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Hypoxia-Induced Vascular Endothelial Growth Factor Expression Precedes Neovascularization after Cerebral Ischemia

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We investigated the hypothesis that hypoxia induces angiogenesis and thereby may counteract the detrimental neurological effects associated with stroke. Forty-eight to seventy-two hours after permanent middle cerebral artery occlusion we found a strong increase in the number of newly formed vessels at the border of the infarction. Using the hypoxia marker nitroimidazole EF5, we detected hypoxic cells in the ischemic border of the neocortex. Expression of vascular endothelial growth factor (VEGF), which is the main regulator of angiogenesis and is inducible by hypoxia, was strongly up-regulated in the ischemic border, at times between 6 and 24 hours after occlusion. In addition, both VEGF receptors (VEGFRs) were up-regulated at the border after 48 hours and later in the ischemic core. Finally, the two transcription factors, hypoxia-inducible factor-1 (HIF-1) and HIF-2, known to be involved in the regulation of VEGF and VEGFR gene expression, were increased in the ischemic border after 72 hours, suggesting a regulatory function for these factors. These results strongly suggest that the VEGF/VEGFR system, induced by hypoxia, leads to the growth of new vessels after cerebral ischemia. Exogenous support of this natural protective mechanism might lead to enhanced survival after stroke. ***(Am J Pathol 2000, 156:965–976)***

Two of the leading causes of death in the western world are associated with ischemia, namely coronary heart disease and stroke. Although some progress in prevention and treatment has been achieved, stroke remains the third most common cause of death.[1] Therefore, studies aiming to elaborate the pathophysiological background of stroke are needed to find novel therapeutical strategies. Stroke often results from focal cerebral ischemia due to occlusion of a cerebral blood vessel. The severe reduction of blood flow to the affected tissue results in a lack of oxygen and nutrient transportation, which ultimately leads to tissue hypoxia and cell death. To compensate for these detrimental effects, the organism responds by trying to increase oxygen delivery to the affected tissue. One potential mechanism used to increase the oxygenation of hypoxic tissue is the induction of angiogenesis. Newly formed vessels would allow increased blood flow, thus increasing the amount of oxygen delivered to the affected tissue. Indeed, analysis of postmortem brain tissues obtained from patients with various survival times after stroke revealed an increase in the number of microvessels in the infarcted brain tissue when compared with the contralateral normal hemisphere.[2] However, no data are available regarding the temporal kinetics and tissue distribution of this angiogenic reaction after cerebral ischemia.

Angiogenesis is defined as the formation of new blood vessels by sprouting of endothelial cells from pre-existing vessels.[3] During the process of sprouting, endothelial cells degrade the underlying basement membrane, migrate into neighboring tissue, proliferate, and assemble into tubes. Finally, tube-to-tube connections are made and blood flow is established. The ability of the mature vasculature to adapt to changing demands requires both soluble factors and cell-cell as well as cell-matrix interactions. Among the factors capable of modulating angiogenesis characterized to date, vascular endothelial growth factor (VEGF) is the best candidate for a specific regulator of endothelial cell growth and differentiation.[4] VEGF, also known as vascular permeability factor, is a dimeric glycoprotein that is mitogenic for endothelial cells and enhances vascular permeability.[5] VEGF is expressed in the normal adult brain, mainly in the epithelial cells of the choroid plexus, but also in astrocytes and neurons, such as granule cells of the cerebellum.[6,7] It binds to two endothelial tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1) (Flt-1) and VEGFR-2 (Flk-1/KDR).[5]

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In memoriam: Werner Risau (1953–1998).

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It has been shown that hypoxia is a strong inducer of VEGF mRNA expression in many cells *in vitro*.[8,9] In addition, *in vivo* experiments have revealed that systemic hypoxia is capable of inducing the expression of both VEGF and VEGFR-1 in various organs, including the brain.[6] Furthermore, VEGF expression is clearly induced in hypoxic regions in the vicinity of tumor necroses[10–12] and in various models of ischemia.[13–18] However, there are conflicting results concerning the temporal kinetics and localization of induction of VEGF and its receptors after cerebral ischemia. Moreover, there is no evidence to connect VEGF to angiogenesis after cerebral ischemia. Finally, very little is known about the mechanisms by which VEGF gene expression is regulated during cerebral ischemia. *In vitro* studies have identified three mechanisms that are responsible for the increase in biologically active VEGF secreted by cells exposed to hypoxia. One is an increased transcription rate mediated by binding of HIF-1 to a hypoxia-responsive element in the 5′-flanking region of the VEGF gene,[19–21] and the second is increased VEGF mRNA stability,[8,22] probably due to binding of the RNA-binding protein HuR.[23] And finally, an internal ribosome entry site ensures efficient translation of VEGF mRNA, even under hypoxia.[24] HIF-1 is a basic helix-loop-helix heterodimeric transcription factor activated by reduced oxygen tension. HIF-1 is composed of a hypoxia-regulated α-subunit and a β-subunit.[25] A homolog of HIF-1, named HIF-2,[25] has recently been cloned[26,27] and shown to be involved in the regulation of VEGF gene expression as well.[28] HIF-2 has an additional role in the regulation of VEGFR-2.[29] However, little is known about the involvement and the activation of these two factors during cerebral ischemia.

In the present study we investigated the hypothesis that the hypoxic environment at the border of an infarcted area can lead to new vessel growth, thereby minimizing the detrimental effects of cerebral ischemia. We demonstrate that the tissue bordering the infarcted area, the penumbra, is indeed hypoxic. Our data further indicate that both VEGF and the VEGF receptors (VEGFR-1 and VEGFR-2) are up-regulated by hypoxia in the brain after cerebral ischemia. These could mediate the angiogenic response, observed in the ischemic border zone and extending toward the core region of the infarcted area.

Materials and Methods

Focal Cerebral Ischemia

Surgical protocols were approved by the local ethics committee and governed by the pertinent national legislation. Focal ischemia was induced in OF1 mice (Iffa Credo, France) by the permanent occlusion of the left middle cerebral artery (MCAO) under chloral hydrate anesthesia as reported previously.[30–32] In this model, ischemia is restricted to the neocortex. At different times after occlusion, mice were anesthetized and the brains were removed.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was prepared from cerebral cortices by phenol-chloroform extraction as described.[32] Reverse transcription (RT) and polymerase chain reaction (PCR) amplification were performed as described.[6] For VEGF, the primers and PCR profile were as described[6]: for mouse HIF-1α, the primers 5′-TGAGGCTCACCATCAGTTAT-3′ (sense) and 5′-TAACCCCATGTATTTGTTC-3′ (antisense) resulted in a 187-bp product (94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes for 35 cycles), and for β-actin the primers 5′-TGTGATGGTGGGAATGGGTCAG-3′ (sense) and 5′-TTTGATGTCACGCACGATTTCC-3′ (antisense) gave rise to a 514-bp product (94°C for 0.75 minute, 60°C for 1 minute, 72°C for 1 minute for 35 cycles).

In Situ *Hybridization*

The techniques and ^{35}S-UTP-labeled single-stranded RNA probes for VEGF, VEGFR-1, and VEGFR-2 used for *in situ* hybridization were essentially as described.[6,33] The HIF-1α and the HIF-2α probe have been described before.[12,34] Hybridization was performed on cryostat-cut coronal brain sections (10 μm) with 2.5–5 $\times$ 10^4 cpm/μl ^{35}S-labeled RNA probe overnight at 48°C. Sections were washed, dehydrated, coated with Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), developed after 12–28 days of exposure, and counterstained with 0.02% toluidine blue.

Immunohistochemistry

The immunohistochemical staining was performed as described previously.[35] The following primary antibodies were used: MEC 13.3 rat monoclonal anti-mouse PECAM-1/CD31 antibody (a gift from E. Dejana, Milan, Italy), polyclonal rabbit anti-mouse Ki67 antibody (Dianova), and rat monoclonal anti-mouse flk-1 (VEGFR-2) antibody (a gift from H. Kataoka, Kyoto, Japan). PECAM-1-positive endothelial cells and Ki67-positive proliferating cells were counted under the microscope in four to eight randomly chosen high-magnification fields ($\times$100) in the ischemic border zone and a mean value was calculated. To map hypoxic regions *in vivo*, 250 μl of 10 mmol/L EF5[36] was administered intravenously to control animals or to mice subjected to cerebral ischemia, 20 hours after MCAO. Four hours later, all animals were killed and the brains were removed. Immunofluorescence for EF5 with Cy3-conjugated ELK3–51 monoclonal antibody (a gift from C. Koch, Philadelphia, PA) was performed as described.[36,37]

Mouse VEGF Immunoassay

Cortical tissue lysates from ipsilateral and contralateral brain hemispheres were prepared at different times after occlusion by homogenization in lysis buffer (100 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 1 mmol/L EDTA) supplemented with a cocktail of proteinase inhibitors (1 μg/ml

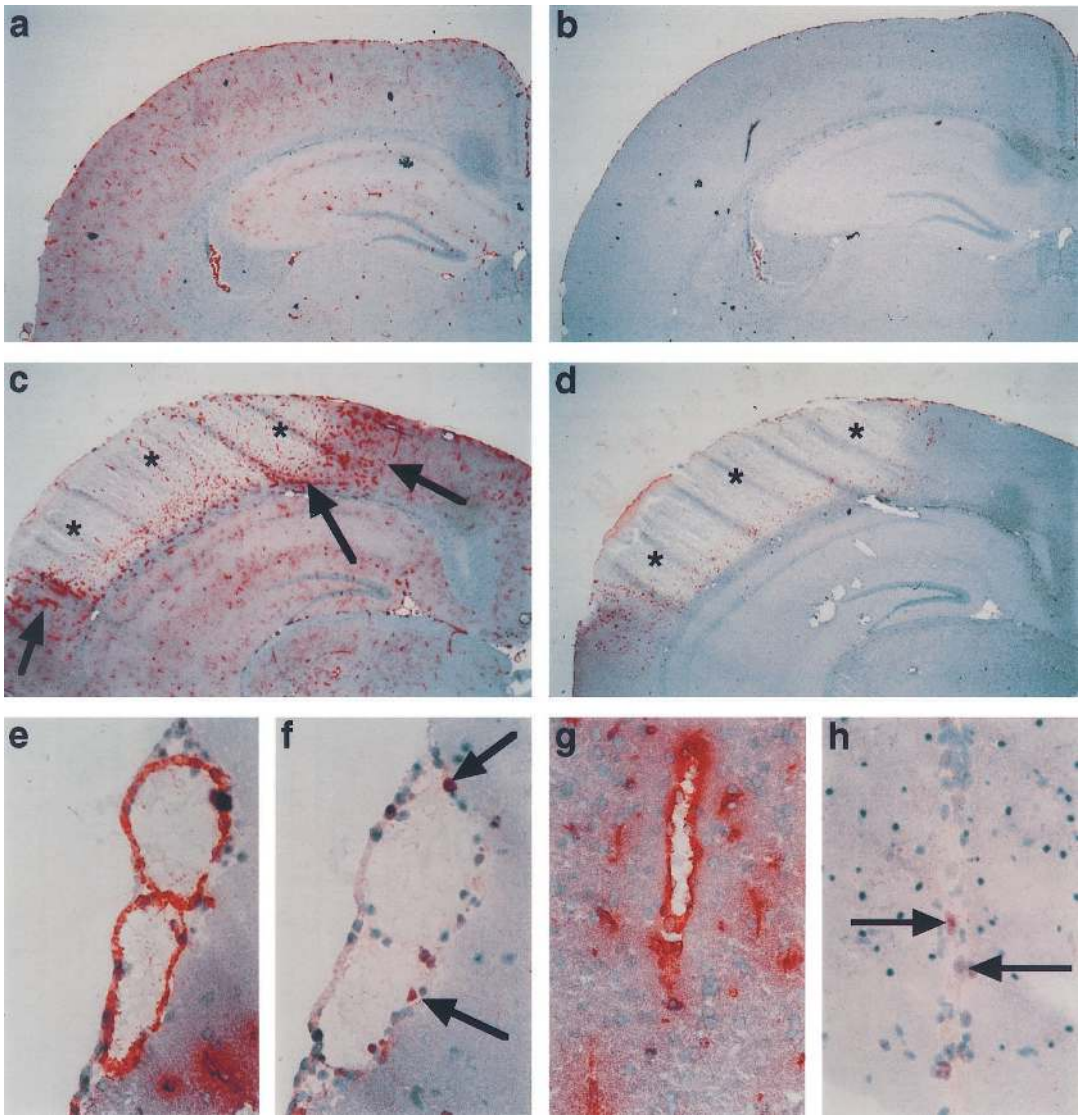

Figure 1. Immunohistochemistry for PECAM-1 (**a, c, e, g**) and Ki67 (**b, d, f, h**) in frontal brain sections of mice at various times after MCAO. **a** and **b:** control brain; **c** and **d:** brain 72 hours after occlusion; **e–h:** brain 48 hours after occlusion. Increased PECAM-1 (**arrows** in **c**) and Ki67 staining (**d**) are visible at the border of the infarction (marked by **asterisks**). PECAM-1 (**e** and **g**) and Ki67 (**f** and **h**) staining were performed on adjacent sections showing a pial vessel (**e** and **f**) and a neocortical vessel (**g** and **h**) invading the infarcted area. **Arrows** point to proliferating endothelial cells (**f** and **h**). Original magnifications: **a–d**, ×6.25; **e–h**, ×100.

pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride). Mouse VEGF was quantitated using a commercially available immunoassay kit (Quantikine M; R&D Systems).

Results

Induction of Endothelial Cell Proliferation and New Vessel Growth after Stroke

To determine the distribution pattern of endothelial cells, we performed immunohistochemistry on frontal sections of adult mouse brain, using an antibody against PECAM-1 as a marker for endothelial cells. Positively stained endothelial cells were detected in the whole brain, mainly in the choroid plexus, in capillaries in the neocortex and hippocampus, and in pial vessels at the surface of the normal brain (Figure 1a). Using an antibody against Ki67, a cell proliferation marker, we confirmed that cell proliferation was absent in normal adult brain parenchyma (Figure 1b). The staining pattern was similar after 0.5, 3, and 12 hours of MCAO, although staining for PECAM-1 was reduced in the ischemic core region (data not shown). After 24 hours, there was a slight increase in the number of PECAM-1-positive cells, but still hardly any Ki67-labeled cells were detected (Figure 2). After 48 and 72 hours of occlusion, however, a major change was observed, for both PECAM-1 and Ki67 staining. PECAM-1 staining was strongly increased at the border zone around the infarcted area and in the ischemic core (Figure 1c), and the number of endothelial cells and proliferating cells increased dramatically (Figure 2). Strong expression of Ki67 in the same area of PECAM-1 staining suggested that endothelial cells had

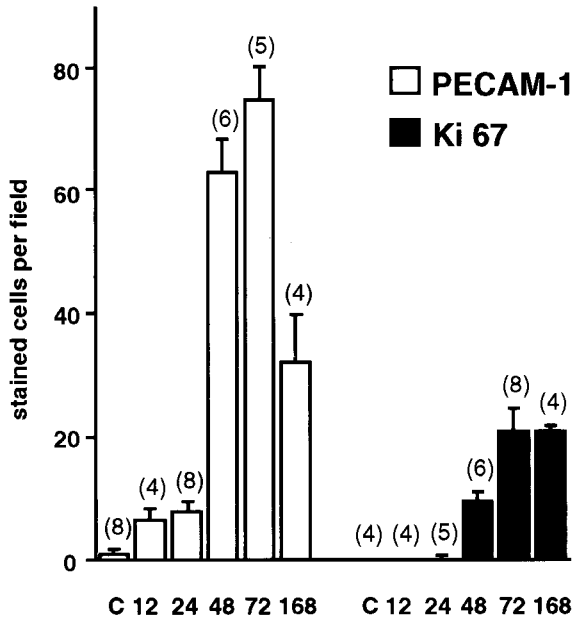

Figure 2. Time course of endothelial cell proliferation after MCAO in the ischemic penumbra. PECAM-1- and Ki67-positive cells were counted under the microscope in randomly chosen high-magnification fields (×100) in the control animals (C) and at various time points (12, 24, 48, 72, 168 hours) after MCAO. Each column and bar represents, respectively, the mean and SD of four to eight examined fields.

resumed cell proliferation (Figure 1d). Immunohistochemistry staining for both antigens was therefore performed on serial sections, where individual vessels could be followed up. Figure 1, e and f, shows the same pial vessel with proliferating endothelial cells. Figure 1, g and h, shows proliferating endothelial cells within a vessel that invades from the ischemic border toward the core region of the infarcted area. At 7 days after occlusion, the number of PECAM-1-positive endothelial cells in vessel-like structures in the ischemic hemisphere was still enhanced compared to the contralateral side (Figure 2). In the contralateral hemisphere, Ki67 staining was absent at all time points, and no change in PECAM-1 staining could be observed (data not shown). These results demonstrate that an angiogenic reaction starts between 24 and 48 hours after cerebral infarction. This angiogenic reaction appears to originate from the borders of the infarcted area and from the pial vessels at the brain surface.

The Border Region of the Infarct (Penumbra) Is Hypoxic

After occlusion of a cerebral artery, oxygenation of the brain tissue supplied by this vessel is impaired, resulting in subsequent tissue hypoxia and even cell death. We hypothesized that tissue hypoxia might be the stimulus responsible for the observed new vessel growth. To map hypoxic tissue *in vivo*, we injected the nitroimidazole compound EF5 intravenously 20 hours after MCAO and analyzed the distribution of this hypoxia marker 4 hours after injection, using immunofluorescence techniques on cryosections of the infarcted brain. We found hypoxic regions around the infarcted area and observed the strongest hypoxia signal in the cortex directly adjacent to the infarction (Figure 3a). This region, known as the penumbra, although perfused, suffers from hypoxia and corresponds completely to the area where the angiogenic response was detected 24 hours later (Figure 1c). This suggests that hypoxia, in fact, could be the inducer of blood vessel formation. In contrast, no EF5 staining was detectable in the contralateral hemisphere (Figure 3b) or in normal brain (Figure 3c).

Cerebral Ischemia Induces Expression of VEGF and Its Receptors

To further delineate the mechanisms by which hypoxia could lead to angiogenesis, we analyzed the expression

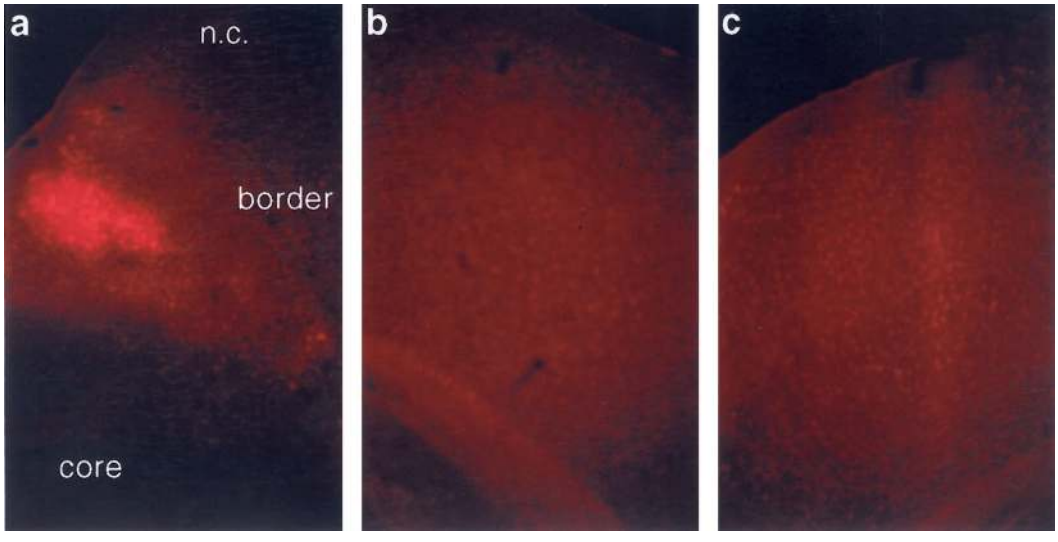

Figure 3. Immunohistochemistry for the *in vivo* hypoxia marker EF5. The nitroimidazole compound EF5 was injected intravenously into mice 20 hours after MCAO (**a** and **b**) or into control animals (**c**). Four hours later, brains were removed and analyzed by immunofluorescence. **a:** Infarcted hemisphere; **b:** contralateral brain hemisphere of the same animal; **c:** brain from control animal. n.c., normal cortex; border and core region of the infarction are marked. Original magnification, ×25.

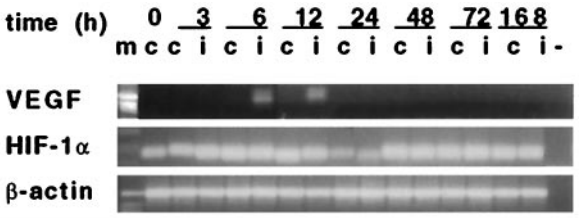

Figure 4. RT-PCR analysis of VEGF ($VEGF_{120}$, 512 bp; $VEGF_{164}$, 644 bp), HIF-1α (187 bp), and β-actin (514 bp) transcripts. cDNA was synthesized from 1 μg of total RNA from contralateral (c) and ipsilateral (i) brain hemispheres at different times after occlusion. The RT-PCR presented is representative of results obtained from three individual experiments. −, no RT; m, molecular weight marker.

pattern of VEGF and its receptors by RT-PCR and *in situ* hybridization. VEGF is the main regulator of angiogenesis during development.[3] We have recently stated that VEGF gene expression is inducible by hypoxia *in vitro* and *in vivo*.[6,8] VEGF mRNA was not detectable by RT-PCR in control brain or in the contralateral hemisphere at any time after occlusion, or in the ipsilateral side after 3 hours of occlusion. After 6 and 12 hours, however, mRNA for both $VEGF_{120}$ and $VEGF_{164}$ was strongly induced in the ischemic hemisphere, but not in the contralateral side (Figure 4). At later time points VEGF mRNA again became undetectable. To identify regional localization of VEGF mRNA expression, *in situ* hybridization was performed. VEGF was expressed throughout the normal brain at a low level and in the choroid plexus at higher levels (Figure 5, a and b). Changes in the expression pattern of the VEGF gene *in situ* were detected 12 hours after occlusion. VEGF gene expression was induced at the border of the infarcted area (Figure 5d), in the same location where we had identified hypoxic tissue by using EF5 staining (Figure 3a). VEGF mRNA levels were strongly increased at a very restricted penumbral area after 24 hours of MCAO (Figure 5e). They remained slightly elevated 72 hours after occlusion (Figure 5f) but returned to normal levels after 168 hours of MCAO (Figure 5g). VEGF gene expression was also induced in the pia above the infarcted area (Figures 5, d and e) but was absent in the ischemic core. To further quantify this reaction, VEGF protein was measured by using a specific enzyme-linked immunosorbent assay. The mean VEGF protein concentration in brain cortex was 4.15 ± 0.69 ng VEGF/g total protein ($n = 4$). Induction of VEGF was not detected in the contralateral hemisphere at any time after occlusion. In the ischemic hemisphere, however, VEGF protein levels increased up to twofold between 12 and 48 hours of occlusion. By 168 hours, levels had returned to basal values (Figure 6). Thus VEGF protein induction

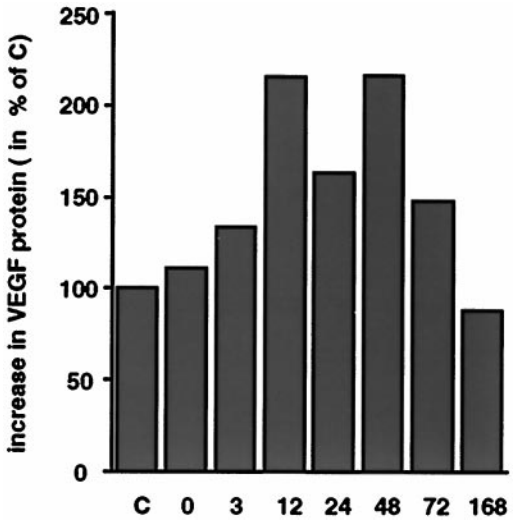

Figure 6. Induction of VEGF protein during cerebral infarction. Brain lysates were prepared from contralateral (C) and ipsilateral cerebral hemispheres at various times after MCAO (0, 3, 12, 24, 48, 72, 168 hours). VEGF production was determined by a commercial immunoassay. Data show values in ipsilateral hemispheres (in percentage of the corresponding contralateral side) and are given as the mean of two independent experiments.

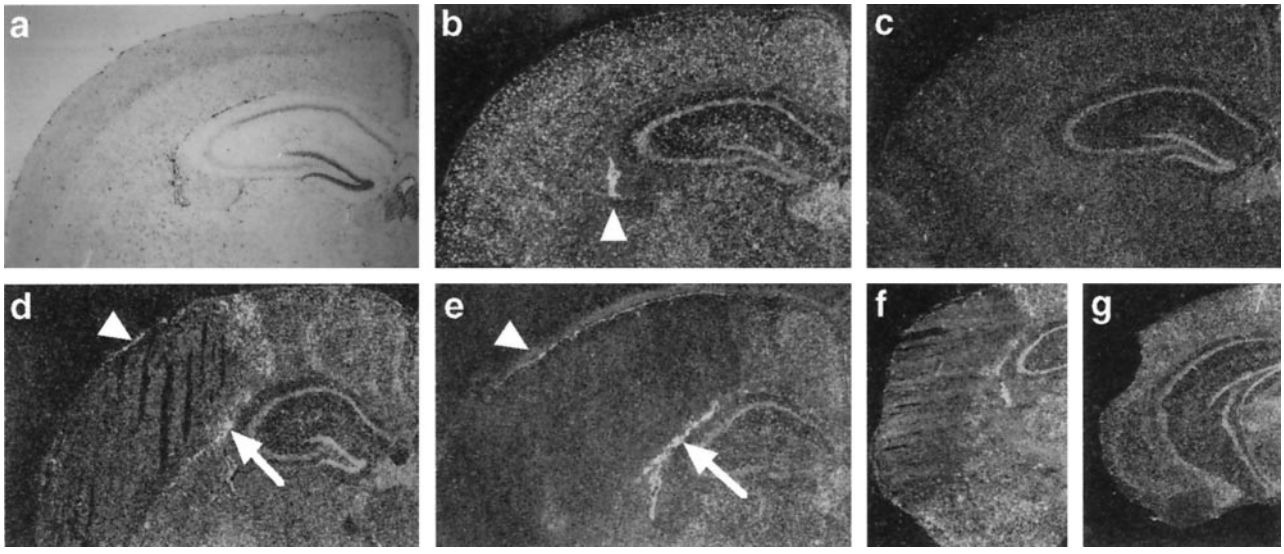

Figure 5. Detection of VEGF mRNA in mouse brain at various times after MCAO. Frontal sections were hybridized *in situ* with ^{35}S-labeled RNA antisense (**a, b, d–g**) or sense (**c**) probes. Shown are (**a**) a bright-field image of a control animal and dark-field images of (**b**) a control animal and of animals (**d**) 12, (**e**) 24, (**f**) 72, and (**g**) 144 hours after MCAO, respectively. Note the strong expression of VEGF in choroid plexus in normal brain (**arrowhead** in **b**) and up-regulation of VEGF in the pia (**arrowheads** in **d** and **e**) and in the penumbra (**arrows** in **d** and **e**) during cerebral ischemia. Original magnification, ×6.25.

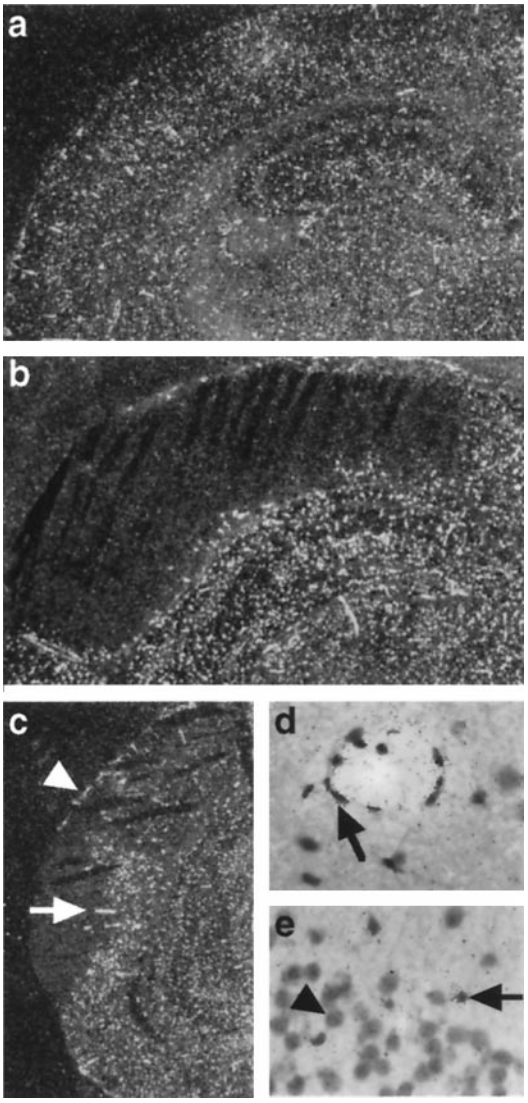

Figure 7. Expression of VEGFR-1 mRNA during cerebral infarction: *in situ* hybridization with a VEGFR-1-specific probe. Shown are dark-field images of (**a**) a control animal and of animals (**b**) 24 and (**c**) 48 hours after MCAO and bright-field images of a control animal, showing VEGFR-1 expression in endothelial cells (**arrow** in **d**) and glial cells (**arrow** in **e**) but not in hippocampal neurons (**arrowhead** in **e**). Note the up-regulation of VEGFR-1 during infarction in pial vessels (**arrowhead**) and in vessels invading the ischemic core (**arrow** in **c**). Original magnifications: **a–c**, ×6.25; **d** and **e**, ×250.

followed the increase seen in RNA levels.

In addition to the ligand, expression of both VEGF receptors was also induced during cerebral infarction. VEGFR-1 was expressed in normal brain in vessel-like structures (Figure 7, a and d), in the choroid plexus, as well as in single glial cells throughout the neocortex and the hippocampus (Figure 7e). Twenty-four hours after MCAO, VEGFR-1 expression was lost in the ischemic core but was otherwise unchanged (Figure 7b). After 48 hours of occlusion, however, VEGFR-1 mRNA levels were induced at the border of the infarct and in the pia, in addition to vessel-like structures invading the core region of the infarcted area (Figure 7c). This suggests that the newly forming vessels express VEGFR-1. By 7 days after occlusion, expression levels returned to normal (not shown).

In the adult brain, VEGFR-2 expression was low, with the exception of the choroid plexus (Figure 8a). Like that of VEGFR-1, VEGFR-2 expression was lost in the ischemic core but remained unchanged in the other brain regions 24 hours after MCAO (Figure 8b). At about 48 hours after occlusion VEGFR-2 was induced at the border of the infarction and in the pia (Figure 8c), with strong expression at 72 hours (Figure 8d). Expression was found in vessel-like structures at the border of the infarction (Figure 8c) and at later times in vessels invading the core region of the infarcted area (Figure 8d). Increased VEGFR-2 mRNA levels persisted for 7 days after MCAO (Figure 8e). These mRNA data were confirmed at the protein level, using a monoclonal antibody against VEGFR-2. In the ipsilateral hemisphere we found vessel-like structures invading from pial vessels and in the core region of the infarct itself that expressed VEGFR-2 (Figure 9a), whereas no VEGFR-2 protein was detectable in the contralateral hemisphere (Figure 9b), where no new vessel growth was observed.

Interestingly, we also found strong expression of VEGFR-2 mRNA in the hippocampus (Figure 8, d and e). Expression in the hippocampus was visible 24 hours after infarction and was sustained for at least 7 days (Figure 8e). This was observed in the hippocampus of the ipsilateral side as well as the contralateral hemisphere (Figure 8f) but was absent in control, sham-operated animals (not shown). A subset of hippocampal cells strongly expressed mRNA for VEGFR-2, as detected by a specific cRNA antisense probe (Figure 8, g, h, and k), whereas signal was absent when the corresponding sense probe was used (Figure 8i). This finding was confirmed by detection of VEGFR-2 protein in cells within the same region by using the anti-VEGFR-2 antibody (Figure 9c), whereas the PECAM-1 antigen, the endothelial cell marker, was clearly present in different cells (Figure 9d).

Taken together, these results demonstrate that VEGF gene expression is induced only in the most hypoxic region adjacent to the infarction. This is followed by up-regulation of VEGFR-1 and VEGFR-2, initially at the penumbra, with subsequent expression along vessel-like structures that invade the core region of the infarcted tissue. We also show that nonendothelial cells in the hippocampus, probably neuronal cells, can express VEGFR-2.

Expression of HIF-1α and HIF-2α

To further analyze the mechanisms by which angiogenesis occurs during cerebral ischemia, we studied the expression of the transcription factors HIF-1 and HIF-2. Both factors are involved in hypoxia-induced transcriptional regulation of both VEGF and its receptors. HIF-1 and HIF-2 consist of two subunits, α and β; the α-subunit is hypoxia responsive.[25] Expression analysis, however, is hampered by the fact that hypoxic regulation of HIF-1 and HIF-2 activity is regulated at the protein level and not at the mRNA level.[25,38] Accordingly, no signif-

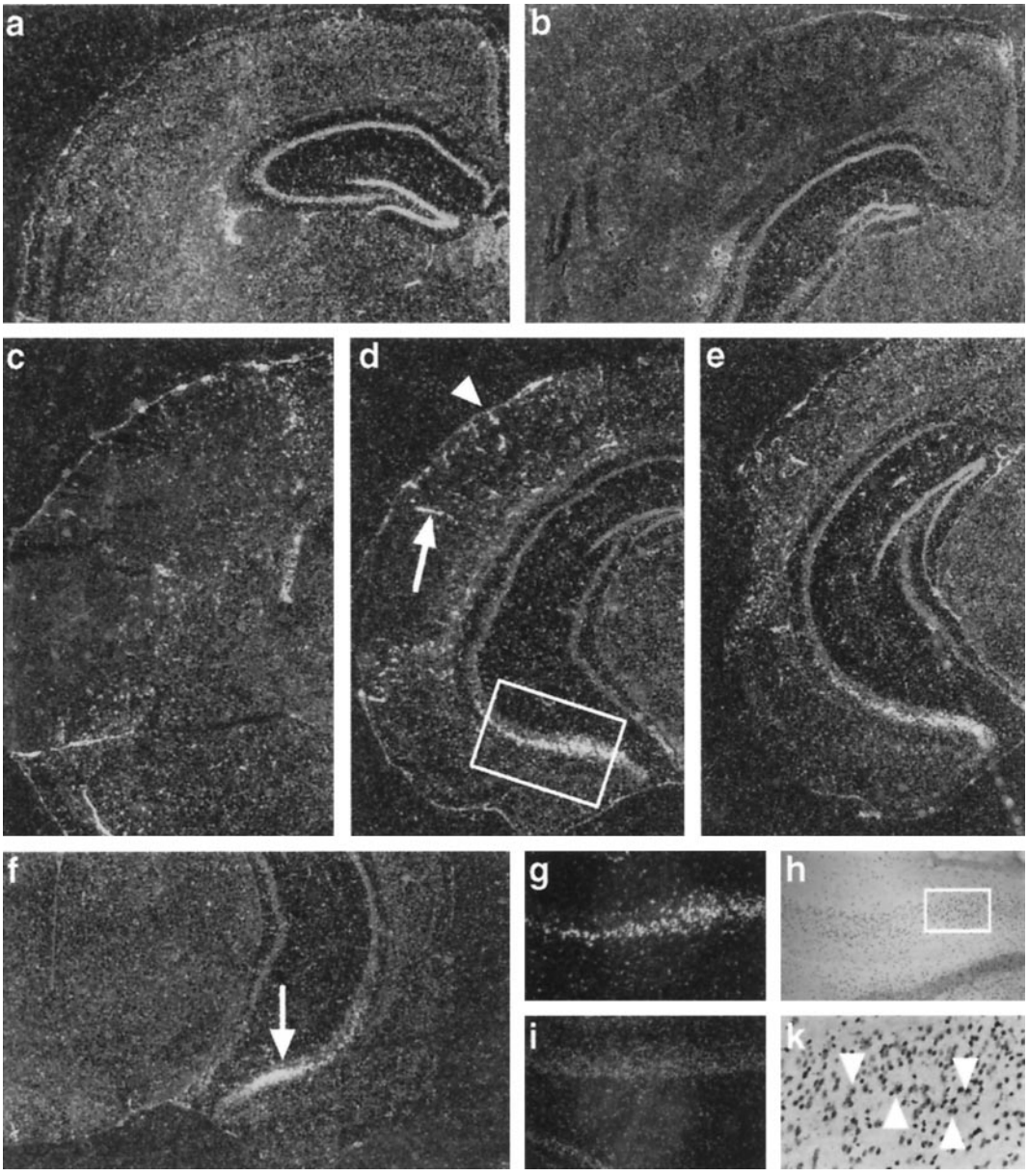

Figure 8. Expression of VEGFR-2 mRNA during cerebral infarction: *in situ* hybridization with a VEGFR-2-specific probe. Shown are dark-field images of (**a**) a control animal and of animals (**b**) 24, (**c**) 48, (**d**) 72, and (**e**) 168 hours after MCAO. Note the up-regulation of VEGFR-2 in pial vessels (**arrowheads**) and in vessels invading the ischemic core (**arrows**) in **d**. **f:** Contralateral hemisphere 24 hours after MCAO. Note strong VEGFR-2 expression in the hippocampus (**arrow**). **g:** Ipsilateral hippocampus 72 hours after MCAO: higher magnification of the area depicted in **d**. **h:** Bright-field image of **g**. **i:** Hybridization with sense probe, same area as in **g**. **k:** Higher magnification of **h**, showing VEGFR-2-expressing cells (**arrowheads**). Original magnifications: **a–f**, ×6.25; **g–i**, ×25; **k**, ×250.

icant changes at the mRNA level of HIF-1α were detected at any time after occlusion by RT-PCR analysis (Figure 4). Nevertheless, *in situ* hybridization analysis showed a remarkable change in the expression pattern. Whereas HIF-1α was expressed ubiquitously at low levels with enhanced expression in the hippocampus and the dentate gyrus, expression of HIF-2α was restricted to vessels (data not shown) as has been reported before.[27,28] Up to 24 hours after occlusion, ie, at a time at which we could clearly identify hypoxic tissue and up-regulated VEGF gene expression, the expression of both HIF-1α and HIF-2α did not change in the penumbra, but again was completely lost in the ischemic core (data not shown), supporting the finding that hypoxia does not activate transcription of these factors. After 48 hours, and more pronouncedly 72 hours after MCAO, though, there was a marked localized increase in mRNA levels for both factors (Figure 10, a and b). Whereas HIF-2α expression was restricted to vessel-like structures at the border of the infarction (Figure 10, d and f), HIF-1α was expressed in cells directly adjacent to the infarction (Figure 10, c and e). Unfortunately, analysis of HIF-1α and HIF-2α protein expression in tissue sections is hampered by the lack of commercially available antibodies against these factors for immunohistochemistry. However, the late, strictly localized induction of both HIF-1α and HIF-2α

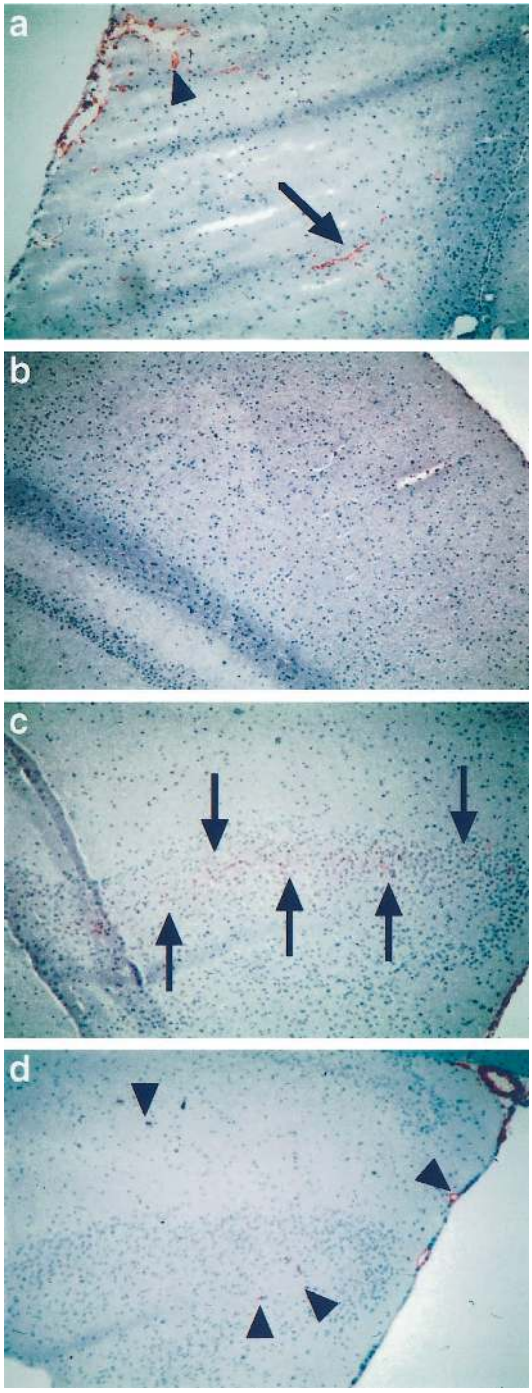

Figure 9. Expression of VEGFR-2 protein during cerebral infarction. Immunohistochemistry with a monoclonal anti-VEGFR-2 antibody of ischemic brain hemisphere (**a**) and contralateral side (**b**) 72 hours after MCAO. Note the up-regulation of VEGFR-2 in pial vessels (**arrowheads**) and in vessels invading the ischemic core (**arrows**) in **a**. **c:** Immunohistochemistry staining with anti-VEGFR-2 antibody (same region as in Figure 8g). The VEGFR-2-positive area is marked by **arrows**. **d:** Immunohistochemistry staining for PECAM-1 (same region as in **c**). Positive endothelial cells are marked by **arrowheads**. Original magnifications: **a** and **b**, ×25; **c** and **d**, ×50.

mRNA expression at 72 hours implies that stimuli, maybe other than hypoxia, are able to induce transcription of these genes.

In summary, our data support the scenario that after occlusion of a cerebral artery, tissue hypoxia leads to increased expression of VEGF and its receptors with subsequent new vessel growth. The pattern of expression suggests that new vessel formation originates from normal brain tissue and pial vessels and that these newly formed vessels grow through the hypoxic border zone into the core region of the infarction.

Discussion

Our study indicates that hypoxia is an important driving force of angiogenesis during cerebral ischemia and is mediated by induced gene expression of VEGF and its two receptors VEGFR-1 and VEGFR-2. It has been appreciated for many years that a hypoxic environment has important implications for both physiological and pathological processes. For example, it has been suggested that the molecular machinery initiating angiogenesis during development may be driven by oxygen deprivation of the expanding tissue.[3] In the model of the developing retina it was shown that hypoxia, caused by the onset of neuronal activity, is detected by astrocytes and Müller cells, which in turn respond by secreting VEGF and thereby induce formation of the retinal vessels. As the vessels become patent, they relieve the hypoxic stimulus, thereby matching vessel formation to oxygen demand.[39] During exposure to systemic or chronic hypoxia (eg, at high altitudes), VEGF gene expression is also induced in various organs[6] and may elicit an angiogenic response as an adaptive mechanism.[40] Furthermore, hypoxia has a major influence on tumor growth by inducing VEGF-mediated angiogenesis,[10,11] which is a prerequisite for continued tumor growth and subsequent metastasis.[41]

With the discovery of HIF-1[42] and HIF-2,[25] the first transcription factors have been identified that mediate hypoxia-induced gene expression at physiologically relevant oxygen tensions.[43] The fundamental role of HIF-1 for angiogenesis was demonstrated by targeted disruption of both HIF-1α and HIF-1β. Mice that are deficient for either subunit die around midgestation and demonstrate defects in angiogenesis as well as reduced levels of VEGF gene expression.[44–46] However, the hypoxic response is not totally abrogated but only partially eliminated.[47] The remaining hypoxic response might be due to hypoxically induced stabilization of the VEGF mRNA, which is probably mediated independently of HIF-1 through an element in the 3′-untranslated region of the VEGF transcript.[12] On the other hand, VEGF mRNA levels were increased in HIF-1α-deficient ES cells by deprivation of glucose,[44] and recently elevated VEGF mRNA levels were also found in HIF-1α-null embryos.[48] As glucose deprivation mainly stabilizes VEGF mRNA,[49] reduced glucose levels may function as a hypoxia- and therefore HIF-1-independent mechanism for VEGF induction. This could occur in situations where tissue perfusion is inadequate and results in combined oxygen and glucose deficiency as it occurs in stroke or even during embryogenesis, when tissues are rapidly expanding.[48] HIF-1 and HIF-2 activities are not regulated at the mRNA level, but at the level of protein stability.[25,38,50] This would explain the lack of HIF-1α and HIF-2α mRNA induction 24

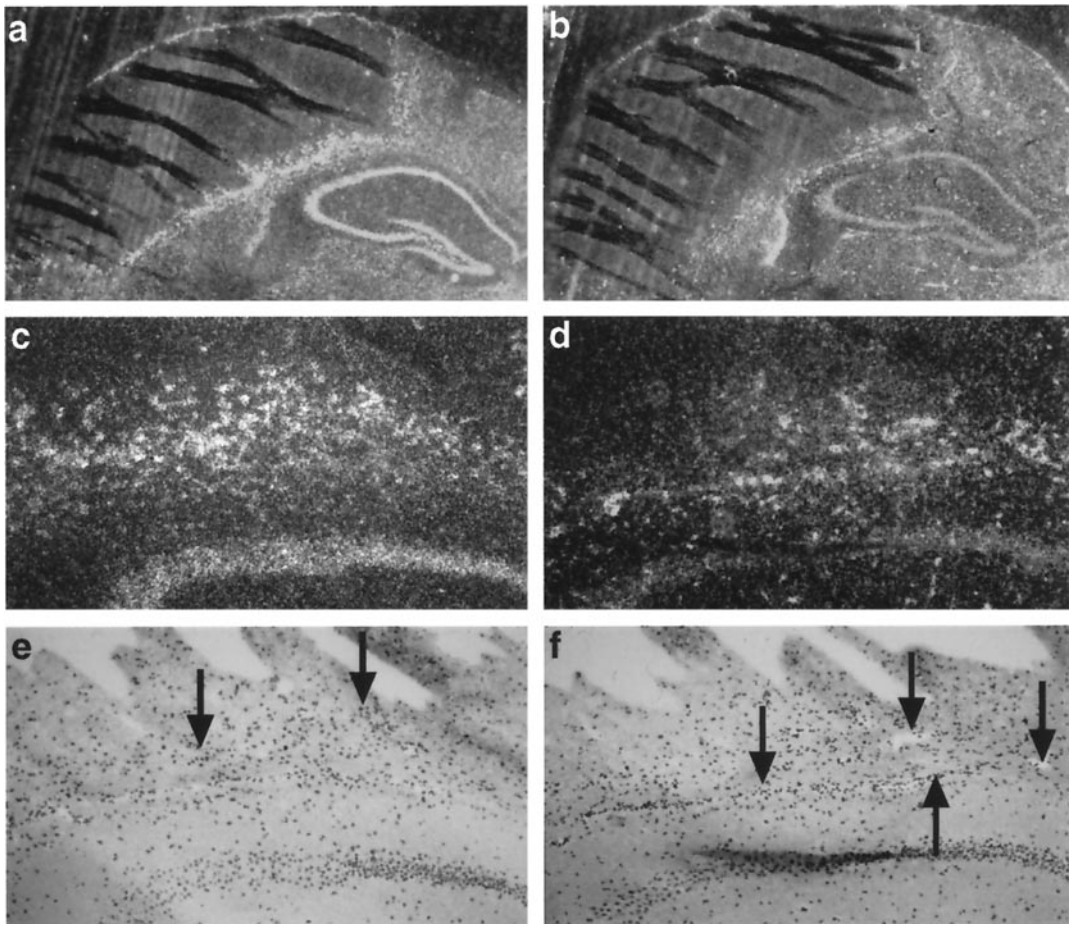

Figure 10. Expression of HIF-1α and HIF-2α mRNA during cerebral infarction: *in situ* hybridization with specific probes for (**a, c, e**) HIF-1α and (**b, d, f**) HIF-2α in animals 72 hours after MCAO. **a:** Dark-field image showing HIF-1α expression; **c**: higher magnification of **a; e:** bright-field image of **c**; **b:** dark-field image showing HIF-2α expression; **d:** higher magnification of **b**; **f:** bright-field image of **d**. Note that HIF-1α-positive cells line the infarction (**arrows** in **e**), and HIF-2α-positive cells are situated in vessel-like structures (**arrows** in **f**). Original magnifications: **a** and **b**, ×6.25; **c–f**, ×50.

hours after occlusion, although we found hypoxic cells and strong VEGF gene induction at this time. Unfortunately, detection of HIF-1α and HIF-2α protein by immunohistochemical methods was hampered by the lack of suitable commercially available antibodies. In addition, the very short half-life of these proteins under normoxic conditions[50,51] makes it extremely difficult to prepare appropriate brain lysates for the detection of this protein. We were unable to detect any HIF-1α or HIF-2α protein by immunoblot analysis in nuclear extracts freshly prepared from ischemic brain hemispheres. However, we found an induction of both HIF-1α and HIF-2α mRNA expression after 3 days of occlusion. Thus there might be factors other than hypoxia involved that are able to induce the expression of HIF-1α and HIF-2α at the transcriptional level. These could be associated with other cellular events ongoing at the time, such as endothelial cell proliferation, astrocyte activation, or microglia invasion.

We found the induction of VEGF gene expression after tissue hypoxia. However, there might be other stimuli involved in this gene activation as well. As mentioned above, glucose deficiency is likely to occur during ischemic disease and may contribute to increased VEGF expression. In addition, a whole range of growth factors and cytokines such as platelet-derived growth factor, transforming growth factor β, tumor necrosis factor α, or interleukin 1β[52] are known inducers of VEGF gene activation. All of these factors are expressed in the brain, become activated during cerebral ischemia,[53] and thus may participate in VEGF gene activation. However, the close temporal and spatial correlation between tissue hypoxia and induction of VEGF gene expression strongly implies hypoxia as a crucial stimulus for increased VEGF gene expression during stroke. Several reports deal with the role of VEGF in focal ischemia. With permanent MCAO, VEGF was detected in macrophages, endothelial cells, glial cells, and neurons. Increased expression was sustained for up to 2 weeks and was observed in both the infarcted core and the periphery.[13,16] Our studies revealed no VEGF gene expression in the ischemic core. However, we found a close spatial and temporal correlation between VEGF gene expression and the occurrence of hypoxic tissue, which was restricted to the penumbra and the pia. After transient MCAO, the increase in VEGF gene and protein expression in neurons and pial cells was generally more rapid and declined earlier than in our model, which might depend on reoxygenation pro-

cesses[14,15] While this work was in progress, Plate et al reported up-regulation of VEGF mRNA in rats specifically in microglial cells in the ipsilateral hemisphere starting 3 hours after the onset of MCAO and peaking after 24 hours.[54] These data are in good agreement with our own observations of strong VEGF mRNA induction between 6 and 24 hours after MCAO.

Expression of VEGFRs was analyzed in three recent reports. Plate et al found an early up-regulation of VEGFR-1 mRNA in peri-ischemic endothelial cells starting 3 hours after MCAO which was sustained for 72 hours, but no expression of VEGFR-2 was found at all.[54] On the other hand, Kovàcs et al found VEGFR-1 expression in endothelial cells in the infarcted core and periphery, with peak expression at 7 days after occlusion,[13] and Lennmyr et al observed expression of VEGFR-1 not only in endothelial cells, but also in neurons and glial cells. Expression of VEGFR-2 was prominent in glial cells between days 1 and 3 after occlusion.[16] Our data suggest that there might be a sequential temporal and spatial induction of both receptors, mainly in endothelial cells but also in glial cells and neurons. Gene expression of VEGFR-1 is directly inducible by hypoxia, as is the case for VEGF itself.[6] Both of these events are mediated by HIF-1.[55] In contrast, gene expression of VEGFR-2 is not directly induced by hypoxia.[6] Accordingly, HIF-1 does not induce gene expression of VEGFR-2 *in vitro*.[55] There are, however, several reports showing up-regulation of VEGFR-2 when endothelial cells were exposed to hypoxia *in vitro*, although this up-regulation does not occur at the level of transcription.[56,57] Transcription of the VEGFR-2 gene, however, is clearly inducible by HIF-2 and by the ligand VEGF itself.[29,58] Up-regulation of the VEGFR-2 gene induced by VEGF may represent an important positive feedback mechanism for VEGF action in ischemia-induced angiogenesis.[59] As a result of cerebral ischemia, expression of both VEGFRs is initially increased in the hypoxic penumbra and the pia, followed by later expression in the ischemic core in vessel-like structures. This suggests that newly forming vessels, invading the core region, express VEGFRs.

Although VEGFR-2 expression is considered to be relatively confined to endothelial cells,[60] VEGFR-2 expression has been described in neurons[16] and neural progenitor cells of the retina.[61] Interestingly, we found expression of VEGFR-2 in the hippocampus after cerebral ischemia, not only in the ipsilateral, but also in the contralateral hemisphere. Furthermore, increased expression levels were sustained for at least 7 days. The remote location of the hippocampus with respect to the ischemic damage suggests that a neuronal signal from the neocortex to the hippocampi, rather than ischemia itself, could have mediated the observed VEGFR-2 induction in this area. Considering that the collapsin/semaphorin receptor neuripilin-1 was recently identified as a coreceptor for VEGF,[62] these results may implicate the VEGF/VEGFR system in neuronal guidance, neurogenesis, and even neuroprotection.

Twenty-four hours after hypoxia-induced VEGF gene expression and in close relation to the expression of the VEGFR-1 and VEGFR-2 genes, endothelial cells started to proliferate. Vessel-like structures were observed that emanate from pial and brain parenchyma vessels and invade the ischemic core. These results support the hypothesis that cerebral ischemia can cause active angiogenesis. Our data are supported by postmortem analysis of human brain tissue from stroke patients, which has revealed a significant increase in the number of microvessels in the ipsilateral hemisphere when compared to the contralateral normal side.[2,63] Furthermore, in these patients higher blood vessel counts correlated with longer survival, suggesting a beneficial effect of this physiological response to ischemia.[2] In addition, exposure to chronic hypoxia, without the occurrence of tissue damage, was sufficient to increase the vascular density in the brain.[40] These findings suggest the presence of an intrinsic mechanism in the brain, the role of which is to protect the organ from hypoxic or ischemic damage.

Based on the aforementioned studies and on our results presented in this study, we propose the following cascade of events for cerebral ischemia: occlusion of a cerebral vessel leads to ischemia and subsequent cell death. At the border between the infarction and the healthy brain tissue, however, there is a zone that suffers from hypoxia (Figure 3), the so-called penumbra. In this particular region, hypoxia is a signal for induction of HIF-1 activity, which in turn activates the expression for both VEGF (Figure 5) and VEGFR-1 genes (Figure 7). Subsequently, transcription of the VEGFR-2 gene also becomes induced (Figure 8). The activation of the VEGF/VEGFR system leads to endothelial cell proliferation and new vessel growth (Figure 1), from healthy tissue toward the penumbra and further into the core region of the infarcted area. The development of new vessels may therefore be regarded as an attempt to counteract the detrimental effects of stroke by increasing perfusion and oxygenation of the suffering tissue.

It is intriguing to speculate that exogenous support of this endogenous emergency response could be useful in enhancing survival after stroke. Atherosclerotic narrowing or occlusion of arteries in the heart or limb, leading to myocardial infarction and gangrene, respectively, are targets for therapeutic angiogenesis.[64] Stimulation of physiological collateral growth and angiogenesis by exogenous VEGF has shown to be a promising strategy for the treatment of these diseases.[65,66] This strategy may also hold a similar promise for the treatment of stroke. Two recent reports emphasize the validity of this concept. Infusion of VEGF via a minipump into the cortex of adult rats produced a remarkable, localized neovascularization,[67] and topical application of VEGF to the surface of ischemic rat brain led to a significant reduction of infarct volume and limited the extent of neuronal damage.[68] It remains to be established, however, whether the beneficial effect of VEGF is due to an angiogenic response or to a putative direct neuroprotective effect.

Treatment of stroke in human patients probably has to combine therapies at different levels of intervention. A combination of different strategies that include direct neuroprotection, stimulation of angiogenesis, and inhibition of inflammation may turn out to be the most successful. We have recently shown that the protective effect of

erythropoietin in stroke can be attributed to a dual function of this growth factor as a direct neuroprotective agent and an angiogenic reagent.[32] Recent reports of the synergistic protection achieved in stroke models by the combined use of caspase-inhibitory compounds and *N*-methyl-D-aspartate receptor antagonists lend further support to this concept.[69,70]

In conclusion, after cerebral ischemia, the penumbra suffers from hypoxia and responds with increased VEGF gene expression, mediated via the transcription factors HIF-1 and HIF-2. Subsequent expression of both VEGFR-1 and VEGFR-2 is induced, and finally an angiogenic reaction, characterized by proliferating endothelial cells and new vessel growth, originates from the penumbra and invades the ischemic core. Enforcing this physiological response by exogenous stimulation with VEGF may prove to have a beneficial outcome for human stroke patients.

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