Angiogenesis in Symptomatic Intracranial Atherosclerosis
Predominance of the Inhibitor Endostatin Is Related to a Greater Extent and Risk of Recurrence
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Background and Purpose—Angiogenesis may be beneficial in chronic myocardial and limb ischemia, but its role in intracranial atherosclerosis remains unknown. We aimed to investigate the relationship between the pro-angiogenic vascular endothelial growth factor (VEGF) and the anti-angiogenic endostatin, and the extent and risk of recurrence of symptomatic intracranial atherosclerosis.

Methods—Of a total of 94 consecutive patients with symptomatic intracranial stenoses, 40 fulfilled all inclusion criteria. Intracranial stenoses were confirmed by magnetic resonance angiography. Magnetic resonance imaging (MRI) including diffusion-weighted sequences was conducted. Plasmatic VEGF and endostatin were determined from blood samples obtained 3 months after stroke onset, and patients were followed-up thereafter.

Results—A total of 144 intracranial stenoses were confirmed (median number per patient=3). Endostatin/VEGF ratio gradually augmented with the increasing number of intracranial stenoses (r=0.35, P=0.02). Diabetes mellitus (OR, 6.04; CI, 1.1 to 32.2; P=0.03) and a higher endostatin/VEGF ratio (OR, 15.7; CI, 2.2 to 112.3; P=0.006) were independently associated with a greater extent of intracranial atherosclerosis. During a median follow-up of 13 months, 8 patients (20%) experienced a new cerebral ischemic event. A higher baseline endostatin concentration was an independent predictor of new events (hazard ratio, 7.24; CI, 1.6 to 33.8; P=0.011) in a Cox regression model after adjustment for age, sex, number of stenotic vessels, and risk factors. Patients with a higher endostatin level had a lower survival free of new events (P=0.01, log-rank test).

Conclusions—A predominance of the inhibitor endostatin within the endogenous angiogenic response is associated with a greater extent and risk of recurrence of symptomatic intracranial atherosclerosis, suggesting that angiogenesis may be beneficial in this condition. (Stroke. 2005;36:92-97.)

Key Words: angiogenesis ■ atherosclerosis, intracranial ■ endostatins ■ intracranial stenosis ■ vascular endothelial growth factor

Patients affected by symptomatic intracranial atherosclerosis are exposed to an elevated risk for recurrent major vascular events and death. Moreover, evidence-based preventive strategies are far from satisfactorily reducing the risk of recurrence in this group of patients. The recently reported Warfarin-Aspirin Symptomatic Intracranial Disease prospective trial showed unacceptably high year-recurrence rates in both warfarin and aspirin arms. This insufficient protection provided by antithrombotics warrants further research on better preventive therapies for this condition.

Angiogenesis is a complex and finely regulated process triggered by hypoxia that consists of the sprouting of new blood vessels from pre-existing vascular structures. Animal and human studies have demonstrated that angiogenesis may play an important role in acute ischemic stroke improving brain tissue recovery and functional outcome. However, its relevance in the natural history of diseases that cause chronic cerebral hypoperfusion, such as intracranial large-artery atherosclerosis, remains unknown.

The endogenous angiogenic response is the result of the balance between many different stimulant and inhibitor factors that interact in an orchestrated manner. Following this premise, we designed a long-term follow-up prospective study to investigate the relationship between the expression of the pro-angiogenic vascular endothelial growth factor (VEGF), the anti-angiogenic endostatin, and the extent and risk of recurrence of symptomatic intracranial large-artery atherosclerosis.
Patients and Methods

Patient Selection

Our study consisted of patients with a first-ever transient ischemic attack (TIA) or ischemic stroke attributable to intracranial atherosclerotic stenoses detected by transcranial Doppler (TCD) and confirmed by magnetic resonance angiography (MRA) in the absence of any other potential causes of cerebral ischemia. Of a total of 834 consecutive first-ever TIA or stroke patients admitted at our Stroke Unit between November 2001 and January 2003, 94 showed intracranial stenosis potentially responsible for the cerebral ischemic event on TCD recordings. Diagnostic work-up included cranial computed tomography scan and MR imaging, cervical carotid ultrasound, immunological study (determination of anti-DNA, ANA, anti-Ro, anti-La, and anti-cardiolipin antibodies), and echocardiography and electrocardiogram–Holter when indicated. Fifty-four patients were excluded for the following reasons: impossibility to perform magnetic resonance imaging (MRI) (n=8); absence of angiographic confirmation of stenoses (n=5); location of ischemic lesion outside the territory supplied by the stenosed artery (n=5); embolicogenic cardiopathy (n=7); cervical internal carotid arteries (ICAs) >30% stenoses (n=13); neoplasms (n=2); inflammatory conditions (n=4); nonatherosclerotic causes of intracranial stenosis (n=4); stroke-related death or severe disability (n=5), and denial of informed consent (n=1). Finally, at inclusion visit, performed 3 months after the qualifying event, informed consent and blood samples were obtained from 40 patients with symptomatic intracranial atherosclerotic stenoses, who were included in this study. This study was approved by the local ethics committee.

Baseline Vascular Risk Factors and Clinical Variables

Cigarette smoking and medical history of hypertension, hypercholesterolemia, diabetes mellitus, diagnosed coronary heart disease, and intermittent claudication were recorded at the inclusion visit. Stroke severity was assessed with the maximum National Institutes of Health Stroke Scale (NIHSS) score obtained during admission. Treatment was allocated after the stenoses were confirmed, following the criteria of the neurologist in charge. The use of acenocoumarol, aspirin, clopidogrel, triflusal, statins, and angiotensin-converting enzyme inhibitors was registered. Functional status at day 90 was assessed by means of the modified Rankin scale.

Ultrasound Protocol

TCD recordings were performed using a Multi-Dop-X/TCD (DWL Elektronische Systeme GmbH) device, with a hand-held transducer in a range-gated, pulsed-wave mode at a frequency of 2 MHz. We used a standard method of insonation through the temporal, occipital, and orbital windows without compression testing, as previously described. Intracranial stenoses were diagnosed according to validated criteria. TCD examinations were performed on admission and repeated at the inclusion visit to confirm the persistence of stenoses. TCD long-term follow-up was conducted in those patients with optimal acoustic windows, with a 6-month periodicity. Progression of intracranial large-artery atherosclerosis was defined either as the progression of pre-existing stenoses or as the appearance of new stenoses during follow-up.

MRI Protocol

All MRI was performed with a 1.5-T whole-body imager system (Magnetom Vision Plus or Magneton Symphony, Siemens Medical Systems). The following sequences were obtained in all patients: T2-weighted turbo spin-echo (13700/90/2) (reception time/echo time/excitations), T2*-weighted gradient-echo (600/26/2), T1-weighted spin-echo (550/1/2), fluid-attenuated inversion recovery (9000/110/2), echo-planar diffusion-weighted (4000/100/2), and MRA [35/7.2/15 (reception time/echo time/flip angle)]. MRI was conducted within the first 5 days after symptom onset in all cases.

Images were interpreted by the same neuroradiologist (A.R.), who was blinded to sonographic and biochemical data. Presence and location of acute cerebral infarctions on diffusion-weighted images (DWIs) were classified as follows: normal (absence of DWI hyperintensities), lacunar, cortical territorial, subcortical territorial, cortico–subcortical fragmented, and watershed areas cerebral infarctions.

MRA was performed using a 3-dimensional time-of-flight sequence, using 1.5-mm-thick sections, 200-mm field of view, 200×512 matrix, and acquisition time that ranged from 7 to 11 minutes. Maximal intensity projection reconstructions were performed at the time of imaging. Intracranial stenosis was defined as a focal narrowing >50% in luminal reduction affecting the main cerebral large arteries. A circumscribed flow gap was considered indicative of severe stenosis when the corresponding TCD signal fulfilled the criteria for intracranial stenosis. The extent of intracranial atherosclerosis was defined by the number of MRA-confirmed stenoses. The symptomatic intracranial stenoses were categorized according to their severity as moderate (50% to 70%) and severe (>70%).

T1-, T2-, and T2*-weighted sequences were performed to evaluate the relationship between coexistent cerebral microangiopathy and the levels of angiogenic factors. The following variables were recorded: presence of chronic lacunar infaracts, presence and degree of white matter hyperintensities, and existence of old microbleeds. White matter hyperintensities were graded according to size and shape into: (1) absent; (2) small, focal with a diameter ≤5 mm (minimal); (3) large, focal, mostly round with a diameter between 5 and 10 mm (moderate); and (4) large, mostly irregular and diffusely confluent or with a diameter >10 mm (severe).

VEGF and Endostatin Level Determination

Blood samples were drawn at the inclusion visit, performed 3 months after the qualifying ischemic event to avoid acute phase changes, always after overnight fast. EDTA tubes were used to collect the blood; plasma was immediately separated by centrifugation at 3500 rpm for 15 minutes and stored at −80°C. Total VEGF (Quantikine; R&D Systems) and endostatin (Chemikine; Chemicon) levels were determined in duplicate by commercially available enzyme-linked immunosorbent assays, and the mean value of both determinations was used. The mean intra-assay coefficients of variation were <10% in all cases.

Clinical Follow-up

Clinical visits were performed every 6 months after blood sampling by a stroke neurologist who was blinded to biological data, and new ischemic strokes or TIAs attributable to intracranial atherosclerosis were recorded.

Statistical Analysis

Statistical analyses were made by use of the SPSS statistical package, version 9.0. Statistical significance for intergroup differences was assessed by the χ² test for categorical variables and the Student t, Mann–Whitney U, and Kruskal–Wallis tests for continuous variables. Angiogenic balance was assessed with the ratio between the levels of endostatin and VEGF. Spearman coefficient was used to study the correlation between the number of intracranial stenoses and the level of angiogenic factors. A multiple logistic regression model was performed to detect independent markers of a greater extent of intracranial atherosclerosis, in which variables showing P<0.1 on univariate testing were included, and adjustment for age, sex, and vascular risk factors was conducted. Likewise, a Cox proportional hazards multivariate analysis was used to identify predictors of further cerebral ischemic events attributable to intracranial stenoses. Receiver–operator characteristic curves were configured, and optimal cutoff values were used to include the variables into the multivariate analyses. Finally, cumulative event-free rates for the time to a new ischemic event were estimated by the Kaplan–Meier product limit method, and comparisons were made with the log-rank test. Results were expressed as adjusted odds ratios.
We included 21 (52.5%) men and 19 (47.5%) women, with a mean age of 69 ± 9.6 years, of whom 34 (85%) had hypertension. Baseline demographic characteristics, risk factor profile, and treatment groups of the study population are shown in Table 1.

### Baseline Variables

Demographic characteristics, risk factor profile, and treatment groups of the study population are shown in Table 1. We included 21 (52.5%) men and 19 (47.5%) women, with a mean age of 69 ± 9.6 years, of whom 34 (85%) had hypertension and 25 (62.5%) had diabetes. The qualifying ischemic event caused by intracranial atherosclerosis was an ischemic stroke in 32 patients and TIA in the remaining 8. The symptomatic intracranial stenoses were located in the middle cerebral artery (MCA) in 15 (37.5%) patients, in the intracranial ICA in 12 (30%), in the posterior cerebral artery in 6 (15%), in the vertebro-basilar arteries in 6 (15%), and in the anterior cerebral artery in 1 (2.5%) patient. Concerning their severity, 30 (75%) stenoses were considered severe and 10 (25%) were moderate. Median NIHSS score was 2 (interquartile range, 0 to 5), and 33 (82.5%) patients had a modified Rankin scale score of 0 to 1 at inclusion visit.

DWI showed fragmented cortico–subcortical infarctions in 12 (30%) patients, lacunar infarctions in 11 (27.5%), single cortical infarctions in 3 (7.5%), single subcortical infarctions in 4 (10%), and watershed areas infarctions in the remaining 2 (5%) patients. Chronic silent lacunar infarctions were visible in 22 (55%) patients. White matter hyperintensities were absent in 12 (30%), mild in 11 (27.5%), moderate in 9 (22.5%), and severe in 8 (20%) patients, respectively. Old microbleeds were present in 10 (25%) T2*-weighted sequences.

### VEGF and Endostatin Concentrations

Median baseline VEGF and endostatin plasmatic levels were 46.7 (interquartile range, 27.5 to 82.01) pg/mL and 125 (114.5 to 163.8) ng/mL, respectively, both higher than the normal range for healthy controls of our laboratory (<27.4 pg/mL for VEGF and 8.4 to 58.9 ng/mL for endostatin). There were no significant differences in VEGF or endostatin concentration regarding age, vascular risk factor profile, therapeutic groups, clinical presentation with TIA or stroke, stroke severity, third-month modified Rankin scale score, DWI lesion pattern, presence and degree of MRI-assessed coexistent cerebral microangiopathy, and severity of the symptomatic stenosis. Women showed a higher VEGF level (P = 0.03).

### Angiogenic Factors and the Extent of Intracranial Atherosclerosis

A total of 144 intracranial stenoses were confirmed by MRA, distributed as follows: 40 in MCA, 40 in intracranial ICA, 39 in posterior cerebral artery, 18 in the vertebro-basilar arteries, and 7 in anterior cerebral artery. Agreement between TCD and MRA was complete for the detection of symptomatic stenoses. The median number of stenoses per patient was 3, ranging from 1 stenosis in 2 patients to 10 stenoses in 1, with 10 patients showing >4 stenoses. A negative correlation was observed between VEGF concentration and the number of intracranial stenoses (r = −0.41, P = 0.008), whereas a positive correlation was found between endostatin/VEGF ratio and the extent of intracranial atherosclerosis (r = 0.35, P = 0.02). Figure 1 illustrates how endostatin/VEGF ratio gradually augments with the increasing number of intracranial stenoses. Univariate analysis with variables potentially associated with a greater extent of intracranial atherosclerosis is shown in Table 2. A multiple logistic regression analysis identified diabetes mellitus (OR, 6.04; CI, 1.1 to 32.2; P = 0.03) and either a VEGF <64 pg/mL (receiver–operator characteristic curve cutoff point, 76% sensitivity, and 73% specificity; OR, 8.2; CI, 1.2 to 52.8; P = 0.02) or an endostatin/VEGF ratio ≥3 (72% sensitivity, 82% specificity; OR, 15.7; CI, 2.2 to 112.3; P = 0.006) as independent markers of a
greater extent of intracranial atherosclerosis, after adjustment for age, sex, and vascular risk factors.

### Angiogenic Factors and Further Events Attributable to Intracranial Atherosclerosis

All patients remained free of ischemic events during the time elapsed between the qualifying episode and the inclusion visit. During a median follow-up time of 13 months (interquartile range, 9 to 18), 8 patients experienced further cerebral ischemic events attributable to intracranial atherosclerotic stenoses, divided in 7 ischemic strokes and 1 TIA. The responsible stenoses were located in MCA in 3 patients, in intracranial ICA in 3, in posterior cerebral artery in 1, and in the basilar artery in 1 patient. These stenoses had been previously symptomatic except for 1 intracranial ICA and 1 MCA stenosis. Those patients that presented a new ischemic event had a significantly higher endostatin level at baseline (183±75.6 versus 135.8±41.1 ng/mL, \( P = 0.02 \)). No significant differences were found regarding VEGF level and all other studied baseline variables in univariate analysis. The study sample was divided in 2 groups attending endostatin median concentration (125 ng/mL), and Figure 2 illustrates how a lower proportion of patients remained free of new ischemic events in the group with a higher baseline level of the anti-angiogenic factor. A receiver–operator characteristic curve identified endostatin >185 ng/mL, with a 50% sensitivity and a 90% specificity, as the best cutoff point to predict new events. A higher baseline endostatin concentration remained a predictor of further ischemic events when a Cox multiple regression model was applied, after adjustment for age, sex, number of stenotic vessels, and vascular risk factors (hazard ratio, 7.24; 1.6 to 33.8; \( P = 0.011 \)). During the same follow-up period, progression of intracranial atherosclerosis was detected in 11 (37%) of 29 patients with optimal acoustic windows. Baseline endostatin level tended to be higher in those patients in whom progression of intracranial stenoses was observed (\( P = 0.06 \)).

### Discussion

Angiogenesis has been shown to play a crucial role in chronic limb and myocardial ischemia;\(^{10,11}\) however, to our knowledge, its importance in intracranial atherosclerosis has not been previously addressed. Our study demonstrates the existence of a direct relationship between endostatin/VEGF ratio and the extent of symptomatic intracranial atherosclerosis, and that patients with higher baseline endostatin level are at an increased risk for recurrent cerebral ischemic events. These results support an important role for angiogenesis in the pathogenesis of intracranial atherosclerosis.

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**TABLE 2. Variables Associated With a Greater Extent of Intracranial Large-Artery Atherosclerosis**

<table>
<thead>
<tr>
<th>Age, y ± SD</th>
<th>Lower Extent (≤2 Stenoses)</th>
<th>Greater Extent (&gt;2 Stenoses)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.1±9.4</td>
<td>70.9±9.1</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>Sex (F), no. (%)</td>
<td>6 (54.5)</td>
<td>13 (44.8)</td>
<td>0.583</td>
</tr>
<tr>
<td>Hypertensive, no. (%)</td>
<td>9 (81.8)</td>
<td>25 (86.2)</td>
<td>0.729</td>
</tr>
<tr>
<td>Diabetes, no. (%)</td>
<td>4 (36.4)</td>
<td>21 (72.4)</td>
<td>0.035</td>
</tr>
<tr>
<td>Hypercholesterolemia, no. (%)</td>
<td>9 (81.8)</td>
<td>18 (62.1)</td>
<td>0.234</td>
</tr>
<tr>
<td>Cigarette smoking, no. (%)</td>
<td>6 (54.5)</td>
<td>5 (17.2)</td>
<td>0.018</td>
</tr>
<tr>
<td>&gt;2 risk factors, no. (%)</td>
<td>7 (63.6)</td>
<td>11 (37.9)</td>
<td>0.145</td>
</tr>
<tr>
<td>Clinical presentation: ischemic stroke, no. (%)</td>
<td>9 (81.8)</td>
<td>21 (72.4)</td>
<td>0.540</td>
</tr>
<tr>
<td>DWI lesion pattern: cortico–subcortical, no. (%)</td>
<td>4 (36.4)</td>
<td>8 (27.6)</td>
<td>0.891</td>
</tr>
<tr>
<td>Presence of chronic lacunar infarcts, no. (%)</td>
<td>6 (54.5)</td>
<td>16 (55.2)</td>
<td>0.971</td>
</tr>
<tr>
<td>Presence of old microbleeds, no. (%)</td>
<td>2 (18.2)</td>
<td>8 (27.6)</td>
<td>0.744</td>
</tr>
<tr>
<td>Presence of white matter hyperintensities, no. (%)</td>
<td>7 (64.6)</td>
<td>21 (72.4)</td>
<td>0.611</td>
</tr>
<tr>
<td>VEGF, pg/mL, median (interquartile range)</td>
<td>82 (46.8–83.3)</td>
<td>38.1 (26.9–68.5)</td>
<td>0.030</td>
</tr>
<tr>
<td>Endostatin, ng/mL, mean±SD</td>
<td>126.1±25.9</td>
<td>152.5±58</td>
<td>0.156</td>
</tr>
<tr>
<td>Endostatin/VEGF ratio, median (interquartile)</td>
<td>1.4 (1.2–2.7)</td>
<td>4.1 (2.2–5.1)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

The table resumes the univariate analysis of variables potentially associated with a greater extent of intracranial atherosclerosis. Results of the multivariate analysis are shown in text.

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**Figure 2.** Kaplan–Meier curves show that a significantly lower proportion of patients with a baseline endostatin level >125 ng/mL (median) remained free of further cerebral ischemic events caused by intracranial atherosclerosis during follow-up. \( P = 0.01 \), log-rank test.
Brain tissue is exquisitely sensitive to hypoxia. In the setting of acute cerebral ischemia, reduction of cerebral blood flow immediately upregulates the expression of angiogenic factors, among which VEGF plays a key role. VEGF induces the development of new collaterals aimed to improve cerebral perfusion, which may result in tissue repair and better functional outcome. Because intracranial atherosclerosis is characterized by the development of multiple stenoses affecting cerebral large arteries, at least in some populations (like Asian and Mediterranean European), it may result in a status of chronic brain hypoperfusion responsible for an enhanced angiogenic response.

We observed a growing predominance of endostatin over VEGF with the increasing number of intracranial stenoses. Therefore, those patients with a greater extent of intracranial atherosclerosis seemed to have a more inhibited angiogenic response. To explain this finding, we hypothesize that the genetic and environmental factors that promote a more severe intracranial atherosclerosis may also lead to a constitutive reduction in the expression of pro-angiogenic factors and to an impairment of collateral vessel development. In agreement with this suggestion, diabetes was found to be an independent marker of a greater extent of this disease. In fact, diabetic patients exhibit impairment in ischemia-induced neovascularization, related to a reduced expression of VEGF in nonretinal tissues and to alterations in the biology of endothelial progenitor cells, which are crucial for correct blood vessel growth. In addition, lipoprotein (a) serum level was also shown to be independently associated with a greater extent of intracranial atherosclerosis. In line with our observation, a high lipoprotein (a) serum concentration impaired collateral vessel formation in lipoprotein (a) transgenic mice. A high baseline endostatin level emerged as an independent predictor of further ischemic events attributable to intracranial atherosclerosis. Endostatin, a fragment of C-terminal domain of collagen XVIII, is a powerful inhibitor of endothelial cell proliferation and migration. Given that atherosclerosis develops in response to endothelial injuries, the decreased re-endothelialization, increased neointima formation, and enhanced apoptosis of endothelial cells promoted by the overexpression of endostatin may explain its deleterious role in intracranial atherosclerosis. Moreover, endostatin level tended to be higher in those patients who experienced progression of intracranial atherosclerosis. These findings differ from the reported observation that intraplaque angiogenesis mediated by angiogenic factors may participate in the progression and destabilization of atherosclerotic lesions affecting other vascular territories. However, the relative contribution of intraplaque angiogenesis to the dynamics of atherosclerotic lesions may vary among the diverse arterial territories. First, arteries from specific regions in the arterial tree may have various developmental origins responsible for differences in the composition of the vessel wall and in its response to angiogenic molecules. Second, only relatively large atherosclerotic plaques may exceed the limit at which they require additional sources of perfusion, and this may not be the case for intracranial large arteries. Thus, the results of our study suggest that the processes of endothelial repair are determinant in the course of intracranial atherosclerosis.

This study has some limitations. First, the conclusions are limited by the low power of the study. Second, diagnosis of intracranial atherosclerosis was based on 3-dimensional time-of-flight MRA images and not on conventional angiography, considered the gold standard for the detection of intracranial stenosis. Moreover, gadolinium, which may improve the discrimination capacity of MRA in the diagnosis of intracranial stenosis, was not administered in our MRA protocol. Third, plasmatic levels of angiogenic factors represent a primary approach to our problem, but information regarding the real origin of these molecules is missing. Furthermore, blood samples were only drawn once. Finally, the evaluation of a functional parameter such as cerebral blood flow or vascular reactivity may be needed to elucidate the relationship between angiogenesis and the hemodynamic compromise caused by intracranial atherosclerosis.

In conclusion, a predominance of endostatin over VEGF within the endogenous angiogenic response is associated with a greater extent and risk of recurrence of symptomatic intracranial atherosclerosis, suggesting that angiogenesis may be beneficial in this condition. Therapeutic angiogenesis has been shown to improve tissue perfusion in animal models of brain ischemia and in clinical trials of chronic limb and myocardial ischemia. Whether therapeutic stimulation of angiogenesis might also be beneficial in patients with intracranial atherosclerosis remains a challenge for future research.

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References


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