Molecular Neurobiology

ISSN0893-7648/01/23(2/3): 121–135/$13.75

Vascular Growth Factors in Cerebral Ischemia

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Abstract

During the past decade, there has been a surge of interest in growth factors (GFs) that act selectively on vascular endothelium and perivascular cells. Studies employing mutant mice or the administration of recombinant proteins have suggested that these factors not only mediate the proliferation of endothelial cells, but also regulate vascular differentiation, regression, and permeability. During and after cerebral ischemia, brain vasculature becomes leaky and unstable, and the normally impermeable blood–brain barrier breaks down. Several days after the ischemic insult, endothelial cells begin to proliferate, and angiogenesis occurs. Expression studies have shown that key vascular GFs are regulated, during these processes, in a complex and coordinated manner. The distinct pattern of regulation exhibited by each vascular GF suggests a unique role for each factor during the initial vascular destabilization and subsequent angiogenesis that occurs after cerebral ischemia. Data from studies in other biological systems support these suggested roles. Thus, manipulation of vascular GFs may prove to be an effective means of stabilizing or enriching brain vasculature after ischemia, and ameliorating the detrimental effects of blood–brain barrier breakdown and vessel regression after stroke.

Index Entries: Vascular endothelial growth factor (VEGF); angiopoietin; Tie receptors; Flt; Flk; VEGFR1; VEGFR2; stroke.

Introduction

During and after cerebral ischemia in animal models, there is a well-documented breakdown of the blood–brain barrier (BBB), which peaks ~1–3 d after the ischemic insult *(1–8).* Edema can be severe after ischemia, and much of the edema is thought to be vasogenic in nature, i.e., caused by leakage of plasma fluids into the brain parenchyma. In addition, ischemic and postischemic vasculature upregulates adhesion molecules, which cause leukocytes to adhere to the luminal wall of vascular endothelium *(9–15).* Chemokine upregulation then leads to the extravasation of leukocytes, which infiltrate the brain parenchyma in an

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inflammatory reaction. These vascular-mediated postischemic responses are likely to contribute to the damage observed after stroke *(12,16–20).*

During the past decade, there has been a surge of interest in vascular growth factors (GFs). During development, these factors have potent effects on endothelial cells (ECs) and perivascular cells, and are thought to regulate proliferation, migration, endothelial tube formation, vascular differentiation, permeability, and regression *(21,22).* Although much still remains to be understood regarding the effects of these factors on adult vasculature, current data suggest that they play similar roles in the changes that occur in both normal and pathological states *(22).* During and after cerebral ischemia, there are many alterations in the cerebral vasculature, including BBB breakdown, EC apoptosis, upregulation of adhesion molecules, and angiogenesis *(1–7,23–30).* Because these alterations may contribute to the brain pathology observed after cerebral ischemia, one must understand the way that decreases or increases in the levels of various vascular GFs may contribute to these pathologies.

Vascular GFs

There are a large number of protein factors that exert effects on vasculature, and the number is growing as interest in their therapeutic potential increases within the scientific community. There are many GFs that can act on vasculature, including, but not limited to, fibroblast GF, platelet-derived GF, transforming GF, and hepatocyte GF (scatter factor). For the purpose of this review, the discussion is limited to two families of vascular GFs: the angiopoietins (Angs) and the vascular endothelial growth factor (VEGF) family. These factors play unique and critical roles in modulating the structure and function of vasculature in both developing and adult organisms. In addition, they differ from many other pleiotrophic GFs that act on endothelium, in that VEGF and Ang receptors are preferentially localized to vasculature *(31–33).* Hence, the actions of these factors are more vascular-specific then most vascular GFs.

Vascular Endothelial Growth Factor

VEGF was originally described as vascular permeability factor *(34),* because of its potent permeabilizing effects on endothelium. Since the discovery of VEGF, four additional VEGF-like proteins have been described. These are placental GF, VEGFB, VEGFC, and VEGFD (the original VEGF has been termed "VEGFA") *(35).* The receptors currently described for the VEGF family are VEGFR1 (Flt-1), VEGFR2 (Flk or KDR), VEGFR3 (Flt-4), and neuropilin *(35,36).* The primary receptors for VEGFA, VEGFR1 and VEGFR2, are localized predominantly to the vascular endothelium, including cerebral endothelium. In addition, several recent papers have reported neuronal localization of VEGFR2 in cultured hippocampal or dorsal root ganglion cells *(37,38).* The neuropilins also bind to VEGFA, and they are expressed in non-ECs, especially in the nervous system *(39,40).* There are five known isoforms of VEGFA in humans, 121, 145, 165, 189, and 206 amino acids in length (one amino acid less in rodents) *(41).* Binding of VEGFA to the neuropilins is isoform-specific (e.g., the 165-amino-acid isoform binds to neuropilin-1, the 121-amino-acid isoform does not *[42]*). In addition, the various VEGF family members have different receptor specificities (e.g., VEGFA binds to VEGFR1 and 2, but placental GF only binds to VEGFR1) (Fig 1; *22*). There is still much to be learned about the roles of the diverse members of the VEGF family of proteins, as well as of the various VEGFA isoforms, particularly in extravascular tissues. However, to date, the vast majority of effects reported for VEGFA (hereafter referred to as "VEGF") have been vascular in nature.

Since VEGF's initial discovery as vascular permeability factor, much research has focused on its role as a potent angiogenic factor that is principally responsible for triggering the develop-

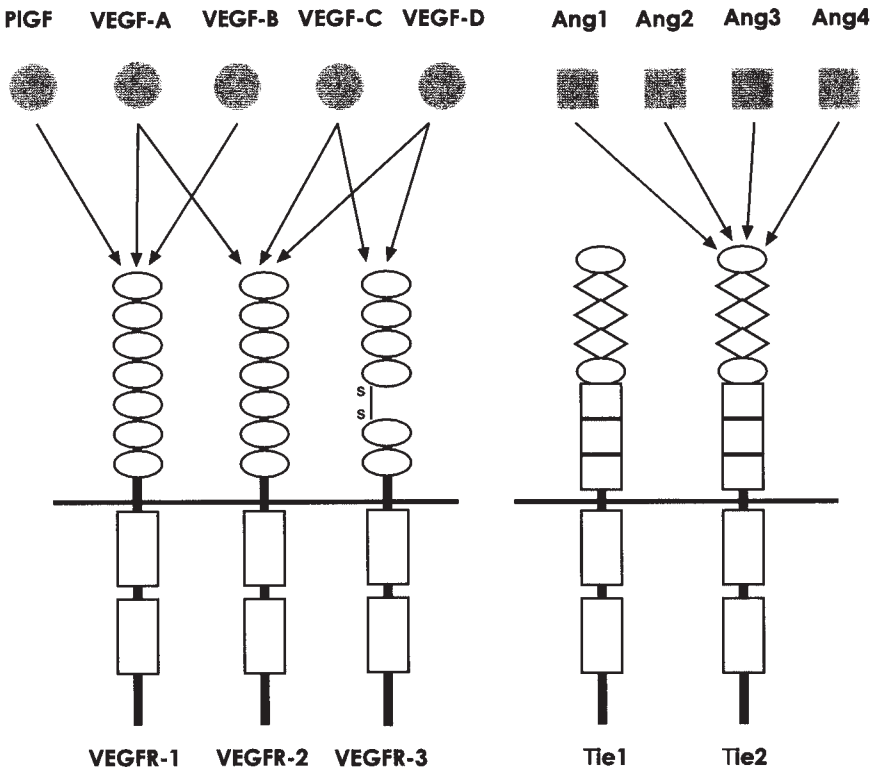


Fig. 1. Schematic illustrating the known members of the VEGF and Ang families of GFs and their primary receptors, the VEGFR family and the Ties. Arrows drawn from a ligand to a receptor indicate that the ligand binds to the receptor with high affinity. The ligand for Tie1 is currently undescribed.

ment of new vessels *(43,44)*. Gene deletion studies have shown that VEGF is critical for angiogenesis during development. Mutant mice, lacking even a single VEGF allele, die during embryonic development, with a striking lack of secondary vasculature *(45,46)*. Both VEGFR1-null *(47)* and VEGFR2-null *(48)* mutants show a profound lack of vasculogenesis, and die early in embryogenesis. The kinase domain of VEGFR1 appears unnecessary for blood vessel formation to occur, because kinase domain mutants develop normal vasculature *(49)*.

In adult animals, application of recombinant VEGF protein has been shown to induce angiogenesis in a variety of normal *(50,51)* and ischemic *(52–54)* tissues. Much excitement has been generated regarding the use of VEGF to treat chronic ischemia by inducing the development of new blood vessels, and human clinical trials have been initiated in ischemic heart and ischemic limb *(55–58)*. However, subsequent animal studies have shown that the blood vessels formed by application of VEGF to adult tissues are typically poorly differentiated, disorganized, and grossly abnormal, characterized by profound permeability and a dilated, tortuous morphology *(50,51,59,60)*. Therefore, VEGF's potential as a self-sufficient revascularizing agent must be considered with caution.

In addition to its role as an angiogenic factor, increasing attention has been given to VEGF's originally described function as a vascular permeabilizing agent. Application of VEGF to adult tissues or cells results in edema and vascular leak. VEGF results in vascular leak in every tissue to which it has been applied,

including, but not limited to, brain, lung, testis, bladder, skin, duodenum, mesentery, and intestine *(59–64).* VEGF is upregulated in animals and humans, in a large number of disease states in which edema and vascular permeability contribute prominently to the pathology *(65–74).*

Angiopoietins

The Angs were first described as ligands for the orphan receptor, Tie2 (Tek) *(75–77).* Like the receptors for VEGF, Tie2 and the related receptor, Tie1, are localized predominantly to the vascular endothelium throughout the body, and, hence, like VEGF, the Angs act relatively specifically on this substrate *(32,76).* Although four members of the Ang family have been cloned *(78),* angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) are the most well-characterized. Studies to date suggest that Ang1 and Ang2 act as agonist and antagonist, respectively, at the Tie2 receptor *(75,77).* Ang1 phosphorylates Tie2 on ECs *(75,77).* Ang2, although it binds to Tie2, does not phosphorylate the receptor in EC cultures. In fact, addition of Ang2 dose-dependently interferes with the phosphorylation of Tie2 by Ang1 in EC cultures *(77).*

Further evidence for the roles of Ang1 and Ang2 as agonist and antagonist at the Tie2 receptor comes from genetic studies in mice. Ang1-null mutants die *in utero,* and exhibit a primitive, poorly differentiated vasculature *(76).* In these mice, it appears that vasculogenesis has occurred, but that the primitive network of vessels does not undergo subsequent differentiation. Mice lacking the Tie2 receptor, like those lacking Ang1, die during embryonic development, with a poorly differentiated, primitive vascular network *(79,80).* Confirmation of Ang2's possible role as an antagonist at the Tie2 receptor came from mice overexpressing Ang2, which show an embryonic lethal phenotype similar to that of Ang1-null mutants *(77),* i.e., excessive expression of Ang2 in the developing vasculature appeared to block Ang1 activity, supporting the notion that Ang2 competes with Ang1 for Tie2 binding, but does not effectively activate the receptor.

Developmental studies suggest that Ang1 plays an important role in the stabilization and differentiation of vasculature (*76,77,79,80)*; recent data in adult animals support this notion. Adult animals overexpressing Ang1, off the K14 promoter in skin, are resistant to stimuli that induce vascular leak. Specifically, vascular permeability induced by topical application of inflammatory agents (mustard oil, serotonin, or platelet-activating factor), or by overexpression of VEGF, are dramatically reduced in Ang1 transgenic mice *(81).* Treatment of adult mice with an Ang1 adenovirus results in a similar resistance to leak *(64).* In addition, recent in vivo data show that treatment of ECs with Ang1 leads to increased junctional adherence between cells, which leads to decreased permeability *(82).* Finally, regulation studies have demonstrated that Ang1 mRNA levels are upregulated during periods of vascular stabilization or recovery from destabilization, such as occurs in the female reproductive system *(77,83,84).*

In contrast to Ang1, Ang2 appears to be upregulated in adult vasculature during periods of vascular destabilization, such as that which occurs before angiogenesis or vascular regression *(26,77,83–88).* Examples of these "destabilized" vessels include leaky postischemic vessels, leaky or regressing tumor vasculature, and regressing ovarian vasculature in atretic follicles and involuting corpora lutea. Therefore, it has been theorized that Ang1 promotes vascular differentiation and stabilization, and that Ang2 destabilizes vasculature in preparation for regression, or for the formation of angiogenic sprouts *(22).*

Regulation of Vascular Factors After Cerebral Ischemia

Early Upregulation of VEGF and Ang2 mRNAs

Within hours after cerebral ischemia, increases in transcripts for both VEGF and Ang2 are consistently evident *(26,88–96).*

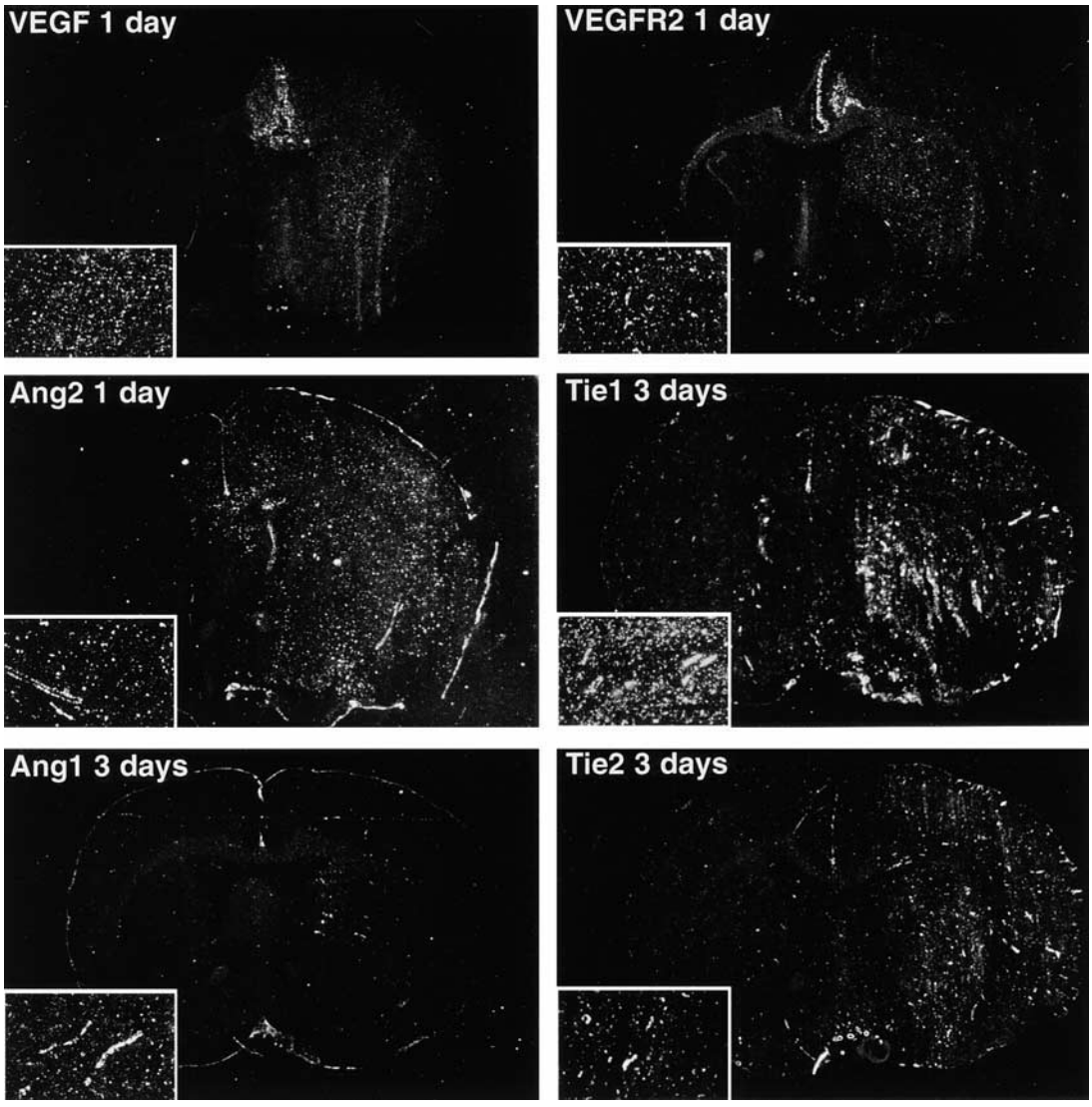
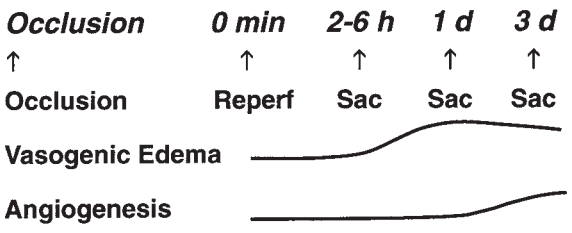


Fig. 2. *In situ* hybridization on brains from normoglycemic, normothermic, adult male Sprague-Dawley rats subjected to 35 min of filamentous MCAO occlusion, then reperfused. Hybridizations are shown for ^{35}S antisense riboprobes to VEGF, Ang1, Ang2, VEGFR1, Tie1, and Tie2. Each panel shows the entire brain, with the postischemic hemisphere on the right. The insets show high-magnification views of the hybridization within the ischemic core in the lateral striatum. For each probe, the maximally upregulated time-point is shown (up to 3 d; 1 d for VEGF, Ang2, and VEGFR1; 3 d for Ang1, Tie1, and Tie2). Sham-operated animals showed hybridization equivalent to that observed on the contralateral hemisphere.

Upregulation of both of these factors has been described as early as 1–3 hours after ischemia, but expression reliably peaks during the period of dramatic vasogenic edema, 24–48 h after ischemia (Figs. 2 and 3). VEGF expression is increased in both glia and neurons of the ischemic brain, as determined by both immunostaining and *in situ* hybridization *(92–94).* In addition, upregulation at these peak time-points sometimes occurs in cells



Probe	Sham	2-6 h	1 d	3 d
VEGF	-	+	+++	+
VEGFR1	+	+	++	+
VEGFR2	+	+	+++	++
Ang1	-	-	-	++
Ang2	-	++	+++	+
Tie2	+	+	++	++
Tie1	+	+	++	+++

Fig. 3. The table summarizes findings across multiple studies, which suggest coordinated regulation of VEGF, the Angs, and their receptors, after ischemia-reperfusion injury. Note that VEGF, Flk, Flt, and Ang2 all show peak upregulation at 1–2 d, during the time of highest vasogenic edema. Ang1 and the Tie receptors, especially Tie1, show peak upregulation after 3 d, when the BBB breakdown is beginning to resolve and angiogenesis is in progress.

not directly affected by the ischemic insult, such as the cingulate cortex and hippocampus (Fig. 2; *97*). Ang2 upregulation appears to occur almost exclusively in cerebral blood vessels within the infarct zone (Fig. 2; *26*). Occasional Ang2-positive vessels have been detected after middle cerebral artery occlusions (MCAO) in the hippocampus and the contralateral hemisphere, regions not directly affected by the ischemic insult *(26).* The mechanisms of the indirect upregulation of VEGF and Ang2 in nonischemic areas remains to be elucidated, but could be caused by damage to neuronal afferents or efferents, pressure effects of edema, or ischemia-related physiological phenomena, such as cortical spreading depression.

VEGFR mRNAs Are Upregulated During Vasogenic Edema

VEGF receptors, like VEGF, appear to be upregulated after cerebral ischemia, primarily at the time of maximal VEGF expression and vasogenic edema (1–2 d after ischemia). VEGF has multiple receptors, but the best-described of these are VEGFR1 (Flt) and VEGFR2. VEGFR1 is expressed constitutively by vascular endothelium throughout the body, and it is modestly upregulated in cerebral blood vessels after ischemia *(89,92,96).* VEGFR2 is dramatically upregulated after ischemia, in a spatial pattern that closely resembles that of VEGF *(92,93).* Upregulation has been described on nonvascular cells, although the functional consequence of VEGF binding to upregulated VEGFR2 on these cells is not well understood (Fig. 2; *92*). However, recent evidence suggests that VEGF can exert trophic effects on neurons, perhaps through VEGFR2 signaling *(37,38,98,99).*

Upregulation of Ang1 and Tie Receptor mRNAs Is Delayed and Prolonged

Message for Tie2 and the homologous receptor, Tie1, are also upregulated by cerebral ischemia (Fig. 2; *26,88,100*). Tie mRNAs first begin to increase ~24 h after ischemia within the ischemic zone, and continue to increase progressively over the first few days after ischemia (Fig. 3; *88,100*). Expression of Tie1 and Tie2 mRNA show long-lasting upregulation after cerebral ischemia, and remain elevated for at least 2 wk *(88,100).* Immunostaining for Tie2 in postischemic brain tissue has failed to reveal a striking increase in Tie2 protein, although more-sensitive methods might reveal increases that would parallel the increases in Tie2 mRNA *(26).* Alternatively, production of Tie2 protein might be concomitantly increased with its mRNA, but turnover of the newly synthesized receptors could be substantially increased as a consequence of binding to elevated levels of Ang2 and Ang1 ligands in the postischemic brain.

Upregulation of Ang1 mRNA is not detected until ~72 h after cerebral ischemia, just before the gradual resolution of the BBB disruption (Fig. 2; *88*). At this time, angiogenesis has also begun to occur, and angiogenic sprouts are clearly evident within the infarct zone *(26).* Ang1 is expressed diffusely in cells throughout the brain, but the upregulation of Ang1 at 72 h postischemia is especially prominent in association with vasculature at the ischemic core (Fig. 2). As for the Tie receptors, Ang1 mRNA remains elevated for approx 2 wk after ischemia, as angiogenesis continues and the vasogenic edema diminishes *(88).*

Coordinated Roles of Vascular Factors After Ischemia

The changing patterns of expression described for VEGF, the Angs, and their receptors are characteristically distinct, and closely correlate with evolving changes in the structure and function of cerebral vasculature (Fig. 3). Although correlational analyses cannot be used to directly deduce the functional roles of these factors in postischemic vascular alterations, these data, in combination with other findings, suggest unique roles for each of these factors in the vascular changes that occur after cerebral ischemia.

Ang2 and VEGF are the earliest mRNAs to show a dramatic upregulation, only 2 h after ischemia, probably in direct response to ischemic injury. For example, Ang 2 has been proposed to be altered as a consequence of shear stress *(101),* and therefore may be sensitive to changes in flow. Ang 2 and VEGF mRNA upregulation are also prominent under hypoxic conditions, in vivo and in vitro *(27,102–109).* For VEGF mRNA, this upregulation has been shown to be associated with the upregulation of the transcription factor, hypoxia-inducible factor 1-α *(103,104,108),* which is also upregulated after cerebral ischemia *(27,110).*

Because Ang2 mRNA previously has been shown to be associated with vascular remodeling (regression or angiogenesis), early increases in Ang2 could cause the cerebral vasculature within the ischemic core and penumbra to become unstable. Yancopoulos et al. *(22)* have proposed that unstable vasculature exposed to VEGF becomes angiogenic, and unstable vasculature in the absence of VEGF regresses. Because VEGF also increases shortly after ischemia, the unstable vasculature is exposed to VEGF, and can enter into an angiogenic phase. Indeed, marked angiogenesis lags behind peak increases in VEGF and Ang2 mRNA by only 1–2 d *(26,27).*

Increases in VEGF mRNA peak during the time of maximal vasogenic edema. Indeed, exposure of adult vasculature to VEGF reliably produces leaky blood vessels, which contribute to edema in surrounding tissues. In fact, mice treated with the VEGF adenovirus develop severe tissue edema *(63,64).* VEGF's effects on vascular leak occur rapidly, within 30 min of exposure to VEGF *(61).* In studies of VEGF-induced angiogenesis, blood vessel formation is evident only after days of exposure to VEGF *(50,51,59),* suggesting that VEGF-mediated changes in vascular permeability precede angiogenesis *(74).*

Increases in VEGFR1, VEGFR2, and Tie2 mRNA lag slightly behind increases in their ligands. Changes in Tie1 mRNA mirror those of Tie2 mRNA, but are more pronounced *(26,88,100).* It is interesting that the increases in Tie1 and Tie2 mRNAs correspond closely to the time-course of postischemic angiogenesis *(26,88,100).*

Ang1 mRNA increases, but Ang2 mRNA decreases, at 3 d, just before decreases in vasogenic edema are observed. This pattern of regulation is consistent with the proposed roles of Ang1 and Ang2 as a vascular stabilizer and destabilizer, respectively, especially regarding vascular leak. In other in vivo models, administration of Ang1 stabilizes blood vessels and decreases vascular permeability *(64,81,82).* Possibly increases in Ang1 starting at 3 d postischemia contribute to the restabilization of the BBB. Ang1 upregulation also occurs coincident with the initiation of postischemic angiogenesis *(88).*

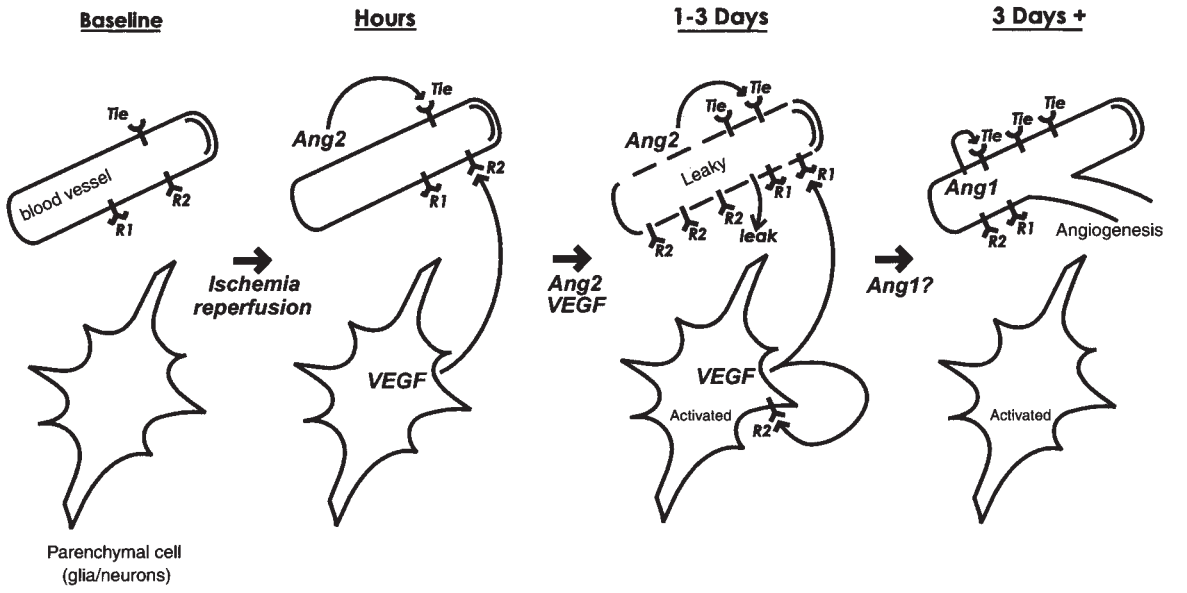


Fig. 4. Schematic diagram illustrating the proposed sequence of events in vascular GF-mediated vascular change after cerebral ischemia-reperfusion. Ang2 and VEGF are upregulated within hours after reperfusion, leading to vascular leak, which peaks 1–3 d after ischemia. Receptors for VEGF show peak upregulation concomitant with VEGF's peak upregulation, and Tie receptors begin to upregulate at 1–2 d after ischemia, also. Continued upregulation of VEGF and Ang2 eventually leads to angiogenesis by 2–3 d. The BBB breakdown begins to resolve after 3 d, coincident with the upregulation of Ang1, Tie1, and Tie2, as well as marked angiogenesis in the necrotic core. Ang1 could contribute to this stabilization of vascular leak, because it has been shown to stabilize leaky vasculature in other animal models of vascular permeability.

A proposed chronology for the effects of VEGF and the Angs in ischemia is diagrammed in Fig. 4. Initially, Ang2 and VEGF are upregulated in response to hypoxia and other stimuli immediately associated with ischemia, causing vascular instability and leak. Furthermore, VEGF and Ang2 collaborate to initiate an angiogenic response in the postischemic tissue. Ang1 levels increase after several days, and the extant and newly proliferating vasculature becomes less leaky.

Therapeutic Potential

Vascular Endothelial Growth Factor

The therapeutic potential for VEGF as a mediator of angiogenesis during chronic ischemia has been repeatedly suggested *(52–54)*. In fact, human trials are currently in progress to evaluate VEGF's potential for inducing neovascularization in ischemic limb and heart *(55–58,111)*. Even so, the therapeutic utility of VEGF monotherapy in ischemia, especially ischemia reperfusion, is less than clear. Endogenous VEGF expression is already markedly increased by ischemia. Although administration of additional exogenous VEGF may induce more angiogenesis or EC survival *(112)*, the quality of the vasculature formed may be highly abnormal *(50,51,59,60,112)*.

In the context of cerebral ischemia, during the first days after reperfusion, the originally extant vasculature is still present within the brain tissue. During those first few days, vasogenic edema, cytotoxic edema, and cell

death appear to be the primary problems, although compromised blood flow is likely to occur as well, because of leukostasis and edema. Given VEGF's demonstrated ability to increase vascular permeability and leukostasis, administration of VEGF, shortly after ischemia, has been demonstrated not surprisingly to increase mortality, morbidity, and cerebral damage in animals *(112).* In marked contrast to these detrimental effects of VEGF, some recent studies have reported that VEGF is neuroprotective in the context of cerebral ischemia *(37,98).* Because VEGFR2 does appear to be upregulated on glia and neurons after cerebral ischemia, possibly VEGF exerts a direct protective effect on these cells *(37,38,98,99).* Direct protective effects of VEGF on any cell containing VEGFRs, including potential induced expression of VEGFR2 on neurons and glia, is possible, given VEGF's activation of the Akt intracellular survival pathway *(113,114).*

Although VEGF may have protective effects on a variety of cells after cerebral ischemia, there is at least as much basis for believing that VEGF could be detrimental after cerebral ischemia. Because VEGF is maximally upregulated after cerebral ischemia in a similar timecourse as vasogenic edema occurs, endogenous VEGF has been implicated in the damaging vasogenic edema that occurs after cerebral ischemia *(112,115).* Therefore, inhibition of endogenous VEGF will possibly ameliorate morbidity and damage after cerebral ischemia. The idea that amelioration of postischemic edema or inflammation may improve the outcome of stroke is not novel, and evidence indeed suggests that various means of inhibiting vascular leak or inflammation decreases lesion volume in animal models of stroke *(9,16–19).* Inhibition of VEGF has been shown to reduce edema in vivo both in ovaries *(116)* and in brain after MCAO *(115).* A recent approach to inhibiting protein ligands has been to use molecular "traps" comprising the extracellular ligand-binding region of the protein's receptor fused to the crystallizable fragment (Fc) portion of human immunoglobulin. The crosslinking of hFc monomers effectively produces a truncated receptor dimer, which further enhances affinity for the protein ligands, which are themselves dimeric in form. These receptor traps have been found to act as powerful high-affinity antagonists to the actions of their ligands. In one recent study, mice were treated with one such VEGF trap, a Flt-Fc, during MCAO *(115).* Treatment with the Flt-Fc significantly reduced cerebral edema after reperfusion. In addition, Flt-Fc significantly decreased the ultimate lesion volume. This approach, although it may obtund angiogenesis, would attack the more immediate and severe problems of leukostasis and vasogenic edema after reperfusion.

The potential drawback to inhibiting VEGF after cerebral ischemia-reperfusion is that, if VEGF is neuroprotective and mediates angiogenesis in the later phases of recovery, inhibition of endogenous VEGF may interfere with other, potentially beneficial, postischemic effects of VEGF. One approach might be to determine if VEGF's diverse effects are mediated by different receptors, and to develop reagents that selectively inhibit specific VEGFRs. Alternatively, vascular leak and leukostasis may be addressed using other vascular GFs that would not obtund the putative beneficial effects of VEGF.

Ang1 has been shown to both stabilize newly formed vasculature and inhibit vascular permeability in diverse animal models *(64,81).* Significantly, Ang1 is upregulated during the period of resolution of the BBB breakdown and angiogenesis after cerebral ischemia *(88).* Thus, Ang1 appears to be a good candidate for inhibiting vasogenic edema after cerebral ischemia. In the animal models studied to date, Ang1 has shown a remarkable ability to inhibit vascular leak, even in the face of potent propermeability compounds, such as mustard oil and VEGF *(64,81).* Ang1 might be able to inhibit the damage that occurs as a consequence of severe vasogenic edema and inflammation, without interfering with the potentially beneficial effects of VEGF to preserve neurons and enhance endothelial health.

Much additional research needs to be conducted before the full extent of therapeutic potential of the vascular GFs will be understood. In addition, there are many factors with trophic effects on vasculature which have not been included within this review, and which may also prove beneficial for cerebral ischemia or other vascular injury. Because of the dramatic breakdown of the BBB that occurs after stroke, and the damage that ensues, the cerebral vasculature is a particularly promising target for therapeutic intervention. The factors discussed in the current review, VEGF and the Angs, have distinct effects on vasculature, and could prove to be excellent targets for therapeutic intervention after stroke.

Acknowledgments

The authors thank many colleagues, both at Regeneron and in the academic community, for stimulating discussions and data exchanges, which have helped to lead to the premises presented in the current review. The authors also are grateful to Evan Burrows and Scott Staton for their expert assistance with the figures.

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