Vascular Endothelial Growth Factor Is Required for Coronary Collateral Growth in the Rat

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Background—The goal of this study was to determine whether the expression of vascular endothelial growth factor (VEGF) is critical for coronary collateral growth. Previous studies have provided an association between coronary collateral growth and VEGF, but none have allowed determination of a causal role.

Methods and Results—We measured coronary collateral growth in rats subjected to repetitive episodes of myocardial ischemia (RI; one 40-second occlusion every 20 minutes for 2 hours 40 minutes, followed by 5 hours 20 minutes of rest, with this 8-hour cycle repeated 3 times per day for 10 days). Collateral growth was measured from blood flow (radioactive microspheres), visualization of arterial-arterial anastomoses (x-ray micro-CT), and maintenance of function during complete coronary occlusion in 3 groups of animals: sham (received instrumentation but no RI), experimental (subjected to RI), and anti–vascular endothelial growth factor (RI+anti-VEGF). The 0.6 mg/100 g per day) to block the endogenous actions of VEGF. In the 3 groups, native collateral flow (measurement for RI or sham protocol) averaged 0.2 to 0.3 mL \( \cdot \) min\(^{-1} \cdot \) g\(^{-1}\) of tissue. In the sham group, collateral flow did not increase during the protocol. Collateral flow in the control RI group increased by \( \approx 6 \) fold to 1.63 mL \( \cdot \) min\(^{-1} \cdot \) g\(^{-1}\) tissue, and in the anti-VEGF group, collateral flow did not increase after the RI protocol \( (0.22 \text{ mL} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) \). In adults, collateral flow was unchanged during vasodilation with dipyridamole, indicating the increases in collateral flow are due to collateral growth and not vasodilation. X-ray micro-CT analysis revealed a 3-fold increase (versus sham group) in the number of arterial-arterial anastomoses per heart after RI, which was prevented by treatment with anti-VEGF. The growth of the collateral circulation was functional in the RI group because complete coronary occlusion did not induce any untoward effects on hemodynamics or arrhythmias. In the sham or anti-VEGF groups, coronary occlusion at the end of the protocol induced many arrhythmias and deterioration of function.

Conclusions—From these results, we conclude that the expression of VEGF is critical to the growth of coronary collaterals.

Key Words: angiogenesis ▪ collateral circulation ▪ coronary circulation ▪ growth substances

Coronary angiogenesis, the de novo formation of capillaries and postcapillary vessels, and collateral growth, the enlargement of and perhaps increase in numbers of arterial-arterial (collateral) connections, are chronic coronary adaptations to myocardial ischemia that restore the coronary flow and can prevent or minimize ischemic myocardial injury.\(^1\)\(^-\)\(^3\) The underlying mechanisms of the “natural process” of coronary angiogenesis/collateral growth are likely a complex orchestration of the expression of numerous growth factors and signaling cascades,\(^4\)\(^-\)\(^6\) although a causal role for any growth factors has not been well elucidated. Among many growth factors, vascular endothelial growth factor (VEGF), a potent angiogenic factor, has been associated with coronary angiogenesis/collateral growth.\(^7\)\(^-\)\(^9\) In a canine model of coronary collateral growth produced by repetitive episodes of myocardial ischemia, Matsunaga et al\(^9\) recently demonstrated an increase in VEGF in myocardial interstitial fluid during early days of ischemic stimulation. Interestingly, the expression of VEGF waned during the course of collateral growth. However, when nitric oxide (NO) production was inhibited, as intervention designed to block VEGF signaling, collateral growth was blocked. This suggests a critical role for VEGF, which signals through NO. However, the inhibition of NO production can affect many aspects of vascular physiology, so the conclusion is somewhat equivocal. Thus, whether endogenous VEGF is essential for coronary collateral growth is still unresolved.

In the present study, we evaluated the causal role of VEGF in coronary collateral growth during repetitive myocardial ischemia (RI) by systemic inhibition of VEGF via adminis-

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tration of a neutralizing antibody against VEGF (anti-VEGF). Our studies were performed in a chronically instrumented rat model to induce collateral growth by producing episodes of RI. We determined the role of VEGF in collateral growth via measurements of blood flow to the collateral-dependent region and images of the coronary vasculature using x-ray micro-CT.

Methods

Animal Preparation
Male Wistar rats (290- to 360-g body weight; n = 50) were used for experiments (preparations were successful in 39 animals). For surgery, rats were premedicated (ketamine 50 mg/mL plus acepromazine 2.5 mg/mL plus torbuterol 2.0 mg/mL, 0.2 mL/100 g body weight IP) and intubated. Oral intubation (16-G polyethylene tubing) was done under direct observation of the vocal cords with an otoscope. General anesthesia was introduced and maintained by sevoflurane inhalation (1.0% to 2.0%, with 100% oxygen). Body temperature was controlled at 37°C by an electric heating table. Surgery was performed using aseptic technique. The animal was initially placed on its dorsal side, and after a neck incision, the right carotid artery was isolated, and a PE-50 catheter filled with heparin (10 U/mL)-saline was inserted. This tubing was used for monitoring of systemic hemodynamics, sampling arterial blood, and maintaining the blood volume. Blood pH, PaO₂, PaCO₂, and systemic hemodynamics were maintained within physiological ranges throughout the surgery. The animal was repositioned on its right side, and the heart was exposed by left thoracotomy. A mini-pneumatic snare occluder (see the Mini-Pneumatic Snare Occluder section for details) was implanted around the mid to proximal left anterior descending coronary artery (LAD). Confirmation that the occluder was functional, ie, producing myocardial ischemia, was determined initially by observation of blanching and hypokinesis of the left ventricle (LV) during inflation. Rats were randomly divided into 2 groups based on the type of measurement: coronary blood flow (CBF) (radioactive microspheres, n = 20) or vascular imaging (micro-CT, n = 13). CBF was measured during coronary occlusion to determine flows to the normal and collateral-dependent zones (see the Measurement of Coronary Blood Flow and the Coronary Vascular Imaging With Micro-CT sections). After instrumentation and measurements, the chest was closed under positive end-expiratory pressure, and the thoracic cavity was evacuated of air. An intraperitoneal catheter (PE-50) for drug administration was inserted, and this catheter and the occluder were tunneled subcutaneously and exteriorized through the scapulae. The chest was closed after a neck incision, the right carotid artery was ligated after the catheter was withdrawn via a carotid artery as a reference. After the first measurement, the carotid artery was ligated after the catheter was withdrawn. Blood withdrawal (0.34 mL/min) was started 15 seconds before microsphere injection. Blood withdrawal was continued 55 seconds during LAD occlusion. During the course of the procedures, systemic pressure and heart rate were recorded (386-BIOS, American Megatrends Inc).

The heart was excised and fixed in 4% paraformaldehyde solution overnight. The fixed LV was sliced along the short axis and observed with a dissecting microscope and fluorescent light source (LT-9800, Lightools Research). The collateral-dependent area (LAD region) was distinguished as the area without fluorescent microspheres. The normal area (non-LAD LV region) was determined by the area distribution of the fluorescent microspheres. The normal and collateral-dependent areas were divided with a blade, and each total weight was measured. CBF (mL·min⁻¹·g⁻¹) in each area was calculated from the following formula: CBF = [(radioactive counts in myocardial specimen)×(blood withdrawal rate)/(radioactive count in blood)]/(weight of myocardial specimen).

Functional Estimation of Collateral Growth
We evaluated collateral growth after completion of the protocol by a simple functional test: total coronary occlusion. The rationale for this
Coronary Microvascular Imaging With Micro-CT

One group of rats (n=13) was prepared for coronary vascular visualization with micro-CT. The coronary circulation was filled with contrast medium by modification of the methodology for micro-CT study in the rats.10 Preparation of contrast medium was according to Wusten et al,11 consisting of barium sulfate (60 g, No. 760, E-Z-EM Inc) and gelatin (12 g, Sigma) in 100 mL saline. The viscosity of the contrast medium enables filling up to coronary arborial level with no or minimal filling of capillaries. The excised heart was immediately connected to a Langendorff’s perfusion apparatus. Warmed contrast medium (42°C) was injected at a rate of 2 mL/min for 40 minutes to unload the LV. Warm saline (42°C) was administered in control group. The heart was perfused for 15 minutes, then the heart was removed and fixed in 4% paraformaldehyde solution (4°C) overnight. Whole hearts were used for micro-CT imaging of coronary collateral growth.

The coronary vasculature was visualized with micro-CT (Imaging Research Laboratory, Mayo Clinic). Details of the micro-CT were given previously.12 In brief, the whole heart was scanned in 1° increments around 360° about its apex-to-base longitudinal axis. The spatial resolution selected in the present study had an 18 μm voxel size to focus on the size of collateral vessels and to minimize the signals from smaller vessels. Finally, CT data were reconstructed as 3D images. The main purpose of these images was to establish the presence or absence of arterial-arterial anastomotic connections. Collateral vessels, ie, arterial-arterial anastomotic connections, were counted by independent observers (r=0.96 between observers) for the 3 groups: sham, RI, and RI plus anti-VEGF.

Experimental Protocol

The RI protocol was introduced by manual inflation of the occluder using the following protocol: 40 seconds of occlusion every 20 minutes for 2 hours 20 minutes, followed by a period of “rest” (deflation) for 5 hours 40 minutes. This 8-hour set was repeated 3 times a day for 10 days. The LAD was occluded manually by remote inflation or deflation through the catheter. In sham rats, the balloon was implanted, but RI was not applied.

Rats under RI protocol were randomly divided into 2 groups: RI without anti-VEGF (control group, n=9) or RI with anti-VEGF administration (anti-VEGF group, n=6). In another group, neutralizing anti-VEGF (13.14 mg/kg, E-Z-EM Inc) was administered via intraperitoneal injection before the repetitive occlusion protocol each day. This daily administration was twice as frequent as that reported by Zheng et al,13 who reported effective blockade of VEGF with administration of this same dose every other day. We chose to double the frequency of administration to ensure adequate neutralization of VEGF. A similar volume of saline was administered in control group. The sham rats (n=5) received no intraperitoneal injections.

In an additional group of rats (n=6), myocardial perfusion and arterial pressure were measured acutely before and after inflation of dipyridamole (0.15 mg · kg⁻¹ · min⁻¹). The purpose of these experiments was to show that flow in the collateral-dependent region was maximally dilated before the repetitive occlusion protocol; therefore, increases in collateral flow would be due to an increase in collateral growth.

Experimental procedures and protocol were approved by Animal Care and Use committees of the Medical College of Wisconsin and Louisiana State University, Health Sciences Center.

Statistical Analysis

Data are expressed as mean±SEM. Comparisons of CBF and hemodynamics at CBF measurement between before and after RI and with or without anti-VEGF were analyzed by 2-way ANOVA, followed by Fisher’s protected least-squares difference test (Stat-View J-5.0 PPC). Numbers of collateral vessels were analyzed by 1-way ANOVA, followed Tukey’s post hoc test. Statistical significance was set at P<0.05.

Results

CBF to the Normal and Collateral-Dependent Zones

In the acute experiments (n=6) designed to test whether vasodilation would increase collateral flow, perfusion to the collateral-dependent zone was not changed from a baseline value of 0.42±0.15 to 0.34±0.10 mL · min⁻¹ · g⁻¹ during dipyridamole. Blood flows to the normal zone were 1.75±0.34 and 1.99±0.32 mL · min⁻¹ · g⁻¹ under baseline conditions and after dipyridamole, respectively. Although the flow to the normal zone did not change significantly during dipyridamole, coronary resistance was reduced from 53±5 to 35±4 mm Hg · min⁻¹ · mL⁻¹ (P<0.05) because arterial pressure fell from 93±10 to 69±12 mm Hg.

In the control repetitive occlusion group, collateral flow was 0.26±0.09 mL · min⁻¹ · g⁻¹ before RI and increased to 1.63±0.30 mL · min⁻¹ · g⁻¹ after RI (P<0.0001; n=9; Figure 2, left). Anti-VEGF administration prevented the increase in collateral-dependent flow; specifically, collateral flow in the anti-VEGF group was 0.15±0.04 mL · min⁻¹ · g⁻¹ before RI and 0.22±0.08 mL · min⁻¹ · g⁻¹ after RI (P=NS; n=6; Figure 2, right). The difference between collateral flow after RI in the control group (1.63±0.30 mL · min⁻¹ · g⁻¹) was significantly different (P<0.0007) than that after RI in the anti-VEGF group (0.22±0.08 mL · min⁻¹ · g⁻¹). CBF in the normal zone was not different before compared with after RI with or without anti-VEGF (before RI in the control group, 1.50±0.22 mL · min⁻¹ · g⁻¹; after RI in the control group, 2.92±0.61 mL · min⁻¹ · g⁻¹; before RI in the anti-VEGF group, 2.72±0.65 mL · min⁻¹ · g⁻¹; after RI in the anti-VEGF group, 4.01±0.63 mL · min⁻¹ · g⁻¹; P=NS, ANOVA). Systemic blood pressure, heart rate, and double product at CBF measurements did not differ between groups (Table). Additionally, hearts from the sham group (with balloon implantation but without RI) did not show any difference in LAD regional flows between the first and second measurements (1.52±0.36 and 1.51±0.20 mL · min⁻¹ · g⁻¹, respectively; P=NS; n=5).

Functional Indices of Collateral Growth

At the end of the protocol and the second measurement of coronary blood flow, the balloon was inflated to occlude the LAD to determine whether cardiac function was maintained during LAD occlusion. All rats in the control group (subjected to RI; n=9) had stable hemodynamics for >2 hours.
during LAD occlusion, whereas all rats in the anti-VEGF group (n=6) had ventricular fibrillation or cardiac arrest between 7 and 13 minutes after the start of occlusion.

Coronary Vascular Architecture

Representative coronary vascular images are shown in Figure 3. An intact heart with complete ligation of the proximal LAD (top right) showed no collateral perfusion into the LAD region. A sham heart with balloon implantation but no RI showed 4 visible collaterals (top left). A heart in the control group after RI showed higher visible vascularity and more connections between the LAD vascular tree and adjacent arteries (bottom left) compared with the sham. However, there was no evidence of collateral growth (4 collateral connections; *P*=0.05 versus sham) in anti-VEGF group after RI (bottom right). These images were typical for each group (sham, n=6; control RI group, n=3; anti-VEGF group, n=4). The average numbers of collateral vessels in each group are given in the legend for Figure 3.

Discussion

In the present study, we made 2 new observations. First, rats develop coronary collaterals in response to repetitive myocardial ischemic stimulation. Attesting to this were our findings that collateral-dependent flow increased and cardiac function was preserved during coronary occlusion after the RI protocol. In addition, visualization of coronary microvasculature (micro-CT) demonstrated the presence of collaterals, ie, arterial-arterial anastomoses. Second, and most important, VEGF plays a causal role in the growth of coronary collaterals. This is based on our observations showing that systemic administration of anti-VEGF abolished all indices of collateral growth. Our work furthers the understanding of coronary collateral growth by moving beyond association, eg, expression of a growth factor increases during collateral growth, to cause and effect, eg, blockade of an endogenous factor corrupts collateral growth. Our conclusions and work depend on several factors, including the model, methodology, and cogent information in the literature.

<table>
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<tr>
<th>Hemodynamic Data at CBF Measurements Before and After IR Protocol</th>
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<tr>
<td><strong>Blood Pressure, mm Hg</strong></td>
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<td>Anti-VEGF group</td>
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Critique of the Model and Methodology

We established an RI protocol in rats: 40 seconds of occlusion every 20 minutes for 2 hours 20 minutes (8 occlusions), followed by a break of 5 hours 40 minutes (total, 8 hours for 1 set) to produce coronary collateral growth. This set was repeated 3 times in a day for 10 days (total, 240 times RI in 10 days). This protocol was based on the RI protocol used in dogs to produce coronary collateral growth9,15: 120 seconds of occlusion every 60 minutes for 8 times for 8 hours in a day. According to the general biological concept of body mass–metabolic rate relationship, metabolic rate is parallel to the body mass with a power of 0.75: \[ E = 4.1W^{0.75} \], where \( E \) is energy and \( W \) is body mass per unit (kg).16 From these variables and equations, rats are estimated to have an \( \approx 3 \) fold-higher metabolic rate than dogs. A general biological concept of the body mass–cardiac cycle relationship indicates that the cardiac cycle is parallel to body mass with a power of 0.25: \[ C = 0.25W^{0.25} \], where \( C \) is cardiac cycle and \( W \) is body mass.17 Because the heart rate of rats is \( \approx 3 \)-fold faster than that of dogs, we assumed that an \( \approx 3 \)-fold-higher metabolic rate would apply to the RI protocol in rats. Thus, we used a shorter occlusion time (reduced by a factor of 3) and 3-fold-higher frequency of ischemic stimulation in the rats than in the dogs.

Collateral-dependent flow after the RI protocol showed some range of variation in the control group (Figure 2). Possible reasons for this scatter may result from limitations of CBF measurement using radioactive microspheres in small animals18 or variable capability of collateral growth against the same ischemic stimulation, ie, associated with genetic background,19,20 etc. We did not consider this to be due to an inconsistency in the effectiveness of the balloon to produced occlusion. This is based on the results of the functional studies showing that inflation of the balloon at the end of the study in the anti-VEGF group caused myocardial ischemia and deterioration of function.

We used 2 indices to evaluate growth of the coronary collateral circulation: collateral blood flow and the number of visible arterial–arterial anastomoses (collateral connections) per heart. Flow provides insight into the net effect of network remodeling and directly reflects the decrease in resistance and increase in conductance of collateral vessels. However, flow measurement does not provide insight into the nature of the remodeling, ie, whether there are a few enlarged connections, many enlarged collaterals, or greater numbers of collateral vessels. To this end, the results provided in Figure 3 provide some insight; namely, the number of visible collateral connections was increased by repetitive occlusions and prevented by anti-VEGF. We cannot say for certain whether this increased number of connections represents de novo formation of collaterals or represents enlargement (and thus better filling) of preexisting native collaterals. Nonetheless, the increased number of visible connections after the RI protocol and the prevention of this increase by anti-VEGF corroborate our results of blood flow to the collateral-dependent region. We would be remiss if we did not mention that although anti-VEGF blocked the growth of coronary collaterals, the antibody did not totally prevent all collateral flow and did not affect the number of native collateral connections; ie, the numbers of connections were similar in the anti-VEGF and sham groups. The presence of collateral flow after administration of anti-VEGF is likely due to these native channels.

Another aspect of our results, namely that collateral flow did not increase after administration of the vasodilator diprydamole, bears emphasizing. This important observation strongly supports our contention that the increase in collateral flow during the chronic occlusion protocol is due to collateral growth rather than a decrease in vascular tone because the collateral-dependent bed was already dilated during the initial coronary occlusions.

Mechanisms of Coronary Collateral Growth

The basis for coronary collateral growth is still unresolved, although roles of angiogenic factors, shear stress, and myocardial ischemia have been investigated. Among many complex cascades of molecular and cellular mechanisms,4 the role of VEGF and its signaling pathway in vivo has been intensively investigated, but the vast majority of these studies have used the hind-limb ischemia model. For example, VEGF has been shown to increase endothelial adhesion and transmigration of monocytes.21 The VEGF homologue, placenta growth factors, induces accumulation and activation of monocyte/macrophages in periphery of collateral vessels via the VEGF receptor 1 (Flt-1).22 Recently, bone marrow–derived cells have been shown to incorporate around collateral vessels and colocalize with VEGF.23 In the coronary circulation, our group9 demonstrated increases in VEGF in myocardial interstitial fluid in the dogs under RI protocol, peaking during the first 3 days of a 3-week RI protocol. We also reported that inhibition of NO synthase prevented the growth of collaterals. These observations suggested a role for VEGF, which signals through NO, but many other factors eg, ephrins,24 also require NO for angiogenesis, so inhibition of NO synthesis can exert antigrowth effects extending beyond VEGF. Additionally, antagonism of NO synthase induces a variety of changes, including redox changes in endothelial cells,25,26 that can complicate a variety of endothelial cell–based responses, including angiogenesis. Thus, our previous results did not provide unequivocal proof for the role of VEGF in collateral growth. In the present study, we demonstrated for the first time that endogenous VEGF is required for coronary collateral growth. We make this statement with conviction because anti-VEGF completely blocked collateral growth.

Recently, adenoviral gene transfer of a soluble VEGFR1 or VEGFR2 inhibited collateral growth and angiogenesis in the ischemic hind limb.27 We must point out that other groups have not found a role for VEGF in collateral growth28 but would likely suggest a likely difference. These investigators studied collateral growth in skeletal muscle, a model in which the ischemic zone is very remote from the site of collateral growth, which contrasts to our model in which the zone of ischemia is juxtaposed to the site of collateral growth. With such differences in models, it is not surprising that the specific growth factors driving the adaptation also differ. Investigators studying collateral growth in the hind limb and in the mesentery have suggested that biomechanical forces imposed onto the vascular wall such as shear stress and stretch promote vascular remodeling of preexisting collateral
vessels.\textsuperscript{28,29} An increase in shear rate in preexisting collaterals that connect obstructed vessel to a neighboring nonobstructed vessel was demonstrated as a primary mechanism of collateral growth in the mesenteric circulation\textsuperscript{30} and in femoral arteries.\textsuperscript{21,23} Our results do not address a role for shear stress in the maturation of coronary collaterals, but it is likely that coronary collateral growth is the net effect of many factors.

Clinical Implications

Takeshita et al\textsuperscript{21} found that VEGF stimulated collateral growth in the ischemic rabbit hind limb. In addition, several subsequent studies reported that VEGF promoted coronary collateral growth in animal models\textsuperscript{3} and patients with ischemic heart disease.\textsuperscript{31} However, therapeutic angiogenesis/collateral growth using exogenous VEGF administration failed in the VIVA phase II trial.\textsuperscript{32} Despite these failures, our results show that endogenous VEGF is crucial for successful coronary collateral growth. One way to reconcile our results with the failed trial relates to the condition of the patient to whom VEGF is being administered. Patients with ischemic heart disease have impaired bioavailability of NO, which would retard the signaling for VEGF, which is dependent on NO. Thus, in hindsight, we speculate that administration of a VEGF to patients with impaired endothelial function would probably not produce a beneficial effect. Although this may be an oversimplification, we believe that the precise kinetics of the expression on VEGF and other angiogenic factors, their receptors, interactions between each angiogenic factors, and the negative influence of angiostatic factors\textsuperscript{13} in the “natural process” of coronary collateral growth should be further investigated.

Acknowledgments

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References


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