Circulating Tissue Kallikrein Levels Correlate With Severity of Carotid Atherosclerosis

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Background—Vascular growth factors are upregulated in stroke patients, but it remains unknown if they correlate with carotid atherosclerosis.

Methods and Results—A case-control study was conducted to determine: (1) possible association between biomarkers of angiogenesis or inflammation and carotid stenosis; and (2) the impact of revascularization on the same biomarkers. Circulating vascular endothelial growth factor (VEGF), basic fibroblast GF (bFGF), tissue kallikrein (tK), and high-sensitivity C-reactive protein (hs-CRP) were measured in 89 patients with carotid obstruction and 45 age-matched controls. Patients were stratified as <50% carotid stenosis (CAS; n=16); 50% to 69% CAS (n=12); 70% to 99% CAS (n=43); and carotid occlusion (CAO; n=18). No association was found between VEGF, bFGF, or hs-CRP and obstruction grading. TK augmented from 360±30 in <50% CAS (P=NS versus controls) to 509±72 in moderate CAS (P<0.05), 1159±178 in high-grade CAS (P<0.02), and 1616±403 pg/mL in CAO (P<0.01). A threshold of 508 pg/mL provided the maximized predictive value of high-grade obstruction. After revascularization, tK decreased from 1410±352 to 782±86 pg/mL (P<0.01), whereas no change was detected in nonoperated cases. Hs-CRP was unaffected by revascularization.

Conclusions—Angiogenic factors are heterogeneously expressed in patients with carotid atherosclerosis. The tK measurement may be useful for the diagnosis and monitoring of atherosclerotic disease. (Arterioscler Thromb Vasc Biol. 2004;24:1104-1110.)

Key Words: brain ▪ growth factors ▪ angiogenesis ▪ atherosclerosis ▪ inflammation ▪ revascularization

Cerebrovascular disease represents a major clinical problem in western countries. Patients at risk for stroke include those with symptomatic, high-grade carotid artery stenosis (CAS), especially if associated with hypertension, dyslipidemia, obesity, and cigarette smoking.1,2 Yet asymptomatic patients with less advanced atherosclerosis are not spared, inasmuch that cerebral thromboembolism often derives from the rupture of small-sized, vulnerable plaque.3 Mass application of angiography or noninvasive imaging techniques for the diagnosis of carotid atherosclerosis is either risky or cost-ineffective.4 Consequently, intense research is focusing on surrogate indicators of critical CAS and/or plaque instability.

A growing understanding of the inflammation mechanisms implicated in atherosclerosis has led to the identification of an expanding array of markers potentially exploitable for patient care. For instance, high-sensitivity C-reactive protein (hs-CRP) showed clinical value in predicting the occurrence or recurrence of future stroke.5 However, hs-CRP does not correlate well with the extent of atherosclerosis.6,7 Furthermore, it remains uncertain whether inflammation biomarkers might help discriminate other characteristics of the plaque, namely its vulnerability.

Angiogenesis, ie, the generation of new vessels from pre-existing capillaries, is also pathogenetically implicated in atherosclerosis. Plaque growth correlates with lesion neovascularization, is enhanced by supplements of vascular endothelial growth factor (VEGF), and is inhibited by antiangiogenic therapy.7,8 However, upregulation of angiogenic GFs is essential for the recovery of tissues downstream to vascular occlusion,9–11 with this healing response being attenuated by atherosclerosis.12 Potentiation of reparative neovascularization by exogenous VEGF reportedly improves postischemic cerebral blood flow recovery,13 and angiogenic tissue kallikrein (tK) prevents stroke in preclinical studies.14 Besides being activated on occurrence of acute complications, circulating angiogenic GFs seem to be heterogeneously upregulated in patients with chronic obstructive arterial disease.15–17 Yet to the best of our knowledge, the possibility that angiogenesis biomarkers might have diagnostic value in atherosclerosis remains unexplored.

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Therefore, we conducted a case-control study to determine whether levels of tK, VEGF, or basic fibroblast GF (bFGF) in peripheral blood correlate with the degree of arterial obstruction, as documented by standard imaging techniques. A side-by-side comparison was made with the inflammatory biomarker hs-CRP. In addition, we evaluated the impact of carotid revascularization on the expression of circulating tK and hs-CRP.

Methods

Study Design

This 2-phase case-control study was performed in agreement with principles of Helsinki Declaration (2000) and according to the regulation of the local ethics committee. We screened all patients admitted at the Department of Vascular Surgery, Medical University of Sassari, Italy for clinical evaluation of carotid obstruction between October 1, 2000 and July 30, 2002 for enrollment in the study. Informed written consent was obtained from each subject. Excluded were subjects who declined to participate or those in whom clinical and laboratory investigation documented symptomatic ischemic disease affecting districts other than the brain or evidence of major cardiac, renal, hepatic, or cancerous disease or infection.

Phase 1: Baseline Measurements of Angiogenesis Biomarkers and hs-CRP

As shown in Figure I (available online at http://atvb.ahajournals.org), after the initial screening, 89 patients (66 men and 23 women; mean age: 69 years; range: 42 to 85 years) with carotid artery obstruction were considered eligible for and were enrolled in the study. Forty-five healthy volunteers (33 men and 12 women; mean age: 66 years; range: 40 to 82 years) were recruited during the same period as a reference group. Venous blood samples for baseline measurements of GFs and hs-CRP were obtained between 8:00 and 9:00 AM from subjects in the recumbent position.

Phase 2: Effect of Revascularization on tK and hs-CRP

Patients entered the second phase of the study aimed at evaluating the impact of carotid endarterectomy (CEA) on biomarkers (Figure I, bottom). Forty-one patients with >50% CAS (25 unilateral and 16 bilateral) underwent CEA. In the remaining 48 patients, revascularization was not executed because of unfavorable benefit/risk index (n=16 with <50% CAS) or patient refusal (n=32). CEA was successful in 39 of 41. Early postoperative complications occurred in the remainder, consisting of 1 ipsilateral stroke and 1 transient ischemic attack. Patients, whether submitted to CEA, were maintained on initial therapeutic regimen except for insertion of low-dosage aspirin (100 mg/d).

At 1-year follow-up, all patients (except 5 dropouts and 3 in post-CEA group) underwent control angiography and were sampled for tK and hs-CRP measurements.

The protocol was initially designed to have biochemical measurements under basal conditions and at the 1-year follow-up visit. During the experimental period, it was proposed by investigators and accepted by the ethics committee that relevant information could be derived from determining GFs at early and intermediate phases after CEA. Therefore, with the study already ongoing, a subgroup of 16 unilateral CAS patients from the global series was proposed and gave consent to be sampled sequentially during the first 3 days after surgery for tK and hs-CRP measurements. Sixteen unilateral CAS patients maintained under medical treatment and 10 subjects, submitted to plastic surgery of the neck, age- and sex-matched with CEA patients, were used as controls. Finally, another subgroup of 17 CAS patients (8 unilateral and 9 bilateral) had tK measured at 3 months from CEA.

Diagnostic Procedures

Clinical examination and complete laboratory testing were performed on admission. Ultrasound examination was performed in all patients. Obstruction grading and plaque instability was based on the criteria established by Tegos. Briefly, images of the plaques were analyzed by 2 independent investigators, not informed of biomarker levels, to distinguish hypoechoic and heterogeneous pattern and recognize the presence of ulceration. Plaque characteristics were also assessed after CEA by direct examination and histology of plaque composition, including hallmarks of instability such as the presence of necrotic core, inflammation, and ulceration.

Selective bilateral angiographies were performed in all patients and reviewed by 2 experienced neuro-radiologists to reach a consensus on obstruction grading on the basis of NASCET criteria. In case of bilateral lesions, the more stenotic side was considered for grading. The degree of contralateral stenosis was taken into account in multivariate analysis.

Risk Stratification

Patients were stratified according to the risk of stroke on the basis of NASCET angiographic criteria and the presence or the absence of previous ischemic brain attacks as follows: high risk, encompassing symptomatic, 70% to 99%, CAS or asymptomatic, 80% to 99%, CAS; moderate risk, symptomatic, 50% to 69%, CAS or asymptomatic, 50% to 79%, CAS; low risk, <50%, CAS. Patients with CAO were considered at high risk if they had contralateral CAS of 70% to 99% or 50% to 69% plus previous ischemic brain attacks.

Biochemical Assays

Samples, collected in EDTA-K3, were immediately centrifuged (1500g, 15 minutes), and plasma was stored at −20°C until assay. VEGF-A and bFGF levels were measured using quantitative ELISA kits (R&D Systems, USA) and CRP by a highly sensitive ELISA kit (Kalon Biological Ltd, UK). Immunoreactive tK was determined by an ELISA (AngioProgen, Italy) specific for the active form of the enzyme. Briefly, microtiter plates (96-well; Corning) were coated with nonlabeled anti-human tK IgG (2 μg/mL, 100 μL per well) overnight at 4°C. The plates were then blocked with 200 μL phosphate-buffered saline at 37°C for 1 hour. After repeated washing, purified human tK standard (0.04 to 2.5 ng/mL) and plasma samples were added to individual wells in a total volume of 100 μL. The plates were incubated at 37°C for 90 minutes. After incubation, the plates were washed 3 times with the washing solution and 100 μL of 1 μg/mL, biotin-labeled anti-human tK IgG diluted in the dilution buffer was added to each well. The reaction was performed at 37°C for 1 hour. After incubation, excess labeled IgG was washed off and 100 μL of 1 μg/mL peroxidase-avidin diluted in the dilution buffer was added to each well. After incubation at 37°C for 1 hour and repeated washing, color reaction was started by adding 100 μL freshly prepared substrate solution (0.03% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.03% H2O2 in 0.1 mol/L citrate buffer, PH 4.31) and incubating at room temperature for 30 minutes. The plates were read at 414 nm on a Titertek Multiskan ELISA reader (Flow Laboratories). The amount of tK in the samples was determined for each test sample by comparison with the calibrator standard curve.

All assays were performed in duplicate by a researcher (E.D.) blind to subject identification. Interassay variation coefficient was <8% for all the aforementioned parameters.

Statistical Analysis

Results are expressed as mean±SEM. Single values across different severity categories are also shown. Because distribution of biomarkers was skewed, logarithmic transformation was applied before statistical analysis. Linear multivariate stepwise analysis was performed to identify possible influence of aggregated risk factors, followed by univariate analysis to check the impact of single risk factors, including age older than 65, gender, smoke, hypercholesterolemia, diabetes, and hypertension as covariates. Interaction between biomarkers and above covariates was assessed within each category.
Clinical and Biochemical Characteristics of Vascular Patients and Controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Healthy Controls</th>
<th>Basal</th>
<th>Follow-up</th>
<th>Surgery Controls</th>
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</thead>
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<td>66/23</td>
<td>7/3</td>
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<td>Mean age, y</td>
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<td>69±1</td>
<td>60±3</td>
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<td>Body mass index</td>
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<td>48%*†</td>
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<td>Current smoking %</td>
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<td>48*†</td>
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<td>Plasma creatinine, mg/dL</td>
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<td>1.09±0.09</td>
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<td>Hemoglobin, g/dL</td>
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<td>Leukocytes, (×10³) per mm³</td>
<td>7.33±0.45</td>
<td>7.31±0.25</td>
<td>7.56±0.15</td>
<td>7.26±0.10</td>
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<tr>
<td>Erythrocyte sedimentation rate, mm/h</td>
<td>ND</td>
<td>21±3</td>
<td>19±2</td>
<td>16±2</td>
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</tbody>
</table>

In vascular patients, biochemical data were measured at baseline and at 1-year follow-up visit. Values are mean±SEM (percent frequency in parentheses). ND indicates not determined.

*P<0.05 vs healthy controls.
†P<0.05 vs surgery controls.

Results

Characteristics of Vascular Patients

Clinical and biochemical characteristics are illustrated in Table. Sixty-two patients showed angiographic evidence of unilateral carotid obstruction, with the remainder having bilateral carotid lesions. According to the severity of obstruction, patients were stratified as having mild-grade, <50%, CAS (n=16); moderate-grade, 50% to 69%, CAS (n=12); high-grade, 70% to 99%, CAS (n=43); and carotid occlusion (CAO, n=18). Contralateral stenosis exceeding 69% was present in 7 (16%) high-grade CAS, and 10 (56%) carotid occlusion (CAO). Plaque instability was documented in 42 patients by ultrasound examination. The accuracy of ultrasound in detecting instability was consistently confirmed by histology in all cases that underwent revascularization.

No difference was observed across groups classified according to the severity of obstruction, as far as mean age, gender, or risk factors are concerned (Table I, available online at http://atvb.ahajournals.org). However, the percentage of patients older than 65 years was greater in high-grade CAS or CAO (82% and 72%, respectively) as compared with mild- to moderate-grade CAS (50%, P<0.05). In addition, previous ischemic cerebral accidents in the form of transient ischemic attack or stroke were more frequent in high-grade CAS or CAO (61% in both groups) than in moderate (25%, P<0.05) or mild-grade CAS (6%, P<0.01).

Baseline Measurements of Angiogenesis Biomarkers and hs-CRP

Circulating Levels of VEGF

Plasma VEGF averaged 208±29 pg/mL (ranging from 6 to 1139 pg/mL, P=NS versus healthy controls), with 3 patients having above and 29 having below the range of distribution of VEGF in healthy controls (from 99 to 775 pg/mL). As shown in Figure 1A, no correlation was found between VEGF and obstruction grading or plaque instability. In addition, no association was detected with gender, neurological symptoms, risk factors, or hematological tests (including other angiogenic GFs [R=0.03 versus hTK; R=0.14 versus bFGF] or hs-CRP [R=0.04]).

Circulating Levels of tK

In healthy subjects, tK averaged 375±33 pg/mL, ranging from 160 to 658 pg/mL. As shown in Figure 1B, tK increasingly augmented as a function of obstruction grading (R=0.80, P<0.01) from 360±30 in <50% CAS (P=NS versus healthy controls) to 509±72 in 50% to 69% CAS (P<0.05 versus controls), 1159±178 in 70% to 99% CAS (P<0.02 versus controls), and 1616±403 pg/mL in CAO (P<0.01 versus controls). The correlation with grading of stenosis was maintained when plotting tK against continuous values of percent CAS (R=0.73, P<0.01). Accordingly, the percent of patients in whom tK exceeded the normal range increased progressively in the 4 grading categories (6%, 42%, 71%, and 77%, respectively). The relation between tK and degree of stenosis was not influenced by gender or risk factors considered as covariates, including plaque instability. Furthermore, within each stenosis category, no interaction was detected between tK and hematological tests (including other angiogenic GFs [R=0.03 versus VEGF; R=0.05 versus bFGF] or hs-CRP [R=0.09]). Among
patients with $>50\%$ CAS, average tK values tended to be higher in those with previous ischemic stroke ($1502 \pm 367$ pg/mL) or transient ischemic attack ($1225 \pm 314$ pg/mL) compared with asymptomatic patients ($833 \pm 128$ pg/mL), but this trend did not reach statistical significance. However, in the subgroup of CAO and previous cerebral ischemic attacks, the percentage of high tK reached 90%.

**Circulating Levels of bFGF**

bFGF averaged 12.2 $\pm$ 1.8 pg/mL (ranging from 2 to 50 pg/mL, $P=0.10$ versus healthy controls). As shown in Figure 1C, 21 patients exceeded the normal range (mean, $6.9 \pm 1.0$ pg/mL, ranging from 1 to 15 pg/mL). An interaction was found with diabetes ($20.2 \pm 6.2$ versus $9.5 \pm 1.4$ pg/mL in nondiabetic patients, $P<0.02$), but not with other risk factors, gender, hematological tests including hs-CRP ($R=0.07$), neurological symptoms, plaque instability, or obstruction grading.

**Circulating Levels of hs-CRP**

hs-CRP averaged 6.7 $\pm$ 0.4 $\mu$g/mL (ranging from 1.3 to 20.4 $\mu$g/mL, $P<0.05$ versus healthy controls). As shown in Figure 1D, except 7 patients, all the others showed values $>3$ $\mu$g/mL, which is considered the high-risk threshold in epidemiological studies. An interaction was detected between hs-CRP and diabetes ($9.8 \pm 1.4$ versus $6.1 \pm 0.4$ $\mu$g/mL in nondiabetic patients, $P<0.01$). A similar association was observed between hs-CRP and body mass index ($P<0.01$). No association was instead detected with other risk factors, gender, obstruction grading, plaque instability, neurological symptoms, or hematological tests (including angiogenic GFs, as shown).

**Comparison of the Diagnostic Power of Biomarkers**

ROC curve analysis indicates that tK is the best indicator of high-degree stenosis (Figure 2), with a threshold of 508 pg/mL for maximized predictive value (75% sensitivity and 67% specificity, area under the curve 0.76).

According to NASCET criteria, 50 of 89 patients were classified as high-risk and the remainder as moderate- or mild-risk. Among high-risk patients, tK was outside normal distribution range in 74%, VEGF in 38%, and bFGF in 22%.

**Biomarker Levels at Follow-Up Visits**

In a subgroup of patients, tK was measured in the first 3 days after CEA. As shown in Figure 3, circulating tK levels rapidly decreased in cases of successful revascularization ($n=14$, $P<0.01$). In contrast, tK increased in the 2 patients with perioperative ischemic complications (from 537 to 841 pg/mL in the 1 who experienced stroke and from 387 to 633 pg/mL in the other).

**Figure 1.** Dispersion graph of serum levels of biomarkers in patients classified according to the degree of carotid artery obstruction. Close circles represent patients with contralateral stenosis $>69\%$. A, Vascular endothelial growth factor (VEGF). B, Tissue kallikrein (tK). C, Basic fibroblast growth factor (bFGF). D, Highly sensitive C-reactive protein (hs-CRP). Shaded area delimited by dotted lines represents normal distribution (mean $\pm$ 2 SD).

**Figure 2.** Receiver-operating characteristic (ROC) curve analysis for the predictive value of biomarkers for carotid stenosis $>69\%$. Sensitivity and specificity were calculated at various levels of each biomarker, followed by definition of threshold levels with maximized sensitivity and specificity. The area under the curve was also calculated.

**Figure 3.** Graph showing the decrease in tK levels during the first 3 days after successful revascularization of unilateral carotid artery stenosis by endarterectomy (CEA). A, Pattern in each single patient. B, Average reduction over time. Values indicated by columns (B) represent mean $\pm$ SEM. *$P<0.05$ versus preoperative value.
From a pathological viewpoint, all stages of atherosclerosis involve cytokines and cells that are characteristic of inflammatory response to injury. Consistent with previous reports, we found that circulating hs-CRP levels are generally elevated in patients with atherosclerotic disease. In our series, the inflammation biomarker did not provide information regarding the extent of stenosis. This finding replicates previous reports showing poor correlation with tests the quantify plaque mass and number of occluded vessels. A correlation between CRP and carotid artery intima-media thickness has been described in subclinical atherosclerosis, but the issue remains controversial.

Here, we report that hs-CRP remains elevated after successful CEA of high-risk CAS. Evidence from clinical trials indicates that carotid revascularization significantly reduces the risk of future stroke in this category of patients. Thus, our results suggest that hs-CRP may not recognize the benefit inherent to CEA. Accordingly, a Consensus Opinion of the American Heart Association concluded that there is no evidence in favor of serial testing of hs-CRP to monitor effect of therapy.

The angiogenic bFGF is reportedly upregulated in stroke patients. A modest increase in circulating levels was observed in our series, mainly associated with concomitant diabetes. No association was found with the degree of vascular stenosis. A growing body of evidence indicates that bFGF favors restenosis via stimulation of vascular smooth muscle cell replication in neointima. High bFGF expression is observed in injured arteries and neointimal proliferation is significantly inhibited by bFGF antibodies. Evaluation of bFGF predictive value with regard to restenosis was beyond the objectives of the present study and further investigation is necessary to address whether high-bFGF patients are at risk for post-CEA late complications.

Human tK, a pleiotropic angiogenic protein, showed relevant therapeutic potential in animal models of ischemia. tK is the only angiogenic biomarker increasingly augmenting as a function of carotid obstruction grade, independently from background risk factors. tK was elevated in 74% of high-risk patients. Thus, the biomarker may represent a simple, noninvasive way to recognize the presence and assess the severity of atherosclerotic disease.

To obtain further insights into the diagnostic usefulness of tK, we evaluated whether revascularization can suppress the biomarker. The early effects of revascularization were assessed in a subgroup of CAS patients sampled repeatedly during the first days after surgery. After successful CEA of unilateral CAS, tK levels promptly decreased, with exception of cases experiencing perioperative ischemic complications.

The decrease of tK levels was confirmed in an additional subgroup of unilateral CAS patients evaluated at 3 months after surgery. In bilateral patients, the trend to reduction was not universal, possibly because of persistent stimulation by contralateral stenotic side. Accordingly, reopening of contralateral carotid resulted in normalization of tK levels. Reversal of tK upregulation was still evident 1-year after CEA in the whole group of 38 patients that concluded the study.

VEGF-A, a prototypical angiogenic cytokine, is reportedly increased in the circulation of stroke patients. Coagulation and thrombus formation increases the expression of VEGF-A.

Discussion

From a pathological viewpoint, all stages of atherosclerosis involve cytokines and cells that are characteristic of inflammatory response to injury. Consistent with previous reports, we found that circulating hs-CRP levels are generally elevated in patients with atherosclerotic disease. In our series, the inflammation biomarker did not provide information regarding the extent of stenosis. This finding replicates previous reports showing poor correlation with tests the quantify plaque mass and number of occluded vessels. A correlation between CRP and carotid artery intima-media thickness has been described in subclinical atherosclerosis, but the issue remains controversial.

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through tissue factor, and VEGF-A is released from aggregated platelets at the site of plaque rupture. In addition, expression of VEGF-A is temporally and spatially correlated with neovascularization in ischemic brain. Unexpectedly, VEGF-A was not upregulated in our series and no association was detected between the GF and carotid obstruction grading. Rather, low VEGF-A levels were observed in approximately one-third of our patients. Because various mechanisms implicated in neangiogenesis are impaired in atherosclerosis, the deficit in VEGF-A may simply represent a hallmark of endothelial dysfunction. Other possible explanations deal with excess of s-Flt1, a soluble receptor that reportedly acts as a potent VEGF antagonist. In addition, VEGF might be rapidly cleared from circulation because of avid binding to vascular receptors.

Perspectives
This case-control study is the first to our knowledge to provide evidence that angiogenesis biomarkers are heterogeneously expressed in patients with carotid atherosclerosis, with tK being significantly correlated with stenosis severity. In contrast, hs-CRP did not help in the recognition of high-grade carotid obstruction. Thus, measurement of tK may add useful information for the diagnosis of atherosclerosis-induced cerebrovascular disease. However, it is clear that similar to other types of accepted diagnostic and monitoring methods, this marker should be used in combination with other diagnostic tests and clinical observations to diagnose the disease and further to develop treatment decisions for each individual patient. Previously, we documented that peripheral vascular patients, without ischemic symptoms at rest, show increased circulating tK, with this upregulation being reversed by revascularization. Therefore, tK may not allow to distinguish whether atherosclerosis is affecting carotid, peripheral vessels, or both. The studies reported also showed a venous-arterial gradient for tK in patients with peripheral vascular disease, suggesting that the angiogenic agent is released from ischemic tissues. It cannot be ruled out that tK is increased by the presence of unstable, possibly inflamed plaque liberating the angiogenic agent. Biochemical and histological studies indicate that kallikrein is expressed in human vascular wall, and levels of expression seem to be variably affected in different forms of hypertension. Preliminary immunohistochemical findings suggest that tK is also expressed in atherosclerotic carotid arteries (Madeddu P, unpublished results 2004). However, the absence of correlation with indicators of plaque instability favors the possibility that tK increases in peripheral circulation as a consequence of chronic hemodynamic changes related to the stenosis.

Finally, similar to most biologic tests, the angiogenesis biomarker has wide range that overlap in persons with disease and in those without it. In the present study, various cutoff points were evaluated for their ability to detect disease, followed by definition of threshold levels with maximized sensitivity and specificity. Longitudinal, large-scale trials are now necessary to validate the accuracy of the threshold values established by the present case-control study and confirm the usefulness of the test for care of cerebrovascular patients.

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