EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS IS INCREASED, BUT MICROVASCULAR RELAXATION IS IMPAIRED IN PATIENTS AFTER ACUTE MYOCARDIAL ISCHEMIA

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Background: Vascular endothelial growth factor, a specific endothelial mitogen, plays an important role in myocardial angiogenesis. Previous work has demonstrated increased expression of vascular endothelial growth factor and its receptors in a rat myocardial infarction model, as well as in a pig model of chronic ischemia. The expression of vascular endothelial growth factor and other growth factors after acute myocardial ischemia in patients has not been examined. In this study we examined the expression of vascular endothelial growth factor and its receptors and the responsiveness of human atrial microvessels to vascular endothelial growth factor before and after acute ischemia.

Methods: Paired specimens of human atrial tissue were harvested before and after atrial devascularization (ligation) in 16 patients undergoing coronary bypass operations.

Results: The messenger RNA (reverse transcriptase–polymerase chain reaction) level of vascular endothelial growth factor and vascular endothelial growth factor receptor 1 were increased by 22.2% ± 4.2% and 30.7% ± 7.6%, respectively ($P < .05$), in the ischemic specimens as compared with the control specimens. Protein expression (Western blotting) of vascular endothelial growth factor and that of vascular endothelial growth factor receptor 1 also were increased significantly by 71.7% ± 27.8% and 68.2% ± 27.6%, respectively ($P < .05$). However, both RNA and protein expressions of another vascular endothelial growth factor receptor, vascular endothelial growth factor receptor 2, and fibroblast growth factor and fibroblast growth factor receptor 1 were unchanged. Reactivity of precontracted atrial vessels was examined with video microscopy. Vascular endothelial growth factor–induced (33.9% ± 2.4% vs 18.3% ± 2.8% in control and ischemic vessels, respectively; $P < .05$), fibroblast growth factor–induced (31.6% ± 3.2% vs 15.8% ± 4.1%, $P < .05$), and substance P–induced (84.5% ± 3.7% vs 54.3% ± 9.0%, $P < .05$) microvascular relaxations were decreased in ischemic samples and in the presence of $N^\text{G}$-nitro-L-arginine, whereas responses to sodium nitroprusside were unchanged (90.9% ± 2.2% vs 91.2% ± 2.0%).

Conclusions: This study suggests that acute myocardial ischemia in patients results in increased expression of vascular endothelial growth factor but not fibroblast growth factor and that the functional activity of vascular endothelial growth factor receptors and that of other growth factors may be impaired.

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dates for coronary artery bypass operations or percutaneous coronary angioplasty because of diffusely diseased or extremely small vessels.4 Either the perivascular or intravascular administration of growth factor proteins produces significant improvement of perfusion in experimental models of chronic myocardial ischemia,5-7 and somewhat lesser degrees of benefit have been reported in human growth factor trials.1,8-13 Reasons for this diminished effect include endothelial dysfunction caused by hypercholesterolemia, diabetes mellitus, or hypertension.14,15 The effect of acute myocardial ischemia on the expression of angiogenic growth factors VEGF and FGF2 and their respective receptors has not been examined in patients. It was hypothesized that expression of VEGF, FGF2, and their receptors would be increased but that the microvascular response (as measured by stimulated vascular relaxation) would be reduced because of a generalized defect in endothelial function.

Methods

Tissue harvest. The atrial appendage was harvested from patients undergoing cardiac operations with cardiopulmonary bypass (CPB). A silk suture (1-0) was placed around the atrial appendage. After administration of heparin, a single, 2-stage atrial cannula was placed and secured with the suture. A piece of right atrial tissue was harvested above the cannulation suture after heparinization but before cannulation (control group). The venous cannula was then secured. Care was taken to assure that the atrial tissue above the suture was deprived of blood as much as possible and totally devascularized. A second piece of atrium was harvested after termination of CPB (ischemic group) but before administration of protamine. Tissue in the pre-CPB (control) and post-CPB (ischemia) groups was immediately frozen in liquid nitrogen deprived of blood as much as possible and totally devascularized. A second piece of atrium was harvested after termination of CPB (ischemic group) but before administration of protamine. Tissue in the pre-CPB (control) and post-CPB (ischemia) groups was immediately frozen in liquid nitrogen.

Messenger RNA analysis by means of reverse transcriptase–polymerase chain reaction. Approximately 1 g of atrial tissue (n = 16) was snap frozen in liquid nitrogen and then homogenized and extracted by using TRIzol Reagent (Sigma Chemical Co, St Louis, Mo). The RNA pellet was dissolved in water and precipitated in ethanol. Complementary DNA was synthesized for 1.5 hours at 37°C by using 1 µg/20 µL total RNA, 600 ng/20 µL oligo T20, 10 mmol/L dithiothreitol, and 200 units/20 µL M-MLV reverse transcriptase (Gibco BRL, Life Technologies, Inc, Rockville, Md). For polymerase chain reaction (PCR), 2.5 U/50 µL RedTaq DNA Polymerase (Sigma), 1× RedTaq PCR buffer (Sigma), and 2 µL of cDNA were used.

For VEGF mRNA studies, the primers of the sense 5′-GGATGTCTATCACGCGAGTACT -3′, corresponding to bases 148-170, and the antisense 5′-TCAGCCCTCAGGCTTGCAC -3′, corresponding to bases 570-592, were used to amplify a 445-base pair fragment of VEGF in 35 cycles (30 seconds at 94°C, 30 seconds at 63°C, and 60 seconds at 72°C). For VEGF receptor 1 (VEGF-R1), the primers of sense 5′-ACGCCCACTCAATTACTTA -3′, corresponding to bases 960-979, and the antisense 5′-TAGAGTCGAGGTTTT -3′, corresponding to bases 1496-1515, were used to amplify a 556-base pair fragment of VEGF-R1 in 30 cycles (30 seconds at 94°C, 30 seconds at 56°C, 60 seconds at 72°C). For VEGF receptor 2 (VEGF-R2), the primers of the sense 5′-AGATTTACCAGGCCAGTAA -3′, corresponding to bases 4737-4756, and the antisense 5′-AAAGGGTTAATCTCTTCTTG -3′, corresponding to bases 5338-5357, were used to amplify a 621-base pair fragment of VEGF-R2 in 30 cycles (30 seconds at 94°C, 30 seconds at 56°C, and 60 seconds at 72°C). For FGF2, the primers of the sense 5′-CAAAGCTTCTCCTTCTCCAGA -3′, corresponding to bases 5881-5900, and the antisense 5′-GAGAAAGGTTGGAAAGAGAT -3′, corresponding to bases 6409-6428, were used to amplify a 548-base pair fragment of FGF2 in 35 cycles (30 seconds at 94°C, 30 seconds at 56°C, and 60 seconds at 72°C). For FGF receptor (FGFR), the primers of the sense 5′-CAGACTGCTCTTGGCAAC -3′, corresponding to bases 1545-1564, and the antisense 5′-CTTGAAAAGTTCCTCCTTG -3′, corresponding to bases 2231-2250, were used to amplify a 706-base pair fragment of FGFR in 35 cycles (30 seconds at 94°C, 30 seconds at 56°C, and 60 seconds at 72°C).

Equal amounts of total RNA were used for reverse transcriptase PCR. For quantification, glyceraldehyde-3-phosphate dehydrogenase was amplified from the same amount of RNA to correct for variation of different samples. The primers of the sense 5′-ATGGGGAAGGTGAAGTGCGAG -3′, corresponding to bases 61-82, and the antisense 5′-CTTGGAGCCATGTGGCCATG -3′, corresponding to bases 1138-1165, were used to amplify a 1105-base pair fragment of glyceraldehyde-3-phosphate dehydrogenase in 35 cycles (30 seconds at 94°C, 30 seconds at 63°C, and 60 seconds at 72°C). The PCR products were subjected to electrophoresis on 1.5% agarose gel, scanned, and quantitated by using Image-Quant software (Molecular Dynamics, Sunnyvale, Calif).

Protein analysis by using Western blotting. Total protein from atrial tissues was obtained by homogenation in a lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS and centrifuging at 12,000 g for 10 minutes at 4°C. Protein concentration of the supernatant was measured by using spectrophotometry at 595 nm (DU640, Beckman) of an aliquot developed for 10 minutes in protein assay dye reagent (Bio-Rad, Hercules, Calif) with bovine serum albumin (Bio-Rad) as standard. Total protein (40 µg/lane) was fractionated on 10% sodium dodecylsulfate–polyacrylamide gel elec-
troscopy gel (Bio-Rad) transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass). Commassie blue staining of the fractionated gel was used to correct for variation of protein loading and transfer efficiency of different samples.

The membrane was incubated with 5% nonfat dry milk powder and 0.05% Tween-20 (Sigma) in Tris-buffered saline (TBS; 2.42 g of Tris base plus 8 g of NaCl/1000 mL, pH 7.6) for 1 hour at 4°C to block nonspecific absorption and then was immuno blotted with the primary antibody at 1:2000 dilution overnight at 4°C. The primary antibodies for VEGF, VEGF-R1, VEGF-R2, FGF2, and FGFR-1 were rabbit anti-VEGF polyclonal immunoglobulin G (IgG), rabbit anti-FLK polyclonal IgG, rabbit anti-FLK polyclonal IgG, and goat anti-FGFR polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, Calif), respectively.

After being washed with TBS-T solution (TBS plus 0.05% Tween-20), the membrane was incubated for 1 hour in 5% milk powder TBS-T containing 1:2000 diluted anti-rabbit IgG conjugated to hors eradish peroxidase (Vector Laboratories, Burlingame, Calif) or anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Peroxidase activity was visualized by exposing an x-ray film to blots incubated with the ECL reagent (Amersham, Arlington Heights, Ill).

Densitometry of digitized images of immunoprobed membranes was performed by using Image-Quant software (Molecular Dynamics, Sunnyvale, Calif).

In vitro atrial microvascular studies. Atrial microvessels (70–156 µm in internal diameter) were dissected by using a 10× to 60× microscope (Olympus Optical, Tokyo, Japan). Microvessels were placed in a microvessel chamber, cannulated with dual glass micropipettes measuring 40 to 80 µm in diameter, and secured with 10-0 nylon monofilament sutures (Ethicon, Inc, Somerville, NJ). Oxygenated (95% oxygen and 5% carbon dioxide) Krebs buffer solution warmed to 37°C was continuously circulated through the microvessel chamber. The vessels were pressurized to 40 mm Hg in a no-flow state by using a burette manometer filled with a Krebs buffer solution. With an inverted microscope (40×–200×; Olympus CK2, Olympus Optical) connected to a video camera, the vessel image was projected onto a black and white television monitor. An electronic dimension analyzer (Living System Instrumentation, Burlington, VT) was used to measure the internal lumen diameter. Vessels were allowed to bathe in the organ chamber for at least 30 minutes before a pharmacologic intervention.

Microvessel study protocols. Relaxation responses of microvessels were examined after development of spontaneous tone with or without supplemental precontraction with the thromboxane A2 analog U46619. Baseline diameter was defined as the internal diameter within minutes of cannulation and placement in the bath when the diameter tended to be at a maximum and spontaneous contraction had not yet occurred. At the completion of an experiment, papaverine (10–4 mol/L) was applied to confirm that the initial diameter reading was similar to the maximally dilated diameter. If the spontaneous contraction was less than 30% of the initial baseline diameter, incremental concentrations of U46619 (10–8 to 10–6 mol/L) were applied so that the final precon traction was 30% to 60% of the initial baseline diameter. Vascular responses to VEGF (10–10 to 10–8 mol/L), FGF2 (10–8 to 10–6 mol/L), substance P (10–15 to 10–6 mol/L), and sodium nitroprusside (10–9 to 10–4 mol/L) were examined. Further experiments were performed in the presence of Nω-nitro-L-arginine (L-NNA; 10–4 mol/L). L-NNA was applied for 20 minutes before a dose-response intervention was performed. All drugs were applied extraluminally. Measurements were made and recorded 2 to 3 minutes after drug administration, when the response had stabilized.

In measuring the response to VEGF, it took 5 to 8 minutes for the response to be stabilized. Once VEGF or substance P was applied to a vessel, the vessel was discarded to avoid tachyphylaxis. One to 4 interventions was performed on each vessel. The order of drug administration was random. The vessels were washed 3 times with a Krebs buffer solution and allowed to equilibrate in a drug-free Krebs buffer solution for 15 to 30 minutes between interventions.

Drugs. Human recombinant VEGF and FGF2 were obtained from Genentech (South San Francisco, Calif). Substance P was obtained from RBI (Natick, Mass). L-NNA, U46619, and sodium nitroprusside were obtained from Sigma Chemical Co. VEGF and FGF2 were dissolved in phosphate-buffered saline solution with 0.1% bovine serum albumin to make stock solutions that were stored at –80°C. L-NNA and sodium nitroprusside were dissolved in ultrapure distilled water and were stored at –20°C. U46619 was dissolved in ethanol to make a 10 mmol/L stock solution that was stored at –20°C. Dilutions were prepared daily.

Data analysis. The relaxation responses were expressed as the percentage of relaxation of the spontaneous vascular contraction of the microvessels, the U46619-induced contraction (mean ± SD) of the microvessels, or both. Responses of vessels were compared with one- or two-factor ANOVA for repeated measures, followed by the Scheffe multiple comparison test. The paired Student t test was used to compare mRNA and protein expressions between control and ischemic tissues. Data are shown as means ± SD versus control.

Results

Clinical characteristics of patients. We studied 16 patients (12 male and 4 female patients), with an average age of 64 ± 12 years. The ischemic time averaged 65 ± 7 minutes. Sixty-nine percent (11/16) of the patients had hypertension, 19% (3/16) had diabetes mellitus, and 19% (3/16) had a recent smoking history. The total cholesterol level of these patients was 169 ± 42 mg/dL, the triglyceride level was 65 ± 32 mg/dL, the high-density lipoprotein level was 56 ± 2 mg/dL, and the low-density lipoprotein level was 101 ± 34 mg/dL. The left ventricular ejection fraction of these patients averaged 48% ± 12%.
Induction of VEGF and VEGF-R1 mRNA in acute ischemic myocardium. The RNA expression was analyzed by using reverse transcriptase PCR and expressed as the percentage of density ratio to the control specimens (100% expression in nonischemic control microvessels) for quantitative analysis. The RNA level of VEGF and VEGF-R1 was increased by 22.2% ± 4.2% and 30.7% ± 7.6%, respectively (P < .05), in the ischemic specimens versus the control specimens. However, the expression of VEGF-R2 (−3.1% ± 3.9% vs control, P > .05), FGF2 (−6.7% ± 3.3% vs control, P > .05), and FGFR-1 (−2.8% ± 2.1% vs control, P > .05) remained unchanged (Fig 1, A and B).

Increased VEGF and VEGF-R1 protein level in acute ischemic myocardium. To further explore the changes in VEGF and VEGF-R1 in human ischemic myocardium, we performed Western blot analysis to determine whether the increase in VEGF and VEGF-R1 in mRNA level was associated with an increase in the level of VEGF protein. The antibodies recognized VEGF with a molecular mass of 23 kd and VEGF-R1 with a molecular mass of 130 kd. Expression of VEGF demonstrated an increase of 71.7% ± 27.8% in ischemic tissue, whereas VEGF-R1 increased 68.2% ± 27.6% in density of protein level. The expression of another VEGF receptor, VEGF-R2 (46.5% ± 28.7%, P > .05), as well as that of FGF2 (21.3% ± 14.0%, P > .05) and FGFR-1 (17.7% ± 15.7%, P > .05), were not different in protein level between ischemic and control myocardium (Fig 2, A and B).

Vessel characteristics. Internal diameter of atrial microvessels ranged from 70 to 156 µm, averaging 118 ± 27 µm in the control group and 120 ± 22 µm in the ischemic group. The percentage of precontraction after spontaneous constriction or after application of the thromboxane A2 analog U46619 (1 nmol/L-1 µmol/L) was 41% ± 9% in the control group and 42% ± 9% in the ischemic group. The concentrations of U46619 required to produce these degrees of precontraction were similar in the 2 groups.

In vitro response to VEGF and FGF2. VEGF caused a potent dilation of atrial microvessels both in the control group and in the ischemic group. The average percentage of dilation of microvessels in the
ischemic group to VEGF was reduced compared with that in the control group ($P < .05$, Fig 3). After treatment of vessels with $10^{-4}$ mol/L nitric oxide synthase inhibitor L-NNA, the VEGF-induced relaxation was markedly reduced in the nonischemic control vessels, suggesting that VEGF caused vascular relaxation in human atrial microvessels largely through the release of nitric oxide (Table I).

Similarly, FGF2 induced a significant relaxation of vessels in both groups, but the response in ischemic vessels was diminished compared with that in nonischemic vessels ($P < .05$, Fig 4). The FGF2-induced relaxation was markedly reduced in the presence of the nitric oxide synthase inhibitor L-NNA ($P < .01$), indicating that FGF2-induced relaxation is mainly through the release of nitric oxide (Table I).

**In vitro response to substance P.** Substance P elicited a potent relaxation response in atrial arterioles that was inhibited in the presence of nitric oxide synthase inhibitor L-NNA (Table I). The relaxation of microvessels to substance P was significantly decreased in the ischemic group compared with that of the nonischemic vessels, suggesting dysfunctioned endothelium after ischemia (Fig 5).

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**Table I. Microvessels relaxation in the presence of NO inhibitor L-NNA**

<table>
<thead>
<tr>
<th></th>
<th>t-NNA, $10^{-4}$ mol/L ($n$)</th>
<th>Control ($n$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF ($10^{-11}$ mol/L)</td>
<td>14.9 ± 8.4 (7)</td>
<td>37.3 ± 16.5 (6)</td>
<td>.019</td>
</tr>
<tr>
<td>FGF2 ($10^{-11}$ mol/L)</td>
<td>5.9 ± 3.2 (6)</td>
<td>30.2 ± 9.3 (6)</td>
<td>.001</td>
</tr>
<tr>
<td>Substance P ($10^{-6}$ mol/L)</td>
<td>10.0 ± 6.5 (6)</td>
<td>48.1 ± 12.2 (8)</td>
<td>.006</td>
</tr>
</tbody>
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Selected experiments were performed on microvessels in the presence of the nitric oxide synthase inhibitor t-NNA. Responses are the percentage of relaxation of spontaneous contractions, U46619-induced contractions, or both. Values are given as means ± SD.

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**Figure 2.** A, Protein expression of VEGF, VEGF-R1, VEGF-R2, FGF2, FGFR-1, and of loading control Commassie blue in human atrium from the nonischemic (control) and ischemic groups, as analyzed with Western blotting. Positions and sizes of the blotting bands in kilodaltons are shown at the right. B, Quantitative analysis of protein expression of VEGF, VEGF-R1, VEGF-R2, FGF2, and FGFR-1 in atrial microvessels in the control ($n = 16$) and acute ischemia ($n = 16$) groups indexed as the percentage of density ratio to control specimens (100% expression in nonischemic microvessels). Data are expressed as means ± SD. *$P < .05$ versus control.
In vitro response to sodium nitroprusside. The relaxation of atrial microvessels to sodium nitroprusside, which operates through an endothelium-independent cyclic guanosine monophosphate–mediated mechanism, was similar in both groups. This suggests that ischemia causes no alteration of the smooth muscle’s ability to relax through the cyclic guanosine monophosphate pathway (Fig 6).

Discussion

The new findings of this study are that the expressions of VEGF and VEGF-R1 were increased after a brief period of acute myocardial ischemia, whereas the expressions of the VEGF-R2, FGF2, and FGFR-1 were not increased significantly. Despite the increased expression of VEGF-R1, the microvascular relaxation was impaired, most likely as a result of impaired endothelial function.

Why are these findings potentially important? First, although it is already well known from studies in rats that the expression of VEGF and its receptors is increased in myocardium early after the onset of acute ischemia, extrapolating a heightened physiologic effect may not be justified because VEGF, FGF2, and several other angiogenic substances may operate through the stimulated release of nitric oxide by activation of tyrosine kinase receptors. Because endothelial dysfunction is known to occur during acute myocardial ischemia, it is plausible that the physiologic actions of endogenous growth factors may be impaired. This was suggested by the findings of this study. The relaxation responses to VEGF, FGF2, and the endothelium-dependent vasodilator substance P were all diminished in the presence of the nitric oxide synthase inhibitor L-NNA, suggesting that the responses are mediated largely through the release of nitric oxide, as they are in other species. The finding that the endothelium-dependent agonist substance P was also impaired suggests that acute myocardial ischemia causes a generalized impairment in endothelial function, at least as far as vasomotor regulation is concerned. Interestingly, in a recent study in pigs subjected to ameroid constriction of the circumflex coronary artery, the expression of VEGF-R1 and VEGF-R2 was increased, and this was associated with an increased relaxation response to VEGF and FGF2, despite impaired endothelium-dependent relaxation to adenosine 5'-diphosphate. Because vessels and atrial tissue were examined after ischemia alone without reperfusion, it is suspected that the increased expression of VEGF and VEGF-R1 may actually underestimate the expression one would encounter after ischemia and reperfusion.

Second, the physiologic effect of the delivery of angiogenic growth factors in patients has generally been disappointing, especially when compared with the marked affect in otherwise normal pigs with chronic myocardial ischemia. This diminished effect may
be due to impaired endothelium function because endothelial dysfunction is observed in patients after acute or chronic myocardial ischemia, atherosclerosis, diabetes mellitus, hypertension, and other factors associated with coronary artery disease. Because the effectiveness of the growth factors is associated with the release of nitric oxide, endothelial dysfunction may diminish the effectiveness of these growth factors. Thus the increased receptor expression may not translate into a heightened physiologic action because of receptor inactivity or a defect in other levels of signal transduction or in nitric oxide synthesis. One limitation of the study is that atrial, rather than ventricular, myocardium was used. Although there are many similarities between these tissues, it cannot be concluded with certainty that identical results would be observed had ventricular myocardium been examined. Furthermore, although the atrial tissue was surrounded by a silk suture and devascularized as much as possible, this tissue may have been exposed to inflammatory mediators, such as cytokines, from a small amount of blood either from the surface or through the vasculature. Thus it cannot be concluded that these cytokine and other inflammatory mediators did not play a small role in the changes observed.

In summary, the expression of VEGF and one of its receptors was increased in acute myocardial ischemia in patients. However, the expression of FGF2 and FGFR-1 was unchanged, and the vascular responses to VEGF and FGF2 were impaired. These findings may not only have implications regarding the mechanism of coronary collateral development after acute coronary occlusion but may also affect the efficacy of therapeutic angiogenesis with angiogenic growth factors or genes encoding the growth factors for the treatment of patients with coronary occlusive disease not amenable to treatment with angioplasty or coronary artery bypass grafting.

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