Reversal of Angiogenic Growth Factor Upregulation by Revascularization of Lower Limb Ischemia

Paolo Porcu, MD; Costanza Emanueli, PhD; Maria Kapatsoris, MD; Julie Chao, PhD; Lee Chao, PhD; Paolo Madeddu, MD, FAHA

Background—Tissue kallikrein (tK) and vascular endothelial growth factor (VEGF) are potent angiogenic agents. Upregulation of tK or VEGF was documented in animal models of acute ischemia, yet it remains unknown whether these endothelial cell mitogens are overexpressed in chronic peripheral vascular insufficiency.

Methods and Results—Circulating tK and VEGF were measured in 36 patients with symptomatic peripheral vascular disease before and after surgical revascularization. In 6 patients without symptoms at rest, tK was assayed after exercise stress test. VEGF levels fell within the normal range in all patients (96±11 versus 109±13 pg/mL in healthy control subjects, P=NS) and remained unchanged after revascularization. In contrast, tK expression was upregulated in 34 of 36 patients (1107±203 versus 85±10 pg/mL in control subjects, P<0.05), with no further increase after exercise. Tissue kallikrein levels in the venous effluent of ischemic limbs were found to be positively correlated with the number of angiographically recognizable collateral vessels (P<0.001). Follow-up studies documented reversal of tK upregulation after revascularization (P<0.01), whereas no change was observed in venous samples from untouched legs.

Conclusions—Induction of tK could represent a compensatory response to chronic arterial insufficiency, attempting to maintain an adequate tissue perfusion. Heterogeneous statement of growth factors may have important implications in reparative and therapeutic angiogenesis. (Circulation. 2002;105:67-72.)

Key Words: angiogenesis ■ atherosclerosis ■ bradykinin ■ revascularization

Ischemic diseases represent a major clinical problem and a field of compelling therapeutic innovation. Accordingly, exogenous supplementation with angiogenic molecules has been proposed to promote reparative collateral growth and accelerate tissue healing.1,2 This approach should overcome the impaired expression of endogenous angiogenic factors that limits spontaneous reparative response to ischemia caused by atherosclerosis-induced vascular obstruction. However, human studies indicate that after acute myocardial infarction or stroke, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), and hepatocyte growth factor are variably modulated.3-5 Furthermore, a differential pattern of expression has been reported for VEGF and its KDR receptors, depending on whether acute ischemia supervenes in the heart or skeletal muscle.6

Information regarding the expression pattern of growth factors in chronic peripheral vascular disease remains elusive. Whether vascular mitogens are modulated under these conditions represents an important issue from a clinical point of view. In fact, most patients are not ischemic at rest. Rather, they have recurrent episodes of ischemia in the form of limb claudication.7 To the best of our knowledge, only bFGF was found to be upregulated in this kind of patient.8 It remains unknown whether other growth factors follow the same pattern or, vice versa, are downregulated because of associated endothelial dysfunction, as suggested by animal studies.9,10 Finally, only a few reports, exclusively limited to the heart, have addressed the relation of growth factor expression with the severity of vascular disease,11 the magnitude of collateral growth, and the impact of reperfusion therapy.12,13

Recently, attention has been drawn to the vasodilator kallikrein-kinin system as a mechanism instrumental to post-ischemic healing. Kinin generation and outflow from the heart (see Reference 14 for review) and myocardial kinin receptor expression are significantly augmented after the induction of myocardial ischemia.14,15 Induction of kinin receptors in ischemic skeletal muscle has been documented to be essential for hemodynamic recovery.16 The therapeutic potential of this observation was then elaborated through an approach that consisted in continuous, local supply of the kinin-generating enzyme tissue kallikrein (tK). Adenovirus-mediated tK gene delivery resulted in a potent angiogenic effect in limb skeletal muscle by a mechanism that involves the stimulation of nitric oxide and cyclooxygenase-2–induced prostaglandin release.16,17 In the experimental setting of

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From the Institute of Internal Medicine (M.K., P.M.) and Vascular Surgery (P.P.), Medical University of Sassari, Sassari, Italy; the Cardiovascular Medicine and Gene Therapy Section of the National Laboratory of the National Institute of Biotecnologies and Biosystems (C.E., P.M.), Osilo, Italy; and the Department of Biochemistry and Molecular Biology, Medical University of South Carolina (J.C., L.C.), Charleston.

Correspondence to Paolo Madeddu, MD, FAHA, Institute of Internal Medicine, Viale S. Pietro 8, 07100 Sassari, Italy. E-mail madeddu@yahoo.com

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ischemia, tK appears to be a safer and more effective therapeutic agent than any other angiogenic factor. Nevertheless, in a field such as vascular gene therapy, the most severe scrutiny is necessary before tK might become clinically applicable. In this context, ascertaining the level of expression of the angiogenic substance in human pathology and the relation with clinical and functional parameters would be valuable information.

Accordingly, we evaluated whether circulating levels of tK and VEGF are modulated by chronic limb ischemia and correlate with the severity of the disease. Furthermore, we explored the impact of reperfusion on tK and VEGF expression. We found that tK but not VEGF is activated in atherosclerosis-induced vascular obstruction. In addition, tK upregulation correlates with the number of angiographically recognizable collateral vessels and is reversed by surgical revascularization.

Methods

Serum tK and VEGF levels were measured in a series of 36 patients admitted to the Department of Vascular Surgery, Medical University of Sassari, for reconstructive surgery of symptomatic peripheral vascular disease. Excluded from the study were patients with major cardiac, renal, hepatic, or cancerous disease or infection. Age-matched healthy subjects (n = 31) were used as control subjects. All recruited subjects were Caucasian. The study was approved by the local ethics committee. Informed consent was obtained from each patient.

Clinical examination and complete laboratory testing were performed on admission. Risk factors such as smoking, hypertension, diabetes, and hypercholesterolemia were also assessed. Severity of the disease was classified on clinical grounds, according to Leriche-Fontaine grading. Doppler-guided Winsor’s Index, defined as limb-to-arm systolic blood pressure ratio, was measured to define the hemodynamic impact of vascular obstruction. Collateral vessel development in the thigh was assessed on angiographic images by the use of a grid overlay with 2-mm squares. Angiographic score was defined as the total number of squares crossed by contrast-opacified vessels divided by the total number of squares covering the ischemic limb multiplied by 100. A single observer, blinded to patient identity, performed all counting.

Blood samples for tK and VEGF measurements were obtained between 8 and 9 AM from an indwelling intravenous catheter inserted into the left brachial vein of patients, who had remained in recumbent position since the night before. In a subset of patients (n = 15), measurements were also performed from both femoral veins and repeated 10 days and 3 months after revascularization. In 6 patients without symptoms at rest, blood was obtained before and after standard exercise stress.

Samples were immediately centrifuged (1500g, 15 minutes), and serum was stored at −20°C until assay. VEGF levels were measured by a standard quantitative ELISA kit (Quantikine, R&D Systems). Immunoreactive tK was measured in duplicate with an ELISA specific for the active form of the enzyme, as previously described,6,17 by a researcher who was blind to subject identification.

Results were expressed as mean ± SEM. Multivariate statistical analysis was performed to identify possible influence of aggregate risk factors on the parameters under study, and then univariate analysis was used to check the impact of each single risk factor. Correlation between variables with other blood parameters, risk factors, or indexes of clinical disability was performed by use of Spearman’s regression analysis, followed by calculation of Spearman’s rank correlation coefficient. Differences within or between groups were determined by means of paired or unpaired Student’s t test, respectively. Data that were recognized to be not normally distributed were analyzed with nonparametric tests for paired (Wilcoxon signed rank test) or unpaired (Mann-Whitney U test) groups.

### Table 1. Characteristics of Patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Control Patients</th>
<th>Vascular Patients</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After Revascularization</td>
</tr>
<tr>
<td>Sex, M/F, n</td>
<td>29/2</td>
<td>33/3</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>61±2</td>
<td>63±3</td>
</tr>
<tr>
<td>Body mass index</td>
<td>22.3±0.3</td>
<td>24.2±0.5</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>0</td>
<td>20 (56%)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0</td>
<td>8 (22%)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>0</td>
<td>15 (42%)</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>5 (16%)</td>
<td>20 (56%)</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dL</td>
<td>0.98±0.05</td>
<td>1.04±0.07</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.6±0.3</td>
<td>14.1±0.3</td>
</tr>
<tr>
<td>Leukocytes, (×10⁶) per mm³</td>
<td>7.31±0.41</td>
<td>7.54±0.46</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate, mm/h</td>
<td>ND</td>
<td>22.7±5</td>
</tr>
</tbody>
</table>

ND indicates not determined.

In vascular patients, biochemical data were measured before and after revascularization. Values are mean ± SEM (percent frequency in parentheses).

respectively. A value of P<0.05 was interpreted to denote statistical significance. The SigmaStat statistical package (Jandel Co) was used for all analyses.

### Results

#### Patients

Thirty-six patients (33 men and 3 women; age, 33 to 79 years; mean age, 63 years) and 31 control subjects (29 men and 2 women; age, 24 to 78 years; mean age, 61 years) were studied. Patients had been previously identified as having angiographic evidence of unilateral or bilateral occlusion (n = 25) or severe stenosis (n = 11) at the level of the iliac or femoral arteries. According to the Leriche-Fontaine classification, they were subdivided as follows: stage IIA (n = 8), stage IIB (n = 13), stage III (n = 8), and stage IV (n = 7). Other characteristics, including risk factors and biochemical data, are shown in Table 1.

#### Serum Levels of VEGF

On admission (Figure 1A), circulating VEGF averaged 96±11 pg/mL (ranging from 19 to 281 pg/mL), being comprised within the limits of normal distribution (109±13 pg/mL, ranging from 32 to 302 pg/mL). No association was detected between VEGF expression and any single risk factor, aggregation of factors, hematological tests, or Leriche-Fontaine grading (Tables 2 and 3). However, a direct correlation was found between VEGF and angiographic score (r = 0.44, P < 0.02). In patients asymptomatic at rest, VEGF was not altered by exercise (from 80±15 to 98±9 pg/mL, P = NS).

#### Serum Levels of tK

In the brachial blood (Figure 1B) tK averaged 1107±203 pg/mL (ranging from 111 to 7031 pg/mL), and in 34 of 36 patients it exceeded the upper limits of normal distribution (85±10 pg/mL, ranging from 7 to 236 pg/mL, P < 0.05, Mann-Whitney U test). No association was detected between
Expression of growth factors was reexamined after unilateral revascularization (from 627±143 to 387±90 and 380±91 pg/mL at 10 days and 3 months after revascularization of the affected side, respectively; n=8, P<0.05 for both comparisons, Wilcoxon signed rank test). A similar pattern was observed in the blood collected from reperfused limbs (Figure 3B), whereas no change was detected in the untouched side (Figure 3C). As shown in Figure 3D, the decrease in tK expression was even more marked after revascularization of bilateral limb ischemia, with special reference to the effluent of revascularized limbs (Figure 3E, P<0.01).

Discussion

Upregulation of angiogenic growth factor has been suggested to be essential for reparative angiogenesis, a host defense process that permits healing and functional recovery of ischemic tissue. VEGF, a dimeric glycoprotein also known as vascular permeability factor and vasculotropin, is considered a prototypical angiogenic cytokine. Besides exerting mitogenic and antiapoptotic activity on vascular endothelial cells, VEGF induces chemotaxis of blood-derived cells, a mechanism implicated in de novo vasculogenesis. Upregulation of VEGF expression after an acute ischemic insult is regulated by hypoxia-inducible transcription factors such as HIF-1α, HIF-1α, and HIF-2α. Kinins, the biological end products of kallikreins, share important features with VEGF, inasmuch as both induce

Table 2. Growth Factor Levels Before and After Revascularization

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
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<tbody>
<tr>
<td></td>
<td>tK</td>
<td>VEGF</td>
</tr>
<tr>
<td>Hypertension</td>
<td>856±215</td>
<td>91±19</td>
</tr>
<tr>
<td></td>
<td>1278±523</td>
<td>99±22</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>830±219</td>
<td>96±18</td>
</tr>
<tr>
<td></td>
<td>1129±377</td>
<td>97±15</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>945±178</td>
<td>76±18</td>
</tr>
<tr>
<td></td>
<td>1234±458</td>
<td>109±29</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (pg/mL). Patients were grouped according to presence (+) or absence (−) of each single risk factor indicated in the raw. Hypertension, diabetes, and hypercholesterolemia was diagnosed in 20, 8, and 15 of 36 patients, respectively.

*P<0.05 vs before revascularization.
plasma extravasation, vasodilation, and vascular endothelial cell proliferation. Either VEGF or kinin outflow from the heart is increased after acute myocardial infarction. However, VEGF receptors and kinin receptors are differentially modulated by myocardial ischemia. Kinin B2 receptor signaling exerts a tonic control of human coronary vasculature, and, on occurrence of ischemia, activation of the pathway that generates kinins could represent a compensatory vasodilatory mechanism to preserve tissue perfusion. ACE inhibitors has been related to the same mechanism as the pathway that generates kinins could represent a compensatory vasodilatory mechanism to preserve tissue perfusion.

The short half-life of circulating VEGF is estimated to be <3 minutes) has been attributed to avid binding of the growth factor to heparan sulfate proteoglycans present on the luminal side of vascular endothelium. Furthermore, receptor upregulation within the region of tissue ischemia, responsible for site-specific effects of VEGF, may enable endothelial cells to act as magnets for circulating VEGF. Therefore, a possible explanation for our results is that augmented utilization of VEGF in the hypoperfused tissue may preclude elevated levels in circulation. It should be mentioned that intermittent ischemia, manifested in the form of exercise-induced claudication, was the only symptom present in a large part of patients with peripheral vascular disease. Since sampling was performed at rest, the lack of VEGF upregulation might be attributable to the absence of critical reduction of limb perfusion. Nevertheless, circulating VEGF levels were normal also in those patients with pain at rest or with ulcerative lesions, indicative of critical chronic ischemia. Furthermore, exercise stress failed to induce VEGF expression, which is in keeping with the possibility that the growth factor is inappropriately modulated in patients with atherosclerosis-related vascular insufficiency. Interestingly enough, peripheral VEGF correlates positively with the number of thigh collaterals visible with angiography. Thus, although falling within the normal range, circulating levels of the growth factor could represent an index of limb angiogenic potential.

At variance with VEGF, tK was strikingly elevated in vascular patients. Rather than with the severity of symptoms, tK expression appears to be related to the extension of vascular disorder, inasmuch as bilateral obstruction led to the highest release from ischemic limbs. Given the potent angiogenic action of tK, it is tempting to link the enhanced endogenous levels to compensatory collateral growth. This is indirectly supported by the observation of a positive correlation between tK in venous effluent from ischemic limbs and angiographic score. However, it should be cautiously noted that neither tK nor angiographic score differs among various Leriche classes. Thus, collateral growth was insufficient to influence symptom grading.

Since the kallikrein-kinin system is implicated in the regulation of vascular tone and in the pathogenesis of inflammatory disorders, it is reasonable to question whether the observed changes in tK levels are in some way linked to hemodynamic alterations or inflammation. It should be pointed out that the kallikrein-kinin system acts in a paracrine/autocrine fashion. Thus, circulating levels of tK do not necessarily reflect the enzyme activity in key organs regulating blood pressure. Furthermore, tK released into the circulation is immediately trapped by the natural inhibitor kallistatin, so that tK contribution in generation of blood-born kinins is negligible. Consistently, only local but not systemic kinins were increased by augmenting tissue concentration of tK to such an extent to lead to significant increase in circulating levels of the enzyme. In addition, reversal of tK upregulation after revascularization was not associated with any change in blood pressure. Thus, it appears unlikely that the observed change in tK can influence systemic hemodynamics. There is instead no doubt that kallikrein, activated by a low-flow situation, could have important implications in maintenance of local blood flow and metabolism of limb skeletal muscle. Another intriguing possibility deals with the observation that kinins stimulate tissue plasminogen activator release from human endothelium, a mechanism that plays a major role in the defense against endogenous thrombosis, particularly in the coronary vasculature. In this way, the kallikrein-kinin system might combat the progression of
vascular occlusion. Finally, upregulation of tK was not associated with any hematological marker of inflammation, as documented by leukocyte count or erythrocyte sedimentation rate. These observations discount the possibility that tissue inflammation has triggered tK overexpression.

Stress-induced ischemia failed to cause any additional increase in tK, suggesting that the endogenous kallikrein-kinin system is already maximally activated under conditions of intermittent ischemia. Alternatively, the rate of synthesis and/or release of tK into the circulation may be too slow to produce significant increases in circulating levels after short-duration exercise.

It must be recognized that correlative results do not suffice to prove a role for tK in angiogenesis or blood flow regulation. We decided to overcome this limitation by evaluating the impact that revascularization may have on the angiogenic factor. Surgical revascularization resulted in a significant increase in tK in brachial effluent (A) as well as in blood from reperfused limbs (B), whereas no change was detected in effluent from untouched limbs (C). In bilateral limb ischemia, revascularization was performed on both sides (n=14 legs) and resulted in striking reduction of tK levels in either brachial (D) or femoral effluents (E). Values are mean±SEM. *P<0.05, **P<0.01 vs before surgery.

**Figure 3.** Bar graph shows levels of tK in patients with unilateral (left, n=8) or bilateral vascular obstruction (right, n=7) before (open bars) and 10 days after successful revascularization (filled bars). In unilateral limb ischemia, revascularization of affected side (n=8 legs) resulted in significant reduction of tK in brachial effluent (A) as well as in blood from reperfused limbs (B), whereas no change was detected in effluent from untouched limbs (C). In bilateral limb ischemia, revascularization was performed on both sides (n=14 legs) and resulted in striking reduction of tK levels in either brachial (D) or femoral effluents (E). Values are mean±SEM. *P<0.05, **P<0.01 vs before surgery.

Together, these results support the possibility that tK expression is modulated by perfusion levels. However, expression in the untouched leg was elevated before surgery and remained higher compared with the revascularized one, which appears to be in contradiction with the previous assumption unless admitting the presence of a certain degree of vascular obstruction and ischemia in the contralateral limb not detectable by angiography.

In conclusion, the differential pattern of expression documented for tK and VEGF in peripheral vascular disease suggests heterogeneity in the response of angiogenic factors to chronic arterial insufficiency. Intermittent ischemia is sufficient to trigger and maintain the upregulated expression of tK, this response being attenuated by restoring tissue perfusion. The degree of upregulation found in peripheral limb ischemia correlates positively with the development of limb collaterals. However, the lack of correlation with LeRiche grading indicates that in a therapeutic perspective this native response might be required to be maximized by delivery of recombinant protein or gene transfer. Altogether, these discoveries may have important implications in reparative angiogenesis and may help in tailoring supplementation strategies aimed at stimulating endogenous healing potential.
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References
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