Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor Differentially Modulate Early Postnatal Coronary Angiogenesis

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Abstract—The roles of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF [FGF-2]) in early postnatal regulation of coronary angiogenesis were investigated by administering neutralizing antibodies to these growth factors between postnatal days 5 and 12. Immunohistochemistry and Western blotting both revealed decreases in VEGF protein in the hearts of rats treated with either antibody. In contrast, bFGF mRNA increased in both treated groups, whereas VEGF mRNA was unchanged. Using stereological assessment of perfusion-fixed hearts, we found that both anti-VEGF and anti-bFGF inhibited the rapid and marked capillary growth that occurs during this time period and that the effects of the two neutralizing antibodies are not additive. Arteriolar growth, as indicated by a lower length density, was inhibited by anti-bFGF, but not anti-VEGF. When both anti-VEGF and anti-bFGF were administered, arteriolar length density was not significantly lower, but the population of small arterioles (15 μm) was markedly reduced, whereas the percentage of large arterioles (26 to 50 μm) more than doubled. Thus, inhibition of both growth factors negated or limited the formation of small arterioles and facilitated an expansion of the largest arterioles. These in vivo data are the first to document that during the early neonatal period, (1) both VEGF and bFGF modulate capillary growth, (2) bFGF facilitates arteriolar growth, and (3) the two growth factors interact to establish the normal hierarchy of the arteriolar tree. (Circ Res. 2001;88:1135-1141.)

Key Words: capillaries • arterioles • myocardium • coronary circulation • development

The early postnatal period is characterized by dramatic growth of the coronary vascular bed. For example, in the rat, aggregate ventricular capillary length more than doubles between postnatal days 5 and 11.1 In humans, the number of terminal arterioles (<20 μm) increases steadily during the last 2 months of gestation and during the first postnatal year.2 Previous work in our laboratory has shown that vascular endothelial growth factor (VEGF) is highly expressed in the rat myocardium during the prenatal and early postnatal periods.3 We also documented two peaks of basic fibroblast growth factor (bFGF [FGF-2]) mRNA expression, ie, at the earliest period of embryonic capillary formation and during the early postnatal period.4 Accordingly, both growth factors are likely to play a major role in myocardial angiogenesis during this period of time. However, the conditions under which they constitute an important stimulus are not clear. Both have been found to stimulate capillary formation and growth in the embryonic chicken heart when injected in ovo.5 However, the timing of their expression during development is an important consideration, given that the effectiveness of bFGF and VEGF in increasing angioblast numbers and facilitating tube formation, respectively, in cultured embryonic hearts depends on the embryonic time point.6

That myocardial growth factors may be triggered by mechanical or metabolic stimuli has been documented in several studies.3 For example, ischemia and/or hypoxia and mechanical stretch of the myocardium have been shown to enhance VEGF expression. In thyroxine-treated rats that undergo a marked myocardial angiogenesis, bFGF mRNA is elevated. We recently tested the hypothesis that myocardial angiogenesis that occurs in response to chronic bradycardia is dependent on VEGF.7 In that study, we documented that the 23% increase in capillary growth during a 3-week treatment with the bradycardia drug alinidine was associated with 2-fold increases in VEGF mRNA and protein. In contrast, bFGF mRNA was not significantly enhanced. Most importantly, this marked angiogenesis was completely negated by administration of VEGF-neutralizing antibodies. Upregulation of VEGF also occurs in response to both static and cyclic stretch.8,9

In consideration of the above, we concluded that both VEGF and bFGF may promote the marked myocardial...
angiogenesis during the early postnatal growth period. We tested this hypothesis by administering neutralizing monoclonal antibodies to neonatal rats during the second week of postnatal life, during which time myocardial growth and capillary formation is so dramatic. Our studies addressed the growth of both capillaries and arterioles to test the hypothesis that VEGF and bFGF selectively regulate the formation and/or growth of these vessels.

Materials and Methods

Experimental Protocol

Five litters of Sprague-Dawley rats were divided randomly into five groups. All protocols were approved by the Animal Care and Use Committee of the University of Iowa and conform to Public Health Service guidelines. Three groups received intraperitoneal injections of VEGF or bFGF monoclonal neutralizing antibodies or nonimmune IgG according to the protocols previously published. Both monoclonal neutralizing antibodies, which were prepared by us, have been previously characterized and their specificity and inhibitory potency described in detail. The antibodies were also tested for cross-reactivity; we found that no cross-reactivity occurred. The fourth group received injections of both neutralizing antibodies. Each rat was injected at postnatal days 5, 7, 9, and 11. The doses of neutralizing antibodies were 300 μg/100 g on day 5 and 600 μg/100 g on the other 3 days. These rats were studied at day 12, at which time the hearts either were fixed by vascular perfusion with a glutaraldehyde mixture or paraformaldehyde or were rapidly frozen for protein and mRNA analysis. The glutaraldehyde-fixed hearts were postfixed in osmium tetroxide and embedded in Spurr’s plastic, and 1-μm sections were prepared and stained with Richardson’s solution. These sections were used for stereological analysis of the myocardial vascular bed. Ultrathin sections were prepared from selected tissue blocks, stained with uranyl acetate and lead citrate, and viewed with a Hitachi 7000 electron microscope. Paraformaldehyde-fixed hearts were embedded in paraffin, and 6-μm sections were prepared and stained with uranyl acetate and lead citrate, and viewed with a Hitachi 7000 electron microscope.

RNA Isolation and Northern Blot Analysis

After removal of the atria, the heart was frozen in liquid nitrogen, and total RNA was extracted using the RNA isolation reagent RNA STAT-60 (Tel-Test Inc) according to the manufacturer’s instructions. Northern blot analysis was performed according to the method previously detailed. We used 10 μg of total RNA, separated on 1.2% formaldehyde-agarose gel, which was then transferred to a Nytran Supercharge nylon transfer membrane (Schleicher and Schuell). Hybridization of the filters was performed for 16 to 20 hours by adding VEGF, bFGF, or 18S RNA probes labeled with [α-32P]dUTP to the solution.

Western Blot Analysis for Growth Factor Protein

Hearts were homogenized in cold PBS containing protease inhibitors, and the extracted proteins (50 μg) were run on a 15% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and blocked with 5% nonfat milk for 1 hour at room temperature. We incubated the blots with VEGF rabbit polyclonal IgG (1:500) and with bFGF rabbit polyclonal IgG (1:500) in 1% milk and 0.05% Tris-buffered saline–Tween 20. Anti-rabbit IgG–horseradish peroxidase (1:5000) and the enhanced chemiluminescence detection system (Amersham) were used to visualize the antigen-antibody complexes. All antibodies were purchased from Santa Cruz Biotechnology. Optical density of the bands was determined using the Photoshop program.

Immunohistochemistry

Sections 6 μm thick from paraformaldehyde-fixed and paraffin-embedded hearts were cut and affixed to Superfrost-Plus slides. The immunohistochemical procedures for bFGF and VEGF have been previously described. Endogenous peroxidases were quenched with 0.3% H2O2, and background staining was blocked by incubation in 5% normal bovine serum. Primary antibody incubations were carried out in a humidified chamber, and the tissues were subsequently incubated with a biotinylated secondary antibody with an avidin-biotin complex (Vector Laboratories) and then reacted with 3,3’-diaminobenzidine–H2O2. The antibodies for VEGF and bFGF were purchased from Santa Cruz Biotechnology. Three hearts from each group were used for histochemical analysis.

Antibody Radiolabeling

Neutralizing antibodies were radiolabeled with Na-125I using the iododead-lactoperoxidase method as previously described. Rat pups were injected with the same amount of radiolabeled antibodies described in the Experimental Protocol section, representing ~1.1×106 cpm/μg.

Capillary and Arteriolar Analysis Using Stereology

One-micrometer sections of left ventricular samples embedded in Spurr’s plastic were prepared, placed on glass slides, and stained with Richardson’s solution (Azure II and methylene blue). Capillary outlines were traced by projecting images of the tissues, mounted on glass slides, onto drawing paper at a magnification of ×1440. The fields from each heart included a total of ~300 to 400 capillary profiles. The outlines were scanned into a computer program for the assessment of various capillary parameters (Image Pro software) as previously detailed. Length density (Lc), ie, total capillary length in 1 mm2 myocardium, was calculated from the long (a) and short (b) axes and capillary numerical density (Nc) using the following formula: $L_c = \frac{(a(b)N_c)}{V}$, where $V$ is the test or planar area of fields.

Arteriolar length densities were calculated as described for capillaries. For this assessment, we systematically scanned four tissue sections from each heart in their entirety and photographed all arterioles. These images, along with outlines of the tissue sections, determined by tracings from a microprojector, were scanned into a computer and the numerical analysis was conducted. One-micrometer sections facilitate the identification of arterioles. These vessels were identified according to the criterion that they contained at least one continuous layer of smooth muscle. An average of 121 arterioles per group (range = 91 to 141) was included in one analysis. The combined sample fields from each rat averaged 2.4 mm2.

Statistical Analysis

A one-way ANOVA followed by a Student t test and the Bonferroni adjustment for multiple comparisons was used to analyze the data. Statistical significance was assumed when $P≤0.05$. All data are presented as mean±SEM.

Results

Delivery of Neutralizing Monoclonal Antibodies to the Heart

We determined radioactivity of 125I-labeled neutralizing monoclonal antibodies in hearts 1 to 5 hours after intraperitoneal injection. Each rat pup (10 g body weight) was injected with 60 μg of antibody representing 6.7×106 counts of radioactivity/min. Five hours after injection, radioactivity counts/min averaged 2.6×104 for the two antibodies. Thus, 233 ng of the antibody had accumulated in the heart by 5
hours. This amount is 0.45% of the total injected and 6 times higher than that expected by nonselective diffusion across the body. To remove blood from the tissue, we perfused the heart with PBS buffer before counting radioactivity.

### Body and Heart Growth and Myocardial Morphology

In control rats, body weight increased 83% between 5 and 12 days, whereas ventricular weight increased by 62%. Treatment with neutralizing antibodies to either bFGF or VEGF did not alter growth, as indicated by similar body and heart weights in these groups and the 12-day controls. Ventricular weights (mg, mean±SEM) in the 12-day-old rats were the following: control, 110±9; anti-VEGF, 114±7; anti-bFGF, 113±8; and anti-VEGF+anti-bFGF, 121±10. Histological examination of 1-μm-thick heart sections did not reveal any differences between the treated groups and the 12-day controls. The normal morphology of the hearts from the treated rats was verified by electron microscopy. Cardiac myocytes and the extracellular compartments in the treated groups resembled their counterparts in the controls.

### Antibody Treatment Decreases VEGF Protein

Immunohistochemistry for VEGF and bFGF protein is illustrated in the micrographs in Figure 1. In sections from hearts of 12-day sham-injected rats, cardiac myocytes show a strong reactivity for both VEGF and bFGF antibodies. Anti-VEGF treatment markedly decreased immunoreactivity for VEGF and slightly decreased immunoreactivity for bFGF. In sections from hearts of rats treated with anti-bFGF, a slight reduction in immunoreactivity for bFGF was observed focally in some sections. The reduction in VEGF protein after anti-VEGF treatment was consistent in all hearts evaluated by Western blotting (Figure 2). In rats treated with both neutralizing antibodies, immunoreactivity for VEGF was decreased and appeared similar to that of rats treated with anti-VEGF alone; a slight focal reduction in bFGF immunoreactivity was noted (data not shown).

### Anti-VEGF and Anti-bFGF Treatment Enhances bFGF mRNA

Expression of bFGF mRNA was enhanced 54% by anti-VEGF and 72% by anti-bFGF treatment (Figure 3). In contrast, VEGF mRNA was not significantly altered by either treatment.

### VEGF and bFGF Regulate Capillary Angiogenesis

The growth of the capillary bed was quantified by image analysis of tissue sections from the left ventricle. Because
Capillary length density tended to be higher (although this difference was not statistically significant) in the 12-day than in the 5-day controls (Figure 4, top), whereas heart mass increased by 61% during this 7-day period, one can appreciate that about one third of the capillary bed was formed during this period of time. VEGF- and bFGF-neutralizing antibodies significantly inhibited capillary growth, given that capillary length density was only 82% and 83%, respectively, of the controls. Mean (±SEM) capillary surface density (mm²/mm³) was also significantly lower in the anti-VEGF (10.6±0.8) and anti-bFGF (11.2±0.5) groups than in the control group (13.4±4). Mean capillary diameter (Figure 4, bottom) was similar in the 5-day and 12-day controls and in anti-VEGF and anti-bFGF groups. However, treatment with a combination of anti-VEGF and anti-bFGF affected a significant increase in capillary diameter. Note that capillary diameter was 5.32±0.09 in the group treated with both neutralizing antibodies, whereas mean capillary diameter ranged between 4.70 and 4.73 in the other 12-day-old groups (anti-VEGF, anti-bFGF, and control).

Capillary Morphology During Decreased VEGF and bFGF

We used transmission electron microscopy to quantitatively evaluate the myocardial microvasculature. Systematic comparisons of micrographs from the treated and control groups did not reveal differences in vascular morphology. High-magnification electron micrographs (Figure 5) document the normal ultrastructural characteristics of capillary endothelial cells. Some maturation of endothelium, primarily an increase in vesicles, between 5 and 12 days was noted (Figures 5A and 5B). Neutralizing antibody treatment (Figures 5C and 5D) did not affect this maturation. In all of the groups studied (data not shown), we consistently and frequently observed endothelial cell processes extending into the lumen and forming partitions. This form of capillary proliferation was first described in 1991.\textsuperscript{15}

Arteriolar Formation and Growth

Arteriolar length density (summarized in Figure 6, top) was not altered by normal growth, nor was it affected by anti-VEGF treatment. In contrast, anti-bFGF treatment lowered length density by ≈42%. Mean arteriolar diameter was not altered by either treatment (Figure 6, bottom). However, in rats administered both neutralizing antibodies, arteriolar length density was not significantly reduced, but mean arteriolar diameter was larger by 50% than the 12-day-old controls (Figure 6). Thus, simultaneous inhibition of both growth factors affects alterations not predicted by inhibition of either growth factor alone. Histograms illustrating the percentage of arterioles within a diameter size class (Figure 7, top) indicated a greater percentage of the smallest arterioles (<10 μm) in the 5-day than in the 12-day rats. Thus, arteriolar growth involves not only lengthening of the arteriolar tree but diameter increases in the smallest arterioles.

Inhibition of VEGF or bFGF with neutralizing monoclonal...
antibodies did not notably alter the distribution of arteriolar diameters (Figure 7, bottom). In contrast, simultaneous inhibition of both growth factors markedly affected a redistribution of arteriolar diameters as illustrated in Figure 8. This dual treatment reduced the percentage of arterioles <10 μm by 78% and increased the largest class of arterioles (26 to 50 μm) 2.5-fold. This shift in arteriolar diameters is consistent with the 50% increase in mean arteriolar diameter seen in Figure 6 and noted above.

The increase in coronary arteriolar diameter in the rats treated with both anti-bFGF and anti-VEGF did not affect wall thickness. Mean wall/lumen ratios (±SD) for arterioles 25 to 50 μm are the following: control, 0.83±0.02, and anti-VEGF+anti-bFGF, 0.09±0.02. For arterioles <25 μm the values are the following: control, 0.12±0.03, and anti-VEGF+anti-bFGF, 0.12±0.04.

Discussion

Our data are the first to document the reliance of myocardial angiogenesis on VEGF and bFGF during the early postnatal period. Moreover, this study provides the first in vivo data on the role and interactions of these growth factors in coronary arteriolar formation and growth. Several salient findings from this study are noted. First, capillary growth during early postnatal life is dependent on both VEGF and bFGF. Second, bFGF facilitates coronary arteriolar formation. Third, VEGF and bFGF interact in determining the hierarchy of the coronary arteriolar tree.

Treatment Protocol

VEGF and its receptors play key roles during embryonic blood vessel development. Mice lacking one VEGF allele die during embryogenesis. Embryonic lethality also occurs inflt-1 or flk-1/KDR knockout mice. That the monoclonal antibodies used in this study did not attenuate heart or body growth is consistent with the finding that newborn mice treated with VEGF polyclonal antiserum are not growth-restricted. That study documented an inhibition of glomerular development. In contrast, inactivation of VEGF via Cre-loxP–mediated VEGF gene ablation or administration of the VEGF receptor chimeric protein mFlt(1–3)–IgG in newborn mice resulted in nearly complete inhibition of somatic growth and lethal metabolic failure. This dependence on VEGF in mice was lost after the fourth week of postnatal life. Given that we did not find that administration of neutralizing monoclonal antibodies to VEGF or bFGF or a combination of the two inhibited increases in heart and body mass, the antiangiogenic effects of the antibodies are not secondary to attenuation of somatic growth. The doses and protocol of VEGF-neutralizing antibodies were identical to those used in our previous work, in which bradycardia-induced myocardial angiogenesis in young adult rats was completely blocked. Similarly, capillary growth during revascularization in the ischemic mouse hindlimb was totally inhibited by VEGF-neutralizing antibodies in the same doses used in our study.

Our 125I labeling of each of the two neutralizing monoclonal antibodies documented their presence in the heart. The finding that VEGF protein was reduced in rats treated for 1 week with VEGF-neutralizing antibodies was identical to those used in our previous work, in which bradycardia-induced myocardial angiogenesis in young adult rats was completely blocked. Similarly, capillary growth during revascularization in the ischemic mouse hindlimb was totally inhibited by VEGF-neutralizing antibodies in the same doses used in our study.

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Capillary Angiogenesis

In this study we focused on the early postnatal growth period when heart and body growth is rapid and proportional.
calculation of ventricular volume from mean ventricular weights (ventricular volume = ventricular weight/1.06) allows the estimation of total capillary length. On the basis of this formula, we estimate that ventricular volume increased from 64 to 104 mm³ between postnatal days 5 and 12. We estimate that total capillary length (capillary length density × ventricular volume) was ≈588 and 1041 m in the 5- and 12-day control groups, respectively. Thus, 453 m, nearly one-half, of the capillary bed was formed during this 7-day period. This is consistent with an earlier study.1 Because in the anti-VEGF and anti-bFGF groups only 310 and 304 m were formed, one can appreciate the magnitude of angiogenesis inhibition by the two monoclonal antibodies. Previous work from our laboratory documented VEGF as an essential molecule for myocardial angiogenesis induced by chronic bradycardia in young adult rats.7 Because bFGF mRNA was not upregulated in this study, we concluded that it was unlikely to be a major stimulus of growth in bradycardia-induced angiogenesis or to play a permissive role. The current study suggests that angiogenesis in the neonatal heart is regulated by both VEGF and bFGF. These data are consistent with our previous in situ experiments that revealed a strong expression of mRNA for both of these growth factors during the early postnatal period.34

Our evaluation with electron microscopy revealed a continuation of endothelial cell maturation between postnatal days 5 and 12, which is most evident during gestation.22 Because a decrease in VEGF or bFGF did not alter endothelial cell ultrastructural characteristics, nor did it affect any morphological changes in other cells in the ventricles, the antiangiogenic effects of the neutralizing antibodies were not secondary to damage due to the treatment protocol.

bFGF is clearly an angiogenic molecule as shown by both in vitro and in vivo experiments23 and is important for vessel assembly in the early embryo.24 Endothelial cells from mouse heart capillaries express both low-affinity heparan sulfate bFGF binding sites and high-affinity tyrosine kinase receptors on their surfaces, and they upregulate urokinase-type plasminogen activator and form capillary tubes in response to bFGF.25 Our finding that anti-bFGF treatment limited myocardial angiogenesis supports an angiogenic role of this growth factor during the early postnatal period. However, we recognize that bFGF may exert its influence on angiogenesis by facilitating the action of VEGF, given that VEGF expression increases when recombinant bFGF is added to cultured endothelial cells or when endogenous bFGF is upregulated.26 Moreover, bFGF has been found to induce expression of the VEGF receptor KDR (flk) via a protein kinase C and p44/p42 mitogen-activated pathway.27

Arteriolar Formation and Growth
Coronary arterioles and arteries develop when smooth muscle cells are recruited by endothelial cell–lined tubes or capillary-like channels.4,28,29 The 62% increase in ventricular weight between 5 and 12 days of postnatal life noted in our study in the absence of a significant decline in arteriolar length density indicates that a substantial portion of the arteriolar tree was formed during this period of time. In 5-day-old rats, 66% of the arterioles were 15 μm or less in diameter. Seven days later, a much larger ventricle had 56% of its arterioles in this size class. Thus, one can recognize that a substantial number of arterioles were formed during this period. At the same time, remodeling of arterioles upstream from the smallest branches must have occurred to preserve the normal arteriolar hierarchy or to increase mean arteriolar diameter. Coronary arteriolar growth and remodeling have been documented in a porcine model of right ventricular hypertrophy.30 That study provided evidence of newly formed small parallel arteriolar vessels. The fact that length density was 42% lower in the anti-bFGF group compared with the controls suggests that arteriolar growth was severely curtailed. Despite this growth inhibition, the arteriolar hierarchy was not notably altered. Thus, anti-bFGF inhibited the overall growth of the arteriolar tree rather than preventing the formation of arterioles, as would be indicated by a reduction in the percentage of arterioles <15 μm. Recent work has shown that FGF-1, or acidic FGF, plays an important role in coronary artery growth.31 Transgenic mice with specific myocardial overexpression of the KFGF-1 gene have increased numerical density of coronary arteries, especially arterioles, as a consequence of increased branching. Thus, another member of the FGF family also contributes to coronary arteriolar formation.

When both anti-bFGF and VEGF were administered, the arteriolar hierarchy was clearly altered, whereas length density was not significantly reduced. Although the number of small arteriole profiles was markedly reduced, an inhibition of arteriolar formation cannot necessarily be assumed, because length density was not lower in the treated group. An alternative explanation is that newly formed arterioles expand rapidly and therefore contribute to the diameter classes >20 μm. Thus, in the group treated with anti-bFGF plus anti-VEGF antibodies, 56% of the arterioles are >20 μm in diameter compared with 26% in the control group. These data precipitate the conclusion that bFGF and VEGF working in concert facilitate the establishment of the hierarchy of the coronary arteriolar tree. Thus, the role of bFGF in arteriolar formation appears to overlap with that of VEGF, given that inhibition of VEGF alone does not affect arteriolar hierarchy or arteriolar length density. The finding that mean arteriolar diameter is increased when two growth factors are inhibited is consistent with our finding in capillaries. Whether other growth factors are stimulated when bFGF and VEGF are inhibited remains to be determined. The physiological consequences of the diameter changes are not clear. One outcome could be a redistribution of vascular resistances in the arteriolar bed. However, in the absence of 3-dimensional data, firm conclusions are not warranted. The major intent of this study was to determine the roles of bFGF and VEGF in arteriolar growth. The consequences of the changes attributed to decreases in these growth factors over longer periods of time are important issues for future studies.

In summary, our experiments provide new evidence that, during the early postnatal period, both VEGF and bFGF modulate coronary capillary angiogenesis, and arteriolar growth is dependent on bFGF. Our data also suggest that VEGF plays some supportive role for bFGF in arteriolar neoformation and in establishing the hierarchy of the coronary arteriolar tree.
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References
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