Background and Purpose—Both vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) are expressed in higher than normal concentrations in the penumbra of patients after ischemic stroke. Because both cytokines are central to the processes of angiogenesis, tissue inflammation, and fibrosis, we performed serial measurements of these cytokines in patients with cerebral infarction and determined their relationship to stroke etiology and volume.

Methods—We serially (at days 0, 1, 3, 7, and 14) measured the serum levels of VEGF and active TGF-β1 in 29 patients with acute ischemic stroke. Age-matched healthy subjects (n = 26) were used as controls.

Results—Expression of VEGF was significantly increased in the majority of patients after acute stroke at each of the time points compared with normal controls. Highest expression occurred at day 7 (588 ± 121 pg/mL; P = 0.005), and it remained significantly elevated at 14 days after stroke. Expression of VEGF correlated with infarct volume, clinical disability (Scandinavian Stroke Scale), and peripheral leukocytosis and was significantly higher in patients with atherothrombotic large-vessel disease and ischemic heart disease (P < 0.05 in all cases). In contrast, expression of active TGF-β1 was not significantly different from control patients at any of the measured time points. When the mean concentration of TGF-β1 from each patient (pooled time points) was compared with the control mean, a significant increase was found in only 2 patients, whereas levels decreased in 12 patients (P < 0.05). There was no correlation between circulating active TGF-β1 and VEGF expression, leukocytosis, stroke subtype, or patient disability as assessed by Scandinavian Stroke Scale score.

Conclusions—VEGF but not TGF-β1 showed a dramatic increase in serum of stroke patients. Correlation between stroke severity and VEGF concentration suggests it could be involved in the subsequent repair processes resulting in partial recovery after stroke. Correlation between VEGF expression and peripheral leukocytosis suggests that these changes may also reflect the immunologic status of the patient. VEGF may play an important role in the pathophysiology of acute ischemic stroke and could be of value in future treatment strategies. (Stroke. 2000;31:1863-1870.)

Key Words: angiogenesis • growth factors • stroke, acute • stroke, ischemic
leukocytes, as well as cholesterol, fibrinogen, urea, and glucose levels, were determined at the time of sampling. VEGF and active TGF-β1 levels were measured by standard quantitative sandwich ELISA (Quantikine) kits, obtained from R&D Systems. Samples from each individual were analyzed in triplicate and subsequently used in all further statistical analysis. The lower limits of detection were 5.0 pg/mL for VEGF and 6.0 pg/mL for TGF-β1. Serum binding of TGF-β1 to α2-macroglobulin was shown not to affect the sensitivity of this assay (information provided by R&D Systems).

Clinical examination was performed on admission (day 0) and 7, 14, and 30 days after ischemic stroke. The examinations were scored according to the 58-point Scandinavian Stroke Scale (SSS).18,19 All patients were evaluated by CT or MRI, and patients were classified according to the 58-point Scandinavian Stroke Scale (SSS).18,19 All patients were also categorized as having a large infarct (LI; largest diameter of infarct >4 cm; SSS >30), a moderate infarct (MI; >1.5 cm and <4 cm; SSS >30), or a small infarct (SI <1.5 cm; SSS >40). Patients were also categorized as having atherothrombotic large-vessel disease (damage to the main anterior, middle, or posterior cerebral artery; n = 14), small-vessel disease (affecting the deep perforating branching arteries; n = 7), or cardioembolic (n = 7) stroke subtypes. According to the Oxfordshire classification, infarction was classified as partial anterior circulation infarct (PACI), total anterior circulation infarct (TACI), or lacunar infarct (LACI). Patients were considered to have ischemic heart disease if they suffered from myocardial infarction, had angina, or had any other signs or symptoms of heart disease demonstrated by additional tests including ECG, echocardiography, or Holter monitoring. Stroke risk factors such as hypertension, smoking, and diabetes were also assessed (Table 1).

Statistical analysis was performed to identify differences in growth factor expression over time after acute stroke, to correlate any changes in growth factor expression in relation to stroke subtypes and etiology, and to correlate stroke volume with growth factor expression and clinical disability, including short-term follow-up. Results are expressed as mean ± SEM. Mean cytokine expression from the 5 measured time points of individual patients was compared with control values by the 1-sample t test. Additional statistical analyses were based on the assumption that the data were not normally distributed, and analysis was performed with nonparametric tests for paired (Spearman rank test) and unpaired (Mann-Whitney U test) groups, respectively. The analysis was 2-tailed unless otherwise specified. The relationships between leukocytes and VEGF or TGF-β1, VEGF and TGF-β1, and both cytokines plus all
Results

Patients
Twenty-nine patients (14 men and 15 women) aged 43 to 89 years (mean age 72 years) and control subjects (n=26; 14 men and 12 women) aged 46 to 85 years (mean age 70 years) were studied (Table 1). Sixteen patients had LIs, 6 had MIs, and 7 had SIs. The patients could be further subdivided into those with atherothrombotic stroke (n=21), embolic stroke (n=7), and stroke of unknown origin (n=1). Patient characteristics, including risk factors, and biochemical and hematological data taken at the time of first sampling are shown in Table 1. The initial SSS score ranged from 4 to 30 (mean 14) in the LI group, 32 to 40 (mean 37) in the MI group, and 41 to 53 (mean 45) in the SI group. After 14 days, the SSS scores peaked after 7 days and were 8 to 52 (mean 23) in the LI group, 43 to 57 (mean 50) in the MI group, and 43 to 58 (mean 54) in the SI group (see Table 1 for further details). Two patients with large-vessel stroke died on days 10 and 30, respectively, from herniation, and 1 patient suffering from cardioembolic stroke died on day 26 from herniation. The initial peripheral leukocyte count was not significantly different in stroke patients compared with controls (Table 1).

Serum Levels of Active TGF-β1
The mean levels of active TGF-β1 in patients with stroke were significantly different compared with controls at all time points (days 0, 1, 3, 7, and 14; P<0.05 in all cases; Spearman rank). There was no correlation of TGF-β1 expression with infarct size, stroke subtype, or leukocyte count. Similarly, there was no relationship between TGF-β1 expression and patients with a history of hypertension (n=21), diabetes mellitus (n=10), smoking (n=10), obesity (n=6), hypercholesterolemia (cholesterol >5.6 mmol/L; n=20), hyperglycemia (glucose >5.8 mmol/L; n=9), and hyperuricemia (n=6) compared with the others (P>0.05 in all cases; Spearman rank).

Serum Levels of VEGF
The mean concentration of VEGF in the serum of patients with stroke was significantly higher than that of the controls at all time points (days 0, 1, 3, 7, and 14; P<0.05 in all cases, Spearman rank; Figure 3). At the time of admission, mean VEGF levels were 410±71 pg/mL; after 24 hours, they were 416±64 pg/mL; and after 3, 7, and 14 days, they were 434±77, 588±128, and 518±80 pg/mL, compared with the control level of 245±28 pg/mL. Mean expression of VEGF peaked after 7 days and was maintained up to 14 days. Comparisons of the mean from pooled time points against the mean control values are shown in Figure 4. Comparison of the subgroups of stroke patients revealed the highest expre-
Discussion

Polypeptide growth factors are likely to play an important role in the cellular and molecular processes underlying wound healing and functional recovery after acute ischemic stroke. Brain injury induces expression of many different growth factors and cytokines that can protect neurones against excitotoxicity, hypoxia, hypoglycemia, acidosis, and pro-oxidants.

In this study, we found that serum levels of the active form of TGF-β1 were not significantly different from those of age-matched controls over a period of 1 to 14 days after acute ischemic stroke. When expression of TGF-β1 from each of the 5 time points (days 0, 1, 3, 7, and 14 after stroke) from each patient was pooled, only 2 of 29 patients had significantly higher mean concentrations than the control patients; however, 12 had mean values significantly below the control levels.

Our own and previous studies have shown upregulation of TGF-β1 protein expression around neural and microglial cells as well as blood vessels after ischemic injury. TGF-β1 is secreted as a latent inactive complex, becoming active only after release; therefore, its functional capabilities are determined by its rate of activation. Our own and previous studies have shown upregulation of TGF-β1 protein expression around neural and microglial cells as well as blood vessels after ischemic injury. TGF-β1 is secreted as a latent inactive complex, becoming active only after release; therefore, its functional capabilities are determined by its rate of activation. TGF-β1 mRNA was detected 3 days after experimental ischemia in rats, which coincides with vascular sprouting, and is involved in vasculogenesis and maintenance of blood vessel integrity. TGF-β1 is secreted as a latent inactive complex, becoming active only after release; therefore, its functional capabilities are determined by its rate of activation. TGF-β1 mRNA was detected 3 days after experimental ischemia in rats, which coincides with vascular sprouting, and is involved in vasculogenesis and maintenance of blood vessel integrity. TGF-β1 is secreted as a latent inactive complex, becoming active only after release; therefore, its functional capabilities are determined by its rate of activation. TGF-β1 mRNA was detected 3 days after experimental ischemia in rats, which coincides with vascular sprouting, and is involved in vasculogenesis and maintenance of blood vessel integrity.
One explanation for our results is that excessive utilization of TGF-β1 in and around the damaged tissues results in lower peripheral circulating levels. TGF-β1 might be produced intrathecally and modulated locally by production of different cytokines, eg, interleukin-6. Circulating TGF-β1 expression was also reduced in patients suffering from *Plasmodium falciparum* malaria infection, which suggests its reduction in ischemic stroke may be a direct result of the ensuing proinflammatory nature of the cytokine network. Because active TGF-β1 is particularly unstable (half-life of ~2 minutes), tightly regulated control of this process would effectively prevent demonstration of excess protein in the blood. Alternatively, we have previously demonstrated a marked expression of CD105, which is a TGF-β1 and TGF-β3 receptor, in angiogenic ECs in stroke tissue. Therefore, it is highly likely that low serum levels of TGF-β1 in stroke patients may be a result of its binding to angiogenic ECs. Blood platelets are a significant source of TGF-β1, and previous studies have shown a reduction after acute ischemic stroke. Unfortunately, we did not measure this parameter. Interestingly, expression of active TGF-β1 in the serum of patients with hypoxic diabetic retinopathy was inversely correlated with retinal proliferation, which suggests that deficient activation of this molecule, possibly as a consequence of blood retina barrier breakdown, can result in improved angiogenesis in hypoxic conditions. We are not aware of any studies comparing TGF-β1 concentration in the brain and in the serum; however, studies comparing cerebrospinal fluid (CSF) expression and serum suggest an association between increased CSF TGF-β1 and reduction in the serum. This could be a result of passage of this cytokine from the peripheral circulation to the intrathecal compartment across the blood-brain barrier.

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**TABLE 2. VEGF (pg/ml) Expression in Serum of Patients After Ischemic Stroke**

<table>
<thead>
<tr>
<th>Stroke Type</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI*</td>
<td>301±183</td>
<td>255±172</td>
<td>237±136</td>
<td>288±192</td>
<td>285±185</td>
</tr>
<tr>
<td>MI</td>
<td>355±159</td>
<td>352±148</td>
<td>352±169</td>
<td>401±160</td>
<td>424±209</td>
</tr>
<tr>
<td>LI</td>
<td>479±185</td>
<td>510±421</td>
<td>552±514</td>
<td>790±875</td>
<td>656±521</td>
</tr>
<tr>
<td>Large vessel</td>
<td>492±466</td>
<td>468±389</td>
<td>524±494</td>
<td>647±574</td>
<td>601±455</td>
</tr>
<tr>
<td>Small vessel</td>
<td>258±180</td>
<td>253±181</td>
<td>231±163</td>
<td>260±175</td>
<td>257±156</td>
</tr>
<tr>
<td>IHD‡</td>
<td>455±392</td>
<td>462±347</td>
<td>477±425</td>
<td>651±722</td>
<td>575±438</td>
</tr>
<tr>
<td>Non-IHD</td>
<td>189±120</td>
<td>185±142</td>
<td>200±161</td>
<td>234±184</td>
<td>170±138</td>
</tr>
</tbody>
</table>

*Values significantly different (P<0.05) at days 3–14 vs LI.
†Values significantly different from small vessel (P<0.05).
‡All values are significantly different (P<0.05) compared with non-IHD.

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**Figure 6.** Sequential serum values from 20 healthy individuals taken on day 1 (VEGF 1) and day 40 (VEGF 2). From the top panel, it is apparent that the median values for the 2 samples were very similar, and as shown in the bottom panel, there was a highly significant correlation between VEGF 1 and VEGF 2 data (r=0.894; P<0.001; Spearman’s r).

**Figure 7.** Serum levels of VEGF in patients with large-vessel (LV; n=15) or small-vessel (SV; n=7) disease at different time points. Data are expressed as mean±SEM. *P<0.05 vs control.
VEGF is a key mediator of angiogenesis, which is an important process leading to reperfusion of ischemic brain tissue after acute stroke. It is well established that under hypoxic conditions, upregulation of VEGF mRNA and protein occurs. VEGF is secreted in significant quantities by activated macrophages and microglial cells in response to hypoxic conditions associated with ischemic stroke. Excess unutilized VEGF has been shown in several studies to be expressed in the serum of cancer patients. Our results showed a significant increase in expression of VEGF, which reached a peak after 7 days and remained elevated after 14 days in stroke patients compared with age-matched controls. We found that the mean VEGF level in control patients (245 pg/mL) was similar to that found in another study (220 pg/mL). Furthermore, expression of VEGF was higher in the serum of patients with the largest infarct volume (LI), in those with large-vessel atherothrombotic disease, and in those with evidence of ischemic heart disease. With respect to stroke volumes, the differences were marginally not statistically significant because of variation in expression from patient to patient; however, an obvious trend could still be seen.

These results are in agreement with our previous findings that neurons, ECs, and astrocytes expressed higher levels of VEGF protein and mRNA in the penumbra surrounding infarct tissue than in the normal contralateral tissue of patients after acute stroke. Other studies using rat models have shown increased expression of VEGF immunoreactivity from day 1 to day 14 and intense angiogenic activity after 3 days coinciding with increased thickness of ECs between day 3 and day 14 after middle cerebral artery occlusion. These results suggest that there is a continuous demand for VEGF during the entire active period of an infarct, and this could be due to or at least beneficial for the long-term requirement for endothelial proliferation and subsequent blood vessel regeneration. The exact relationship between kinetics of VEGF expression and angiogenesis is impossible to clarify at this stage because we do not have reliable markers of angiogenesis in the serum or CSF.

Mean VEGF expression was lowest in the serum of patients with SI, increasing in MI and being the greatest in LI patients, which suggests that VEGF could be a marker indicating the size of the infarct. Perhaps not surprisingly, peripheral leukocyte count was also greatest in those patients with LI, probably as a consequence of altered immunologic status caused by extensive tissue damage. A pathogenic role has previously been suggested for leukocyte adhesion and migration in acute cerebral ischemia. In the present study, a strong correlation was found between leukocyte expression and VEGF concentration in the serum of patients after acute stroke. A similar correlation was shown between monocytes and interleukin-6 after ischemic stroke, which suggests that peripheral leukocytes could be a possible origin of increases in cytokine levels. Activated macrophages have previously been shown to be a source of VEGF; however, astroglia also express VEGF, which is upregulated in hypoxic conditions, whereas levels of VEGF are increased during angiogenesis in the embryonic neuroectoderm and are not associated with leukocytosis. Our results showed that those patients with ischemic heart disease expressed significantly higher VEGF levels than those without. Taken together, these results do not allow us to determine the exact cellular source of VEGF after ischemic stroke but do suggest a relationship between tissue damage, hypoxia, and VEGF expression.

In the present study, there was no overall correlation between increased VEGF expression and improvement in SSS rating or reduction in stroke volume within the 30-day test period. We noticed, however, that 3 of the 29 patients expressed exceptionally high mean VEGF levels (>1000 pg/mL) and that these patients presented with the highest improvements in SSS rating (data not shown). Previous studies have shown that the addition of bFGF to rats after the onset of focal cerebral ischemia, although it did not affect stroke volume, produced a striking degree of recovery of contralateral forelimb and hindlimb function over a period of several weeks. Increased VEGF expression may provide more long-term beneficial effects as a result of continued angiogenesis over several months. Additional longer-term studies are required in which recovery from stroke is compared with overall activities of multiple growth factors/cytokines that are modified during ischemic stroke (including PDGF and bFGF). These studies should be performed with patients who have similar initial disability levels.
There was no correlation between VEGF and TGF-β1 expression, which suggests that although TGF-β1 can have a synergistic effect on VEGF secretion (for example, in human synovial fibroblasts), this is not an important feature in the production of high VEGF levels after ischemic stroke.

In conclusion, this longitudinal study of cytokine levels in serum after acute ischemic stroke indicates that levels of TGF-β1 were not significantly different from an age-matched control group. On the other hand, VEGF concentration was lowest in the serum of patients with SI and highest in those with LI. We also found that VEGF was further elevated in subjects with atherothrombotic large-vessel disease as well as ischemic heart disease. It is well established that recovery from stroke is associated with angiogenesis, and we have demonstrated a relationship between stroke volume and expression of the angiogenic molecule VEGF. Additional studies may help to clarify the therapeutic potential of VEGF administration after stroke.

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References


Serial Measurement of Vascular Endothelial Growth Factor and Transforming Growth Factor-β1 in Serum of Patients With Acute Ischemic Stroke
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