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Patterns of brain angiogenesis after vascular endothelial growth factor administration *in vitro* and *in vivo*

(cerebrovasculature/neovascularization/receptor/astrocyte)

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ABSTRACT **Vascular endothelial growth factor (VEGF) is a secreted endothelial cell mitogen that has been shown to induce vasculogenesis and angiogenesis in many organ systems and tumors. Considering the importance of VEGF to embryonic vascularization and survival, the effects of administered VEGF on developing or adult cerebrovasculature are unknown: can VEGF alter brain angiogenesis or mature cerebrovascular patterns? To examine these questions we exposed fetal, newborn, and adult rat cortical slice explants to graduated doses of recombinant VEGF. The effects of another known angiogenic factor, basic fibroblast growth factor (bFGF), were evaluated in a comparable manner. In addition, we infused VEGF via minipump into the adult cortex. Significant angiogenic effects were found in all VEGF experiments in a dose-responsive manner that were abolished by the addition of VEGF neutralizing antibody. Fetal and newborn explants had a highly complex network of branched vessels that immunoexpressed the *flt-1* VEGF receptor, and *flk-1* VEGF receptor expression was determined by reverse transcription–PCR. Adult explants had enlarged, dilated vessels that appeared to be an expansion of the existing network. All bFGF-treated explants had substantially fewer vascular profiles. VEGF infusions produced both a remarkable localized neovascularization and, unexpectedly, the expression of *flt-1* on reactive astrocytes but not on endothelial cells. The preponderance of neovascularization *in vitro* and *in vivo*, however, lacked the blood–brain barrier (BBB) phenotype marker, GLUT-1, suggesting that in brain the angiogenic role of VEGF may differ from a potential BBB functional role, i.e., transport and permeability. VEGF may serve an important capacity in neovascularization or BBB alterations after brain injury.**

Understanding the factors that control brain angiogenesis is critical for determining developmental aspects of the blood–brain barrier (BBB) as well as cerebrovascular changes after injury. Several factors have been shown to be angiogenic *in vivo* (1, 2), but only vascular endothelial growth factor (VEGF) is a secreted mitogen that is specific for the vascular endothelium (3, 4). VEGF is a dimeric polypeptide that exists in four forms that are generated by alternative splicing from a single gene (4, 5). It appears to play an important regulatory role in endothelial growth and differentiation in several organ systems during embryonic development and is a significant factor in both vasculogenesis and angiogenesis particularly within tumors (6–9). Previous studies examining VEGF mRNA in the embryonic rodent brain found expression in the neuroepithelium and in the ventricular and choroid plexus epithelium at embryonic and early postnatal times (10, 11). The VEGF receptors, *flt-1* and *flk-1*, are expressed in the vascular networks surrounding the neural tube (10, 12), and it has been suggested that early brain angiogenesis may be a result of directed growth toward a diffusible signal, perhaps VEGF, generated from the subventricular zone (10, 11, 13). In the adult, only the choroid plexus, area postrema, and cerebellar granule cells continue to produce low levels of VEGF or receptor mRNA (14, 15). VEGF receptor mRNA is expressed by blood vessels in the embryonic cerebral cortex and is down-regulated after angiogenesis ceases (8, 11).

Recently, the direct cellular effects of growth factors such as brain-derived neurotrophic factor (BDNF) or glial-derived neurotrophic factor (GDNF) on central nervous system (CNS) tissue have been well studied. Considering the importance of VEGF and its receptors to early vascularization and embryonic survival (16, 17), to date the effects of administered VEGF on developing or adult mammalian brain tissue are unknown. Application of $VEGF_{165}$ has improved blood flow clinically in ischemic limb (18) and in myocardial infarction in animal models (19). Insufficient blood flow may be a significant cause of cerebral palsy and mental retardation in the neonate, and adult stroke remains a leading cause of death. To date, no direct therapy has addressed vascular or barrier components after brain injury. Can VEGF alter brain angiogenesis or mature cerebrovascular patterns and does it have any influence on nonvascular cells, particularly astrocytes, which are believed to play an important role in barrier functions? Understanding the capabilities and mechanisms of action of VEGF in the brain could lead to strategies for the control and direction of cerebral angiogenesis, for example, by gene therapy application (18). Of equal importance is the elucidation of VEGF cell biological effects on cerebrovascular growth or BBB permeability. Because VEGF protein therapy presently is not an option for human application (18), we examined the direct effects of the cytokine in rodent brain tissue models *in vitro* and *in vivo*.

To determine whether administration of VEGF could increase the vascularity of brain tissues we exposed fetal (E18), newborn, or adult rat brain slice explants in serum-free media to graduated doses of recombinant VEGF. In separate sets of experiments, the effects of another known angiogenic factor, basic fibroblast growth factor (bFGF), also were examined in a comparable manner. We have qualitatively and quantitatively evaluated the angiogenic response to VEGF and also have evaluated the angiogenic response after direct VEGF infusion in the adult cortex. The results show that VEGF administration to brain tissue *in vitro* greatly enhances vascular proliferation in a dose-dependent manner and differs markedly from the effects of bFGF both in degree and vascular

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Abbreviations: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; BBB, blood–brain barrier; CNS, central nervous system; RT-PCR, reverse transcription–PCR.

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conformation. *In vivo*, exogenous VEGF produces significant cerebral angiogenesis as well as the immunoexpression of the *flt-1* receptor on adjacent reactive astrocytes.

MATERIALS AND METHODS

Explant Cultures. Pregnant Wistar dams (E18–19) were anesthetized with ketamine (60 mg/kg)/Rompun (6 mg/kg). Fetuses were removed individually under aseptic conditions, and the neocortex was dissected in ice-cold Earle's balanced salt solution (EBSS; GIBCO). Newborn and young adult rats were overdosed with Nembutal (0.1 ml/100 g body weight). Explant cultures were prepared by using a slight modification of the method of Stoppini (20). Four hundred-micrometer slices were prepared by using a Stoelting manual tissue slicer. Slice explants were placed on Millicell-CM culture plate inserts (Millipore), which were placed in culture dishes with 1 ml of serum-free medium consisting of Neurobasal medium (a modification of DMEM F12) with B27 supplement (GIBCO) and 0.025 mM glutamate, and 0.5 mM glutamine was applied to the dishes. A 1-μg/ml stock solution of human recombinant VEGF (Sigma) was made up in 0.1 M PBS, pH 7.4, with 0.1% BSA. VEGF was added to the medium at dosages of 1, 10, 25, 50, or 100 ng/ml. In a separate set of experiments, human recombinant bFGF (Sigma) was added to the medium at doses of 0.1, 1.0, 5.0, 10, or 20 ng/ml (21). Control explants received vehicle (0.1 M PBS with 0.1% BSA) alone. To determine the specificity of potential VEGF effects, a polyclonal neutralizing antibody to VEGF (R & D Systems) was added to some of the cultures at the 10-, 25-, and 50-ng/ml doses. Each dose of recombinant VEGF was incubated for 1 hr at room temperature with the appropriate neutralizing antibody concentration, i.e., 12, 22.5, and 45 μg/ml as indicated by R & D Systems.

All explant cultures were incubated at 37°C with 5% CO_2 and 95% air for 3 days (normoxic conditions), after which the explants were processed for qualitative and/or quantitative analysis.

Immunocytochemistry. Explants were fixed overnight in 4% paraformaldehyde fixative in 0.1 M sodium cacodylate buffer with 3% sucrose added, pH 7.2. After washing in TBS, whole explants were processed for immunocytochemistry with the following antibodies: laminin (polyclonal; Sigma, 1:200); collagen type IV (polyclonal; Sigma, 1:2,000), GLUT-1 (polyclonal; East Acres Biologicals, Southbridge, MA, 1:200), *flt* (polyclonal; Santa Cruz Biotechnology, 1:200), *flk-1* (polyclonal: Santa Cruz Biotechnology, 1:100–2,000 or Sigma monoclonal, 1:200–1,000), and GFAP (monoclonal; Chemicon 1:1,000). All antibodies were diluted in 0.05 M TBS containing 1% normal goat serum. Explants processed for GLUT-1 immunocytochemistry were dehydrated and then rehydrated by passing them through a series of methanol solutions (50–100%). All explants, except those being processed for GLUT-1 immunocytochemistry, were blocked for endogenous peroxidase activity with 3% peroxide in 10% methanol. The explants then were incubated with the appropriate dilutions of primary antibodies for 48 hr at 4°C. After washing with 0.5 M TBS, the explants were incubated in the appropriate secondary antibody for 30 min, followed by exposure to peroxidase–anti-peroxidase. Visualization of the reaction product was done by using either diaminobenzidine (DAB) alone or by nickel intensification of the DAB reaction product. For fluorescence microscopy, primary labeled sections (GFAP and *flt-1*) were exposed to a solution of fluorescein isothiocyanate-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG for 60 min at room temperature and studied in the Bio-Rad 1000 laser confocal microscope.

Image Analysis. Semi-quantitative analysis of angiogenic activity was accomplished by digitizing the images of laminin-immunostained explants by using the IMAGE-PRO image analysis system. The contrast was adjusted manually so that the labeled blood vessels were above threshold, whereas the unstained regions were below threshold. Six regions within each image (explant, $n = 8$) were sampled by using a per area ratio measurement. The data were analyzed by the Mann–Whitney test to determine significant differences between control and VEGF-treated explants.

[^{3}H]Thymidine Application. To monitor endothelial proliferation within the explants, some were incubated with 0.2 μCi/mmoll [^{3}H]thymidine (40–60 Ci/mmol; Amersham) for 3 days. After washing with PBS, the explants were immersed in 4% paraformaldehyde fixative in 0.1 M sodium cacodylate buffer, pH 7.2. After overnight fixation at 4°C, the explants were rinsed in 0.1 M cacodylate buffer, dehydrated through a series of alcohol solutions, and processed for paraffin embedding. The explants were sectioned at 7 μm and immunocytochemically stained for the basal lamina component laminin. The slides were then dipped in NTB2 emulsion (Kodak) and developed after a 3.5-week exposure period.

Reverse Transcription–PCR (RT-PCR). Total RNA was isolated (Purescript, Gentra Systems) from control and VEGF-treated E18 cortical explants. One microgram of total RNA from each sample was reverse-transcribed and amplified by PCR (Perkin–Elmer) and resulted in a 286-bp PCR product. Samples were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. The *flk-1* primers and PCR conditions used here have been described previously (22).

Minipump Infusions of VEGF. Osmotic minipumps designed to deliver 1 μl/hr for 7 days (Model 2001, Alza) were filled with 0.05 μg/ml recombinant VEGF (Sigma) in PBS, pH 7.1, and each was attached to a brain infusion cannula. Control pumps contained PBS alone. The filled pumps were incubated overnight in sterile saline at 37°C. Young adult Wistar rats were anesthetized with ketamine (60 mg/kg)/xylazine (5 mg/kg). After a skin incision over the right side of the skull, a pocket was formed over the neck and shoulder blades to hold the minipump. A 2-mm hole was drilled in the skull 1 mm posterior to the coronal suture and 5 mm lateral to the sagittal suture. The cannula was placed in the cortex at a depth of 3 mm, and a sterile anchor screw was inserted. The cannula was cemented in place and the incision was sutured. After a 1-week survival, the animals were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M cacodylate buffer with 3% sucrose added. The cannulae were removed and the brains were blocked and processed for routine paraffin embedment. To visualize the vasculature, sections were immunostained with the basal lamina component laminin and the BBB marker GLUT-1. Sections were also immunostained with GFAP and *flt-1* for analysis of the astrocytic reaction to the infusions.

RESULTS

VEGF-Induced Angiogenesis. Fetal and newborn neocortical explants responded strongly to specific doses of VEGF exposure. As indicated by both laminin or GLUT-1 immunostaining, control explants that did not receive VEGF contained only a small number of small, discontinuous vascular segments (Figs. 1 and 2*a*) In contrast, there was a dose-dependent increase in the ratio of vascular area/total explant area within the explants at VEGF dosages up to 25 ng/ml. At higher VEGF concentrations, angiogenesis appeared to taper off, resulting in a decrease in vascular/total explant area at 50 ng/ml, which further decreased at 100 ng/ml (Figs. 1 and 2*c*). Qualitatively, the vascular network became increasingly complex at exposures of up to 25 ng VEGF/ml. The laminin-immunostained vessels appeared somewhat dilated, particularly in newborn explants. Numerous branch points resulted in a dense vascular meshwork (Fig. 2 *b* and *e*) with many of the vessel segments labeled with [^{3}H]thymidine (Fig. 2*h*). In

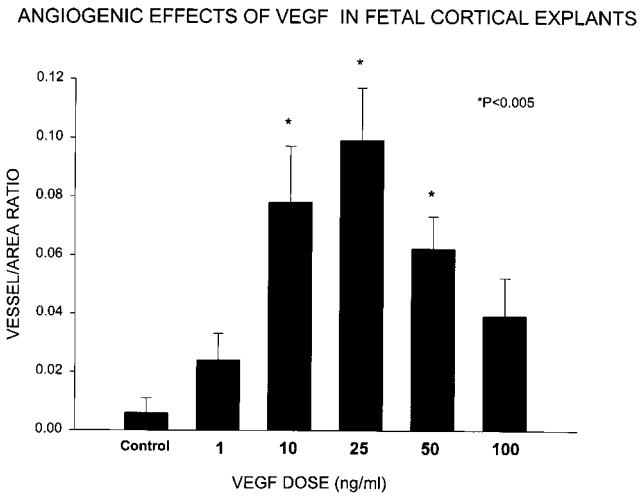

FIG. 1. Analysis of vessel profile area within six sampled areas of fetal cortical explants ($n = 4$). Significant angiogenic values peak at the 10- to 25-ng dose and then taper off. Note that the control ratio is markedly lower than in the adult explant (see Fig. 3).

explants in which neutralizing antibody to VEGF was added to the media, laminin or GLUT-1 immunostaining of the vasculature was undetectable at all dosages (Fig. 2*d*).

Interestingly, GLUT-1 immunostaining, which depicts the BBB-competent vascular phenotype, did not directly correspond to laminin immunostaining. In control fetal explants, a small number of vessels appeared to have light, patchy staining. The greatest number of vessels were immunolabeled at the 1-ng/ml dosage, and the staining pattern corresponded roughly to that seen with anti-laminin at this dose (Fig. 2*f*). However, at the 10- to 50-ng/ml dosages, a much smaller number of GLUT-1-immunostained vessels were observed primarily at the edges of the explants. There was no detectable staining at 100 ng/ml. *flt-1* receptor immunostaining was not detected in control explants but showed patchy expression in the vascular network up to the 25-ng/ml dosage of VEGF (Fig. 2*g*) after which it was reduced. No immunoexpression for *flk-1* was found with the commercial antibodies we used. However, *flk-1* was clearly present at all VEGF doses as shown by RT-PCR methods with possible increased expression over control (Fig. 3).

In adult cortical explants VEGF administration also showed a significant quantitative increase in vessel/area ratio but only at the 10- and 25-ng/ml dosage (Fig. 4). In contrast to the fetal explants, vessel profiles in non-VEGF-treated adult explants were depicted easily by laminin immunostaining. These vessels had a reasonably normal *in vitro* appearance in that they consisted of small, somewhat flattened segments (Fig. 5*a*). However, the addition of VEGF, particularly again at the

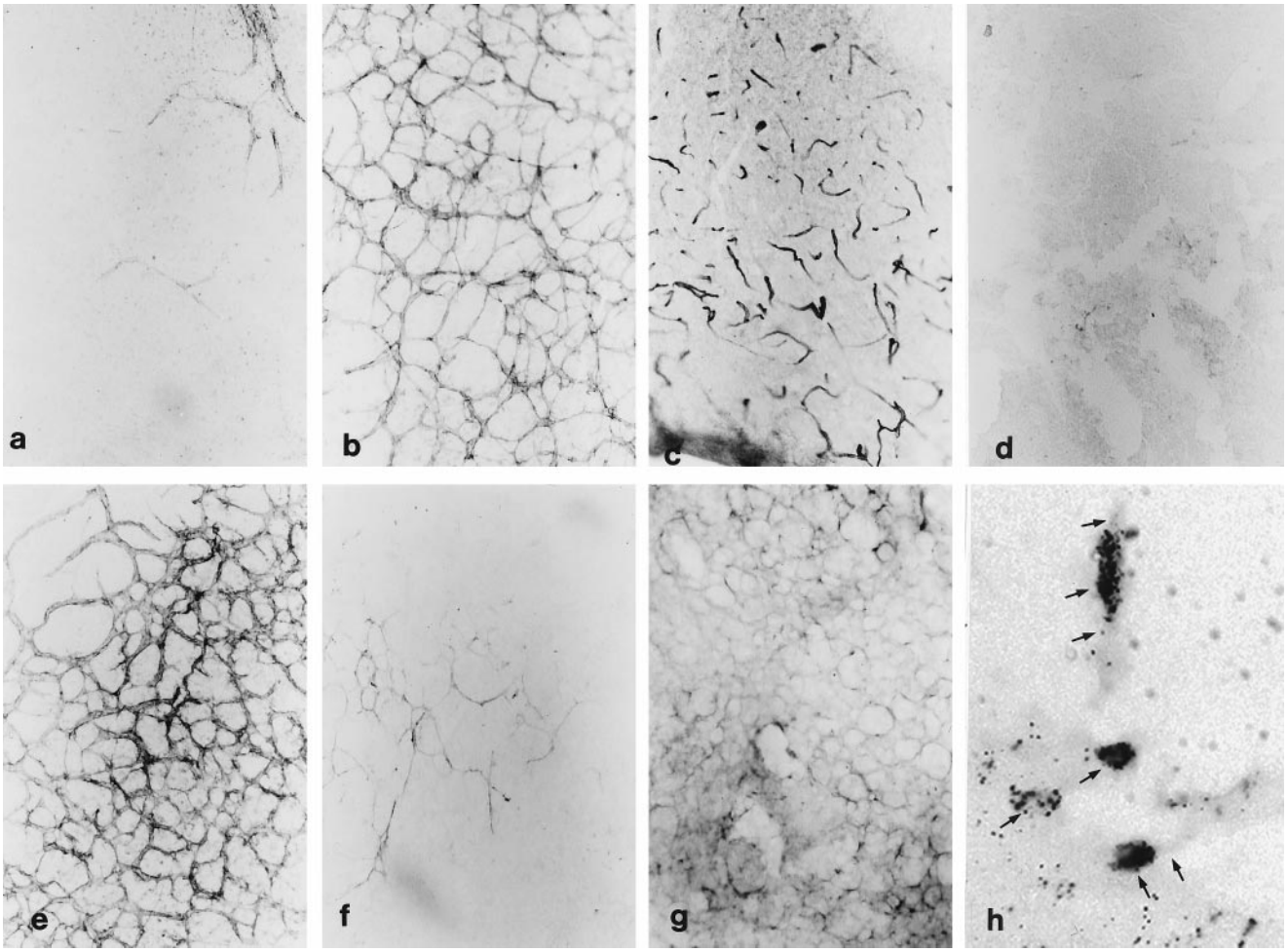

FIG. 2. Depiction of fetal cortical explants after VEGF treatments. Immunostaining for laminin (*a–e*) shows only a few vascular profiles in control explant (*a*) whereas the 25-ng dose produces a significant angiogenic network in fetal (*b*) and newborn (*e*) (nickel intensification) explants. At the 100-ng dose angiogenesis is tapered off (*c*) (nickel intensification) and addition of neutralizing antibody to VEGF abolishes all angiogenic effects (*d*). Immunoexpression of GLUT-1 depicts substantially fewer vessel profiles than laminin and is most prominent at the 1-ng dose (*f*) whereas patchy immunoexpression of the *Flt-1* receptor is found on most vessels at the 25-ng dose (*g*). After [^{3}H]thymidine administration, anti-laminin (+) vascular profiles are labeled in a 7-μm paraffin section (arrows in *h*). All micrographs except *h* are identical magnification.

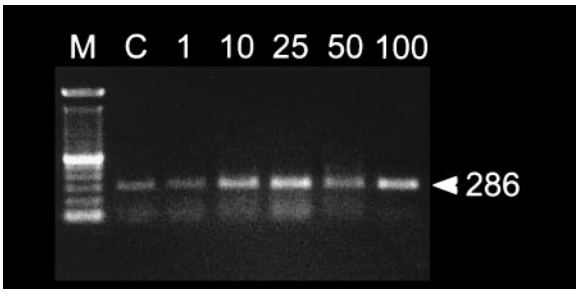

FIG. 3. RT-PCR analysis of *flk-1* mRNA expression in E18 cortical explant cultures incubated with VEGF doses for 3 days. Each lane was loaded with 10 μl of PCR product. M, marker (100-bp DNA ladder, Life Technologies); C, control explant, which received no VEGF. Size of expected *flk-1* fragment, in bp, is at right.

25-ng dose, produced what appeared to be a great expansion of the vascular network. The vascular profiles were much longer, formed large arching palisades, and were much more rounded, possibly dilated (Fig. 5*b*). Although the adult explants had a significant increase in vessel area after VEGF treatment at the 10- and 25-ng/ml dose, it was qualitatively different from the treated fetal or newborn explants in that the adult explants lacked the unique vascular network or "geometric" appearance (i.e., Fig. 2*e*). As in fetal explants, GLUT-1 immunostaining in the adult VEGF-treated explants was not comparable to the laminin immunostaining (data not shown).

bFGF-Induced Angiogenesis. In all experiments bFGF exposure produced substantially fewer vessels than VEGF exposure and none of the area measurements were statistically significant. In most explants, there appeared to be a distinct morphological difference in network/branching between bFGF- and VEGF-induced vasculature. Fetal explants were most responsive to the 1-μg bFGF administration and produced a sparse network of longer, practically straight vessels that had a smaller diameter compared with the extensive vascular network produced by VEGF exposure (Fig. 6*a*).The adult explants appeared most responsive to 20 ng/ml bFGF and showed a qualitative change in the vascular patterns compared with control. The vessels in these explants also appeared to be longer and had a looping and cascading appearance (Fig. 6*b*).

VEGF Infusions *in Vivo*. The control PBS infusions produced a cavitation within the striatum, and the density of

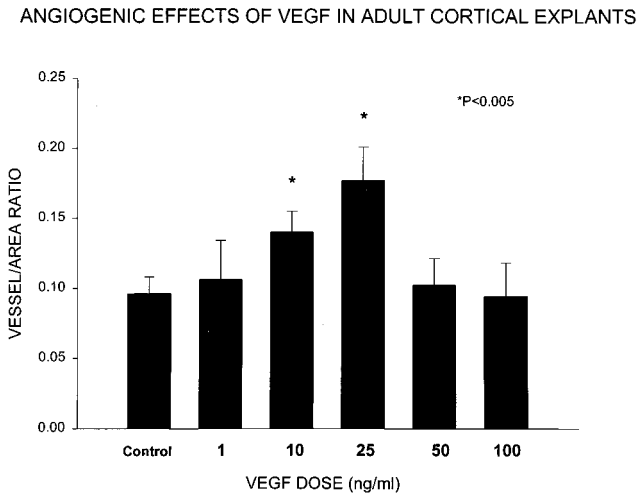

FIG. 4. Analysis of vessel profile area within six sampled areas of adult cortical explants ($n = 4$). Significant angiogenic values are found only at the 10- to 25-ng doses with other doses showing no effects. Note that the control ratio is markedly higher than in the fetal explants (see Fig. 1).

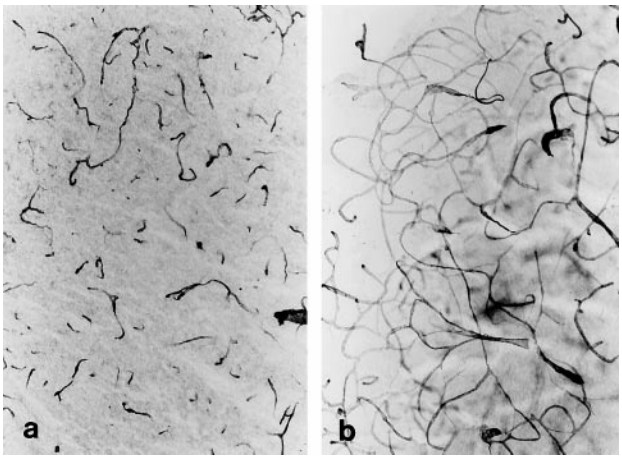

FIG. 5. Control adult cortical explant (*a*) has small, flattened vascular segments whereas the administration of the 25-ng VEGF dose (*b*) produces a significant expansion and elongation of the cerebrovascular patterns.

vessels around the infusion wound showed no measurable changes as assessed by laminin and GLUT-1 immunostaining when compared with surrounding normal tissue (Fig. 7*a*). After VEGF infusion, instead of a cavitation the infusion site was filled with what appeared to be remarkably vascular tissue as shown by laminin immunostaining. When compared with the normal cerebral vasculature, the VEGF-responsive vessels were tortuous and dilated and lacked uniform spacing (Fig. 7*b*); the extensive proliferation made it difficult to assess a vessel/area ratio. Interestingly, reactive (GFAP+) astrocytes were widespread after VEGF infusion and unexpectedly showed colocalization with *flt-1* (Fig. 7 *c* and *d*). Control infusions had a much more localized GFAP (+) population, and few cells had *flt-1* immunoexpression.

DISCUSSION

Previous studies have suggested that endogenous VEGF production is a mediator of hypoxia-initiated angiogenesis in brain tumors (7, 8), retina (23, 24), heart (25), and cell cultures (26). We present evidence that exogenous application of the growth factor directly causes angiogenesis in mammalian CNS tissue *in vitro* and *in vivo*. The explant cultures thrived in the

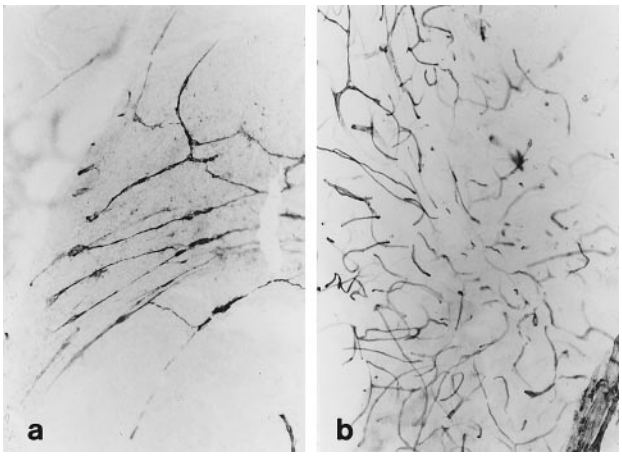

FIG. 6. Fetal neocortical explants treated with 1 ng of bFGF (*a*) produced a sparse network of elongated vessels. Adult cortical explant treated with 20 ng of bFGF (*b*) shows qualitative change in the cerebrovasculature compared with control; the vessels appeared somewhat longer with more branch points although less extensive than VEGF-treated explants.

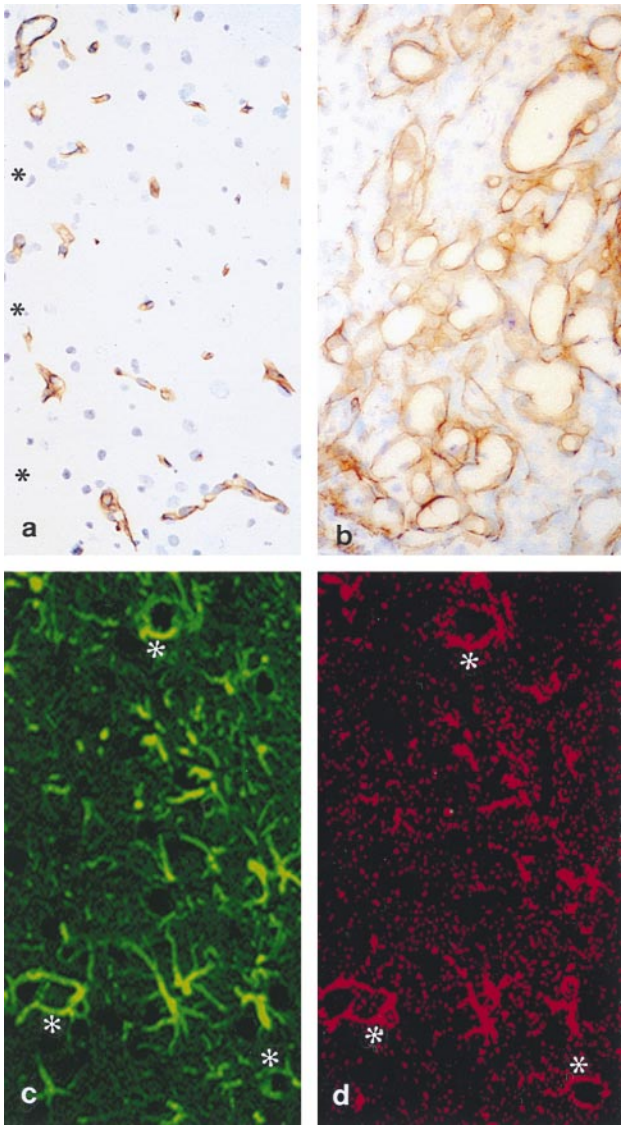

FIG. 7. Minipump infusions of VEGF into the adult cortex. Control (saline infusion) (*a*) shows no measurable changes in the cerebrovasculature as shown by laminin immunostaining along the wound edge (∗). After VEGF infusion a robust angiogenic effect produces a substantial number of tightly packed, dilated vessels (*b*). *a* and *b* are at identical magnification. The VEGF *flt-1* receptor is expressed by reactive astrocytes after VEGF infusion. Laser confocal microscopy shows that immunoexpression of GFAP (*c*) near the infusion site mostly colocalizes with the *flt-1* receptor (*d*). Note the dual labeling of perivascular astroglia (∗ in *c* and *d*).

serum-free medium supplemented only by VEGF. The significant angiogenic effects were observed in both developing and adult CNS tissues and were blocked by the application of neutralizing antibody to VEGF. The configurations of the enhanced vascular patterns were markedly different in that the developing vessels responded to the optimum dose by the formation of a regularly arranged vascular reticular network whereas adult CNS vessels became elongated and dilated and appeared to be an elaboration of the established vascular tree. The increased vessel/area ratio coupled with [^{3}H]thymidine labeling the *flt-1* receptor immunoexpression and *flk-1* expression by RT-PCR strongly indicates that VEGF is a significant mitogen for the cerebrovasculature. By contrast, the administration of bFGF, by all accounts a potent angiogenic factor (1, 2, 27, 28), produced only comparatively modest effects in explants of both developing and adult CNS tissue.

VEGF plays an important role in vasculogenesis, the *de novo* formation of a vascular network from angioblasts, because its mRNA is widely distributed in rodent tissues including brain (10, 11, 29). VEGF receptors are associated only with early developing vascular patterns and are significantly downregulated in the adult brain (10, 11). Further evidence for the early regulation of vascularization by VEGF is underscored by the embryonic lethality of VEGF gene knockouts (16, 17). VEGF injected into the early quail embryo caused malformed vessel formations in the cardiovascular system that were not considered to be mitogenic but involved cellular motility and elongation (30). Because of the profile of VEGF expression and actions in very early cerebrovascular development, the proliferation in the perinatal explants, particularly the elaborate vascular meshwork production, was surprising. Fetal brain at E18+ is considerably beyond the vasculogenesis stage at this time; although angiogenesis slowly proceeds, the capillary patterns are already set. [Angiogenesis in developing brain peaks at postnatal days 5–9 as neuronal metabolism increases (31)]. Perinatal explants have a loose neuropil and still have some dividing endothelium. These proliferating cells might provide particular targets for the exogenous VEGF, and subsequent branching and fusing of the nascent vessels produced a reticular network, not normally found in brain, that probably maximizes the vascular profiles within a given (explant) space. The exogenous VEGF might be bound to the extracellular matrix or perhaps to basement membrane components shared by endothelial cells and astrocytes. In this position, VEGF may cause endothelial mitogenic and migrational activity because it is well known that basement membrane components play an important role in endothelial cell proliferation (32, 33). The explants responded to the VEGF in a dose-dependent manner with optimal effects produced in the middle range. Although the lower doses may not have reached a threshold for receptor activation, the lack of appreciable angiogenesis at high doses suggests either a saturation or self-inhibition of the *flt-1* or *flk-1* receptors. Indeed, it is possible that VEGF and/or its receptors, after its initial activity in early vasculogenesis, subsequently may be produced only after tissue insult requiring wound healing and angiogenesis.

The significant neovascularization of the adult brain both *in vitro* and *in vivo* presents a different set of circumstances than those in developing brain. Angiogenesis in normal adult brain is practically nil, and although ^{125}I-VEGF-binding sites on adult brain vessels have been shown (34) neither the *flk-1* or *flt-1* receptors have been demonstrated on normal rat or human brain endothelium. That the VEGF application caused marked vascular growth in mature brain would suggest an induction of a receptor-mediated mechanism of either the *flt-1* [found on tumor endothelium (8)] or the *flk-1* receptor, which has been only recently demonstrated in retinal progenitor cells (35) and in the hypoxic retina (24). Interestingly, after *in vivo* infusion we found strong expression of *flt-1* on reactive astrocytes and not on endothelium as might be expected. Because perivascular astrocytes are thought to induce BBB function and have other homeostatic roles (36), they could serve some intermediary role in brain angiogenesis particularly after injury. In addition, we have found recently both VEGF mRNA and protein in activated astrocytes adjacent to brain injuries (unpublished observations).

Direct brain infusion of VEGF caused substantial vascular growth in a manner similar to the adult explants and appeared to be a far better effector of angiogenesis than intraventricular infusion of bFGF (37). After 1 month of continuous VEGF infusion, however, the vascular proliferation is not nearly as great [M. Brightman, personal communication and ref. 38], indicating that the angiogenic changes may not be permanent. A comparable mechanism of receptor saturation that we observed in the high-dose explants could also occur *in vivo*.

These findings further suggest a clue that might link VEGF and cerebrovascular angiogenesis either during development or particularly after brain injury. Secreted VEGF, produced by as yet unknown cell types, may cause an angiogenic cascade resulting in supernumerary vessels that ultimately recede. Cessation of VEGF production and/or down-regulation of VEGF receptors may be signaled when a normalized blood flow is established that meets the metabolic needs of the developing/injured brain tissue such as that which may occur with the GLUT-1 protein (39). We have observed such a scenario in the early stages after neural grafting to the brain where clusters of large vessels were found at the host–graft interface that were not present at later times (40, 41).

An intriguing aspect of this study is that the extensive VEGF-produced vascularization of brain tissue demonstrated by a structural marker (laminin or type IV collagen) was not accompanied by a concurrent increase of a functional BBB marker (GLUT-1). That the GLUT-1 immunostaining was present at VEGF doses lower than those producing optimal vascularization of the explants indicated that many of the neovessels lacked the BBB hallmark for glucose transport. In explants, it is not possible to reach conclusions about protein permeability. However, only a portion of vessels formed after *in vivo* VEGF infusions were GLUT-1 (+), and these tissues had a larger BBB deficit to serum protein than control (unpublished observations). VEGF also is believed to be a vascular permeability factor increasing endothelial cell leakage in skin, retina, and particularly within tumor tissue in response to hypoxia (6, 7, 14, 24, 42, 43). Although VEGF affects the vasculature of most all organ systems, in brain its effects on the integrity of the BBB regulatory functions are still unclear. It is possible that fine coordination of VEGF and its receptors may serve to dissociate its angiogenic and potential permeability functions. Whether VEGF plays a role in the breakdown and restoration of the BBB awaits further study, but it appears that it serves a key role in the development, control, and maintenance of brain angiogenesis.

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