Expression of VEGF and angiopoietins-1 and -2 during ischemia-induced coronary angiogenesis

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CORONARY CAPILLARIES are comprised of endothelial cells that form the tube and a discontinuous layer of vascular pericytes. The capillary network plays a critical role in the transport oxygen and nutrients to myocardial cells (18, 21). An increase in capillary number enhances the transfer of oxygen and nutrients to myocardium under hypoperfusion (18). Despite the importance of this adaptation to ischemia, the mechanisms in the adult heart have not been identified. The potential importance of coronary angiogenesis was highlighted by observations that downregulation of vascular endothelial growth factor (VEGF) and angiopoietin (Ang)/Tie-2 were involved in capillary growth as an adaptation to ischemia. To test this hypothesis we measured capillary density, and the expressions of VEGF, Ang-1, Ang-2, and the Tie-2 receptor and its phosphorylation state during repetitive episodes of myocardial ischemia in chronically instrumented canines. Repetitive episodes of ischemia were induced by multiple (once/hour; 8/day), brief (2 min) occlusions of the left anterior descending coronary artery for 1, 7, 14, or 21 days. A sham group received the same instrumentation as the experimental groups but not the occlusion protocol. Collateral blood flow (microspheres) progressively increased from 9 ± 3 to 83 ± 10 ml·min⁻¹·100 g⁻¹ on day 21. Capillary density increased at day 7, 2,378 ± 53 (sham) to 2,962 ± 60/mm², but it decreased to 2,594 ± 39/mm² at day 21. Both VEGF and Ang-2 expression in myocardial interstitial fluid (Western analyses) peaked at day 3 of the repetitive occlusions but waned thereafter. In contrast the expression of Ang-1 remained relatively constant at all times in the occlusion groups. In shams, the expression of VEGF and Ang-2 was low and constant at all times. Tie-2 phosphorylation myocardial decreased decreased at day 7 but increased at 21 days of occlusions (P < 0.05). Our results indicate that capillary density was augmented by myocardial ischemia, but after development of collaterals and restoration of flow to the ischemic zone, capillary density returned to control levels. The change in capillary density paralleled with VEGF and Ang-2 expression but was inversely related to Tie-2 phosphorylation. We speculate the coronary angiogenesis is a coordinated event involving the expression of both VEGF and Ang-2 and that therapeutic angiogenic strategies may ultimately require treatment with more than a single factor.

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on coronary angiogenesis by assessing the time course of expression of these factors and relating it to capillary density using a canine model of repetitive episodes of myocardial ischemia.

METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Repetitive coronal occlusion model. Mongrel dogs of either sex (25–30 kg) were anesthetized with propofol (50 mg/kg iv) and isofluorane (1.5–2.0%) in 100% O₂. A left thoracotomy was performed under sterile conditions, and the following were implanted, as described previously (11, 15): 1) a heparin-filled catheter in the thoracic aorta for measurement of arterial pressure; 2) a Doppler ultrasonic flow transducer (20 MHz) on the left anterior descending coronary artery (LAD) for measurement of coronary blood flow velocity; 3) a balloon-cuff vascular occluder (In Vivo Metric) around the LAD for production of brief coronary occlusions; 4) a heparin-filled catheter in the left atrial appendage for administration of drugs and radioactive microspheres; and 5) an intramyocardial catheter (0.8 mm OD, 0.44 mm ID) in the LAD perfusion territory for sampling of interstitial fluid. The catheter had 40 holes (25 gauge) punched in a 2-cm segment that was situated in the ventricular wall.

All of the catheters and leads were secured, tunneled subcutaneously, and exteriorized between the scapulae, the wounds were repaired, and the dog was postoperatively treated, as described previously (11). Dogs recovered from surgery for 10 days before experimentation, and during this time period the animals were trained to stand quietly in a restraining sling. Systemic and coronary hemodynamics and reactive hyperemic responses were monitored immediately before, during, and after each coronary artery occlusion.

Myocardial interstitial fluid. Samples of myocardial interstitial fluid (MIF) were collected from the LAD region each morning before subsequent experimentation. Isotonic saline (4 ml) was flushed into the catheter as 4 ml of fluid was withdrawn. The sample was immediately placed on ice, aliquoted, frozen, and stored at −80°C until analysis as described previously (15).

Experimental protocols. Repetitive myocardial ischemia was induced by multiple 2-min occlusions (once/hour, 8/day) of the LAD with the use of the pneumatic vascular occluder. Samples of myocardial interstitial fluid were obtained at days 1–2 (n = 4), days 3–5 (n = 4), days 7–9 (n = 6), days 12–15 (n = 3), and days 19–21 (n = 7). In a sham group (n = 6) occlusions were not performed, and these animals were used as time controls for analysis of VEGF and Ang-2 in MIF. Samples of tissue were obtained from sham animals (n = 6) and from animals in the repetitive occlusion protocol at day 1 (n = 5), day 7 (n = 6), and day 21 (n = 6). Tissue samples were used for morphometric analyses, estimates of mRNA transcripts for Ang-1 and -2, and analyses of Tie-2 phosphorylation.

Regional myocardial blood flow. Carbonized plastic microspheres (15 ± 2 μm diameter, New England Nuclear; Boston, MA) labeled with 141Ce, 103Ru, 51Cr, or 99Nb were used to measure regional myocardial blood flow to the normal and collateral-dependent regions at days 0, 7, 14, and 21 of the occlusion protocol with the use of standard techniques (11, 15).

Capillary density. Frozen myocardium was sliced into 10-μm-thick sections with a cryocut device (Leica) and fixed in acetone for 10 min at −20°C. After being air dried, the sections were incubated FITC-conjugated lectin (Griffonia simplicifolia agglutinin I, Vector Laboratories, 1:100 dilution in PBS) for 30 min at room temperature (8). After the incubation period, the section was washed three times with PBS and was then mounted in aqueous mounting medium (Biomedica). Capillaries and myocardial muscle fibers were counted with the use of a fluorescent microscope at ×400 (Nikon). Vessels with a diameter <8 μm were considered capillaries, and only sections with capillaries oriented perpendicularly were analyzed.

Tie-2 receptor expression and phosphorylation. Transmural myocardial samples were homogenized in a lysis buffer (1% Igepal CA-630, 0.1% SDS, 10% glycerol, 138 mM NaCl, 20 mM Tris, and 5 mM Na fluoride at 4°C). Supernatants were collected after centrifugation at 14,000 revolutions/min for 15 min and were used for protein determination (bicinchoninic acid protein assay, Bio-Rad). Protein A-Sepharose CL-4B (150 μl, Amersham Pharmacia) (diluted 1:10 in lysis buffer) and 0.5 μg of polyclonal anti-Tie-2 antibody (Santa Cruz) or 5 μg of recombinant Tie-2/Fc (R&D) were mixed and incubated at 4°C for 2 h (6, 27). After this procedure, 500 μg of protein from the samples were added and incubated at 4°C for overnight. Beads were washed with lysis buffer four times and collected by centrifugation at 5,000 revolutions/min for 5 min at 4°C. After the final wash, the supernatant was aspirated and discarded, and the pellet was resuspended in 20 μl of ×2 SDS sample buffer. Samples were boiled for 5 min and separated on a 10% SDS-PAGE gel by electrophoresis. Proteins were transferred to nitrocellulose membranes (Bio-Rad) by electroblotting. Blots were blocked overnight at 4°C with 2.5% bovine serum albumin (Factor V, protease free) in Tris-buffered saline (13 mM Tris, pH 7.6, and 150 mM NaCl) and then incubated with antiphosphotyrosine antibody (Santa Cruz, 1:500 in blocking solution) for 2 h at 4°C. After being washed in blocking solution, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted 1:1,000 in blocking solution. The blots were washed five times with 13 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20, and twice with Tris-buffered saline. Bands were detected with a nonradioactive detection system (ECL from Amersham). After visualizing the immobilized protein, the antibody complex was stripped in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl (pH 6.5) for 30 min at 50°C. The blot was then reprobed with anti-Tie-2 antibody with the use of the same procedure described above. All blots were performed in triplicate, and the average of the three blots was used in the final analyses.

Analyses of VEGF, Ang-1, and Ang-2 expression in MIF. VEGF and Ang-1 and -2 expressions in MIF were determined by via Western analyses. MIF was collected and diluted in SDS sample buffer. MIF (30 μl) was separated in a 10% SDS-PAGE gel. We used mouse monoclonal VEGF primary antibody (Santa Cruz Biotechnology) and goat polyclonal antibodies for Ang-1 and Ang-2 (Santa Cruz Biotechnology). A 50-μg standard for human VEGF₁₆₅ (R&D systems), a 50-ng standard for Ang-1 (Regeneron), and a 100-ng standard for human Ang-2 (R&D systems) were also electrophoresed for comparison to the probed proteins in the sample. Signals were digitized using a charge-coupled device camera-
frame digitizer system, analyzed with the use of NIH Image software (density and band area), and expressed as a ratio to the standard. All blots were performed in triplicate, and the average of the three blots was used in the final analyses.

Reverse transcriptase-polymerase chain reaction. RNA was isolated from cardiac tissue by the acid guanidinium isothiocyanate/phenol method. Transmural samples (∼500 mg) of ventricle of sham and after 1 day, 7 days, and 21 days of repetitive occlusions were excised and cleaned of adherent fat in ice-cold PBS. RNA was extracted, precipitated, washed in 70% ethanol, and stored in diethyl pyrocarbonate-treated H2O at −80°C until analysis.

Total RNA (2 µg) was reverse transcribed with an oligo dT18–24 primer (1 µg) with the use of 20 µl 1 x reaction buffer composed of (in mM) 50 Tris·HCl, pH 8.3, 75 KCl, 3 MgCl2, and 10 dithiothreitol containing 200 units of Moloney murine leukemia virus reverse transcriptase, 10 pmol 2-deoxyxycytidine 5’-triphosphate, and 40 units of RNase inhibitor (Pharmacia). All samples were carried out at 37°C for 2 h, followed by incubation at 75°C for 15 min to inactivate the enzyme. We added 20 µl of diethyl pyrocarbonate water (total volume 40 µl) and used 2 µl of this cDNA solution for PCR. The resultant cDNA was amplified in 48 µl reaction buffer containing 25 pmol sense and antisense primer, 22 mM Tris·HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 220 µM dNTPs, and 22 U recombinant Taq DNA polymerase/ml. The thermal profile used a Gene Amp PCR system 2400 (Perkin-Elmer) consisted of a denaturation step of 94°C for 1 min, an annealing step of 55°C for 1 min for Ang-1 and Ang-2 (an annealing step 58°C for GAPDH), and an extension step of 72°C for 1 min. All samples were initially denatured for a total of 5 min (94°C).

The sequence of Ang-1 sense primer was 5’-TGTTGTCCAT-CAGCTCCAGTGC-3’ and that of antisense primer was 5’-CGGCTACATGCTGACAGATAG-3’. The sequence of Ang-2 sense primer was 5’-GATGGCCAGGTGATTTCA-3’ and that of antisense primer was 5’-ACATGATCAACAC-CACCAGC-3’. We cloned and sequenced PCR products to verify that the products we amplified with angiopoietin primers corresponded to the known sequences of Ang-1 and -2 in Genbank. The PCR products of 401 and 363 bp amplified by our Ang-1 and -2 primer, respectively. The sequence of GAPDH sense primer was 5’-GCCAAA-AGGGTCATCATCTC-3’ and that of the antisense primer was 5’-GCCCATCCACAGTCTTCT-3’, which amplifies the 225 bp product.

In preliminary studies, we found that the amount of PCR product increased exponentially from 28 to 38 cycles for Ang-1, from 26 to 36 cycles for Ang-2 and from 22 to 32 for GAPDH. Saturation was reached after 40, 38, and 34 cycles, respectively. Accordingly, Ang-1 products were amplified for 32 cycles, Ang-2 products for 30 cycles and GAPDH products for 27 cycles. Each PCR reaction product was electrophoresed through a 1.5% agarose gel, and the product was visualized by incubation for 20 min in a solution containing 10 ng/ml ethidium bromide. The resulting gel bands were imaged with the use of a Fluor imager (Molecular Dynamics). The relative intensities of the bands, expressed as optimal density units, were quantitatively analyzed using NIH Image software. Ang-1 and -2 signals (density and band size) were normalized to the GAPDH mRNA signal of which the latter served as internal standard.

Data analysis. The capillary density was expressed as millimeters squared. Diffusion distance (radius of the Krogh cylinder) was calculated from the area of the field and number of capillaries according to the procedure described by Rakusan and Turek (22).

All data are expressed as means ± SE. The changes in the parameters between the groups and over time were compared with two-way ANOVA for repeated measurements. The level of significance was P < 0.05.

RESULTS

Hemodynamic parameters. Myocardial blood flow to the normal zone [left circumflex coronary artery (LCX) region] was unchanged at the various times. Coronary collateral blood flow progressively increased during the repetitive occlusions. However, in the sham group, it did not change during the entire protocol. Blood pressure and heart rate did not change in both groups (Table 1).

Capillary density in ischemic myocardium. Figure 1 depicts capillary density in the myocardial tissue after repetitive myocardial ischemia. In ischemic LAD region, capillary density increased from 2,378 ± 53 to 2,964 ± 60/mm² (P < 0.05) at day 7. However, it diminished additional occlusions (Fig. 1B). We calculated the capillary-to-myocardial fiber ratio to exclude the effect of section orientation. The capillary-to-myocardial fiber ratio also increased from 1.66 ± 0.04 to 2.14 ± 0.03 (P < 0.05) at day 7 and decreased additional occlusion days (Fig. 1C). Furthermore, diffusion distance decreased from 12.6 ± 0.14 to 11.4 ± 0.10 µm (P < 0.05) at day 7 and slowly returned to baseline additional occlusion (Fig. 1D). In the nonischemic LCX region, these factors did not change during entire protocol.

VEGF and Ang-1 and -2 expression in MIF. Figure 2 shows the results of the Western analyses for VEGF, Ang-1, and Ang-2 in MIF. Both VEGF and Ang-2 protein expressions peaked 3 days after the initiation of repetitive occlusions and then waned after additional coronary occlusions. Ang-1 expression remained relatively constant at the various time points. In the sham

Table 1. Myocardial flow and hemodynamic parameters

<table>
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<th>Occlusion Dogs</th>
<th>Sham Dogs</th>
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<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
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<tr>
<td>Collateral flow, ml·min−1·100 g−1</td>
<td>9 ± 3</td>
<td>29 ± 6*</td>
</tr>
<tr>
<td>Normal zone flow, ml·min−1·100 g−1</td>
<td>121 ± 10</td>
<td>109 ± 13</td>
</tr>
<tr>
<td>CZ/NZ, %</td>
<td>9 ± 3</td>
<td>34 ± 11*</td>
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<tr>
<td>Mean BP, mmHg</td>
<td>107 ± 5</td>
<td>103 ± 4</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>103 ± 8</td>
<td>86 ± 5</td>
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Values are means ± SE. CZ, collateral zone; NZ, normal zone; BP, blood pressure. *P < 0.05 vs. day 0; †P < 0.05 vs. day 7.
group, expression of the growth factors did not vary throughout the protocol.

Tie-2 phosphorylation level in myocardial tissue. Tie-2 protein expression did not significantly change with repetitive ischemia. In contrast, Tie-2 phosphorylation level significantly decreased at 7 days of occlusion compared with that in shams \((P < 0.05)\) but increased at 21 days of occlusion \((P < 0.05)\).

RT-PCR analysis for Ang-1 and -2 mRNA. Figure 4 depicts RT-PCR for Ang-1 and Ang-2 mRNA in myocardium from ischemic \((\text{LAD perfusion area})\) regions in sham and after 1, 7, and 21 days of repetitive occlusions. The PCR product of Ang-1 did not significantly change at day 1 and day 7. However, it increased modestly but significantly at day 21 \((P < 0.05)\). The PCR product of Ang-2 significantly increased at day 1 and gradually diminished additional occlusions \((P < 0.05)\). The ratio of Ang-1 to Ang-2 showed a modest but significant decrease at day 1 compared with that in sham. However, it increased at the late stage of occlusion protocol \((P < 0.05)\).

**DISCUSSION**

We have made several new observations in this study: 1) capillary density increased in response to repetitive episodes of myocardial ischemia, but as collateral conductance increased and the intensity of ischemia waned during the repetitive occlusions, capillary density returned to baseline levels; 2) the change in capillary density correlated with the expression of VEGF and Ang-2, and the phosphorylation state of the Tie-2 receptor; and 3) expression of Ang-1 and the Tie-2 receptor was not altered by repetitive episodes of myocardial ischemia. Our data provide compelling results for the involvement of VEGF and Ang-2 in coronary angiogenesis in the adult heart in response to ischemia.

Capillary density and VEGF in ischemic heart. VEGF expression is stimulated by hypoxia, and the levels of this growth factor are substantially increased in ischemic myocardium \((3, 15)\). We observed that VEGF expression increased during the early occlusion days and waned as collateral flow and the severity of ischemia was ameliorated by the increase in collateral conductance \((15)\).

Recently, Carmeliet et al. \((4)\) reported that in VEGF\(_{164}\) and VEGF\(_{188}\) knockout mice angiogenesis is impaired and ischemic cardiomyopathy develops. Although this certainly reflects the role of VEGF in normal cardiac development and growth in the embryonic
Fig. 2. Time course of vascular endothelial growth factor (VEGF) (A), angiopoietin-2 (Ang-2) (B), and angiopoietin-1 (Ang-1) (C) protein expression in myocardial interstitial fluid (MIF) on sham and repetitive occlusion dogs. Ang-2 was identified by the immunoprecipitation with Tie-2/Fc at \( \pm 50 \) kDa. The expression VEGF and Ang-2 peaked at day 3 and waned after additional occlusion. Ang-2 expression was well paralleled with that in VEGF. The bands are from representative samples, and the graphs are from all samples (\( n = 5–8 \) at each time point). \(*P < 0.05\) vs. sham; \(†P < 0.05\) vs. day 3.

Fig. 3. Western analysis of Tie-2 protein expression and Tie-2 phosphorylation (P-Tyr) in myocardial lysate in sham (lanes 1 and 2), 7 (lanes 3 and 4), and 21 days (lanes 5 and 6) of repetitive occlusion-received dogs. A: Tie-2 protein expression did not significantly change with repetitive ischemia. B: In contrast, Tie-2 phosphorylation level significantly decreased at day 7 compared with sham, but it robustly increased at 21 days of occlusion. The bands are from representative samples (different animals for the paired samples), and the graphs are from all samples (\( n = 5–6 \) at each time point). IP, immunoprecipitated. \(*P < 0.05\) vs. sham; \(†P < 0.05\) vs. day 7.
and neonatal period, the result may have some bearing on our observations. We infer that if angiogenesis is abrogated, ischemic cardiomyopathy may result. Interestingly, in patients with dilated cardiomyopathy, capillary density is reduced compared with normal patients, and expression of VEGF165 and the kinase domain receptor was reduced (1). These results suggest that VEGF, especially VEGF164–165, is a key factor for the regulation and preservation of capillary density in adult heart. In addition, Tomanek et al. (27, 28) reported that VEGF is importantly involved in coronary angiogenesis in the developing heart. In the present study, capillary density paralleled in VEGF164 expression: increasing and decreasing with the levels of VEGF. We believe that the increase in collateral flow and the waning of ischemia during the repetitive occlusions cause the diminishment of VEGF expression, which then induces capillary regression.

**Role of Ang-1 and -2 in coronary capillary growth.**

Ang-1 and Ang-2 bind with similar affinity to the endothelial cell tyrosine kinase receptor, Tie-2 (4, 23). However, Ang-1 induces Tie-2 phosphorylation, whereas Ang-2 is a competitive antagonist of Ang-1 and, consequently, blocks Ang-1-induced Tie-2 phosphorylation (13, 20). In Ang-1−/− mice or with Ang-2 overexpression, studies (13, 26) have revealed embryonic defects because of poorly organized subendothelial matrix, loose endothelial cell contacts with the basement membrane, and few perivascular cells. These results have provided an insight into the functions of the angiopoietins in angiogenesis. Ang-1 released from perivascular cells contributes to vessel stability (5, 7, 12, 19, 25). In contrast, Ang-2 destabilizes the vessel and promotes disassembly of the cellular components (2, 7, 12). The expression of Ang-2 is augmented by hypoxia, which is similar to that for VEGF (14, 17), and these two factors are frequently coexpressed at sites of angiogenesis, such as the forefront of invading vessels in the developing corpus luteum or growing vessels in a glioblastoma (10, 25). Because of common factors dictating expression of Ang-2 and VEGF and because they produce complementary effects, these two factors and their receptors are thought to act in concert (7, 12). Our results demonstrated that the profile of Ang-2 expression parallels that of VEGF in the heart during angiogenesis. In contrast, expression of Ang-1 remained constant and Tie-2 phosphorylation level decreased when expression of VEGF and Ang-2 increased. Our results suggest that capillary density in heart is directly linked to the expression of VEGF and Ang-2 and inversely related to Tie-2 phosphorylation.

**Clinical implications.** Recently, there has been much interest in therapeutic angiogenesis for the treatment of the patient with ischemic heart disease (24), especially in the context of stimulating coronary collateral growth. In addition, heart failure in some patients may be related to a decrease in capillary density (1, 4), thus this condition may also be receptive to therapeutic angiogenesis. Several trials of coronary therapeutic angiogenesis have failed, and we propose that these failures are related to inadequate knowledge concerning the myriad mechanisms of coronary angiogenesis. Hence before therapeutic angiogenesis can be realized, the mechanisms of coronary angiogenesis must be elucidated. In the present study, we demonstrated the time course of capillary density and the expression of VEGF, Ang-1, Ang-2, and Tie-2 phosphorylation during ischemia-induced coronary angiogenesis and collateral development. We believe that our results will be useful for the elaboration of strategy about therapeutic angiogenesis for patient care, perhaps via incorpora-
tion of therapies involving treatment with more than a single factor.

In conclusion, we found that myocardial capillary density paralleled the changes in VEGF and Ang-2 expression, increasing and decreasing concomitantly with the levels of these two factors in the cardiac interstitium, and was inversely related to Tie-2 phosphorylation. We speculate that the expression of Ang-2 and VEGF is casually related to coronary angiogenesis.

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