VEGF and bFGF stimulate myocardial vascularization in embryonic chick

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Tomanek, Robert J., Kapildeo Lotun, Edward B. Clark, Padma R. Suvarna, and Norman Hu. VEGF and bFGF stimulate myocardial vascularization in embryonic chick. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1620–H1626, 1998.—We tested the hypothesis that early vascularization of the embryonic heart is enhanced after bolus injections of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) into the vitelline vein before the onset of myocardial vasculogenesis (3.5 days, stage 21). Electron and light microscopy were utilized to obtain morphometric data. At stages 29 and 31, myocardial vessel volume or numerical density were higher in embryos injected with 50 ng bFGF than in the saline-injected controls. A VEGF injection increased vascular volume density at stage 29 and both volume and numerical density at stage 31. bFGF, but not VEGF, was associated with an enhancement of the sinusoidal system (spongy layer of the ventricle) at stage 29. This effect disappeared by stage 31. In conclusion, 1) enhancement of bFGF or VEGF before myocardial vascularization increases vascular growth, but the initial effect of bFGF is greater; 2) the effects of these growth factors on vascular volume and numerical density are temporally dependent; and 3) bFGF, in addition to its effects on the coronary vasculature, influences ventricular modeling by apparently acting on myocytes as well as endothelial cells.

ALTHOUGH THE ANGIGENIC effects of various growth factors in disease states, e.g., tumors and retinal myopathy, are currently receiving considerable attention, fewer studies have addressed the effects of growth factors during normal development. In this regard, our understanding of the regulation of coronary vasculogenesis and angiogenesis in the embryo and fetus is especially poor. Of the various growth factors, which may be implicated in vascular growth, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the two most probable regulators of coronary growth and, accordingly, were selected for this study. Both growth factors are not only mitogenic for endothelial cells but also enable basement membrane degradation and stimulation of cell migration (2, 9). VEGF, bFGF, and acidic fibroblast growth factor are the only growth factors known to have all three of these characteristics (20).

Early vessel formation, before coronary vasculogenesis, is dependent on VEGF, since homozygous VEGF-deficient mice die in midgestation and no viable VEGF heterozygous offspring are obtained at birth (4). Recepiors for this growth factor are present during early embryogenesis. In mice, flt-1 mRNA is specifically localized in blood vessels of developing organs (3). Flk-1 has been shown to be essential for blood island formation and vasculogenesis, since mice deficient in this receptor die between 8.5 and 9.5 days postcoitum (23). In the rat heart, VEGF is expressed by the 11th or 12th day of gestation (12), just before the onset of vasculogenesis (22, 26).

bFGF, which targets several cell types, is higher in bovine hearts during fetal and neonatal development than in the adult (5). We have found that bFGF mRNA is elevated at two time points: at the onset of vasculogenesis and during the first week of postnatal life when capillary angiogenesis is marked (26). Coronary vasculogenesis is susceptible to fibroblast growth factor (FGF) signaling, since abnormal branching of coronary arteries develops in chickens in which FGF and a reporter gene had been introduced during the third day of incubation (16). Moreover, the bFGF receptor peaks in the chick at stage 24 (24), which corresponds to the time of early ventricular vasculogenesis (19).

Our previous in vitro experiments with embryonic rat hearts indicate a role in vasculogenesis for both of these growth factors (21). bFGF was found to enhance proliferation of presumptive endothelial cells, whereas VEGF was shown to stimulate tube formation. Each growth factor exerted its influence at a specific time point: bFGF in embryonic day 12 hearts and VEGF in embryonic day 14 hearts. On the basis of these data, and the findings cited above, we hypothesized that increases in these growth factors in vivo, just before the onset of vasculogenesis, would increase vessel formation. Injections of the growth factors were performed on day 12 hearts and VEGF in ovo in chickens just before the onset of coronary vasculogenesis.

MATERIALS AND METHODS

Animals and experimental protocol. Fertilized White Leghorn chicken eggs were incubated, blunt end up, to Hamburger-Hamilton stage 21 (3.5 days). The embryo was exposed via a window in the shell and an incision of the inner shell membrane. Growth factor protein, 50 ng in 5 µl PBS buffer, was injected via a microcannula introduced into the vitelline vein with the aid of a micromanipulator. Immediately after injection, the window in the shell was sealed with paraffin film, and the egg was returned to the incubator. Controls (sham) consisted of embryos injected with 5 µl PBS buffer. The eggs were removed from the incubator at either point: bFGF in embryonic day 12 hearts and VEGF in embryonic day 14 hearts. On the basis of these data, and the findings cited above, we hypothesized that increases in these growth factors in vivo, just before the onset of vasculogenesis, would increase vessel formation. Injections of the growth factors were performed on day 12 hearts and VEGF in ovo in chickens just before the onset of coronary vasculogenesis.

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stage 29 or 31, i.e., 6 or 7 days, respectively. A microcannula was inserted into the ventricle, and the heart was perfused with a 2.5% glutaraldehyde solution buffered with 0.1 M sodium cacodylate. To maintain a diastolic state, we added 0.025 μg verapamil to the perfusate. The heart was placed in the same fixative for 6 h at 4°C and subsequently weighed and postfixed in 2% osmium tetroxide. The left ventricle was then embedded in Spurr’s plastic. Sections for light and electron microscopy were prepared from the blocks and utilized for morphometric analysis. To define normal ventricular and vascular growth during the 3.5- to 7-day period, we also obtained nontreated specimens at various stages (21–31).

To determine whether a gradual introduction of growth factor would be more efficacious, bFGF was introduced via suffusion utilizing an osmotic pump. To accomplish the suffusions, a cannula was placed near the heart, and the growth factor was delivered for a 24-h period, at a rate of 5 ng protein/h.

bFGF and VEGF. Human recombinant bFGF and VEGF were purchased from Collaborative Biomedical Products (Bedford, MA).

Morphometric analysis. For light microscopic analysis, we utilized tissue sections (1 μm in thickness) of the ventricular free wall stained with Richardson’s solution. Images of cross-sectional fields were projected onto drawing paper with a microprojector, and images of the tissue were traced. Equidistant points were marked along the epimyocardial border, and the distance from each point to the nearest endocardial point was measured and the mean of these values calculated. Approximately 30 measurements were made for each sample.

Coronary vascular volumes were based on electron microscopic and point-counting methods. Ultrathin sections from the compact region of the left ventricular free wall, prepared using a Reichart Ultratome and a diamond knife, were placed on single oval slot grids that contained a formvar film to support the sections. The sections were stained with uranyl acetate and lead citrate. At least eight fields were photographed at a magnification of ×1,000, and prints were enlarged to ×2,500. Microvessels were identified and point-counted using an overlay grid with intercepts every 6 mm (1,008 points/micrograph). All of the vessels were capillaries or venules, since arteries were not yet formed at the stages studied. Artery formation commences at stages 32–33, as previously documented by Waldo et al. (30) and confirmed in this study. Nonvascular cells were point-counted using a grid with intercepts every 12 mm; these counts were multiplied by four to match those of the finer grid used to determine vascular density.

Because sinusoids are also endothelium-lined structures, we took precautions to avoid including these structures in our measurements of vascular densities. In preliminary experiments, we examined channels in serial sections to determine if they were sinusoids, i.e., open to the ventricular lumen. Our data showed that sinusoid channel widths were nearly always >15 μm. Therefore, in our analysis, profiles of this dimension or larger were excluded from our data. Although we recognize that some profiles smaller than 15 μm could be the ends of sinusoids, their number in relation to vascular profiles is quite small and therefore not likely to bias the data.

Statistical analysis. The data were analyzed by ANOVA and a Bonferroni adjustment for multiple comparisons where appropriate. Significant intergroup differences were based on a P value <0.05. The data are based on seven to nine hearts from each experimental group and six hearts from each sham group.

RESULTS

Growth factor treatment did not significantly affect ventricular weight, as indicated by data from stage 31 embryos. Ventricular weights were as follows: sham, 4.60 ± 0.13 mg; bFGF, 4.81 ± 0.16 mg; VEGF, 5.1 ± 0.15 mg.

Structural characteristics of normal and sham hearts. At stage 18, the chicken heart ventricle develops trabeculae, which by stage 21 have expanded to form a mesh. The spaces between the trabeculae are “sinusoids” and serve to minimize the distances between the ventricular lumen and the myocardial cells. Therefore, sinusoids are invaginations of the ventricular lumen lined by endocardial endothelial cells. The mesh formed by trabeculae is called the “spongy” zone of the ventricle, whereas the more peripheral layer lacking sinusoids is the “compact” zone. At stage 21, the compact zone is only about two myocardial cells thick and is avascular. As this zone expands via cardioblast proliferation and the thickness consists of three or four cells, angioblasts begin forming vascular tubes. This occurs at about stage 23. The compact zone is ∼5–6 cells thick at stage 29 and ∼8–10 cells thick by stage 31 (Fig. 1). Although the major growth occurs in the compact region, the spongy zone continues to grow during this time period as well.

Effects of exogenous bFGF. Stage 29 hearts from embryos that had received a bolus injection of bFGF were characterized by an enhanced sinusoidal system as illustrated in Fig. 1. The invaginations of the endocardium generally extended deeper into the compact region than those of sham hearts. In one series of serial sections, we found an endothelial-lined connection between the epicardium and endocardium (Fig. 1, A–C). Quantitation of the distances between the epimyocardium and the endocardium is illustrated in Fig. 2. Compared with the sham control group, hearts from embryos injected with bFGF had mean epicardial-to-endocardial distances that were 30% shorter. However, further growth of the ventricle between stages 29 and 31 completely abolished this earlier effect of bFGF. In a group of eight embryos in which bFGF was suffused at 5 ng/h for 24 h, epicardial-to-endocardial distance was similarly reduced (37%) compared with the shams.

bFGF stimulated vascular growth as indicated by data illustrated in Fig. 3. At stage 29, vessel volume percent in the treated group was nearly double (96%) that of the sham group. Numerical density tended to be higher in the treated group but was of borderline significance. Suffusion of bFGF also increased mean vessel volume percent by a similar magnitude, i.e., 91% (data not shown). By stage 31, the difference in vessel volume density between sham and bFGF-injected embryos was no longer statistically significant (P = 0.10). In contrast, numerical density was significantly higher in the treated group. Thus the major effect of bFGF on vessel volume expansion occurred at stage 29, whereas its effect on numerical density was manifested later.
i.e., stage 31. Figure 4 is an electron micrograph illustrating vascular tubes (capillaries) in various phases of development. The micrographs illustrate the point that many capillary profiles are small and require electron microscopy for detection.

Effects of exogenous VEGF. In contrast to bFGF (Fig. 2), exogenous VEGF did not influence the modeling of the sinusoidal system as indicated by epicardial-to-endocardial distances provided in Table 1. These values are similar in the VEGF and sham groups. However, VEGF affected an accelerated growth of myocardial vessels, as illustrated in Fig. 5. Vessel volume percent was elevated at both time points (stages 29 and 31). In contrast, vessel numerical density at stage 29 was similar in the VEGF and sham groups. However, numerical density was 62% higher in the VEGF group than in shams at stage 31. These data indicate that the initial effect of VEGF was volume expansion of vessels, rather than their proliferation. The effect of VEGF (50-ng dose) on the vascular parameters was less than that observed with bFGF treatment, i.e., compared with the shams vascular volume was 40% higher in the VEGF group at stage 29, and 26% higher in the VEGF group at stage 31. As previously noted, vessel volume percent nearly doubled by stage 29 with bFGF treatment. In chicks injected with 100 mg VEGF and studied at stage 29 (data not shown), vessel volume percent was 8.4 ± 0.70 (n = 7), compared with 8.1 ± 0.50 for the group treated with 50

Fig. 1. Light micrographs of ventricular wall of stage 29 embryos. A-C: representative serial sections illustrating occurrence of an endothelium-lined transmural vascular channel (Ve) from an embryo injected with basic fibroblast growth factor (bFGF). This channel connects epicardial surface (Ep) with ventricular lumen (*). bFGF also initially (stage 29) enhanced sinusoidal system (S) as seen in E, and compared with a ventricular section from a sham-injected embryo shown in D. Bar indicates 20 µm; all micrographs are at same magnification.

Fig. 2. Epicardial-to-endocardial distance (means ± SE). bFGF transiently (stage 29) reduced this distance by enhancing sinusoidal system as shown in Fig. 1. By stage 31, this effect was totally negated.
ng VEGF. Therefore, a maximal effect was achieved with the 50-ng dose.

**DISCUSSION**

Although previous work has shown that VEGF plays a vital role in the early formation of the embryo, the role of this growth factor and that of bFGF in the subsequent early formation of the coronary vasculature had not been elucidated. The current study provides evidence that administration of bFGF or VEGF just before myocardial vasculogenesis enhances the formation of coronary vessels. Our data support three conclusions.

1. bFGF has a more marked effect on vessel growth, at this early time point, than VEGF.
2. Vessel numerical density and size appear to be differentially affected over time by these growth factors.
3. bFGF, but not VEGF, transiently enhances the sinusoidal system and therefore influences the modeling of the ventricle during development. These data are the first to demonstrate that exogenous bFGF and VEGF enhance the formation of coronary vessels in the embryo.

Previous studies have explored the angiogenic potential of exogenous bFGF and VEGF in the adult ischemic heart. Intracoronary administration of VEGF over a 28-day period to dogs with coronary ligation increased the density of precapillary vessels >20 µm in diameter by 89% but had no effect on capillary density (1). VEGF administration failed to increase collateral development in this dog model (15) but was found to increase the number of collaterals in the pig (11). Intracoronary injection of bFGF in dogs with gradual constriction of a coronary artery enhanced the numerical density of distribution vessels and endothelial synthesis in the ischemic zone (29). Systemic administration of bFGF after coronary ligation increased collateral conductance and collateral zone vascularity (14, 15). These studies suggest that angiogenesis, i.e., formation of vessels by sprouting, occurred in these adult hearts. Our work addressed the onset of vascularization of the myocardium, which includes primarily vasculogenesis, i.e., formation of vessels from angioblasts. The vascular structures formed during the time period investigated are capillaries and venules.

Our findings indicate that exogenous bFGF enhances both the formation of vascular channels and the extent of the sinusoidal system. The latter is transitory, since the mean distance between the epicardial junction and endocardium in the bFGF group is no longer shorter by stage 31. Because bFGF affects several cell types, its influence on modeling of the trabecular (spongy) region of the ventricle is not surprising. To accomplish this selective growth of the trabecular...
region, the spatial arrangement of both endocardial cells and ventricular myocytes had to be directed. That bFGF did not enhance the overall growth of the ventricle, as indicated by the ventricular weights, is consistent with evidence that overexpression of FGFR1 did not enhance cardiocyte proliferation (17). In the heart, FGFR peaks at stage 24 (24), which corresponds to early coronary vascularization. Thus the data indicate that bFGF increased growth of the spongy layer (i.e., increased the proportion of myocytes in this ventricular component). The consequences of this arrangement are an increase in the surface area of the endocardium and a reduction in oxygen diffusion distances.

Table 1. Epimyocardial-to-endocardial distances in embryos treated with VEGF

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<thead>
<tr>
<th>Group</th>
<th>Epimyocardial-to-Endocardial Distance, µm</th>
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<tbody>
<tr>
<td></td>
<td>Stage 29</td>
</tr>
<tr>
<td>Sham</td>
<td>29.73 ± 1.90</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
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<tr>
<td>50 ng</td>
<td>29.89 ± 1.79</td>
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<tr>
<td>100 ng</td>
<td>30.80 ± 2.00</td>
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Values are means ± SE. None of the intergroup differences are statistically significant. VEGF, vascular endothelial growth factor.

Evidence that coronary vasculogenesis is susceptible to FGF signaling is indicated by a study that reported abnormal branching patterns of coronary vessels in which FGF and a reporter gene had been introduced into the epicardial space of chickens on day 3, before the onset of vasculogenesis, and the hearts studied on day 18 (16). This study differs from ours in that bFGF expression was precocious and ectopic and in that a retrovirus was used. However, as illustrated in Fig. 3, we did observe an anomalous coronary channel between the epicardium and the endocardium.

Recent evidence indicates that the VEGFR2 gene product is essential for the precursors of both hematopoietic and vascular endothelial lineages and that the latter is dependent on VEGF for differentiation (8). Our previous work on explanted embryonic rat hearts showed that tube formation by endothelial precursors migrating onto a collagen gel did not form tubes unless VEGF was added to the medium (21). In contrast, bFGF did not stimulate tube formation in this model. The finding that vascular volume density in the VEGF group was increased at stage 29, while numerical density tended to be lower than the controls, suggests that the initial effect of VEGF was to enhance the coalescence of vascular tubes. By stage 31, multiplica-
tion of vascular channels was increased by VEGF, as indicated by a higher numerical density compared with the shams. Vascular patterning has been found to be influenced by VEGF (7). When VEGF was injected at the onset of vasculogenesis (5-somite stage), inappropriate neovascularization in normally avascular areas and unregulated excessive fusion of vessels occurred. Similarly, when a retroviral vector was used to overexpress VEGF-122 in quail and chicken limb buds, hypervascularization occurred (10).

Because hypoxia is a powerful stimulus for VEGF in the heart (1, 13), we previously proposed the hypothesis that the growth of the compact region of the ventricle extends the diffusion distance for oxygen from the endocardium and thereby renders part of this region hypoxic (28). Although the current study did not address this issue, the evidence that exogenous VEGF enhanced vascularization during this early period is consistent with this hypothesis. Ventricular mass is a determinant of myocardial vascularization as indicated by our experiments on pressure-overloaded chick embryonic hearts (unpublished data). When accelerated ventricular growth occurs in response to conotruncal banding, vascularization is accelerated to match the enhanced ventricular mass.

The current study is the first documentation of enhanced coronary vascular growth in the developing heart in response to VEGF and bFGF. In addition, bFGF has been found to play a role in modeling of the ventricular sinusoidal system. However, this study focused on the early development of the coronary vascular channels, which is the first phase of the developmental sequence of the coronary vascular system (for a review, see Refs. 25, 27, 28). This phase concerns primarily endothelial cells and pericytes. We recognize that the later events contributing to the formation of the coronary arteries and their branches and their subsequent remodeling may be regulated by other or additional factors. For example, platelet-derived growth factor and epidermal growth factor may play important roles in the development of the tunica media of coronary vessels, since in vitro experiments have demonstrated that they are mitogens for smooth muscle cells (6). Other key questions concern the regulation of spatial and directional growth of the coronary vasculature. One important question concerns potential chemotactic factors, which direct the ingrowth of a vascular plexus into the aorta to form the coronary ostia.

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