Role of Angiogenesis in Patients With Cerebral Ischemic Stroke

Jerzy Krupinski, MD; Jozef Kaluza, MD, PhD; Pat Kumar, PhD; Shant Kumar, PhD, FRC Path; J.M. Wang, PhD

Background and Purpose Stroke is one of the most common causes of mortality and morbidity in the Western world. It results from the occlusion of a cerebral artery followed by severe disturbances in blood supply through microvessels to brain tissue. Despite an extensive literature its pathophysiology is poorly understood, and this has severely impeded the logical development of therapy.

Methods Brains were obtained from 10 patients aged 46 to 85 years with survival times of 5 to 92 days after their stroke. Infarcted areas and representative control tissues from the contralateral uninvolved brain hemisphere were collected. Microvessel density was measured microscopically. A total of 6520 microvessels were scored in 10 801 areas. The level of activation of the endothelial cells was studied by immunohistochemistry using three monoclonal antibodies, viz, E-9, raised against activated endothelial cells; IG11, recognizing vascular cell adhesion molecule-1; and anti-proliferating cell nuclear antigen. Angiogenic activity in tissue extracts was examined using an in vivo chicken chorioallantoic membrane assay.

Results There was a statistically significant increase in the number of microvessels (Wilcoxon log-rank test; P≤.01) in 9 of 10 infarcted brain tissues when compared with their contralateral normal hemisphere. In these patients the higher blood vessel counts correlated with longer survival, as ascertained by Spearman's ρ analysis (P<.02). The number of microvessels filled with blood cells was significantly lower in the infarcted hemispheres (P<.01). In contrast, statistically significant increased numbers of empty microvessels occurred in infarcted tissues compared with the contralateral hemisphere. Monoclonal antibody E-9 reacted weakly with normal-brain vascular endothelial cells; anti-proliferating cell nuclear antigen and IG11 were virtually negative. All three antibodies strongly stained the blood vessels of stroke tissues. The stroke tissues contained angiogenic activity, as shown by the induction of new blood vessels in a chorioallantoic membrane assay.

Conclusions We have shown that stroke causes active angiogenesis that is more developed in the penumbra. Further experiments are needed to determine if this angiogenesis has beneficial effect. (Stroke. 1994;25:1794-1798.)

Key Words • angiogenesis • cerebral circulation • cerebral ischemia

Although there has been a significant decrease in the incidence of stroke in the last few decades, it is still the third leading cause of mortality and remains the most common cause of morbidity in the Western world.1 Much of the recent research has been conducted with the aim of ensuring or improving survival of neurons; very little attention has been directed toward investigating the role of angiogenesis. The latter might be a crucial determinant of neuronal survival after stroke. Indeed, the findings of positron emission tomography and single-photon emission tomography have implied that a higher blood-vessel density indicated a better prognosis in stroke patients.2,3 Therefore, it was suggested that angiogenesis in stroke tissue should be investigated.4 Ischemic stroke results from a reduction in cerebral blood flow after the occlusion of an artery, often the middle cerebral artery. As a result, some areas become ischemic, while others become hyperemic. Hyperemia usually occurs in the border zone known as the penumbra of the infarct and may be associated with hyperperfusion after the ischemic insult. In the penumbra one tends to see an increase in the number of blood vessels. Recurrent hypoxic conditions are associated with a considerable increase in the cerebral microvascular network in children perinatally and the elderly.5 Examination of autopsied brains of South American Indians living at high altitudes and permanently exposed to hypoxia has revealed increases in microvessel density.6 Furthermore, the different time courses of ischemia caused variability in the number of surviving microvessels, and this prompted us to undertake a quantitative study on the morphology of blood vessels and vascular density. The findings were correlated with patient survival time after stroke.

Materials and Methods

Source of Tissues and Vascular Density

Brains were obtained within 12 hours of death from 10 patients aged 46 to 85 years who had survived for 5 to 92 days after their stroke (Table 1). Infarcted areas and representative control tissues from the contralateral uninvolved brain hemisphere were collected. They were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin or for hemoglobin after Pickworth. A microvessel was counted if its profile was separated from other profiles. (There is the possibility that multiple profiles close together could be of the same microvessel, but this is difficult to exclude. Vascular casting would demonstrate the three-dimensional arrangement of new vessels. We intend to carry out such studies in the future.) Microvessel density was measured microscopically. A total of 6520 microvessels were scored in 10 801 areas. The level of activation of the endothelial cells was studied by immunohistochemistry using three monoclonal antibodies, viz, E-9, raised against activated endothelial cells; IG11, recognizing vascular cell adhesion molecule-1; and anti-proliferating cell nuclear antigen. Angiogenic activity in tissue extracts was examined using an in vivo chicken chorioallantoic membrane assay.

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References


4. Cerebral microvasculature in children perinatally and the elderly. Examination of autopsied brains of South American Indians living at high altitudes and permanently exposed to hypoxia has revealed increases in microvessel density. Furthermore, the different time courses of ischemia caused variability in the number of surviving microvessels, and this prompted us to undertake a quantitative study on the morphology of blood vessels and vascular density. The findings were correlated with patient survival time after stroke.

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Extraction of Angiogenic Activity

The slides were counterstained with Mayer's hematoxylin and mounted in gelatin gel. The angiogenic activity of endothelial cells was determined rather than quiescent. The latter antibody stains vascular endothelial cells in fetal, embryonic, and adult tissues. The E-9 antigen is a dimeric protein with a molecular weight of 170 kD under nonreducing conditions and 96 kD under reducing conditions. VCAM is a glycosylated cell molecule-1 [VCAM-1], Immunotech) and E-9, raised against activated/proliferating human umbilical vein endothelial cells. Thus, stroke tissues contain a higher proportion of empty microvessels than normal brain, either because they may be no longer functional or because they represent new blood vessel sprouts that have not yet been perfused. The intensity of staining with E-9 was greater in stroke tissues than in the contralateral normal hemisphere (Fig 2A), implying an activation of the endothelial cells in stroke, which is further evidence for the angiogenesis demonstrated in Table 1. The presence of VCAM was observed only in the blood vessels of the infarcted hemisphere (Fig 2B). This observation is probably connected with a large infiltration of granulocytes, lymphocytes, and macrophages into infarcted brain tissues, which is a commonly measured microscopically (×400 magnification) in each area of disease and normal brains using an eyepiece with a calibrated 1-μm squared field. Each measure was of an area equal to the field of view at ×400 magnification, approximately 0.2 mm². The number of areas counted for each patient was determined by the size of the tissue section and extent of the stroke damage. A total of 6520 microvessels of approximately 200 μm or less in diameter were studied in 10801 areas. The microvessels were also scored as “empty,” “filled with red blood cells” (RBCs), or pathological. The latter category included microvessels with an abnormal appearance, ie, lumen occluded by endothelial cells or budding microvessels or multiple lumens. Statistical analysis was performed using the Wilcoxon rank-test, and a difference was considered significant only when *P<.01. Spearman's ρ analysis was used to determine the correlation coefficient between blood vessel number and survival.

In view of the fact that angiogenesis is likely to be occurring, immunohistochemistry was performed on the microvessels to investigate the level of endothelial cell activation. Cryostat sections (5 μm) from tissues were snap-frozen, fixed in cold acetone for 10 minutes, and stained with two mouse monoclonal antibodies (MAbs), IG11 (anti-vascular cell adhesion molecule-1 [VCAM-1], Immunotech) and E-9, raised against activated/proliferating human umbilical vein endothelial cells. The latter antibody stains vascular endothelial cells in fetal, regenerating, and inflamed tissues and tumors but in very few normal tissues. The E-9 antigen is a dimeric protein with a molecular weight of 170 kD under nonreducing conditions and 96 kD under reducing conditions. VCAM is a glycosylated cell surface adhesion molecule. It belongs to the immunoglobulin superfAMILY and binds to one of the integrins, very late-acting antigen-4 (VLA-4), on leukocytes. VLA-4 is absent in normal endothelial cells but is upregulated on activated endothelial cells. A monoclonal antibody to proliferating cell nuclear antigen (PCNA; DAKO) was used to determine how many endothelial cells were in cell cycle rather than quiescent. The indirect immunoperoxidase method of Sternberger et al was used for staining, using the DAKO AEC substrate system as chromogen. The slides were counterstained with Mayer's hematoxylin and mounted in gelatin gel.

**Extraction of Angiogenic Activity**

Aliquots of normal brain and infarcted brain were extracted following the method of Kumar et al. The angiogenic activity of extracts was examined using a chorioallantoic membrane assay described in the same publication. A sample was scored as positive when a definite spoke-wheel formation of blood vessels was seen at the site of its application.

**Results**

Details of the 10 patients studied and their survival times after stroke are given in Table 1. The same table also shows the results of the quantification of microvessels in infarcted and contralateral normal hemispheres. A statistically significant increase in microvessel density in the former compared with the latter was observed in 9 of 10 patients. Data on the survival of patients were analyzed for a possible correlation with increase in microvessel density. Although there were only 10 patients with a rather short survival period (5 to 92 days), the use of Spearman's ρ analysis demonstrated a statistically significant correlation (ρ=.73, *P<.02) between blood vessel count and the survival period (Fig 1). Table 2 shows the densities of empty microvessels and microvessels containing RBCs in normal and infarcted tissue. Within the same microscopic field some blood vessels contained RBCs and others did not. The ratio of microvessels containing RBCs versus empty microvessels in the infarcted hemisphere was 0.17:0.11 (1.55:1), whereas the ratio in normal brain was 0.35:0.06 (6.0:1). This difference was statistically significant (*P<.01). Thus, stroke tissues contain a higher proportion of empty microvessels than normal brain, either because they may be no longer functional or because they represent new blood vessel sprouts that have not yet been perfused. The intensity of staining with E-9 was greater in stroke tissues than in the contralateral normal hemisphere (Fig 2A), implying an activation of the endothelial cells in stroke, which is further evidence for the angiogenesis demonstrated in Table 1. The presence of VCAM was observed only in the blood vessels of stroke tissues (Fig 2B); normal brain was negative (Fig 2C). This observation is probably connected with a large infiltration of granulocytes, lymphocytes, and macrophages into infarcted brain tissues, which is a commonly
observed early event in stroke. MAb to PCNA stained microvessels in infarcted tissue and penumbra, but there was hardly any staining of normal tissue. This indicated that endothelial cells in stroke were no longer quiescent but were in cell cycle.

Extracts of infarcted brain induced a typical angiogenic response in the chick chorioallantoic membrane assay; normal brain tissues produced no such response.

**Discussion**

This is the first study that has quantified microvessel density and demonstrated a correlation with survival in stroke. Our studies included only cases with thrombembolic pathology; there is no evidence in the published literature as to whether this or other types of stroke can result in increased numbers of microvessels. A number of important findings that have emerged are discussed, and their possible significance is evaluated.

Stroke is a vascular disease involving a disturbed blood supply to the brain. Even a short period (5 minutes) of experimental ischemia causes disturbances in capillary blood flow, ie, hypoperfusion in one region and hyperperfusion in another. The repeated occurrence of ischemia causes a progressive increase in edema of endothelial cells and brain tissue, infiltration of white blood cells

### Table 2. Densities of Empty Microvessels and Microvessels Containing RBCs in Normal and Infarcted Tissue

<table>
<thead>
<tr>
<th>Source of Tissue</th>
<th>Mean No./Area±SD†</th>
<th>Empty Blood Vessels</th>
<th>Vessels Filled With Blood Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarcted hemisphere</td>
<td>0.111±0.054</td>
<td>0.170±0.108</td>
<td></td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
<td>0.056±0.057*</td>
<td>0.350±0.057†</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly lower (P<.01) than Infarcted hemisphere.
†Significantly higher (P<.01) than Infarcted hemisphere.

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into nerve tissue, and damage to neurons. Fewer perfused capillaries remain even though total blood flow and total vascular volume return to control levels. This is in agreement with our results, in which the ratio of microvessels with RBCs to empty microvessels was 6.0:1 in normal brain but only 1.5:1 in infarcted brain. The inner parts of an infarct contain dead neurons with a capillary "no-reflow" phenomenon, ie, endothelial cells swell to obstruct the lumen. However, the outer area or penumbra still has blood flow between the functional and metabolic borders so that most neurons remain alive for some time. Restoration of appropriate perfusion in the penumbra partly through collateral capillaries (non-sprouting angiogenesis) can ameliorate the ischemia, as indeed can initiation of new vessel formation (sprouting angiogenesis), which previously has been observed within hours of experimentally induced ischemia. Our study of survivors several days after a cerebral infarct demonstrated a significant increase in microvascular density in infarced areas compared with the contralateral normal hemispheres in 9 of 10 patients. Although infarct and penumbra were counted together, the highest densities of microvessels were actually observed peripheral to the infarct in the penumbra. Higher blood vessel counts were correlated with improved patient survival (P<.02). However, the possibility exists that increased microvessel formation may well be the consequence of a longer delay, leaving time for the vessels to develop in a way that may not influence or direct prognosis. This demands further studies. We suggest that sprouting angiogenesis might be occurring after collateralization in response to brain ischemia just as it has been demonstrated to occur in survivors of myocardial infarction.

Angiogenesis, first described by Folkman et al in 1971, may occur by the action of growth factors, proteolytic enzymes, or other extracellular matrix factors that stimulate the growth of endothelial cells. Three growth factors are especially relevant to brain angiogenesis. (1) Vascular endothelial cell growth factor or vascular permeability factor is a glycosylated heparin-binding protein and a specific endothelial cell mitogen. Recently, an ischemia-induced angiogenesis factor has been demonstrated in myocardium and brain tumors. Endothelial cell growth factor-β, a heparin-binding growth factor, is a precursor of endothelial cell growth factor-α and fibroblast growth factor-α. (3) Transforming growth factor-β (TGF-β) induces angiogenesis in vivo but inhibits endothelial cell proliferation and migration in vitro. Injection of a mixture of antibodies to TGF-β and TGF-β5 causes a marked decrease in scarring during skin wound healing. In contrast, administration of TGF-β increases the anaggressiveness of angiogenesis.

The positive staining of endothelial cells with E-9 and IG11 in the majority of microvessels in the infarcted tissue supports the idea of their activation after the occurrence of stroke. MAb E-9 has been raised against activated/proliferating human umbilical vein endothelial cells. In vivo, it is present on the vascular endothelial cells of tumors, wounds, fetal organs, and inflamed tissues, but it generally stains only a few normal tissues very weakly. Therefore, the greater intensity of E-9 protein staining in stroke tissue compared with normal brain is an expected but novel finding.

The presence of VCAM in the vascular endothelial cells in stroke tissue and its absence in normal brain are
Fig 2. A, Photomicrograph showing blood vessels in the infarcted brain with heavy infiltration of granulocytes, lymphocytes, and macrophages. Stained after Pickworth and counterstained with hematoxylin and eosin; original magnification ×400. B, Monoclonal antibody E-9 stained vascular endothelial cells in infarcted brain (original magnification ×400). C, Localization of vascular cell adhesion molecules (VCAMs) in blood vessels of infarcted brain (original magnification ×400). D, Unlike panel C, normal brain shows almost complete lack of staining for VCAM (original magnification ×400).
also new and important findings. Leukocytes have been implicated in the pathogenesis of cerebral ischemia, although their role has not been clearly identified. For instance, there is an association between risk of future stroke and elevated leukocyte count in patients with transient ischemic attack. In our studies vascular cuffing by leukocytes was a prominent feature of infarct and penumbra but not normal brain. This, thus, might explain the increased expression of VCAM on these endothelial cells. VCAM is an adhesion molecule and plays a role in the binding of leukocytes to endothelial cells. Antibodies that can prevent the adhesion to and penetration of endothelium by leukocytes have been used with beneficial effects in diseases such as myocardial infarction, and their use has been advocated in the treatment of cerebral ischemia. We now have three lines of evidence that, taken together, suggest that sprouting angiogenesis is involved in recovery from stroke: (1) our observation of a large increase in microvessel profiles that would not be expected after collagenization alone, (2) angiogenic activity demonstrated in chorioallantoic membrane assay was isolated from stroke tissue but not from normal brain, and (3) immunohistochemistry using MAbs to E-9 and VCAM-1 indicates that endothelial cells are at least "activated" in stroke tissue. An anti-PCNA stains only stroke tissue, implying that endothelial cells in this tissue are no longer quiescent but are in cell cycle.

In conclusion, despite a rapidly expanding literature, the pathogenesis of stroke is poorly understood. Blood vessels undoubtedly are a key component in stroke. This study has shown that an increase in neovascularization is correlated with longer survival of the patients. Further studies are warranted to examine if a therapeutic approach aimed at stimulating angiogenesis and/or decreasing leukocyte adherence to endothelial cells might lessen nerve tissue damage after the occurrence of ischemia or prevent subsequent stroke in patients with transient ischemic attack.

References

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