Myocardial Contrast Echocardiography Can Be Used to Assess the Microvascular Response to Vascular Endothelial Growth Factor-121

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Background—Therapeutic angiogenesis is a new approach to treating ischemic heart disease, and the optimal method for assessing its efficacy is unclear. We used myocardial contrast echocardiography (MCE) to evaluate the therapeutic response to the angiogenic agent, vascular endothelial growth factor-121 (VEGF121).

Methods and Results—After placement of an ameroid constrictor (day 0) around the left anterior descending artery (LAD), dogs were given intracoronary VEGF121 protein (108 μg, n=6) or placebo (n=6) on days 7 and 21, and subcutaneous VEGF121 (1 mg) or placebo on days 8 to 20 and 22 to 27. On day 48, MCE was performed during rest and dobutamine stress. Videointensity (y) and pulsing interval (t) were fit to an exponential model (y=A[1−e−βt]) used to derive indices of red cell velocity (β) and capillary area (A), and parameters were compared with radiolabeled microsphere flow data. VEGF121 treatment resulted in higher resting left anterior descending artery/left circumflex flow ratio compared with placebo (P<0.03) and improved collateral flow reserve. β was 0.94±0.37 in VEGF121 dogs versus 0.38±0.31 in controls (P<0.02), with the greatest difference in the endocardium. The parameter A was comparable in both groups, suggesting that microvascular changes did not alter capillary cross-sectional area, and histology indicated a trend toward higher arteriolar density in VEGF121-treated animals.

Conclusions—VEGF121 protein improves collateral flow and reserve. MCE can evaluate the transmural location and structural and functional responses of the microvasculature to angiogenic interventions. (Circulation. 2002;105:759-765.)

Key Words: echocardiography ■ angiogenesis ■ coronary disease

Therapeutic angiogenesis represents a new approach to treating coronary artery or peripheral vascular disease using exogenous delivery of angiogenic growth factors (or genes encoding these factors) to enhance new blood vessel development or promote remodeling of existing vessels.1 This treatment is based on studies demonstrating that angiogenic interventions improve perfusion in experimental models of ischemia.2–8

Vascular endothelial growth factor (VEGF) is one of the most intensively studied angiogenic agents,2–6 partly because its mitogenic activity is almost entirely specific for endothelial cells and its expression is induced by hypoxia, indicating that it is a key natural mediator of angiogenesis in response to ischemia.8,9 Most commonly, the protein is a homodimer of polypeptides extending 165 or 121 amino acids in length (VEGF165 and VEGF121, respectively), which binds to high-affinity tyrosine kinase receptors.9 VEGF165, but not VEGF121, also binds to heparin-like molecules and to the receptors neuropilin-1 and -2.9 Despite these differences, VEGF165 and VEGF121 both enhance collateral blood flow in experimental models.2–6,8 In the category of coronary models, positive effects for VEGF121 have been reported using a localized gene therapy approach in which adenoviruses encoding VEGF121 were injected directly into ischemic porcine myocardium.4,6

With regard to the clinical application of angiogenic agents such as VEGF, numerous questions remain, including the following: (1) Will single or multiple agents be required? (2) Does the agent need to be delivered locally? (3) Is the agent best delivered as a recombinant protein, or via gene therapy? Additionally, the appropriate end point for establishing the efficacy of angiogenic therapy has been debated,1,10 partly because the optimal method for measuring the blood vessel response is unclear, the microvascular level at which growth factors act is incompletely understood, and there are no in vivo imaging methods for evaluating vessels <200 μm in diameter.
Myocardial contrast echocardiography (MCE) is an intravascular tracer technique using intravenously injected microbubbles as red cell tracers during ultrasound imaging.\textsuperscript{11–13} New approaches to the quantification of MCE data\textsuperscript{13} may uniquely allow us to noninvasively interrogate the microcirculation and evaluate the effects of therapeutic angiogenesis.

The purpose of this study was 2-fold. First, as an extension of results previously reported with VEGF\textsubscript{121} gene therapy, we tested the hypothesis that sustained exposure to VEGF\textsubscript{121} protein achieved through repeated subcutaneous and intracoronary injection would enhance collateral development in a canine model of chronic myocardial ischemia. Second, we tested the hypothesis that MCE can assess the effects of angiogenic therapy on microvascular structure and function in vivo. With histology and radiolabeled microsphere flow data as standards, we report that treatment with VEGF\textsubscript{121} protein was efficacious in producing a therapeutic angiogenic response, that MCE could detect a subset of the effects, and that MCE can yield unique insights into the physiology of the response.

Methods

Surgical Preparation

**Day 0: Recovery Surgery**

This protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and complied with American Heart Association Guidelines. After premedication with transdermal fentanyl (75 \mu g/kg) and IV thiopenthal sodium (10 mg/kg), 13 dogs were intubated and ventilated. General anesthesia was maintained with 0.5% to 2.0% inhaled isoflurane. A left lateral thoracotomy was performed. Catheters were placed in the left atrium for microsphere injection, in the cephalic vein for drug and ultrasound contrast delivery, and in a femoral artery for pressure monitoring and radiolabeled microsphere reference withdrawal. A 2.5- to 2.75-mm-diameter amiderm constrictor was placed around the proximal anterior descending artery (LAD) to cause total occlusion 2 to 3 weeks later.\textsuperscript{5,12} All catheters were removed before chest closure, the dogs were recovered, and aspirin (325 mg PO) was given daily for 24 days to prevent coronary thrombosis.

**Day 48: Terminal Surgery**

On the 48th postoperative day, the dogs were anesthetized with sodium pentobarbital (30 mg/kg), intubated, and ventilated. A left lateral thoracotomy was performed, and catheters were placed in the left atrium, cephalic vein, and femoral artery.

Myocardial Contrast Echocardiography

ECG-triggered end-systolic harmonic B-mode imaging was performed in short-axis views at incremental pulsing intervals using 1.8- to 3.6-MHz transmit/receive frequencies (Sequoia, Acuson Corp). Gain, depth, mechanical index (0.8 to 1.0), focus, and dynamic range (75 dB) were held constant.

The contrast agent (Definity, Du Pont Pharmaceuticals Co) was a microbubble preparation (mean diameter 2.3 \mu m) containing perfluoropropane gas and air inside a phospholipid bilayer shell.\textsuperscript{12,13} Microbubbles (1.5 mL) were placed in 50 mL 0.9% saline and intravenously infused at a constant rate (120 to 160 mL/h) initially adjusted to optimize myocardial opacification.

Digitally acquired images were aligned and background subtraction and color coding were performed offline, where shades of red, orange, yellow, and white represented increasing videointensity change.\textsuperscript{12} Videointensity at each pulsing interval was measured in LAD and left circumflex (LCX) territory regions of interest. Videointensity-pulsing interval data were fit to a monoexponential function (y=A[1−e^{−βt}]), where videointensity (y) is a function of bubble replenishment rate (β) with time (t) after bubble destruction, and A=peak plateau videointensity. In this model, β marks red cell velocity and A is the index of the intramyocardial vascular cross-sectional area\textsuperscript{13} predominantly comprising capillaries.\textsuperscript{14}

Cardiac Catheterization

Dogs were given IV thiopeptol sodium (15 mg/kg), intubated, and maintained under gas anesthesia. A femoral artery was percutaneously cannulated with a 5F coronary catheter that was placed subselectively into either the LAD or LCX for infusions.

**VEGF\textsubscript{121} Synthesis**

VEGF\textsubscript{121} was expressed recombinantly in *Pichia pastoris*\textsuperscript{15} as secreted protein. The protein was purified by serial chromatography on an Octyl-Sepharose FF column (Pharmacia), a Chelating-Sepharose FF column (Pharmacia) charged with nickel, and a C18 preparative HPLC column (Vydac, 10×25 mm, 10 \mu m). The VEGF\textsubscript{121} was lyophilized and dissolved in PBS and 10 mmol/L citrate, pH 5.5 (vehicle). Protein concentration was determined by amino acid analysis.

Measurement of Regional Myocardial Blood Flow

Approximately 3×10\textsuperscript{11} 11-\mu m radiolabeled microspheres (New England Nuclear) were suspended in 3 mL 0.9% saline/0.01% Tween 80, injected into the left atrium, and flushed with saline during 90-second arterial reference sample withdrawal. One of the following radiolabels was used for each injection stage: \textsuperscript{125}Sn, \textsuperscript{185}Ru, \textsuperscript{161}Ce, \textsuperscript{99m}Tc, \textsuperscript{99m}Nb, \textsuperscript{51}Cr, and \textsuperscript{46}Sc. Postmortem, the slice paralleling the MCE image was sectioned into endocardial and epicardial pieces. Samples were placed in a gamma counter (Packard Instruments), and blood flow (mL/min · g\textsuperscript{−1}) was calculated.\textsuperscript{15}

**Immunohistochemistry**

Myocardium from the midventricular LAD and LCX beds was fixed in 4% paraformaldehyde, paraffin embedded, sectioned, deparaffinized, incubated with blocking serum, and exposed to mouse anti-α-smooth muscle actin antibody (1 \mu g/mL, Santa Cruz Biotechnology). Bound antibody was detected with biotin-conjugated anti-mouse secondary antibody using the ABC immunoperoxidase method.\textsuperscript{16} For each dog, 54 microscopic fields from 27 samples were digitized. An investigator blinded to treatment counted the number of arterioles (per field), defined as 10- to 200-\mu m-diameter vessels with actin-stained muscle.

Experimental Protocol

On day 0, MCE and radiolabeled microsphere measurements were performed at baseline and during transient LAD occlusion. Animals were randomly assigned to receive 3 weeks of daily VEGF\textsubscript{121} or vehicle (placebo). Animals were infused over 30 minutes with 108 \mu g of VEGF\textsubscript{121} or an equivalent volume of vehicle into the LAD (day 7) or LCX (day 21) during hemodynamic monitoring. On days 8 to 20 and 22 to 27, animals were given 1 mg of VEGF\textsubscript{121} or equivalent volume of vehicle via subcutaneous injection. On day 48, microsphere and MCE measurements were made at baseline and during IV dobutamine (20 to 40 \mu g/kg · min\textsuperscript{−1}). Dogs were euthanized with pentobarbital and potassium chloride.

**Statistical Analysis**

Data are expressed as mean±SD. Comparisons between the VEGF\textsubscript{121}-treated and control dogs were made by unpaired \(t\) testing. Significance was defined as \(P<0.05\) (2-tailed).

Results

One dog died postoperatively. Data from 12 dogs (6 VEGF\textsubscript{121}-treated, 6 controls) are presented here.
Native Collateral Flow: Day 0
Baseline resting LAD/LCX flow was essentially identical in both groups on day 0 (Figure 1C). LAD risk area size as judged by MCE (see examples in Figure 1A and 1B) was also similar (Figure 1C, right). Radiolabeled microsphere flows during acute LAD occlusion were not different in the 2 groups (Figure 1C), confirming similar pretreatment native collateral flow.

Collateral Development
The risk areas detected by MCE during acute occlusion on day 0 were no longer present on day 48 (compare Figure 1A and 1B and Figure 2), indicating restoration of resting perfusion in the LAD bed via collaterals. Nonetheless, overall there was a modest but statistically significantly higher resting LAD/LCX microsphere flow in the VEGF121-treated dogs (0.92±0.04) compared with controls (0.86±0.04) on day 48 (P<0.03).

Collateral Flow Reserve
Collateral flow reserve with dobutamine stress on day 48 was impaired in both groups, but less so in the VEGF121-treated dogs. Figure 3 depicts images at increasing pulsing intervals in a VEGF121-treated and control dog at similar rate-pressure products during dobutamine stress. In both animals, there was an LAD endocardial defect at the shortest pulsing interval (panels B and F), because insufficient time had elapsed since the previous destructive pulse for bubbles to replenish this region of reduced reserve. As bubble replenishment increased with higher pulsing intervals, this defect filled in (Figure 3C through 3D and 3G through 3H).

Figure 1. Regional perfusion on day 0. MCE images demonstrate risk area during LAD occlusion in VEGF121 dog (A) and control dog (B). C, Graph summarizes radiolabeled microsphere flow at baseline and during LAD occlusion (left axis) and MCE risk area size (right axis) in all 12 dogs. LAD flow is normalized to LCX flow. Risk area is expressed as percentage of the left ventricular short axis (% LV).

Figure 2. MCE images on day 48 from dogs in Figure 1, demonstrating absence of previously seen risk areas in VEGF121 dog (A) and control dog (B).

Figure 3. Sequential images in the same VEGF121-treated dog (A through D) and control dog (E through H) shown in Figure 2, at incremental triggering intervals during dobutamine stress. Time (seconds) represents pulsing interval duration. There is more rapid resolution of the endocardial defect (region between arrows) in the VEGF121-treated dog.
Despite the initial delay in LAD endocardial enhancement in the VEGF121 dog (Figure 3, upper panels), intensity in both LAD and LCX was equivalent by 2.2 seconds, suggesting a similar capillary cross-sectional area in the 2 beds (Figure 3D). In the control dog (Figure 3, lower panels), however, the endocardial defect persisted at 2.2 seconds (Figure 3H). Later frames (not shown) demonstrated resolution of this defect, suggesting equivalent LAD and LCX capillary volumes even in this control dog. These images thus suggest that despite restoration of resting flow, flow reserve was impaired, with the more severe impairment in the control dog manifesting as decreased endocardial red cell velocity.

Figure 4 plots LAD and LCX bed videointensity versus pulsing interval data for the dogs shown in Figure 3. In the VEGF121 dog, the initial endocardial defect resulted in decreased LAD intensity relative to the LCX at short pulsing intervals, but this defect quickly resolved and videointensity equalized in both beds by 2 seconds. In the control dog, the rate of LAD videointensity change was markedly delayed as compared with that of LCX. When these data were fit to an exponential function, plateau LAD and LCX intensity (A value) ultimately equalized (not shown in graph). Overall, bubble velocity (β) in the LAD relative to LCX bed was higher in the VEGF121 group as compared with controls (Figure 5A), with differences most evident in the endocardium, where β values for the endocardium and endocardial/epicardial ratios were higher in VEGF121 dogs (Figure 5B). Plateau videointensity (A) was similar in both groups (Figure 5A).

The radiolabeled microspheres corroborated the MCE data. On day 48, there was no difference between control and VEGF121-treated dogs with respect to resting LAD/LCX endocardial flow (0.82 ± 0.10 versus 0.88 ± 0.06, P = 0.22) and LAD/LCX endocardial/epicardial flow ratios (0.96 ± 0.22 versus 0.96 ± 0.08, P = 0.99). With dobutamine, however, normalized endocardial flow was higher in the VEGF121 dogs compared with controls, resulting in higher endocardial/epicardial flow ratios (Figure 6).

The number of arterioles per field in the endocardium (Figure 7) tended to be higher in VEGF121 dogs (12.6 ± 5.1) as compared with controls (7.6 ± 2.8, P = 0.058).

Hemodynamics and Safety

There was no difference in rate-pressure product between VEGF121 and control groups during dobutamine stress (45 ± 7 × 10^3 versus 38 ± 8 × 10^3, P = 0.20), indicating similar myocardial oxygen demand.

Heart rate and mean blood pressure were stable during intracoronary infusion in the controls (Table). In the VEGF121 dogs, heart rate increased in association with a slight rise in blood pressure (Table). In 3 conscious dogs receiving VEGF121, cuff blood pressure was measured before and 5 minutes after subcutaneous injection. There was no difference between mean blood pressure before (92 ± 6 mm Hg) and after (89 ± 2 mm Hg) VEGF121 injection (P = 0.37). Serum hemoglobin, leukocyte and platelet counts, blood urea nitrogen, creatinine, and serum transaminases remained unchanged.

Discussion

Using a canine model of chronic myocardial ischemia, this study demonstrated that (1) sustained exposure to recombinant VEGF121 protein via subcutaneous and intracoronary delivery is feasible and safe, (2) VEGF121 delivered in this fashion enhances collateral flow and reserve, and (3) MCE can assess physiological and structural effects of VEGF121 on the microvasculature.

Therapeutic Effect of VEGF121

This is the first study to show that administration of VEGF121 protein is associated with a therapeutic angiogenic effect in ischemic myocardium. Unlike VEGF165 used in previous studies, VEGF121 does not bind to heparin-like molecules in extracellular matrix.8,9 We reasoned that a subcutaneous depot of the protein would be systemically absorbed and ultimately bind to endothelial VEGF121 receptors overexpressed in ischemic tissue.8,9 Indeed, in previous studies in rats, a subcutaneous VEGF121 dose of 100 μg, even less than the weight-adjusted dose used in our study, resulted in therapeutically effective peak plasma levels of 50 ng/mL at 100 minutes and 5 ng/mL at 6 hours.17 We also reasoned that sustained exposure to VEGF121 afforded by repetitive dosing would confer therapeutic benefit.

The treatment resulted in modestly higher resting flow (measured by radiolabeled microspheres) to the collateral-dependent bed in VEGF121 dogs as compared with controls at day 48. Microspheres injected during LAD occlusion on day 0 confirmed that pretreatment collateral perfusion to the LAD...
bed was similar in both groups. Thus, resting differences in preexisting collaterals were unlikely to have biased the day 48 results.

We assessed flow reserve in the collateral-dependent bed to further characterize physiological effects of VEGF121 treatment and found that flow reserve was better in VEGF121-treated dogs. In particular, endocardial reserve was higher in VEGF121-dogs as compared with controls, resulting in improved endocardial/epicardial flow ratios and better preservation of transmural flow distribution during stress.

Although there are numerous investigations of therapeutic myocardial angiogenesis in animal models, few have used clinically applicable methods to assess the effect of treatment on coronary flow reserve. Harada et al reported that VEGF165 administered to ameroid-constricted pigs improved endocardial flow reserve during pacing stress, as judged by microspheres. Adenovirus encoding for VEGF121 injected into ischemic porcine myocardium augmented hyperemia during pacing stress as measured by \([99m\text{Tc}]\)Sestamibi single-photon emission computed tomography (SPECT).

Transmural flow distribution was not analyzed, as SPECT cannot evaluate endocardial/epicardial flow distribution. Our study builds on these results and underscores the importance of defining collateral flow reserve as a therapeutic end point. Moreover, our data suggest that the transmural distribution of flow is an important criterion for evaluating angiogenic success.

**Advantages of MCE for Assessing Angiogenesis**

A major finding of our study is that quantification of myocardial perfusion using MCE can be applied to the evaluation of therapeutic angiogenesis. The unique power of MCE for physiological assessment of therapeutic angiogenesis derives from several attributes of the technique when performed during continuous contrast infusion. First, because intramyocardial blood volume comprises predominantly capillaries, the videointensity on MCE represents tissue concentration of bubbles within the capillary compartment. The videointensity on MCE represents tissue concentration of bubbles within the capillary compartment. Second, microbubble destruction and replenishment of the ultrasound beam during microbubble infusion can be used to derive insight into the microcirculation. After a destructive ultrasound pulse, the rate of bubble replenishment of the beam, manifested as the rate of videointensity increase with incremental pulsing intervals (β), relates to capillary red cell velocity. At sufficiently long intervals, the beam will completely replenish between pulses, further increases in pulsing interval will not change videointensity, and the
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... diameter have increased in number in pigs^3^ and dogs^5^. Angiography has been used in angiogenic treatment trials,^1^ but the optimal method for assessing the effect of therapeutic angiogenic strategies on tissue perfusion is unclear.^1^ An incremental therapeutic effect; and (3) the positive effects were preferentially seen in the endocardium, where there are few native canine collaterals.

**Clinical Implications**

The exogenous administration of growth factors to stimulate development of new or preexisting vessels is a promising approach to enhancing tissue perfusion in settings of ischemia. If our results in dogs hold true in the clinical setting, VEGF^121^ should exert a therapeutic effect that is most evident when collateral flow reserve is tested. Abnormal collateral flow reserve parallels symptoms of angina in patients with coronary artery disease,^2,0^ and alleviation of these abnormalities would be expected to translate into alleviation of symptoms. Multiple angiogenic strategies are being evaluated in humans,^5,1,8,12,21,22^ and there is a need to determine what end points and tools are best suited for assessing the therapeutic response. This study suggests that MCE can provide a relatively simple approach to quantifying changes in tissue perfusion in response to angiogenic stimuli, and moreover, that it has the unique ability to delineate the transmural and microvascular level at which these structural and physiological changes occur.

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**Alternatives for Evaluating Angiogenic Therapy**

The positive effect of VEGF^121^ on bubble velocity was most evident in the endocardium, paralleling the preserved endocardial/epicardial flow ratios measured with radiolabeled microspheres and the endocardial location of arteriolar increase in the VEGF^121^-treated group. These findings suggest that VEGF^121^ has a differential effect on endocardium as compared with epicardium. Conceivably, a transmural gradient in therapeutic effect could result from higher expression of VEGF receptors by the endocardium in response to the more severe endocardial versus epicardial ischemia typically seen in dogs with occlusion.^^11^**

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**Study Limitations**

Canines have a natural predilection for collateralization during ischemia as a result of the presence of preformed collaterals.^^12^ This attribute should not undermine our conclusions because (1) most humans with coronary disease have collaterals^^16^; (2) natural collateralization in dogs would bias against finding an additional benefit, yet VEGF^121^ had an incremental therapeutic effect; and (3) the positive effects were preferentially seen in the endocardium, where there are few native canine collaterals.
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