Angiogenic-Induced Enhancement of Collateral Blood Flow to Ischemic Myocardium by Vascular Endothelial Growth Factor in Dogs

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Background Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that is angiogenic in vitro and in vivo. It has been hypothesized that VEGF plays a role in myocardial collateral formation; however, the effects of VEGF on collateral flow to ischemic myocardium are unknown.

Methods and Results We studied the effect of VEGF on collateral blood flow in dogs subjected to gradual occlusion of the left circumflex coronary artery (LCx). Beginning 10 days after placement of an LCx-constricting device, VEGF 45 µg (n=9) or saline (n=12) was administered daily via an indwelling catheter in the distal LCx, at a point just beyond the occlusion. Treatment was maintained for 28 days. Collateral blood flow was determined with microspheres 7 days before treatment, immediately before treatment (day 0), and 7, 14, 21, and 28 days into the treatment period. Collateral blood flow was quantified during chromanor-induced maximal vasodilation and expressed as a collateral zone/normal zone (CZ/NZ) ratio. Treatment with VEGF was associated with a 40% increase in collateral blood flow (final CZ/NZ blood flow ratios of 0.49±0.06 and 0.35±0.02 in the VEGF-treated and control groups, respectively, P<.0037) as well as an 89% increase in the numerical density of intramyocardial distribution vessels (>20 µm diameter) in the CZ (6.6±1.4 versus 3.5±0.7 vessels/mm² in VEGF-treated and control dogs, respectively, P<.05).

Conclusions We conclude that intracoronary VEGF enhances the development of small coronary arteries supplying ischemic myocardium, resulting in marked augmentation of maximal collateral blood flow delivery. These results demonstrate the feasibility of pharmacological enhancement of collateral growth and suggest a new therapeutic approach for the treatment of myocardial ischemia. (Circulation. 1994;89:2183-2189.)

Key Words • circulation, coronary • growth factor, vascular endothelial • endothelium • angiogenesis

Coronary collaterals improve myocardial blood flow and preserve myocardial function in the condition of coronary obstruction. The presence of collateral vessels ameliorates myocardial ischemia, and well-developed collaterals diminish the functional derangements that occur as a consequence of acute coronary occlusion.1,2 In dogs, gradual occlusion of a major coronary artery induces collateral formation.3 In general, the resulting collateral blood flow provides adequate metabolic support under resting conditions and is sufficient to prevent myocardial infarction; however, maximal blood flow is compromised. Under conditions of increased metabolic demand or during pharmacologically induced coronary vasodilation, the expected increase in blood flow in the collateral-dependent area is blunted, creating a disparity between the normally perfused and collateral-perfused areas.

Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor that is angiogenic in vivo, possessing unique target cell specificity for vascular endothelial cells.4-6 A 46-kDa homodimeric peptide, VEGF was originally purified from media conditioned by bovine pituitary folliculostellate cells. VEGF is structurally related to the A and B chains of PDGF, and the protein was purified independently as vascular permeability factor, a glycoprotein that promotes vascular fluid and protein extravasation.7 Unlike acidic and basic fibroblast growth factors, the VEGF sequence is preceded by a signal peptide, a characteristic consistent with extracellular secretion.8 High-affinity VEGF binding sites have been localized to endothelial cells in adult rat tissue sections,9 and expression of VEGF mRNA has been shown to be related temporally and spatially to physiological angiogenesis in a number of cell types in the murine female reproductive system.10 Moreover, VEGF mRNA levels are reversibly increased in cultured cells subjected to hypoxia,11 and VEGF has been reported to be expressed by cardiac myocytes in response to ischemia in vitro and in vivo.12 Together, these findings suggest that the peptide is a regulator of angiogenesis under physiological conditions as well as during the adaptive response to pathological states. We speculated that in the setting of progressive coronary occlusion, exogenously administered VEGF would accelerate myocardial angiogenesis, thereby improving collateral blood flow, and tested this hypothesis in a canine model.

Methods

The experimental methods were approved by the Animal Care and Use Committee of the National Heart, Lung, and

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Fig 1. Schematic of experimental animal model used to engender coronary collateral formation and provide for direct administration of vascular endothelial growth factor or placebo into the collateral-dependent zone vasculature. Ao indicates aorta; LCCA, left circumflex coronary artery; and LAD, left anterior descending coronary artery.

Blood Institute and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication No. [NIH] 86-23, revised 1985) and NIH issue 3040-2, "Animal Care and Use in the Intramural Program." The results of the placebo-treated control dogs were also used as the control group of a concurrently performed study.

**Surgical Procedures**

Twenty-six mixed hounds of either sex, average weight 18 kg, were obtained from Hazelton Laboratories, Cumberland, Va. Dogs were anesthetized with acepromazine 0.2 mg/kg IM and thiopental sodium 15 mg/kg IV and inhaled methoxyflurane. A thoracotomy was performed using sterile technique. An ameroid constrictor13 was placed on the proximal left circumflex coronary artery (LCx) before the takeoff of the first marginal branch, and a hydraulic balloon occluder was placed immediately distal to the encircling ameroid (Fig 1). A Silastic catheter was inserted into the first circumflex marginal branch (distal to the ameroid) with its tip positioned in the main body of the LCx, establishing access for intracoronary drug infusions into the ischemic zone. A second catheter was positioned in the left atrial appendage for microsphere blood flow determinations. The tip of the balloon occluder and the infusion ports of the LCx and left atrial catheters were secured in the subcutaneous tissue of the left lateral chest wall. The pericardium and chest were closed, and the dogs were allowed to recover. The LCx and left atrial catheters were flushed with saline daily to maintain patency.

**Angiography**

Radiopaque contrast material was injected into the LCx catheter 9 days after surgery to confirm proper catheter position in the distal LCx and to rule out an obstruction or leak in the catheter system.

**Treatment Protocol and Preparation of VEGF**

Dogs were randomized to receive VEGF (n=9) or placebo (n=12) beginning 10 days after the placement of the ameroid device. VEGF 45 µg/d or placebo was administered on a daily basis (5 d/wk) for 4 weeks. Aliquots of stock human recombinant VEGF, 0.5 mg/mL, were dissolved in 10 mmol/L citrate, 125 mmol/L NaCl, pH 6.0, and stored at -70°C until used. The peptide was diluted in 2 mL phosphate-buffered saline immediately before administration. Injections were made into the distal LCx, into the circulation supplying the ischemic myocardium. Routine hematologic and biochemical studies were performed weekly.

**Microsphere Blood Flow Measurements**

Microsphere blood flow studies were performed with the animal in the conscious state during maximal coronary vasodilatation. Myocardial blood flow studies were conducted weekly. The first two determinations were made before randomization to treatment, and four subsequent determinations were made during the treatment period. Thus, collateral blood flow was quantified 3, 10, 17, 24, 31, and 38 days after ameroid placement. Chromonar 8 mg/kg (Hoechst-Roussel Pharmaceuticals) was administered into the left atrial catheter to effect maximal coronary vasodilatation. This dose elicits a five- to fivefold increase in myocardial blood flow and abolishes the reactive hyperemic response to 20-second coronary occlusions without an appreciable effect on arterial blood pressure.14,15 Approximately 3×105 radiolabeled microspheres, 15 µm in diameter, were injected into the left atrial catheter as described previously.16 A different isotope was randomly selected each week: 141Ce, 113Sn, 85Sr, 99Nb, 48Sc (New England Nuclear), or 125I, (3M Co). Collateral blood flow was expressed as the ratio of collateral zone (CZ) to normal zone (NZ) blood flow, CZ/NZ. During the first few weeks after ameroid placement, arterial constriction was incomplete, and LCx flow was only partially arrested; thus, CZ blood flow was the sum of residual antegrade LCx flow and collateral flow (provided primarily through anastomoses originating from the left anterior descending coronary artery). Thus, for microsphere determinations of CZ blood flow, the LCx hydraulic balloon occluder was temporarily inflated to interrupt antegrade LCx flow such that collateral flow alone could be quantified. In addition, the balloon occluder was permanently inflated on day 24 to ensure complete and timely LCx occlusion.

**Tissue Preparation**

Animals were killed with an overdose of sodium pentobarbital and KCl. The myocardium was preserved using perfusion fixation with McDowell-Trump solution at physiological pressure.17 It was verified that the proximal LCx was encircled by the ameroid constrictor and that the vessel was completely occluded. Three central short-axis slices were removed from the left ventricle, and each was divided into eight circumferential wedges that were further subdivided into endocardial and epicardial portions. The midequatorial slice and a second slice on the apical side of the central slice were used for microsphere blood flow analysis (Fig 2A). A third slice (to the base of the central slice) was used for estimation of myocardial infarct size. Based on plots of blood flow versus position (Fig 2B), two CZ wedges and two NZ wedges were selected from each slice for further analysis, as we have done previously.16 Thus, four CZ and four NZ transmural wedges were used to compute mean CZ and NZ blood flow, respectively, and vascular density was quantified in these samples.

Vascular density was assessed in tissue sections using computer-based image analysis. Samples were embedded in glycol methacrylate. Two-micrometer sections were stained with toluidine blue and examined using a microscope interfaced with a video camera and computer-controlled motorized stage, such that each slide was systematically analyzed in its entirety.
Each section was examined at ×40, ×10, ×4, and with a macro lens to match system magnification with the size of the object(s) to be detected. Thus, vessels ≤10 μm in diameter were considered to be capillaries, and capillary density was determined using a ×40 objective lens. Vessels 20 to 60 μm in diameter were quantified at ×10, and vessels 60 to 120 μm, 120 to 180 μm, and >180 μm were analyzed at ×4. Total tissue area, quantified using a macro lens, served as the denominator for all vessels. Vascular profiles were discriminated and counted using a computer-based image analysis system (Advanced Imaging Concepts, Inc). To exclude tangentially sectioned vessels, the circularity of each vascular profile was calculated as 4π×area/perimeter². (A vessel cut in perfect cross section [a circle] would have a circularity of 1, whereas the elliptical profile of a tangentially cut vessel would have a circularity <1.) Inclusion of vascular profiles with circularity >0.5 provided consistency and objectivity to the analyses of vascular density and provided excellent agreement with manual vessel counting. For estimation of myocardial infarct size, all 16 endocardial and epicardial sections of one short-axis slice were stained using the Masson’s trichrome method and analyzed at ×4 magnification. Planimetry was performed on the sections: Blue-stained areas were considered scar; pink-stained areas were considered viable. All counting and planimetry were performed by a single observer who was blinded to treatment group.

**In Vitro Experiments**

Endothelial and smooth muscle cells were harvested from the carotid arteries of two dogs. Purity of the cultures was confirmed by morphology, appropriate immunoperoxidase staining for von Willebrand factor using a polyclonal rabbit anti-human antibody, and fluorescent staining for the presence of acetylated low-density lipoprotein receptors. Assays were performed with second or third passage cells. Endothelial cells and smooth muscle cells were seeded on day zero at a density of 5000 cells/cm² in M-199 with 10% fetal bovine serum. Endothelial cells were seeded on 1% gelatin-coated dishes; smooth muscle cells were seeded on uncoated dishes. The medium was not changed for the duration of the experiment. Cells were fed with VEGF 10.0 ng/mL, basic fibroblast growth factor (FGF) 10.0 ng/mL, or 0.2% gelatin (control) on days 1, 3, and 5. Triplicate wells were trypsinized and counted by two independent observers on days 2, 4, 6, and 8.

**Statistical Analyses**

Data are presented as mean±SE. For analysis of the CZ/NZ blood flow ratios in VEGF-treated versus control dogs, blood flow measurements on days 3 and 10 were considered to constitute baseline measurements for each dog (obtained before randomization to treatment). A repeated-measures ANOVA model was used that included the following effects: treatment, time, and time by treatment interaction. In this model, the time by treatment interaction effect was significant; therefore, it was necessary to compare treatment means with control means at each time point instead of comparing treatment means with control means across time. A Bonferroni correction was used to adjust for multiple comparisons (ie, repeated measurements on the same dogs).18

**Results**

Twenty-one of 26 dogs completed the studies. Three dogs died before randomization: One dog died during
chromonar infusion on day 3, one died during LCx angiography on day 9, and one died during temporary LCx balloon occlusion on day 10. An additional dog sustained full cardiopulmonary arrest during the LCx balloon occlusion on day 10. This dog was resuscitated and assigned to treatment with VEGF; however, the dog had chronic, almost incessant ventricular tachycardia during the weeks after recovery and was excluded before blood flow results were available. A fifth dog died suddenly during the initial VEGF injection on day 10.

Complete blood count, platelet count, differential leukocyte count, serum electrolytes, glucose, urea nitrogen, creatinine, albumin, lactate dehydrogenase, alkaline phosphatase, creatinine phosphokinase, SGOT, SGPT, and bilirubin were similar in VEGF-treated and control dogs throughout the course of treatment. By light microscopy, no significant histopathologic abnormalities were observed in lung, skin, gut, kidney, liver, spleen, or adrenal.

**Systemic Hemodynamic Data**

At the conclusion of the study (day 38), basal heart rates were 115±12 and 131±7 beats per minute in VEGF-treated and control dogs, respectively, P=NS. Mean blood pressure was also similar in the two groups (mean arterial pressure, 98±7 mm Hg in VEGF-treated dogs versus 109±8 mm Hg in control dogs). Administration of chromonar was not associated with a reduction in blood pressure in either group; however, chromonar caused a significant increase in heart rate in VEGF-treated dogs (from 115±12 to 161±15 beats per minute, P<.05). The change in heart rate in control dogs was not significant (131±7 beats per minute before versus 145±9 beats per minute after chromonar).

**Collateral Blood Flow**

Collateral blood flow data are summarized in Fig 3. On days 3 and 10 (before randomization to treatment), there was no difference in collateral flow between the two groups. After 1 week of treatment (day 17), collateral flow was also virtually the same in the two groups. After 2 weeks of treatment, collateral flow in VEGF-treated dogs diverged from that of controls, with CZ/NZ ratios of 0.36±0.07 and 0.25±0.04, respectively, on day 24 (P=.039) and ratios of 0.39±0.07 and 0.32±0.02 on day 31 (P=.10). At the 38-day conclusion of the study, CZ/NZ blood flow ratios were 0.49±0.06 and 0.35±0.02 in VEGF-treated and control dogs, respectively, a 40% difference (P=.0037). The effect of VEGF treatment was apparent in both the endocardium and epicardium: Final endocardial CZ/NZ ratios were 0.48±0.09 and 0.30±0.05 in treated and control dogs, respectively; final epicardial CZ/NZ ratios were 0.62±0.12 versus 0.48±0.04 in treated and control dogs, respectively.

**Infarct Size**

As a percentage of a representative short-axis left ventricular slice, infarct size was 0.51±0.20% in VEGF-treated dogs and 1.3±0.4% in control dogs (P=NS).

**Vascular Density**

For determination of vascular density, hearts were perfusion-fixed at physiological pressure. Given that the LCx was occluded, the CZ could be subjected to lower perfusion pressure than the NZ during this process, which could lead to some underestimation of vascular density in the CZ. Thus, we believe that comparisons between groups are valid (CZ of treated versus CZ of control, NZ of treated versus NZ of control), whereas direct comparisons between CZ and NZ are not.

**Capillaries**

In the CZ, capillary density (vessel diameter ≤10 μm) was 4030±280 and 3970±350 vessels/mm² in VEGF-treated and control dogs, respectively (P=NS). Likewise, NZ capillary density was similar in VEGF-treated and control dogs (4260±230 versus 4830±250 vessels/mm², respectively, P=NS) (see Fig 4, left).

**Distribution Vessels**

The numerical vascular density of distribution vessels (diameter >20 μm) was 6.5±1.4/mm² in the CZ of VEGF-treated dogs and 3.5±0.7/mm² in controls (P<.05) (see Fig 4, right). Increased vascular density was apparent in the endocardium as well as the epicar-
ment are inextricably related to the capacity of collateral vessels.\textsuperscript{1,2} Collateral development is incompletely understood: Mechanical factors, chemical mediators, and angiogenic growth factors all have been implicated in the process.\textsuperscript{11,12,19-23} In the setting of progressive coronary occlusion, the augmented transcollateral pressure gradient leads to increased flow across collateral vessels, with attendant increases in radial wall tension and shear stress. These changes by themselves or possibly accompanied by metabolic alterations in hypoperfused tissue could lead to synthesis or release of peptide growth factors or upregulation of their receptors. Such growth factor–receptor interactions are postulated to play an active role in stimulating cellular proliferation and locomotion, requisites of new vessel growth. We hypothesized that intracoronary administration of VEGF, a potent, endothelial cell–specific angiogenic growth factor, would enhance the formation of coronary collaterals during the complex circumstances induced by coronary occlusion. Our results demonstrate that treatment with exogenous VEGF augments the angiogenic process induced by coronary occlusion: VEGF administration was associated with a 40% increase in collateral blood flow after 4 weeks of treatment as well as an 89% increase in the numerical density of intramyocardial distribution vessels (ie, vessels larger than capillaries).

The mechanism of action of the peptide cannot be deduced from our data. VEGF is a potent inducer of vascular permeability,\textsuperscript{7} and the peptide has been found to express interstitial collagenase in endothelial cells; both properties facilitate cell migration, a crucial step in the angiogenic process. Using canine arterial endothelial and smooth muscle cells, we found that the mitogenic effects of VEGF were specific for vascular endothelial cells in vitro (Fig 5), confirming that the results of previous studies using cells derived from other species are also applicable to canine cells.\textsuperscript{4-6} If the specificity of VEGF noted in vitro can be extrapolated to the in vivo situation, then it appears that selective stimulation of endothelial cells provides sufficient inducement for the promotion of angiogenesis. This implies that direct stimulation of fibroblasts and smooth muscle cells is unnecessary to induce angiogenesis, although VEGF may have indirect effects on these cell types. For example, activated endothelial cells express PDGF, which in turn could provide a mitogenic stimulus to underlying smooth muscle cells.

Studies of the effects of exogenous growth factor administration on the response to coronary occlusion have been limited. We have administered acidic FGF via an epicardial sponge in dogs subjected to amerox-induced coronary occlusion and demonstrated marked vascular smooth muscle cell proliferation in areas of myocardial infarction.\textsuperscript{24} We also found that chronic intracoronary administration of acidic FGF had no effect on collateral flow in dogs with LCX amerox constrictors when the peptide was given 5 to 9 weeks after LCX amerox placement.\textsuperscript{25} There have been two positive reports of the effect of basic FGF in myocardial ischemia/infarction. We have demonstrated that intracoronary administration of basic FGF can enhance coronary collateral development in dogs,\textsuperscript{26} and Yanagisawa-Miwa et al\textsuperscript{27} have reported that basic FGF administration reduces infarct size and preserves global left ventricular function after acute myocardial infarction in

**Fig 5.** Line plots show in vitro mitogenic effect of vascular endothelial growth factor (VEGF) on canine arterial endothelial and smooth muscle cells. Basic fibroblast growth factor (bFGF), a nonselective mesenchymal cell mitogen, was used as a positive control for smooth muscle cells and endothelial cells. Top, Media containing VEGF or bFGF potentiated the proliferation of canine arterial endothelial cells (ECs). The potency of VEGF was roughly half that of bFGF. Bottom, Addition of VEGF had no effect on canine arterial smooth muscle cells (SMCs), whereas bFGF markedly potentiated proliferation, confirming the selective mitogenicity of VEGF for endothelial cells. (VEGF and control curves are superimposed.) Experiments were run in triplicate.

**In Vitro Studies**

The mitogenic effect of VEGF on canine arterial endothelial and smooth muscle cells in vitro is illustrated in Fig 5. Basic FGF, a nonselective mesenchymal cell mitogen, was used as a positive control for smooth muscle cells and endothelial cells. Both VEGF and basic FGF potentiated the proliferation of canine arterial endothelial cells, and basic FGF was approximately twice as potent as VEGF (Fig 5, top). Unlike basic FGF, VEGF was not mitogenic for canine arterial smooth muscle cells, confirming the selectivity of VEGF for endothelial cells (Fig 5, bottom).

**Discussion**

As coronary artery narrowing progresses, myocardial viability and the extent of myocardial functional derange-
dogs, although the mechanism of action of an angiogenic peptide in the context of acute infarction is difficult to explain. (To prevent infarction, blood flow to acutely ischemic myocardium must be restored within hours, in far less time than would be required for basic FGF to induce neovascularization).

Administration of VEGF enhanced the development of coronary arteries supplying collateral-dependent myocardium; however, the role of ischemia and the potential effect of VEGF on normal myocardium are matters of speculation. A recent investigation in miniature subjects to amiodar-induced LCx occlusion demonstrated that the presence of ischemia is not a prerequisite for the development of collaterals, consistent with the concept that local factors (ie, increased endothelial stretch or shear) are responsible for collateral development.28 It is possible that such events may render the endothelium responsive to exogenous VEGF; alternatively, it is possible that VEGF could exert an angiogenic effect in the presence of ischemia and/or increased shear stress. If so, the peptide could lead to increased vascularity without an appreciable effect on blood flow, and it would be interesting to evaluate the effect of VEGF on coronary blood flow in the normal myocardium.

In the present study, blood flow measurements were obtained during maximal coronary vasodilatation to increase the sensitivity of the analysis and, most importantly, to eliminate autoregulatory influences that could confound the interpretation of the data. In the maximally vasodilated state, vascular tone is reduced to an absolute minimum. Therefore, at any level of arterial pressure, coronary blood flow is limited exclusively by anatomic determinants of vascular resistance. Thus, the increase in collateral blood flow in treated dogs could not be the result of a vasodilator property of VEGF because blood flow was determined in a state of maximum vasodilatation at all times. In addition, we observed an 89% increase in the numerical density of intramyocardial distribution vessels in the CZ of VEGF-treated dogs, providing the anatomic correlate of the increase in maximal collateral blood flow found in these dogs.

Under conditions of maximal coronary vasodilatation, perfusion to the territory of the occluded LCx is dependent on the series resistance of collateral vessels and the coronary microvasculature. The microsphere perfusion measurements used in this study provide an assessment of total CZ vascular resistance but do not distinguish between its individual components. The detection of an increased number of intramyocardial distribution vessels in the CZ of VEGF-treated dogs suggests that the decrease in overall resistance resulted at least in part from alterations within the microvasculature. In future studies, these two resistive components could be discriminated by performing coronary blood flow measurements using an isolated heart preparation.29

We obtained measurements of coronary perfusion on a serial basis; however, an inherent limitation of our study design was that anatomic evidence of neovascularization could be obtained only once: at the conclusion of the study and subsequent to the period of peak vascular growth. Undoubtedly, the increased number of small coronary distribution vessels in VEGF-treated dogs had a salutary effect on myocardial perfusion; however, the experimental design was such that it was not possible to determine whether the increase in small coronary arteries resulted from the radial growth of preexisting arterioles or, alternatively, whether they originated de novo as a result of vascular sprouting.

The results we report, demonstrating a major effect of VEGF on increasing collateral flow to ischemic myocardium, were obtained in a canine model. It must be emphasized that the canine coronary circulation differs in several respects from that of humans.30 Dogs have a strong tendency to develop collaterals in response to gradual coronary occlusion, such that resting collateral flow is normal or near normal; however, maximal perfusion is compromised. Thus, at the end of our study, maximal collateral flow in untreated dogs was only 35% of normal. Such a deficit allows the detection of the angiogenic effects of an intervention, as demonstrated in the present study. Another factor to consider about our model is that VEGF was administered during a period of active collateral growth. In the setting of chronic coronary insufficiency (in humans), when collateral growth has reached a plateau and vascular proliferation has ceased, it is possible that VEGF would not exert a positive effect. Nonetheless, these results suggest a novel and potentially important therapeutic approach to patients with ischemic heart disease; it remains to be established, however, whether VEGF will exert a salutary effect on collateral blood flow in humans.

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