed in hyperplastic and nonhyperplastic vessels in human astrocytomas, suggesting an early role of this cytokine in glioma progression.⁵³ In an animal model of glioma, Ang-2 expression, endothelial apoptosis, and vascular involution have been shown to precede tumor necrosis.⁵⁴ Further investigations are necessary to demonstrate whether subsequent microvascular hyperplasia and glomeruloid vascular proliferation are related to these phenomena in human brain tumors.

An Experimental Model with a Future

One might wonder why a new animal model of angiogenesis such as the one introduced by Sundberg et al is necessary, since numerous experimental models of tumor angiogenesis already exist. First, this new model could allow much more detailed investigations of vascular growth and regression in the absence of confounding effects of neoplastic cells. Pharmacological studies of

anti-angiogenic therapies, in particular, can be difficult to interpret because anti-angiogenic effects of a drug are not always easily separable from primary or secondary effects of the drug on tumor cells. As has been described, the steps involved in angiogenesis are numerous and intricate. A pure experimental system offers a greater chance for accurate interpretation of underlying mechanisms.

Secondly, some investigators have questioned the validity of the most commonly used animal models of tumor angiogenesis.⁴ These studies typically transplant rapidly growing tumors into subcutaneous tissue of mice and investigate effects of therapies, including anti-angiogenic drugs. It has been suggested that these models necessarily exaggerate responses of anti-angiogenic therapies due to the location of the transplanted neoplasm and to their inordinate number of new blood vessels, which are especially vulnerable to anti-angiogenic drugs. New animal models of angiogenesis, especially ones that demonstrate a reproducible sequence of vessel formation and regression in response to VPF/VEGF, are a welcome addition because of the potential they hold for future investigation.

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