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Role of Vascular Endothelial Growth Factor in Blood-Brain Barrier Breakdown and Angiogenesis in Brain Trauma

SUKRITI NAG, MD, PHD, FRCPC, JENNIFER L. TAKAHASHI, BSC, AND DANIEL W. KILTY, BSC

Abstract. The role of vascular endothelial growth factor (VEGF) in blood-brain barrier (BBB) breakdown and angiogenesis, observed previously in the cerebral cortical cold-injury model, was investigated. Immunohistochemistry was used to assess BBB permeability to plasma fibronectin and to localize VEGF protein in the cortical cold-injury model over a period of 10 min to 14 days post-injury. BBB breakdown to fibronectin in lesion vessels was observed at 10 min post-injury, was maximal between 2 and 4 days and declined gradually thereafter, while occasional perilesional vessels remained permeable up to 6 days. Increased VEGF immunoreactivity occurred later— it was observed in pial vessels after 6 hours (h), and persisted up to day 14. Arterioles within the cold lesion showed VEGF immunoreactivity at 36 h, thus preceding the onset of endothelial proliferation and angiogenesis that occurred from day 3 to day 5. VEGF immunoreactivity was also observed in inflammatory cells and astrocytes. These results indicate that the immediate breakdown of the BBB in the cold lesion is unrelated to VEGF. The presence of mural VEGF in permeable pial vessels and lesional arterioles suggests that VEGF is one of several factors that mediates BBB breakdown in this model. The association of maximal VEGF immunoreactivity with endothelial proliferation and neovascularization suggests that VEGF promotes angiogenesis and repair following brain trauma.

Key Words: Angiogenesis; Blood-brain barrier; Fibronectin; Trauma; Vascular endothelial growth factor.

INTRODUCTION

Numerous angiogenic growth factors have been isolated in the past 2 decades (1). Of these, vascular permeability factor (2, 3), or vascular endothelial growth factor (VEGF) (4), is a cytokine with multiple functions, with most attention directed to its mitogenic and permeability properties. VEGF is a specific endothelial cell mitogen (5, 6) during embryogenesis (7, 8) and pathological states (9–11). In addition, VEGF can enhance endothelial permeability without leading to endothelial cell damage, being >50,000 times more potent than histamine (12). This dual function of VEGF led to studies of its role in tumor angiogenesis in both systemic (9) and brain (13, 14) tumors. Overexpression of VEGF at both the protein and mRNA level was observed in the pseudopalisading cells around areas of necrosis in glioblastomas (10, 13, 14). In addition, upregulation of the VEGF receptor (flt) mRNA was observed in endothelial cells in glioblastomas (14), suggesting a paracrine mechanism of angiogenesis through VEGF and its receptor.

There is increasing interest in the role of VEGF in the repair of non-neoplastic conditions of the brain (15, 16). The cortical cold-injury model, which is characterized by both blood-brain barrier (BBB) breakdown leading to vasogenic edema and angiogenesis at the lesion site (17, 18), is an ideal model to test the hypothesis that VEGF has a role in these processes in a non-neoplastic setting. In the present study, temporal and spatial immunolocalization of VEGF was undertaken over a period of 10 min to 14 days following cortical cold-injury to determine whether VEGF is associated with BBB breakdown and/or angiogenesis. Fibronectin immunolocalization was used as a marker of BBB permeability.

MATERIALS AND METHODS

Eighty female wistar rats (100 to 120 grams) were anesthetized by methoxyflurane inhalation. Cortical cold injuries were produced as described previously (18). Rats were sacrificed in groups of 3 along with 2 controls at 10, 30, and 60 min, 3, 6, and 12 h, and 1, 2, 3, 4, 5, 6, 7, 8, 10 and 14 days. At the time of sacrifice, rats were anesthetized by methoxyflurane. A thoracotomy was performed for perfusion of fixative via a cannula in the ascending aorta at a pressure of 110 mm Hg. The fixative contained 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). India ink was used to mark the meninges under the craniotomy site to ensure that the correct area was sampled for morphological studies. Two coronal slices of brain containing the cold-injury site were processed for paraffin sectioning using standard techniques. Sections having a thickness of 5 μm were stained with hematoxylin and eosin and adjacent sections were used for immunohistochemistry.

The indirect streptavidin-biotin peroxidase method was used and paraffin sections were incubated overnight in primary antibodies at 4°C. For VEGF and fibronectin demonstration, sections were pretreated with 0.5% pepsin for 30 min at 37°C. Rabbit antibodies and the dilutions used were: VEGF (Santa Cruz Biotechnology Inc, CA) 1:80, Fibronectin (Gibco BRL) 1:700, and glial fibrillary acidic protein (Dakopatts, Glostrup, Denmark) 1:5000. The immunohistochemical specificity of VEGF was confirmed by substituting rabbit nonimmune IgG for the primary antibody and by using anti-VEGF IgG (1 μg/ml) adsorbed with an excess of VEGF-165 peptide (25 μg/ml; Santa Cruz Biotechnology, Inc).

From the Department of Pathology, Division of Neuropathology, University of Toronto, and the Playfair Neuroscience Unit of The Toronto Hospital.

Correspondence to: Dr Sukriti Nag, Division of Neuropathology, The Toronto Hospital, Western Division, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada.

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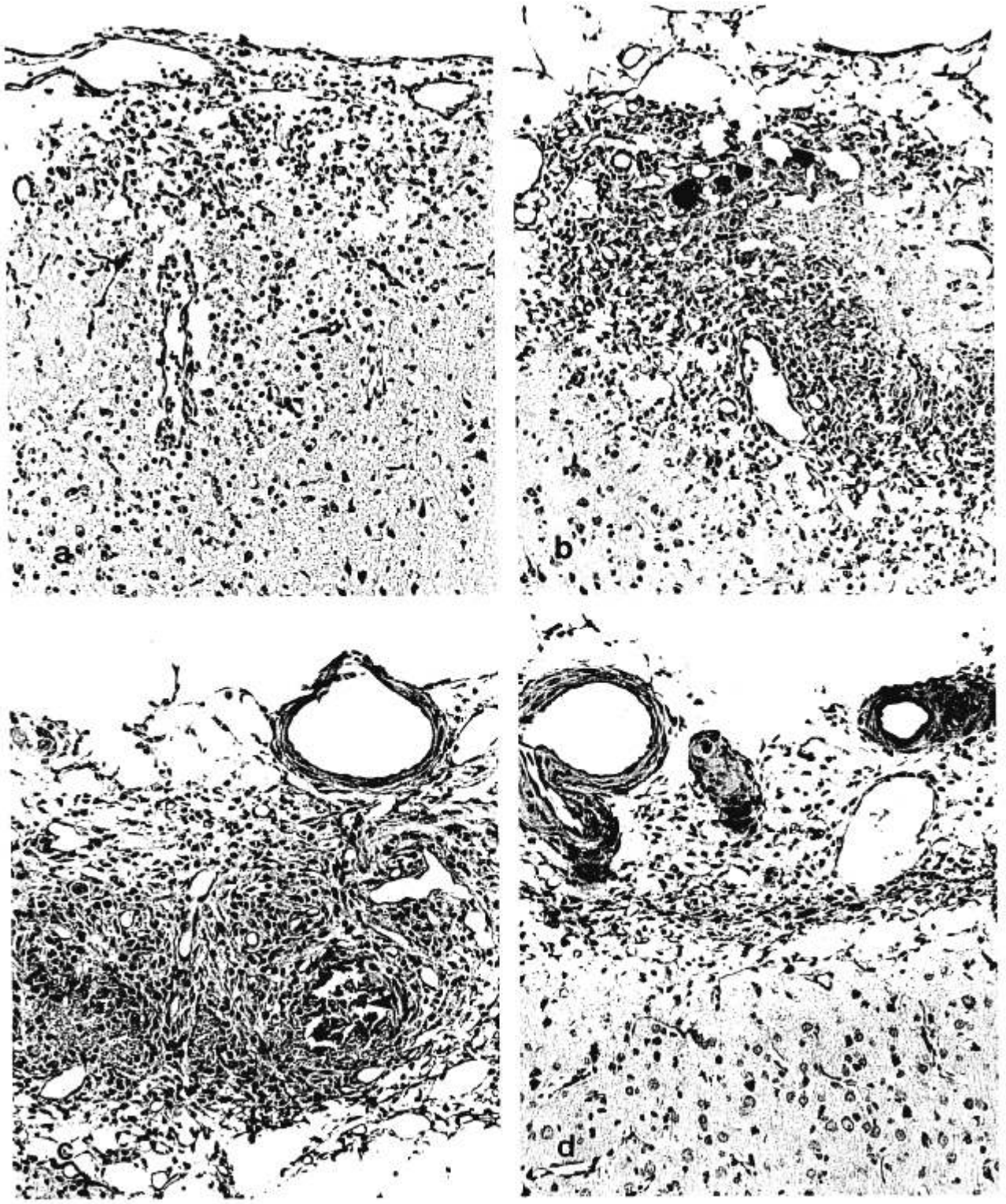


Fig. 1. Light microscopic appearance of the cortical cold injury at 2 (a), 4 (b), 6 (c) and 10 (d) days. a. At day 2, there is a mild increase in macrophages at the margin of the area of necrosis and surrounding brain. b. At day 4, there is a moderate increase in cellularity due to presence of macrophages and endothelial cells. c. At day 6, profiles of neovessels are present at the lesion site. A focal area of calcification is present (arrowhead) and there is mild medial hyperplasia of the pial artery overlying the lesion area. d. At day 10, the medial hyperplasia of the pial arteries overlying the lesion is striking. The underlying cortex appears normal. H&E stain, a–d, ×145.

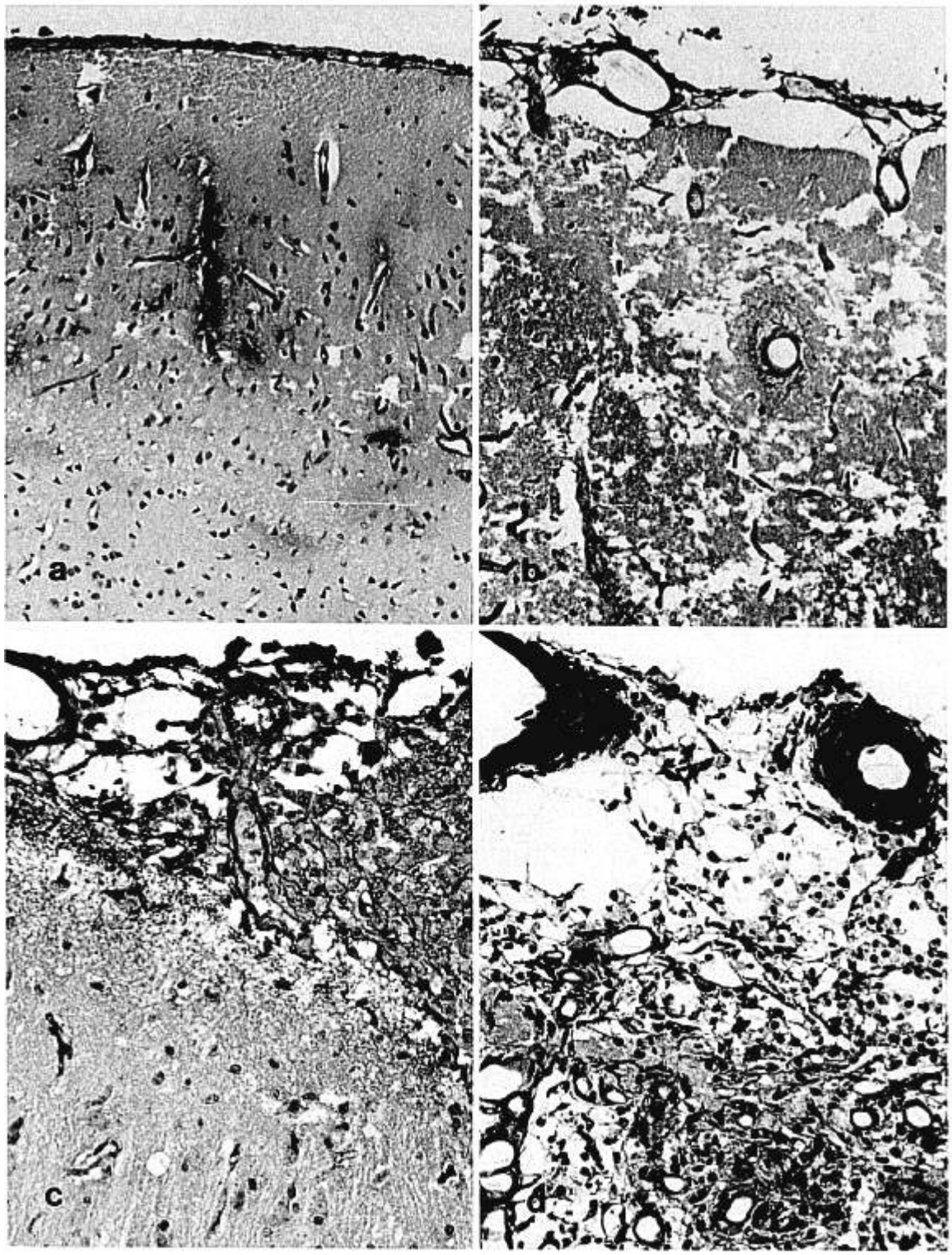


Fig. 2. Immunolocalization of fibronectin (a, b, c, d) and VEGF (e, f, g, h) in adjacent sections at 30 min, 2, 4 and 6 days following a cortical cold injury. a. At 30 min there is fibronectin extravasation from arteriolar and capillary walls at the lesion site. The same vessels fail to show immunoreactivity for VEGF (e). b. At day 2, there is extravasation of fibronectin from pial and intracerebral vessels. Only pial vessels and intracerebral arterioles show mural VEGF (f). c. At day 4, there is extravasation of fibronectin from a pial vein and arteriole and penetrating arteriole. Fibronectin extravasation is confined to the lesion area and surrounds the proliferating endothelial cells, forming a meshlike pattern. The same pial vessels show mural VEGF (g). Endothelial mitoses (arrowheads) are also present in this area (g). d. At day 6, the basement membranes of all vessels show fibronectin, including those of the neovessels. Residual extracellular fibronectin is still present at the lesion site. The pial artery and vein show marked mural thickening due to medial hyperplasia and presence of fibronectin in the extracellular matrix between the smooth muscle cells. These same vessels show VEGF in the endothelial and smooth muscle cells (h). a, b, e, f, ×125; c, d, g, h, ×250.

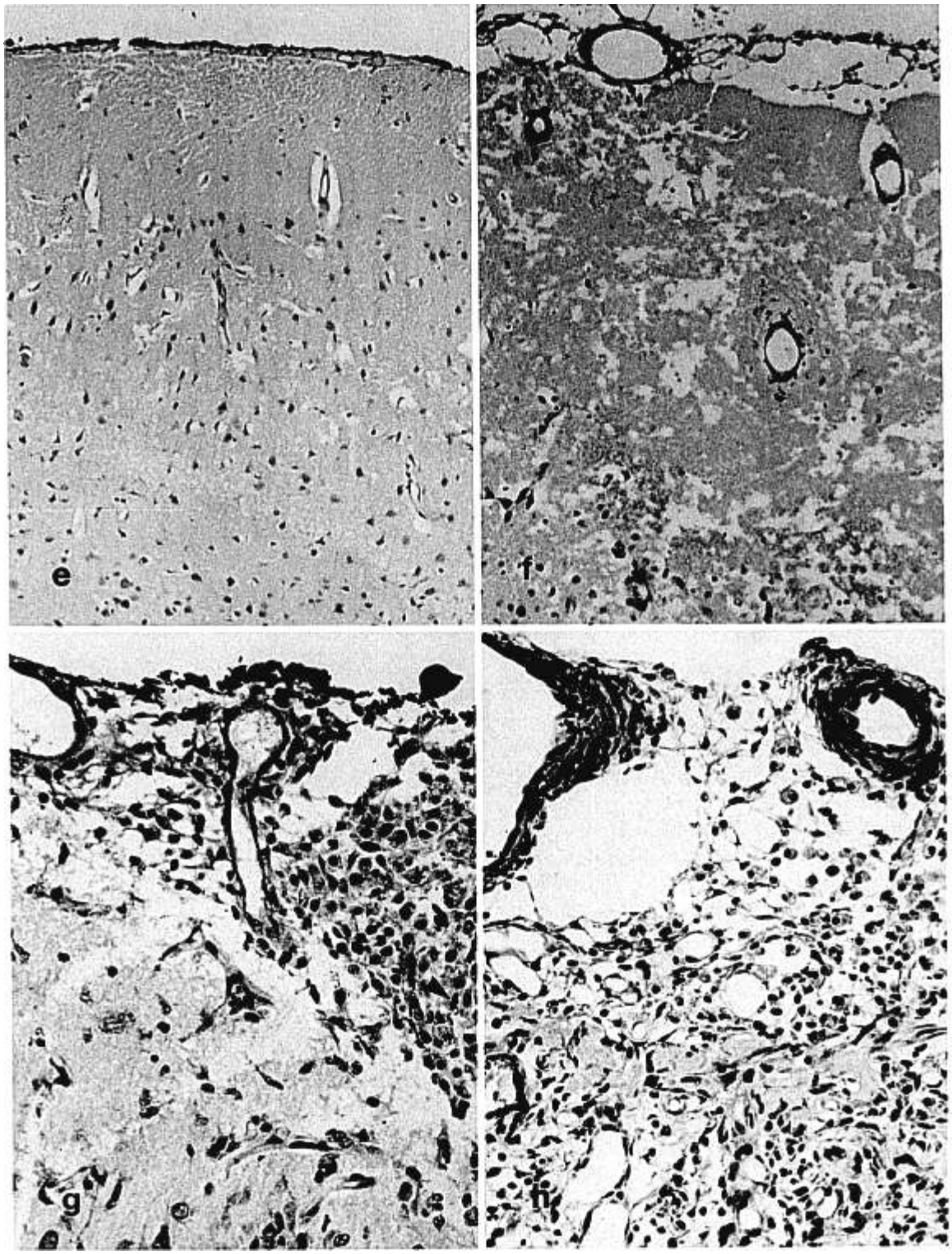


Fig. 2. Continued

Selected sections were double-labeled with VEGF antibody, and 3,3'-diaminobenzidine tetrahydrochloride was used as a substrate followed by immunostaining with anti-GFAP antibody at 1:1600 dilution for 1 h at room temperature. The substrate for the latter reaction was 3-amino-9-ethylcarbazole (Sigma-Aldrich Canada Ltd. On.), which produced an insoluble red reaction product. Following the immunohistochemical reaction, sections were stained with hematoxylin only.

The number of vessels permeable to fibronectin and those containing mural VEGF shown in Figure 3 were counted from sections immunostained with fibronectin and VEGF, respectively. Counts were done at the light microscopical level at a total magnification of 100×. In each rat, 3 microscopic fields were used for the counts—the right and left margins and the center of the cold lesion. Only pial arteries, veins and intracerebral arterioles were counted, as most of the microvessels (capillaries and venules) in the lesion and perilesional areas were permeable to fibronectin but rarely showed mural VEGF. The intensity of immunostaining and changes in cellular numbers were assessed semiquantitatively in a blinded manner by assigning the following scores: -, no change; 1+, mild increase; 2+, moderate increase; 3+, marked increase. This scoring was used to prepare Figure 4.

RESULTS

Control Rats

Fibronectin: Marked immunoreactivity was observed in the coagulated serum proteins in the lumen of occasional poorly perfused vessels, the pia-arachnoid membrane and endothelial basement membranes of pial vessels, while only mild immunoreactivity was observed in the endothelial basement membrane of intracerebral vessels. The BBB was intact and leakage of plasma fibronectin into the brain was not observed.

VEGF: Immunoreactivity was observed in smooth muscle cells of the posterior cerebral arteries, in the subependymal astrocytes around the third ventricle, ventricular ependyma and choroid plexus epithelium. No immunoreactivity was observed when VEGF antibody adsorbed with an excess of VEGF peptide was used.

GFAP: Astrocytes having delicate processes located in the deep cortex, white matter, stratum lacunosum and dentate gyrus of the hippocampus and in the subependymal region of the third ventricle showed cytoplasmic immunoreactivity for GFAP.

Cold Injury

The morphological appearance of the cold injury was similar to previous observations (17, 18) (Fig. 1a–d). At 10 min, a central area of coagulative necrosis that extended from the surface to the fourth cortical layer was present (Fig. 1a, b). Calcification was noted in the center of the lesion between 4 and 6 days (Fig. 1c).

Vascular Changes

BBB Permeability: Extravasation of fibronectin from intracerebral vessels in the lesion site was observed from 10 min post-injury (Fig. 2). Immunoreactivity was maximal in and around vessel walls and decreased away from the wall, indicating leakage from the vessel into the surrounding neuropil (Fig. 2a, b). The number of lesion vessels showing fibronectin leakage was maximal between 2 to 4 days, declining thereafter (Fig. 3). In contrast, an occasional vessel in the brain bordering the lesion (the perilesional area) showed increased permeability to fibronectin at 60 min. The number of permeable perilesional vessels increased up to 4 days and decreased thereafter so that only a few perilesion vessels showed BBB breakdown beyond 5 days.

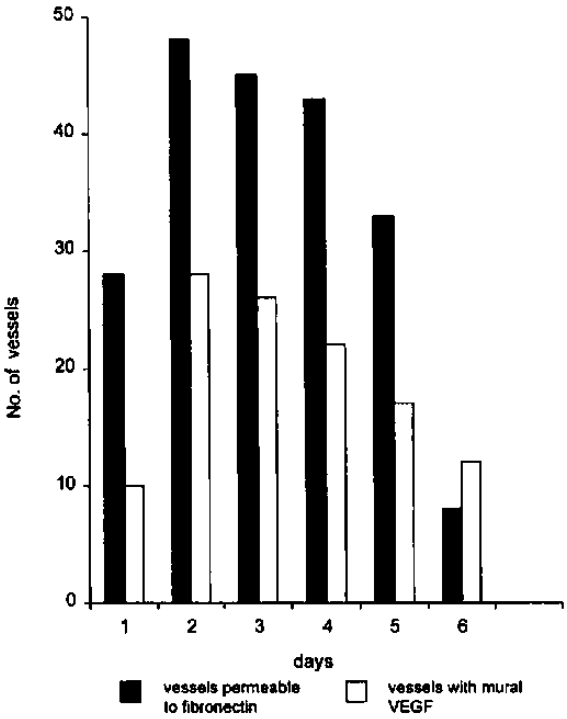


Fig. 3. The total number of pial arteries and veins and lesional arterioles with BBB breakdown to fibronectin and those with mural VEGF present from 1 to 6 days are shown.

Spread of fibronectin from the lesion site into the neuropil underlying the cold lesion was observed in all rats at 3 h, and by 6 h protein extravasation was present in the white matter underlying the lesion. By 24 h, extension of edema into the white matter of the contralateral hemisphere was noted. The latter condition was observed in all rats sacrificed up to day 3, and after this period, fibronectin extravasation remained localized to the lesion site.

Neurons present in the cold-injury site up to hour 24 had pyknotic nuclei and shrunken cytoplasm immunoreactive for fibronectin (Fig. 2a). In addition, viable neurons in the deep cortex adjacent to the edematous white matter and astrocytes in the white matter showed cytoplasmic uptake of fibronectin up to day 5.

Angiogenesis: Endothelial proliferation occurred as early as day 2 in one rat and in all rats on day 3. Single endothelial cells were large and occasionally multinucleate (Fig. 1b), and occasional cells showed mitotic figures (Fig. 2g). Extracellular fibronectin was observed around these cells, forming a meshlike pattern (Fig. 2c). Neovessels were present on days 4 to 6, and the basement membranes of these vessels were immunoreactive with antifibronectin antibody (Fig. 2d). After day 6 any residual necrotic cortex was

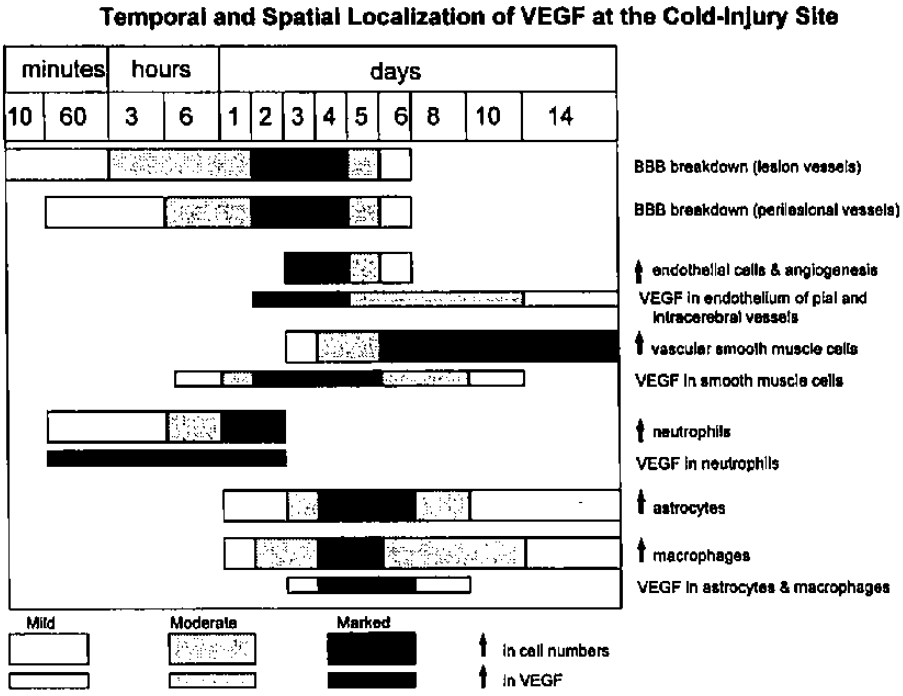


Fig. 4. The temporal and spatial localization of VEGF is shown in relation to BBB breakdown, angiogenesis and cellular responses in the cortical cold-injury model from 10 min to 14 days.

pushed to the surface and the underlying cortex showed a normal vascular pattern (Fig. 1d).

Pial Arteries: Medial hyperplasia of pial arteries overlying the cold-injury site and some arterioles within the lesion occurred as early as day 3 (Fig. 1c). Marked mural thickening of pial arteries and occasional veins were striking features, especially after day 6 (Fig. 1d). These vessels showed mural fibronectin immunoreactivity (Fig. 2d), which persisted up to day 14.

Cellular Response

Neutrophils: Margination of neutrophils was observed in pial vessels as early as 30 min postinjury. By 12 h these cells were present in a perivascular location and in the necrotic neuropil. The neutrophil response persisted up to day 2, and at this time period there was evidence of a breakdown of polymorphonuclear leukocytes and presence of nuclear debris in the cold-injury site (Fig. 2f).

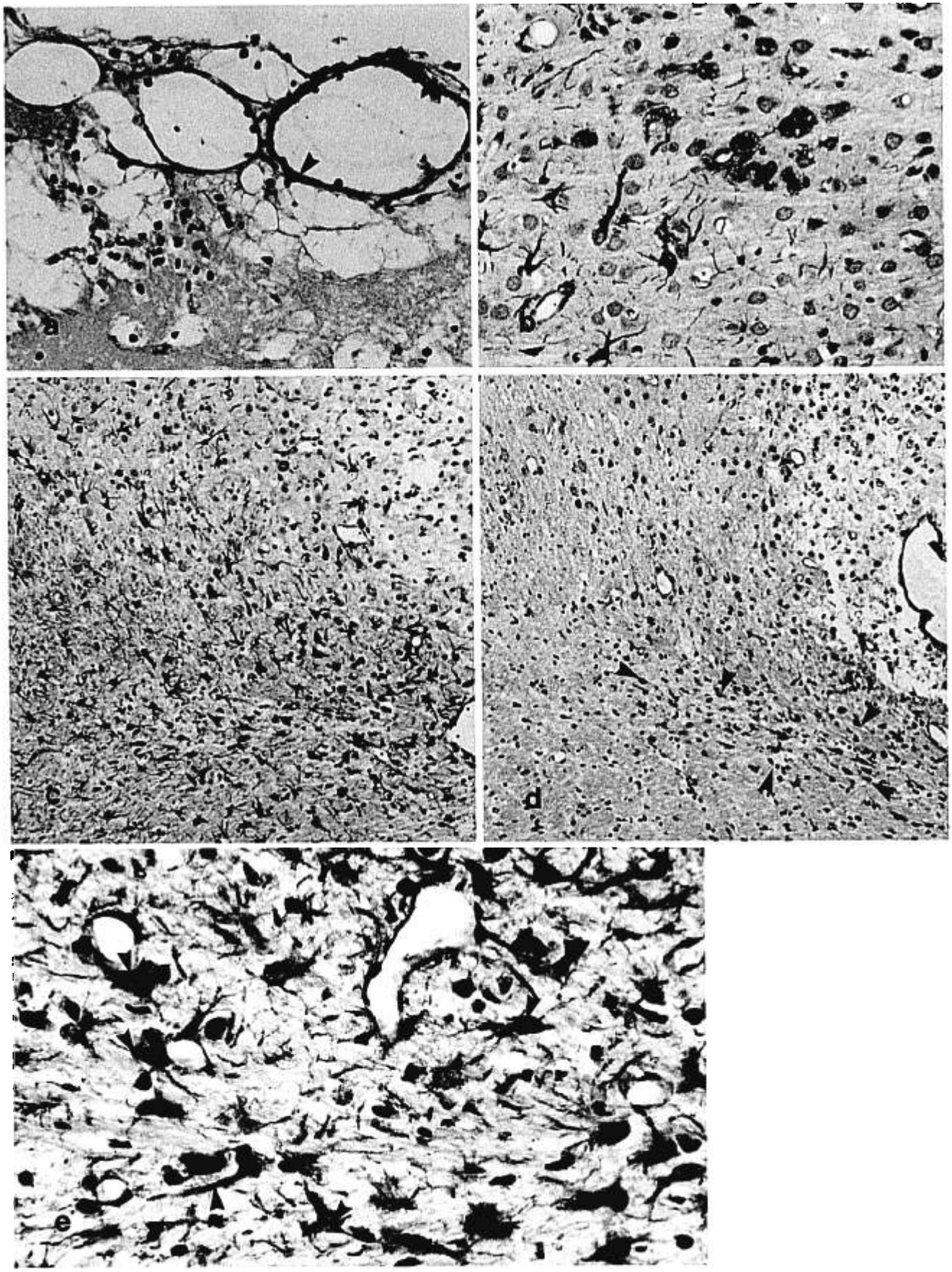
Macrophages: Few macrophages were present at the junction of the cold-injured site and normal brain at 24 h. The numbers of macrophages increased progressively (Fig. 1a), with maximal numbers observed at days 4 and 5 in both the lesional and perilesional areas. Their numbers decreased thereafter and only occasional macrophages were present at 14 days.

Astrocytes: Astrocytes at the lateral and basal margins of the lesion underlying white matter and the subpial region increased in size and number starting by day 1. By day 2, there were a larger number of astrocytes and an increased amount of immunoreactivity for GFAP. Moderate astrocytosis was also noted in the hippocampus ipsilateral to the lesion. These changes were maximal between days 4–6 and declined thereafter. However, mild astrocytosis persisted up to day 14.

VEGF: The presence of VEGF protein in relation to BBB breakdown, angiogenesis and the cellular response in the cold-injury model from 10 min to 14 days is shown in Figure 4. The earliest VEGF immunoreactivity was observed in polymorphonuclear leucocytes marginating in permeable pial vessels overlying the lesion site (Fig. 5a). This was observed in 1 of 3 rats at 30 min, and in all rats at 60 min and thereafter up to 2 days.

Pial vessels: VEGF protein was not demonstrable in cerebral vessels until 6 h, when immunoreactivity was observed in the smooth muscle cells of pial arteries and veins permeable to fibronectin in all rats (Figs. 2f, 5a). Pial arteries showing medial hyperplasia showed marked VEGF immunoreactivity in smooth muscle cells, which persisted up to 10 days (Figs. 2h). The endothelium of pial arteries and veins showed VEGF immunoreactivity at 36 h, which persisted up to 14 days (Fig. 2g, h).

Lesion vessels: Few arterioles within the lesion that were permeable to fibronectin also showed VEGF in smooth muscle cells at 12 h and in endothelium at 36 h (Fig. 2f). This finding was present in all rats sacrificed up to day 5. Permeable microvessels in the lesion and perilesional area rarely showed mural VEGF.



Cytoplasmic immunoreactivity for VEGF was observed only in focal collections of macrophages and astrocytes present in the perilesional areas from day 3 up to day 10 (Figs. 5b, d). In sections immunostained for both VEGF and GFAP, the red reaction product for GFAP was seen along with the brown reaction product for VEGF in the cytoplasm of focal groups of astrocytes (Fig. 5e). Occasional cortical neurons in the perilesional area showed both nuclear and cytoplasmic VEGF. Proliferating endothelial cells showed cytoplasmic VEGF. However, once the vascular tubes were formed, endothelial cells lost VEGF immunoreactivity (Fig. 2h).

DISCUSSION

This study provides a temporal and spatial localization of VEGF protein in brain at the site of a cortical cold injury. Increased VEGF was observed in endothelium and smooth muscle cells of pial vessels and lesion arterioles with BBB breakdown to fibronectin and in association with angiogenesis.

Distribution of VEGF in Normal Cerebral Vessels

Intracerebral vessels of control rats did not show mural VEGF. Similar findings were reported by others in rat (16) and human (14) cerebral and retinal (19) vessels at the protein level and at the mRNA level (20). Smooth muscle cells of the large meningeal arteries, such as the posterior cerebral arteries, showed moderate immunoreactivity for VEGF. Although localization of VEGF in normal vascular smooth muscle cells was not mentioned in the studies cited above, smooth muscle cells are known to synthesize VEGF, since low levels of VEGF mRNA were detected in cultured vascular smooth muscle cells by northern blots (21–23).

VEGF and BBB Permeability

The pattern of BBB breakdown to fibronectin in the lesion and perilesional areas was similar to what we observed previously using serum protein antibody to demonstrate BBB breakdown (18). Fibronectin extravasation at the lesion site occurred immediately following induction of the cold lesion. This was attributed to dissolution of cells in the vessel wall produced by the cold lesion (17) and is unrelated to the effects of permeability mediators. In keeping with this view, VEGF was not demonstrable immediately after induction of the cold injury. However, lesion and later perilesional arterioles and microvessels demonstrated BBB breakdown to fibronectin up to day 6. From days 2 to 5, VEGF was present in smooth muscle and endothelial cells of 52 to 58% permeable pial vessels and intracerebral arterioles, suggesting that the increased permeability is mediated by VEGF. In addition, since VEGF is known to be diffusible, having the signal sequence for secretion (5, 24), VEGF derived from polymorphonuclear leukocytes, macrophages, and astrocytes in addition to endothelial and smooth muscle cells could mediate the BBB breakdown noted in adjacent lesional and perilesional vessels that did not show mural VEGF. Similar association of VEGF with increased permeability to albumin was noted in retinal vessels of diabetic rats (25) and in vessels of noncerebral tumors (26).

VEGF induces vascular permeability rapidly, and plasma leakage was observed within minutes following an intradermal injection of this protein into rabbit skin (27). Similar information is not available for cerebral vessels. However, a recent in vitro study demonstrated a 3-fold increase in permeability of brain microvascular endothelial cells to [^{14}C] sucrose within 5 h of exposure to VEGF, and this effect lasted for over 48 h (28). A recent ultrastructural study demonstrated that 10 minutes (min) after application of VEGF to the cremaster muscle and skin, fenestrations developed in small veins and capillaries that normally have nonfenestrated endothelium (29). Possibly, VEGF induces a change in cerebral endothelial structure, since a previous study (17) demonstrated patent interendothelial channels in perilesional vessels 3 days after a freeze-injury at a time when VEGF was present locally.

Immunoreactivity for VEGF was observed in endothelial cells of pial veins and arteries and intracerebral arterioles starting from about 36 h. In the case of cerebral infarcts, VEGF and flt immunoreactivity was noted in endothelial cells from 1 to 14 days (16). Overexpression of VEGF at both the mRNA and protein levels is well documented in endothelium of cerebral (14) and noncerebral (26, 30) tumor vessels. The intensity of vascular labeling with VEGF antibody in the present study is curious, especially since VEGF exerts its effects on vascular endothelium at nanomolar to picomolar concentrations, which are well below levels detectable by immunohistochemistry. It was suggested that endothelial accumulation of VEGF may be a mechanism for concentrating VEGF locally, therefore maximizing its activity on immediately adjacent vessels while preventing its spread and action at more distant sites (31). These authors suggested that some of the immunohistochemical staining

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Fig. 5. VEGF immunoreactivity (brown) is present in polymorphonuclear leukocytes, endothelium of a pial arteriole (a) (arrowhead) and a focal collection of macrophages (b). A 4-day lesion immunostained for GFAP (c) and VEGF (d) shows marked astrocytosis deep in the lesion and the underlying white matter. Note that only a focal group of astrocytes (arrowheads) shows cytoplasmic VEGF (d). Double labeling for VEGF and GFAP shows colocalization of both proteins in the cytoplasm of astrocytes at day 4. The brown reaction product indicates presence of VEGF (arrowheads). a, b, ×250; c, d, ×125; e, ×500.

represents VEGF bound to specific endothelial receptors, and since VEGF is known to bind to heparin (2), much of the staining could reflect VEGF binding to heparin-like proteoglycans present in the vascular basement membrane or associated with the endothelial surface.

Pial arterioles with marked mural thickening due to medial hyperplasia showed fibronectin in the extracellular matrix of the vessel wall up to day 14. This likely represents increased fibronectin production by the hyperplastic smooth muscle cells rather than persisting BBB breakdown, since previous studies (17, 18) using tracers as markers of permeability demonstrated restoration of the BBB at 5 to 6 days after production of a cold lesion. Despite the presence of structural differences between cerebral and noncerebral endothelium, the time course of BBB breakdown after cold injury is similar to that seen in noncerebral vessels during wound healing in skin (32).

VEGF and Angiogenesis

Our previous study demonstrated that the majority of cells present at the margin and within the cold lesion were immunoreactive for glucose transporter and are therefore endothelial in nature (18). These cells demonstrated VEGF immunoreactivity at 3-4 days. Once vascular tubes were formed, the endothelium lost VEGF immunoreactivity, thus resembling the pattern of VEGF localization in normal adult vessels.

Breakdown of the BBB to fibronectin and presence of VEGF protein in endothelium of pial vessels and intracerebral arterioles preceded endothelial proliferation. The finding that vascular hyperpermeability precedes and accompanies angiogenesis is well documented in noncerebral tumor vessels (26) and wound healing in skin (32). Plasma proteins form a new provisional extracellular matrix that permits and favors the migration of endothelial cells. Of the plasma proteins, fibrin is known to exert its proangiogenic effect at least in part by providing a favorable surface for cell adhesion and migration, presumably via its arg-gly-asp sequence (24). A similar sequence is present in fibronectin, which was noted to form an extracellular matrix in the present study.

Localization of VEGF in Other Cell Types

Smooth muscle cells: Increased VEGF in smooth muscle cells of pial arteries and intracerebral arterioles was quite striking. This finding has received little attention in the literature relating to cerebral vessels, although a study of arterio-venous malformations of the brain reported increased VEGF immunoreactivity in the subendothelial layer of abnormal vessels (33). Illustrations in the latter study showed VEGF localization in the smooth muscle cell layer of abnormal vessels. At the mRNA level, overexpression of VEGF mRNA occurred when cultured smooth muscle cells were stimulated by exposure to serum (20) or IL-1β (23). It was suggested that VEGF synthesized by vascular smooth muscle cells played a role in maintaining the vascular endothelium in steady states and in repair following endothelial damage (22).

Astrocytes: Presence of VEGF in neoplastic astrocytes is well documented (10, 13, 14). Whether reactive astrocytes synthesize VEGF or take it up from the extracellular space in the same manner as they phagocytose serum proteins and fibronectin in areas of vasogenic edema (18) is uncertain. However, overexpression of VEGF mRNA was demonstrated in retinal astrocytes of diabetic rats in areas of blood-retinal barrier breakdown, indicating that astrocytes are capable of synthesizing this protein in response to injury (25). Another interesting finding noted previously (15) and in the present study is that only focal collections of astrocytes in each lesion show cytoplasmic VEGF.

Inflammatory Cells

Polymorphonuclear Leukocytes: Cytoplasmic VEGF immunoreactivity was first noted in the vessel lumina in polymorphonuclear leukocytes that were marginating and those in a perivascular location at the cold-injury site. VEGF immmunoreactivity in polymorphonuclear leukocytes was also noted in the retina of diabetics (19).

Macrophages: Cytoplasmic VEGF was present in focal collections of macrophages at the cold injury site, indicating that it is a subset that expresses VEGF. Localization of VEGF protein in macrophages was also noted in cerebral infarcts (15, 16). Stimulated macrophages are known to overexpress VEGF mRNA, while resident macrophages do not (21). In addition, macrophages are known to promote all phases of angiogenesis by virtue of their secretory products (34).

There are differences in VEGF immunolocalization in the cold-injury model and cerebral infarcts (16). The latter study did not report VEGF localization in polymorphonuclear leukocytes and pial and intracerebral vascular smooth muscle cells. The same study noted VEGF immunoreactivity in ischemic neurons. In the present study, occasional rats showed both nuclear and cytoplasmic staining for VEGF in neurons in a few focal areas; this is attributed to artifact.

In summary, this study provides a temporal and spatial localization of VEGF protein in brain at the site of a cortical cold injury. The presence of mural VEGF in permeable pial and lesional arterioles suggests that VEGF is one of several factors that mediates BBB breakdown in this model. The association of maximal VEGF immunoreactivity with endothelial proliferation and neovascularization suggests that VEGF promotes angiogenesis and repair following brain trauma. Thus, there is increasing evidence for a role for VEGF in permeability and

angiogenesis not only in brain tumors but also in non-neoplastic conditions of brain.

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