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Tie Endothelial Cell-specific Receptor Tyrosine Kinase Is Upregulated in the Vasculature of Arteriovenous Malformations

ERIKA HATVA, MSc, JUHA JÄÄSKELÄINEN, MD, HARRI HIRVONEN, MD,
KARI ALITALO, MD, AND MATTI HALTIA, MD

Abstract. Arteriovenous malformations (AVMs) are congenital lesions composed of abnormal vasculature, with no capillary component, and are clinically significant due to their tendency to spontaneously hemorrhage. The mechanisms regulating the genesis and progression of these lesions are unknown. In order to study the role of angiogenesis in AVMs, we have analyzed the expression of the endothelial cell mitogen vascular endothelial growth factor (VEGF) and a novel endothelial cell-specific receptor tyrosine kinase, Tie, by in situ hybridization and immunohistochemistry in these malformations and surrounding brain tissue. We have previously shown upregulation of Tie accompanying wound healing and tumor progression. In this study, we demonstrate significantly elevated levels of Tie mRNA and protein in AVM and surrounding brain vasculature. Upregulation of VEGF mRNA was observed in the cells of brain parenchyma adjacent to the AVM, and VEGF protein was detected in this tissue as well as in AVM endothelia. Normal brain, in comparison, expressed little or no Tie or VEGF. The significant upregulation of VEGF and Tie in AVMs may indicate some ongoing angiogenesis, possibly contributing to the slow growth and maintenance of the AVM, and could be of potential use in the therapeutic targeting of these lesions.

Key Words: Angiogenesis; Arteriovenous malformation; Blood vessels; Brain; Tie; VEGF

INTRODUCTION

Vascular malformations of the central nervous system are generally held to be congenital disorders. They are thought to result from aberrant differentiation of the mesoderm during embryonic development, leading to lesions that bear morphological resemblance to the early anastomotic plexuses formed during CNS vascular embryogenesis (1). Clinically, the most significant group is the arteriovenous malformations (AVMs), as they are commonly associated with intracranial hemorrhage (2, 3). AVMs are microscopically characterized by the absence of a capillary network, and are composed of abnormally developed dilated arteries and veins. Many of the abnormal vascular characteristics are due to direct shunting of blood at arterial pressures, resulting in arterialized veins with walls thickened by fibroblast proliferation (3–5). It has been suggested that AVMs are composed of relatively mature vessels, as they express laminin but not fibronectin, whereas another type of vascular malformation, cavernous hemangiomas, are composed of dilated immature vessels (3) which express fibronectin but no laminin (6). Few studies have been carried out on the basic biology of these lesions.

A variety of growth factors, both stimulatory and inhibitory, regulate CNS vascular development during embryogenesis in a process which includes vasculogenesis (7), the formation of blood vessels de novo, and angiogenesis, the sprouting of blood vessels from pre-existing vasculature (8). One of the critical factors is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) (9–14), a secreted dimeric glycoprotein that acts as a highly specific mitogen for endothelial cells. During CNS embryogenesis, VEGF is synthesized in the ventricular neuroectoderm and secreted into the surrounding vascular plexus, resulting in the sprouting of capillaries towards the neuroectoderm (15). Abundant VEGF expression and endothelial cell proliferation are key features in the developing embryonic brain. In the adult brain, however, VEGF is significantly downregulated and endothelial cells remain quiescent (15, 16). Under pathological conditions such as tumorigenesis, high VEGF levels are accompanied by uncontrolled endothelial proliferation (17–22). Two novel VEGF-related growth factors, VEGF-B (23), with unknown receptor, and VEGF-C, which binds FLT4/VEGFR-3 (24), have recently been discovered.

We have identified a novel endothelial-cell-specific receptor, Tie, a tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (29), and an unknown ligand. Tie is an early endothelial cell marker highly expressed in developing embryonic vasculature (30) and downregulated in adult tissues, but induced during physiological (30, 31) and pathological (26, 27, 33) neovascularization. Tie knockout studies in mice show disruption of endothelial cell integrity and no microvessel

From the Neurobiology Laboratory (EH, MH) and the Molecular/Cancer Biology Laboratory (EH, KA), Haartman Institute, and the Department of Neurosurgery (JJ), University of Helsinki, P.O. Box 21, Haartmaninkatu 3, SF-00014 Helsinki, Finland, and the Departments of Medical Biochemistry (HH) and Neurology (HH), University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland.

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Correspondence to: Matti Haltia, MD, Neurobiology Laboratory, Department of Pathology, University of Helsinki, P.O. Box 21, Haartmaninkatu 3, SF-00014 Helsinki, Finland.

TABLE 1
Human Tissue Specimens for In Situ Hybridization and Immunohistochemical Analysis

#	Tissues	Sex	Age	Bleeding*	Previous treatment
1	AVM (P)	F	31	no bleeding	none
2	AVM (C)	F	45	no bleeding	none
3	AVM (P)	M	42	at 6 days	none
4	AVM (P)	M	37	at 9 days	none
5	AVM (C)	F	46	at 13 days	none
6	AVM (C)	M	63	at 13 days	none
7	AVM (P)	M	34	at 18 years	1. none
				at 18 days	2. operation (sample)
8	AVM (P)	M	35	at 30 days	none
9	AVM (C)	M	31	at 4 years	1. operation
				at 99 days	2. embolization + removal 7 d later (sample)
10	CH	F	30	no bleeding	none
11	CH (P)	F	40	no bleeding	none
12	CH (P)	F	41	no bleeding	none
13	Brain cortex	M	11	—	—

AVM = arteriovenous malformation; CH = cavernous hemangioma; P = paraffin sample; C = cryostat sample.
*: Time from bleeding to operation.

formation, culminating in embryonic death (32), suggesting a critical role for Tie in embryonic vascular development.

We and others have analyzed the expression of vascular growth factors and endothelial cell-specific receptors in CNS tumors (18, 25–28) and observed upregulation of VEGF with increasing tumor grade or vascularity, with the highest expression in glioblastomas (18, 26) and capillary hemangioblastomas (19, 20, 27), respectively. It is unknown whether angiogenesis occurs in arteriovenous malformations or contributes to the genesis of these lesions.

In this study we have examined the expression of VEGF and Tie in developing human embryonic brain, normal adult brain and arteriovenous malformations, and correlated these results with proliferative activity.

MATERIALS AND METHODS

Human Tissue Specimens

Fresh surgical samples (Table 1) of 9 arteriovenous malformations (AVMs), seven of which had bled, and 3 nonbleeding cavernous hemangiomas (CHs) were obtained from the Department of Neurosurgery, Helsinki University Central Hospital. Five AVMs and 2 CHs were processed as paraffin samples, and 4 AVMs and 1 CH were immediately frozen in liquid nitrogen after surgical excision and stored at −70°C. A neuropathologist (MH) assessed hematoxylin/eosin-stained sections, and most malformation samples were found to include some adjacent brain tissue. A control brain tissue sample (Table 1) was obtained during functional hemisphaerectomy for intractable epilepsy.

Fetal brain tissue was obtained from therapeutic abortions at 17 to 20 weeks of gestation, with the approval of the Joint Ethical Committee of the Turku University Central Hospital and the University of Turku (34). An adult cortical brain specimen was obtained from a patient operated for an arteriovenous malformation (35). RNA was extracted by the guanidine thiocyanate protocol (36).

In Situ Hybridization

The Tie cDNA template was generated by subcloning a 2.2 kb fragment encoding the Tie receptor extracellular domain (nucleotides 1–2190) into pGEM 7Z (−). The plasmid was then linearized with Pvu II or Sma I and, with incorporation of (^{35}S)-UTP (Amersham) on addition of SP6 or T7 RNA polymerases (Promega Riboprobe Gemini II Core System), sense and antisense radiolabeled RNA probes were generated, respectively. VEGF probe was obtained by linearizing a pGEM 3Z f(+) plasmid containing an Eco RI fragment of VEGF cDNA (nucleotides 57–639), and by transcription with T7 RNA polymerase. After treatment with DNase I, the Tie probes were partially hydrolyzed to 400 bp fragments, whereas alkaline hydrolysis was omitted for the VEGF probes.

In situ hybridization of cryostat sections was carried out as previously described (37, 38) with the following modifications: (a) 5-μm cryostat sections were cut onto sterile slides pretreated with a solution of 2% 3-aminopropyltriethoxysilane (TESPA) in acetone, and then fixed in 4% paraformaldehyde in PBS; (b) post-hybridization washes included low stringency (2× SSC, 20mM dithiotreitol or DTT) for 1 hour (h) at 50°C and high stringency (1× SSC, 30mM DTT, 50% formamide) for 30 minutes (min) at 65°C. In situ hybridization of paraffin sections was modified as follows: (a) the proteinase K pretreatment was replaced by 10 min of microwave treatment in 10mM sodium citrate buffer; (b) the sections were washed at low stringency (1× SSC, 40mM DTT) for 1 h at 50°C and at high stringency (1× SSC, 50mM DTT, 50% formamide) for 30 min at 65°C. The slides were dipped in NTB2 emulsion (Kodak), exposed at 4°C for 2 weeks (wk) (VEGF) or 8 wk (Tie), developed in D19 (Kodak), stained with Mayer's hematoxylin and mounted in Permount.

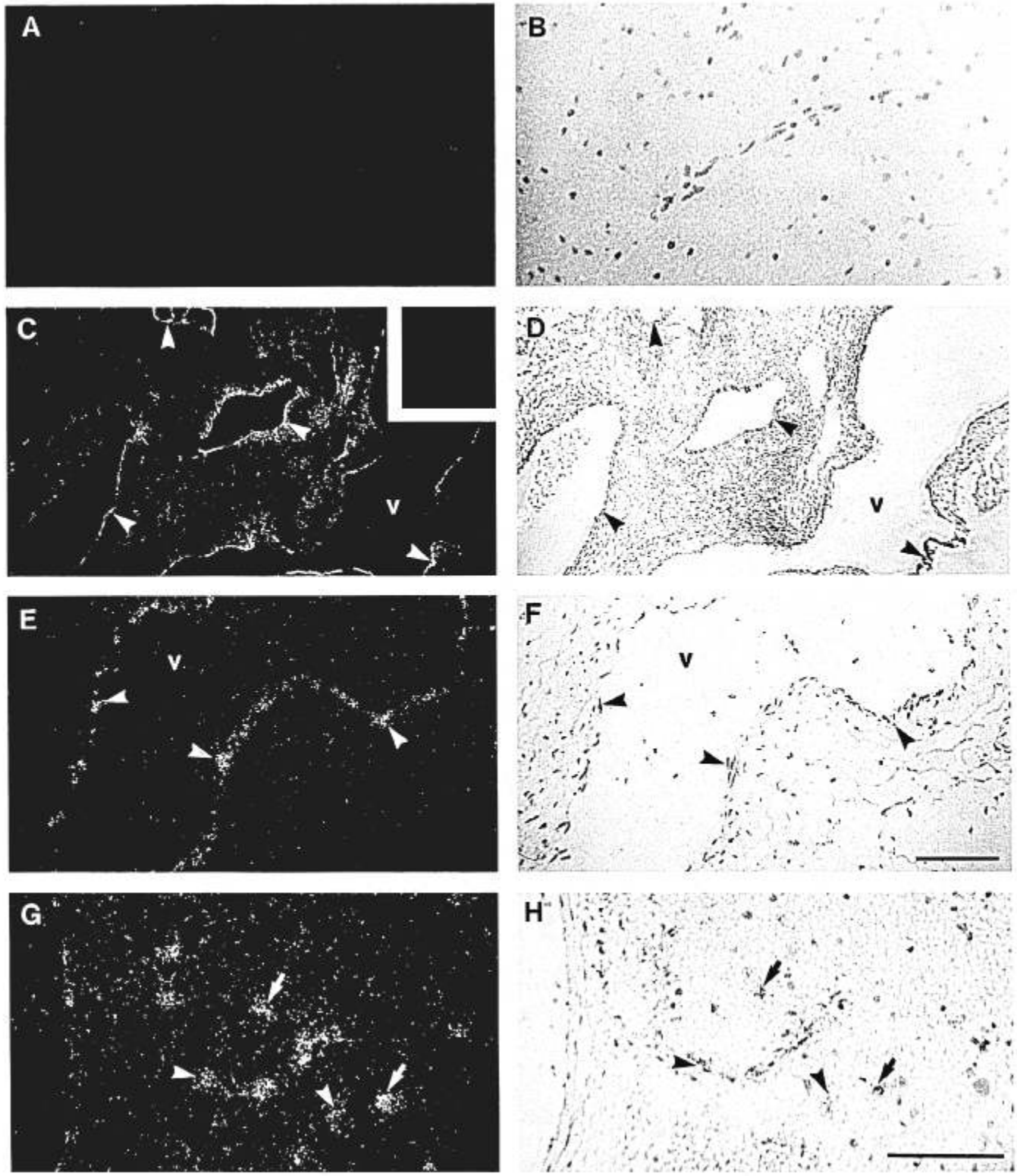

Fig. 1. Expression of Tie mRNA in arteriovenous malformations. Normal cortical brain tissue analyzed by in situ hybridization is negative for Tie (A, B) (dark field is shown on the left and the corresponding light field is shown on the right), whereas strong expression of Tie mRNA can be observed in the vasculature (v) (arrowheads, C–F) of the AVM nidus. Intense clustering of grains also occurs on endothelial cells lining the small vessels (arrowheads, G, H) and capillaries (arrows, G, H) of surrounding brain tissue. A Tie sense control is included in panel C, inset, showing no specific signal above background levels. Scale bar, A, B, E–H = 0.1 mm; C, D = 0.2 mm.

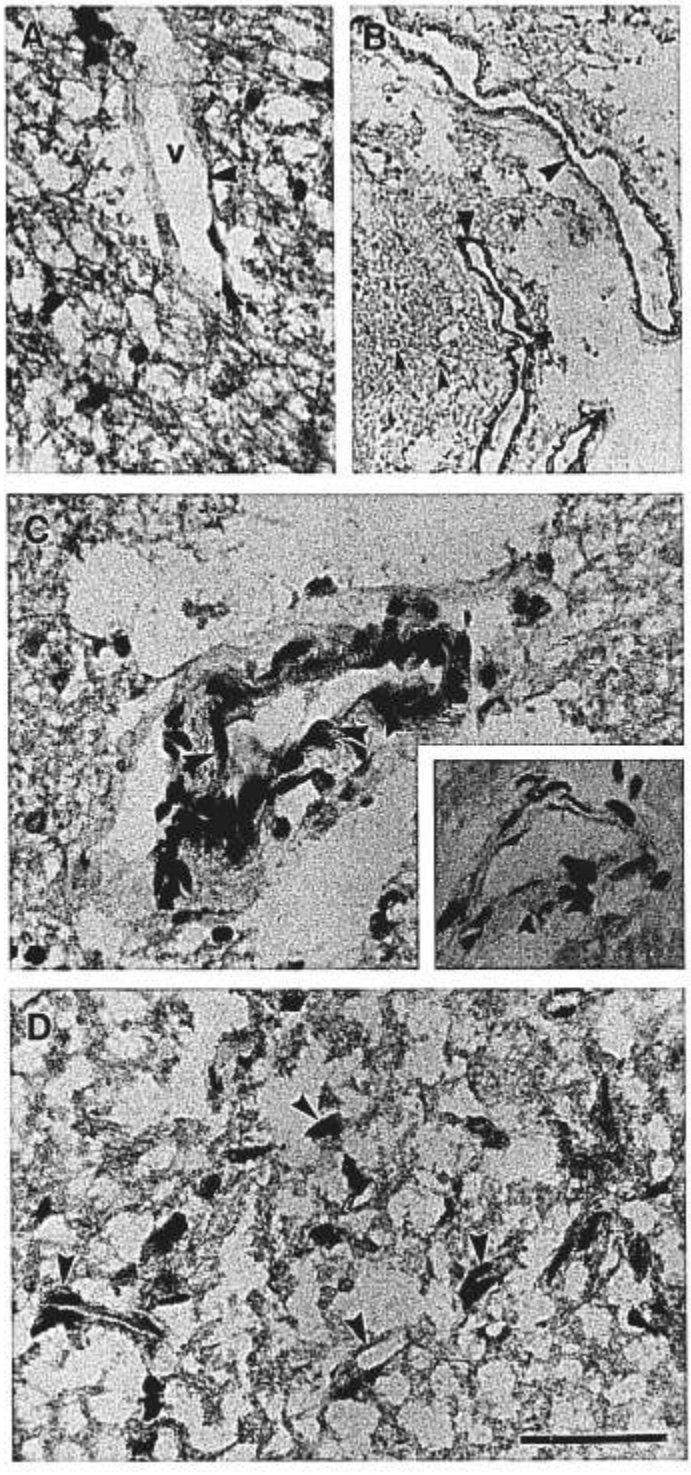

Fig. 2. Immunohistochemical detection of Tie protein. Very low levels of Tie protein are expressed in the endothelia of normal brain vessels (v) (arrowheads, A). Intense staining of the endothelia lining the large abnormal AVM vessels (arrowheads, B), and smaller vessels in the interstitial tissue (small arrowheads, B, and arrowheads, C) can be observed. Strong expression of Tie protein is also seen in the capillaries (arrowheads, D) of bordering brain parenchyma. A control staining is shown in panel C, inset. Scale bar, A, C, D = 0.05 mm; B = 0.2 mm.

Immunohistochemistry

Mouse monoclonal antibody 7E869 against human Tie extracellular domain (a kind gift from Dr Juha Partanen) was used at a 1:4 dilution for staining of 5-μm cryostat sections. Staining was also carried out with the following antibodies: mouse anti-VEGF mAb (R&D Systems Europe, UK) at 1:50, mouse anti-MIB-1 mAb against the Ki-67 proliferation antigen (Dakopatts, Denmark) at 1:500, and rabbit antibodies (Dakopatts, Denmark) against von Willebrand factor (vWF) at 1:50 (39) and CD34 at 1:50 (40). Controls were stained using normal serum or non-related anti-human mouse IgG. To detect bound antibody, the Vectastain ABC Elite biotin/avidin peroxidase stain (Vector Laboratories, Burlingame, CA) was developed with 0.2 mg/ml 3-amino 9-ethylcarbazole (AEC), 0.03% H_2O_2, 14 mM acetic acid and 33 mM sodium acetate. The sections were counterstained with hematoxylin and mounted in Aquamount. Endothelial proliferation indices were calculated as the percentage of MIB-1 positive nuclei per 500 endothelial cells.

RESULTS

Tie mRNA and Protein in Vascular Malformations

Normal cortical brain vasculature expressed little or no Tie mRNA (Fig. 1A, B), as previously described (26). All 8 AVMs analyzed expressed Tie mRNA, 6 of them at very high levels, in endothelial cells lining arteries, veins, and arterialized veins (Fig. 1, arrowheads, C–F). High expression was also observed in the arterioles, venules (Fig. 1, arrowheads, G, H) and capillaries (arrows, G, H) of adjacent brain parenchyma. In some samples, the highest levels of Tie mRNA appeared to occur in the endothelia lining arterialized veins and surrounding brain capillaries, and lower levels were seen in thin-walled veins. Tie sense probe hybridizations did not show any signal above background (Fig. 1, inset, C).

Immunohistochemistry showed strong Tie protein expression in AVM vasculature in all 4 samples analyzed. In parallel to Tie mRNA expression, little or no Tie protein was observed in normal adult cortical endothelia (Fig. 2, arrowheads, A). Intense staining for Tie was observed in the endothelial cells lining the arteries and veins of the AVMs (Fig. 2, large arrowheads, B), but also in the smaller vessels and capillaries in brain tissue bordering the malformation (Fig. 2B–D). Tie mRNA and protein codistributed with vWF and CD34 in adjacent sections (data not shown), with most vasculature within and immediately surrounding the AVMs positive for Tie. Control immunostainings for Tie were negative (inset, C).

Cavernous hemangiomas expressed Tie mRNA (Fig. 3, arrows, A–D) and low levels of Tie protein (data not shown) in their endothelia with little variation between vessels.

VEGF mRNA and Protein in Vascular Malformations

In situ hybridization showed low VEGF mRNA expression in normal cortical brain tissue (Fig. 4, arrows,

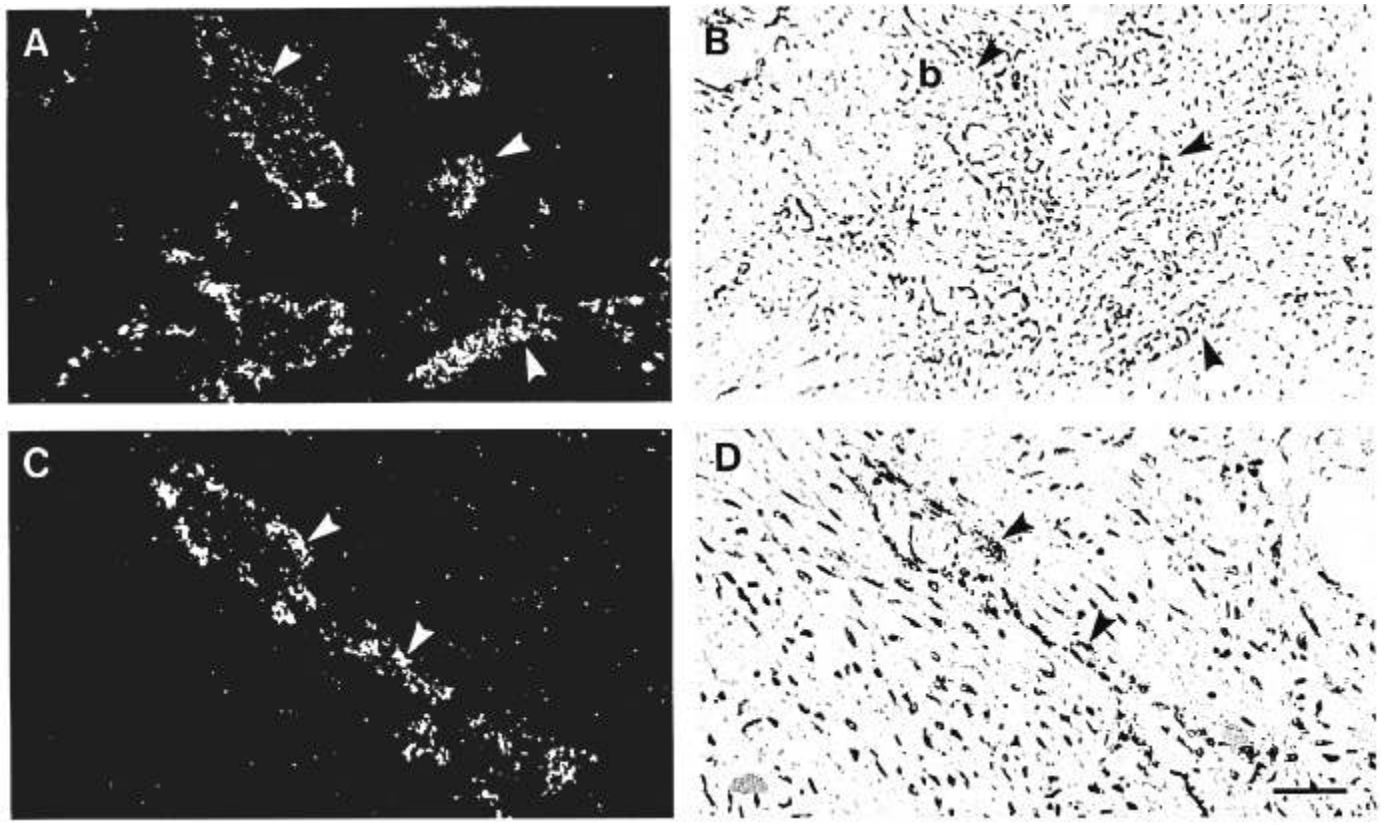

Fig. 3. Expression of Tie mRNA in cavernous hemangiomas. In situ hybridization analysis of cavernous hemangiomas (dark field on the left and corresponding light field on the right), showing that the endothelial cells lining the CH vascular spaces are strongly positive for Tie mRNA (arrows, A–D), with expression in all vessel types. Positive signal in blood cells within the vessels (b, panel B) is due to unspecific hybridization. Scale bar, A, B = 0.1 mm; C, D = 0.05 mm.

A, B), mainly adjacent to vasculature. In comparison, there was significant upregulation of VEGF mRNA in brain tissue immediately surrounding the AVM (Fig. 4, C–H), in stellate cells bordering the malformation vasculature (Fig. 4, arrowheads, E, F), and close to capillaries and small vessels (v) of the adjacent brain (Fig. 4, arrowheads, G, H). Glial fibrillary acidic protein (GFAP) staining of adjacent sections (data not shown) identified the tissue surrounding the AVM vessels as mainly gliotic, with most of the stellate cells staining positive for GFAP. The endothelia in all samples were negative for VEGF mRNA. Control hybridizations did not show any specific signal (inset, G).

Immunostaining of normal brain tissue showed very low levels of VEGF protein lining the vasculature (Fig. 5, arrowhead, A), as previously noted, whereas all 9 AVM samples expressed VEGF protein, 4 at very high levels, mainly in the cytoplasm of cells bordering small and large AVM vessels (Fig. 5, arrowheads, B, C), in a similar pattern to VEGF mRNA expression. Endothelia of the large AVM vessels expressed slightly lower levels of VEGF (Fig. 5, small arrowheads, C, D), in comparison to capillaries in brain tissue directly bordering the malformation (Fig. 5, arrowheads, D). Relatively few cells in the CH samples were positive for VEGF protein (Fig. 5, arrowheads, E), and the endothelia appeared to be mainly negative. Control sample immunostainings were negative (inset, E).

Expression of the Cell Proliferation Marker MIB-1 in Vascular Malformations

MIB-1-positive cells were detected in both AVMs and CHs, and the mean endothelial proliferation indices were 2.5% for 9 AVMs, 1.9% for 3 CHs, and < 0.5% in normal brain. Endothelial proliferation varied between and within samples, but in AVMs was mainly restricted to the arterioles, venules and capillaries of surrounding brain tissue (Fig. 6, arrowheads, A), with some positive cells in the large vessels within the nidus (Fig. 6, arrowheads, B). In some samples, single AVM vessels showed a high number of positive endothelial cells (Fig. 6, arrowheads, C) and cells in the vascular wall (arrows, C). The few MIB-1 positive endothelial cells in the CH samples occurred mainly in the large vascular channels (Fig. 6, arrowhead, D), which had low proliferative activity in the

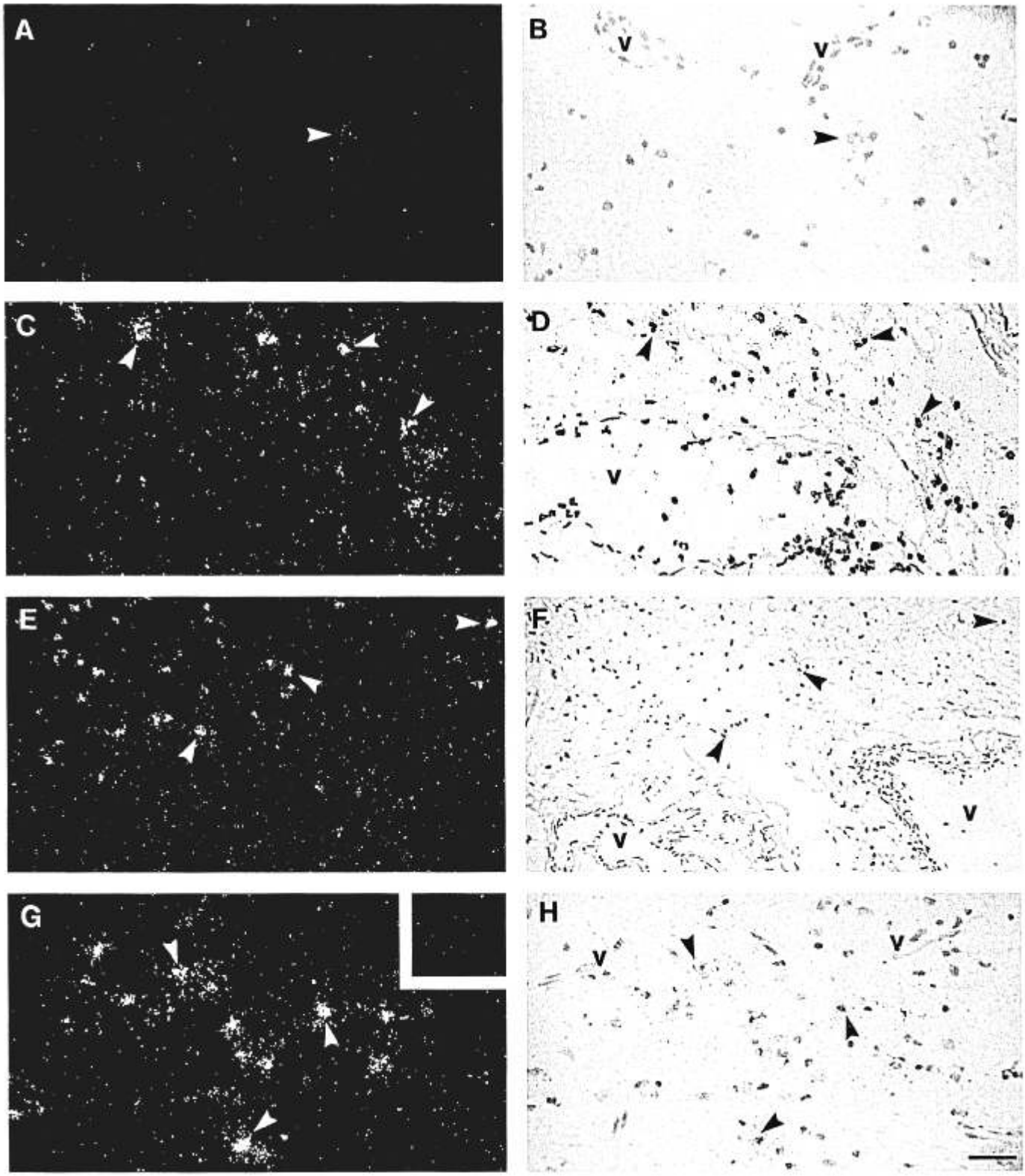

Fig. 4. VEGF mRNA expression in AVMs. In situ hybridization of normal brain tissue shows low VEGF signal in some cells adjacent to vessels (v) (arrowheads, A, B). Clusters of stellate cells with large nuclei in the gliotic tissue surrounding AVM vessels (v) (arrowheads, C, D) and bordering the malformation (arrowheads, E–H) show strong upregulation of VEGF mRNA. Endothelial cells are negative for VEGF (C–H). Sense controls show low background levels (inset, G). Scale bar, A–D, G, H = 0.04 mm; E, F = 0.1 mm.

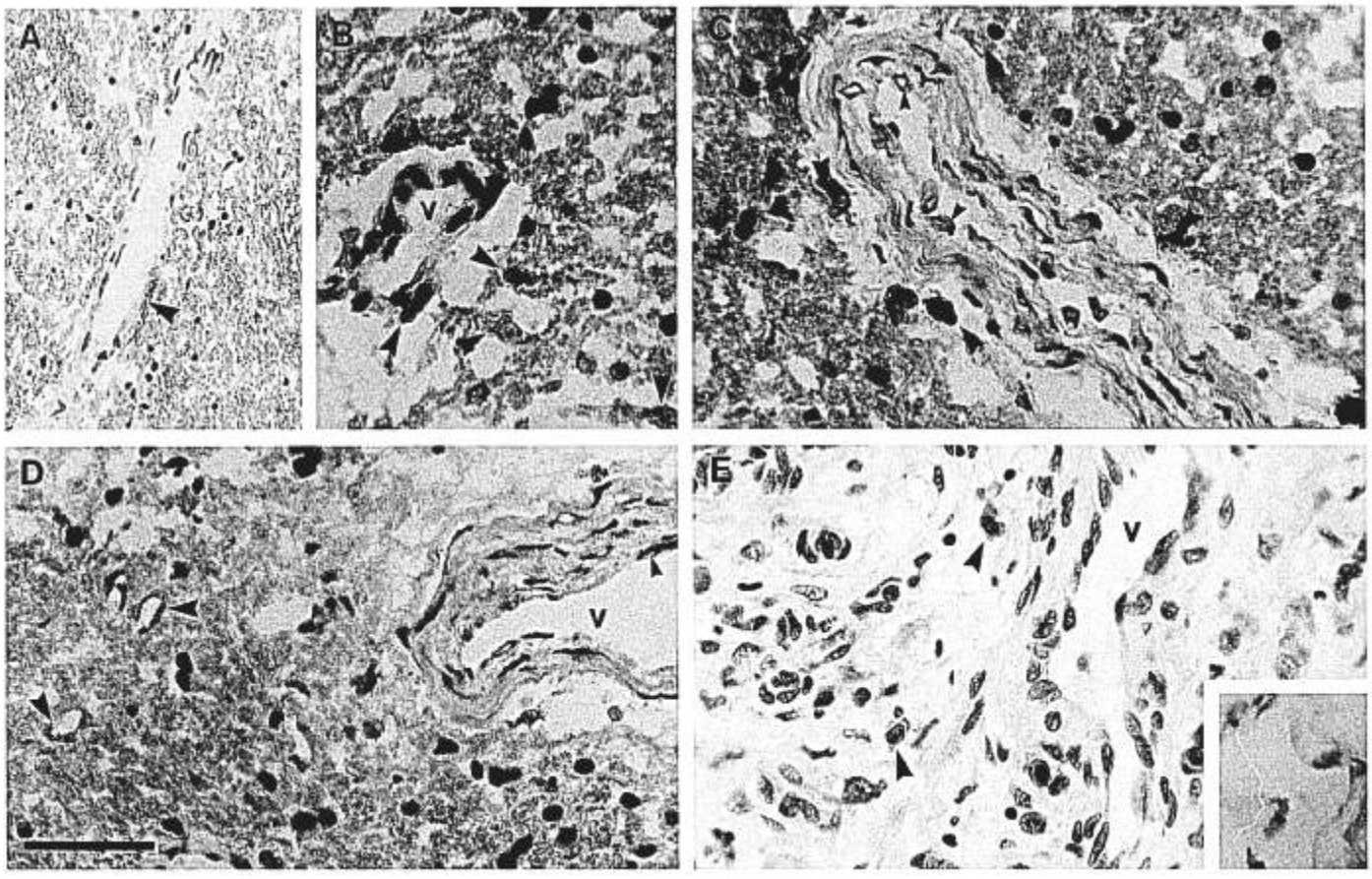

Fig. 5. VEGF protein expression in AVMs. Immunohistochemical staining of normal brain shows low levels of VEGF protein lining some vessels (arrowhead, A). Cells with large nuclei and stellate morphology in interstitial tissue bordering small vessels (arrowheads, B) and large vessels of the malformation (arrowheads, C) are strongly positive for VEGF. In addition, high levels of VEGF protein can be observed in the endothelial cells lining capillaries of brain tissue adjacent to the AVM (arrowheads, D), whereas there are relatively lower levels in the AVM vessels (v) (small arrowheads, C, D). A few cells in cavernous hemangiomas express VEGF protein (arrowheads, E) and the endothelial cells lining the vasculature (v) are mainly negative. A control staining for VEGF is included in panel E, inset. Scale bar, A = 0.1 mm; B–E = 0.05.

vessel walls (arrow, D). Little or no endothelial cell proliferation was observed in normal cortical brain.

DISCUSSION

The molecular biology of arteriovenous malformations of the brain, including the role of angiogenic factors in their pathobiology and maintenance, has not been explored. We have therefore undertaken a study of AVMs of the brain for the expression of Tie, an early endothelial cell-specific receptor tyrosine kinase, and VEGF, a highly potent vascular mitogen, both of which have been implicated in developmental and pathological angiogenesis (26, 30, 33, 41, 42). It is generally held that AVMs of the brain arise during embryogenesis as a result of aberrant vascular development (1). The treatment for cerebral AVMs currently includes operative resection, endovascular embolization and radiosurgery. Complete removal or obliteration is difficult if the AVMs are large, eloquently located, and drain into deep veins (43). Any residual malformation may slowly enlarge, and one study has shown that post-operative angiogenesis occurs in AVMs over embolization material (44).

The aberrant AVM vasculature, characterized by absence of connecting capillaries, is suggestive of an embryonic stage of vascular development, when extensive remodeling of vascular channels occurs. The Tie receptor tyrosine kinase is of critical importance during embryonic angiogenesis, as homozygous Tie knockout mice die before day 14.5. The embryonic vasculature develops, but the integrity of the endothelial cell lining is not maintained, resulting in oedema followed by hemorrhage (32, 45). Tie is highly expressed in all vessels of developing rat and mouse embryos (30, 46), including continuous and discontinuous vasculature, particularly in the meninges, cerebellum and choroid plexus, and is strongly downregulated in adult endothelia (30, 47). Northern

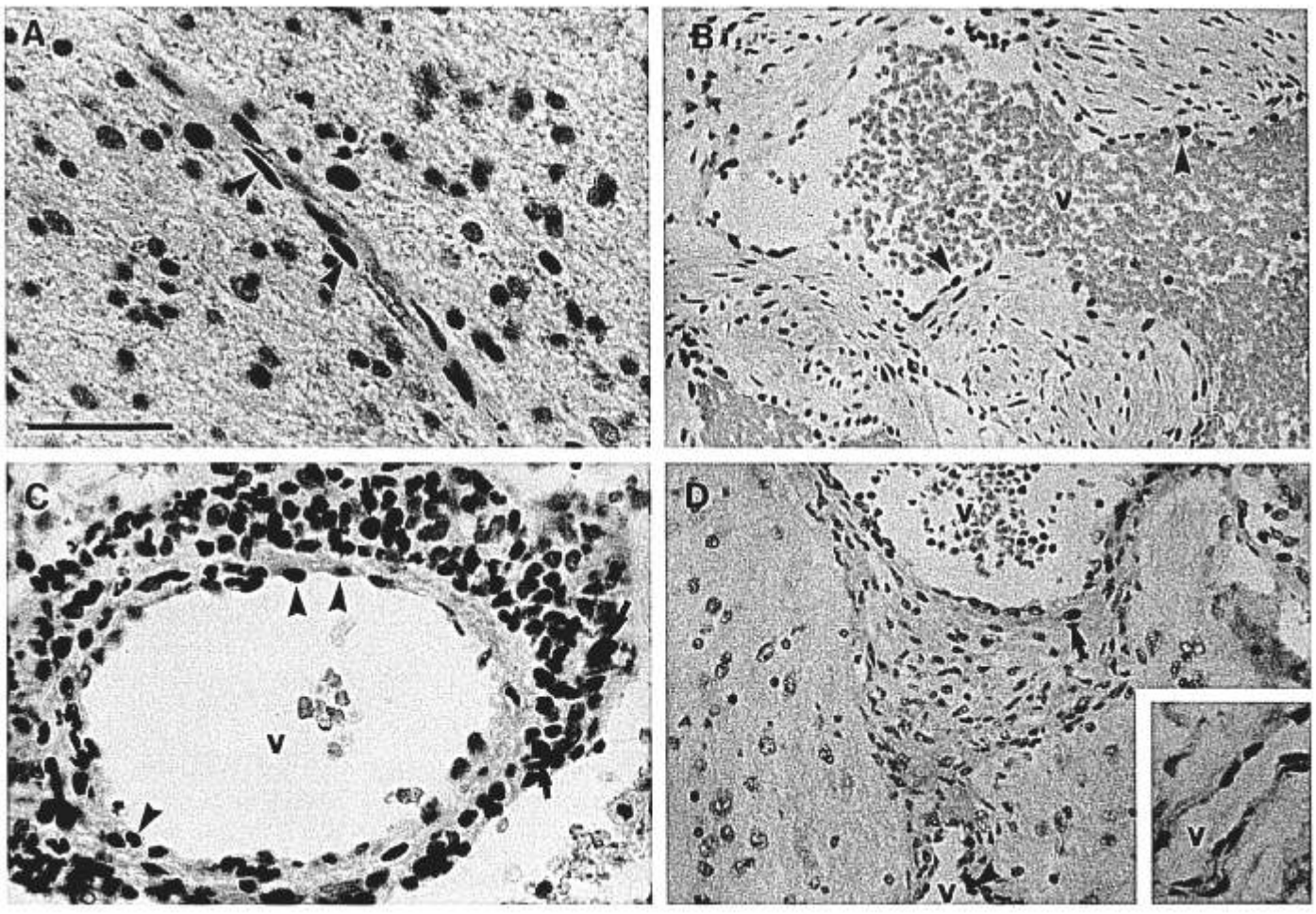

Fig. 6. Immunohistochemical detection of the MIB-1 cell proliferation marker. Endothelial cell proliferation in AVM samples (A–C) occurs mainly in capillaries of adjacent brain tissue (arrowheads, A), and to a lesser extent in large vascular channels (v) of the AVMs (arrowheads, B). Focal areas of increased cell turnover were seen in some samples in the endothelium (arrowheads, C) and vessel walls (arrows, C). This pattern can also be observed, to a lesser extent, in cavernous hemangiomas (D). Normal brain tissue shows little or no endothelial cell division (inset, D). Scale bar, A, C = 0.05 mm; B, D = 0.1 mm.

analysis of fetal and adult brain (data not shown) showed similar results, and agreed with our in situ hybridization findings of downregulated Tie expression in human adult cortex. Our results show high levels of Tie mRNA and protein in the endothelia lining all the abnormal arteries and veins of the AVM nidus, and the capillaries of brain tissue bordering the malformation. This expression gradient between the AVM and normal brain could be of potential use in therapeutic radioimmunological targeting of AVM vessels.

Although the ligand and exact function of Tie is currently unknown, the unique structure of Tie extracellular domains may indicate that it has evolved for multiple protein–protein interactions. Another indication of function may derive from analysis of the Tie receptor promoter structure, which shares a weak homology with the Tek promoter. Several domains are well conserved, including binding sites for PEA3 and ets-1, implicated in the transcription of urokinase plasminogen activator (matrix degradation) and various collagenases associated with degradation and remodeling in vessel-branching morphogenesis (47, 48). Interestingly, ets-1 shares a similar pattern of expression to that of Tie during CNS development (48). We have observed upregulation of Tie during physiological and pathological neovascularization, including ovulation and wound healing (30, 31) and very high levels of Tie in the endothelia of malignant brain neoplasms (26, 33). The high expression of Tie in AVMs and adjacent brain tissue may therefore be associated with vascular maintenance and the remodeling of vasculature during angiogenesis, and may be a physiological response to the hemodynamic stress caused by increased

flow and pressure due to arteriovenous shunting. The expression of Tie in areas peripheral to the nidus as well as within the malformation itself is an indication of the dynamism of AVMs, with possible angiogenesis and vascular remodeling occurring in surrounding brain vasculature, which may contribute to vessel "recruitment" by the malformation.

The progressive symptoms caused by AVMs may occur as a result of local change in the lesions' size and organization, possibly due to vascular proliferation. There have been few studies on angiogenic growth factors in vascular malformations. We and others have previously shown that VEGF is significantly upregulated in glioblastoma (18, 20, 26) and capillary hemangioblastoma (19, 27, 28), both characterized by abundant vasculature. In this study, we show the presence of high levels of VEGF mRNA transcripts and protein in the brain tissue within and bordering the AVM. The abnormal AVM vessels and vasculature of brain directly bordering the malformation were positive for VEGF protein, but did not express VEGF mRNA. Together, these results may indicate secretion of VEGF from reactive astrocytes and subsequent binding/activity of VEGF in AVM vessels. VEGF has been shown to be upregulated in reactive and neoplastic astrocytes (49) and in astrocytic cultures under hypoxic conditions (50). The high levels of VEGF in AVMs may be induced by hypoxia (51), as vascular thrombosis and tissue necrosis are associated with the malformation (3). VEGF upregulation could be seen as a protective mechanism against hypoxia due to shunting of arterial blood through the nidus of the AVM (steal syndrome).

Our results show specific AVM vessels with high levels of endothelial cell proliferative activity, suggesting that focal areas in the AVM and particularly in surrounding brain tissue may be the sites of active angiogenesis and vascular remodeling. The overall proliferation index of endothelia appeared to be higher in AVMs than in normal brain, possibly indicating some endothelial cell turnover and angiogenesis occurring in the AVM nidus. VEGF activity may be instrumental in the maintenance and slight increase in vasculature and size of the AVM during life. Long-term angiographic follow-up may show slow enlargement of the AVM complex, but this could also be due to dilation and elongation of the arteries and veins, with little change in the nidus.

Our findings show that, in terms of angiogenesis, arteriovenous malformations of the brain are not static lesions, but are in dynamic interaction with the adjacent brain. Further studies are necessary for a greater understanding of the pathogenesis and nature of these malformations and the role of angiogenic factors in their development and maintenance, which may lead to specific therapies for currently untreatable AVMs.

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