Acidic Fibroblast Growth Factor and Heart Development
Role in Myocyte Proliferation and Capillary Angiogenesis

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Proliferative growth of the ventricular myocyte (cardiomyocyte) is primarily limited to fetal and early neonatal periods of development. In concert with the neonatal “transition” from proliferative to hypertrophic growth, ventricular remodeling of the nonmyocyte compartment is characterized by increased extracellular matrix synthesis/deposition and capillary angiogenesis. A role for locally generated and bioactive ventricular acidic fibroblast growth factor (aFGF) in these processes is proposed and substantiated by the following: 1) colocalization of aFGF peptide and fibroblast growth factor receptor (fig) transcripts to the developing fetal cardiomyocyte by immunohistochemistry, immunoelectron microscopy, and in situ hybridization, 2) continued localization of aFGF peptide and transcripts to the neonatal/mature cardiomyocyte, and 3) localization of fig immunoreactivity and transcripts to specific neonatal ventricular nonmuscle cell types. Specific ventricular cell types at distinct developmental stages appear to be responsive to ventricular myocyte-derived aFGF (myocytes in the fetal heart and nonmyocytes/endothelial cells in the neonatal heart). These data indicate that expression of aFGF and one of its receptors (fig) are most pronounced in the fetal to early neonatal ventricle, the presence of both suggesting an autocrine/paracrine growth regulatory function. As the animal matures, ventricular capillary angiogenesis may be facilitated by “release” of cardiomyocyte-derived fibroblast growth factors into the surrounding extracellular space/matrix functioning as a “paracrine” angiogenic stimuli. Therefore, the results of our study suggest that myocyte-derived aFGF may function to increase the fetal ventricular cardiomyocyte population in absolute number as well as to facilitate the subsequent increase in capillary angiogenesis that occurs during cardiomyocyte maturation and ventricular remodeling. (Circulation Research 1993;72:7-19)

KEY WORDS • acidic fibroblast growth factor • fibroblast growth factor receptor • angiogenesis • myocytes • growth factors

Growth and maturation of the ventricular myocyte (cardiomyocyte) are rapid processes that appear to be under partial control by locally generated growth factors. The expression of specific growth factors during fetal and neonatal periods of heart development may play distinct roles in initiating and modulating the proliferation and maturation of this unique cell type. Growth and development of the mammalian ventricle during the fetal and neonatal periods is, to a large extent, dependent on underlying changes occurring in the cardiomyocyte population. Cardiomyocyte growth in cell number in mammals is primarily limited to fetal periods of development.1,2 A limited number of studies have focused on any specific growth factors and/or their cognate receptors during the critical fetal to neonatal “transition” period of cardiomyocyte development.3–10 Composite analysis of the presence of both the peptide growth factor or associated transcript as well as the cognate receptor(s) or associated transcript are necessary to support the hypothesis of a specific autocrine/paracrine growth factor “loop” within a developing organ. Subsequent increases in ventricular mass during the neonatal period are primarily the result of increased cellular size (hypertrophy) of a finite population of cardiomyocytes. Cardiomyocytes occupy the bulk of the heart tissue yet compose only 20–40% of the total cellular populations of the ventricle.1,11 In addition, the ventricle of the neonate undergoes rapid tissue remodeling that is associated with capillary angiogenesis and the formation of an extensive extracellular matrix (ECM).2,12,13 During this “transition” from fetal to neonatal development,14,15 cardiomyocytes exit the cell cycle, complete additional rounds of DNA synthesis associated with the processes of binucleation and polyploidy, and begin their long-term maturational process of cellular hypertrophy. In concert with the terminal proliferative events in the cardiomyocyte population, the length and numbers of ventricular capillaries increase as the mechanisms of oxygen/nutrient exchange switch from diffusion in the fetal heart to vascular-mediated exchange in the maturing and adult myocardium.16,17 Although

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ventricular angiogenesis of the capillary network occurs primarily during neonatal development in rodents and humans, the stimulus or stimuli necessary for this angiogenic response remain to be determined. Multiple stimuli may originate from the cardiomyocyte population of the maturing ventricle itself and function within the microenvironment of the growing vascular bed of the heart.

One specific well-defined angiogenic stimulus for the neonatal heart may be ventricular acidic fibroblast growth factor (aFGF), a peptide that is produced by the cardiomyocytes themselves. Cardiomyocyte-produced fibroblast growth factors (FGFs, both aFGF and basic FGF [bFGF]) would then be "deposited" in the surrounding ECM and could thereby function as a "paracrine" angiogenic stimulus during this critical period of ventricular remodeling. To implicate such a relation, we have shown that the fetal/neonatal cardiomyocyte and surrounding ECM are rich sources of aFGF peptide and transcripts, as determined by immunohistochemistry, immunoelectron microscopy, in situ hybridization, and total ventricular RNA hybridization analyses. In addition, we have identified and colocalized the ventricular expression of one of the FGF receptor family genes (fgf) to the developing myocardium. We present evidence to indicate that aFGF may function during fetal development of the heart as an important locally generated growth factor stimulating cardiomyocyte proliferative growth. This response is postulated to be mediated via cardiomyocyte cell-surface aFGF receptors as evidenced by high levels of FGF receptor (fgf) gene expression in the fetal ventricle. We present evidence to suggest that aFGF plays a dual role in heart development, stimulating both cardiomyocyte proliferation and subsequent capillary angiogenesis.

Materials and Methods

Neonatal animals were obtained from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats bred in our AAALAC-approved facility. Fetal tissues of timed pregnant SHR and WKY females were obtained from Harlan Sprague Dawley, Inc., Indianapolis, Ind. Nucleic acid extraction and isolation materials were as follows: guanidine isothiocyanate and phenol from Ambrosco Chemical Co., Solon, Ohio; cesium chloride and oligo dT-cellulose from Bethesda Research Laboratory, Gaithersburg, Md.; and beta-mercaptoethanol, Sigmacote, and sodium dodecyl sulfate from Sigma Chemical Co., St. Louis, Mo. [alpha-32P]dCTP, [alpha-35S]dCTP, and [3H]deoxyribonucleotide triphosphates (dCTP, dGTP, dATP, and TTP), a random-prime labeling kit, and Auroprobe 30-nm gold-labeled goat-anti-rabbit antisera were obtained from Amersham Corp., Arlington Heights, Ill. Ilford K.5D emulsion diluted 1:1 with diethyl pyrocatechol (DEPC)-water that had been prewarmed to 42°C for 1 hour and exposed for 4–7 or 14 days (3H or 32P, respectively) at 20°C. Routine autoradiographic grain development was with Kodak D-19. Samples were counterstained with hematoxylin for adequate nuclear detail with minimal cytoplasmic staining. Clarification of potential nonspecific binding of the cDNA probes to any cellular target was determined by ribonuclease A (RNase) pretreatment (incubated with 20 mg/ml RNase in PBS at 37°C for 1 hour, rinsed in PBS twice, dehydrated, and air-dried before hybridization) with subsequent reduction of autoradiographic signal to indicate the specificity of the hybridization reactions to cellular RNAs (data not shown).

Tissue Immunohistochemistry

Ventricular tissues from fetal and neonatal rat pups or adult rats were removed, rinsed in Hanks’ balanced salt solution, and fixed overnight in 4% paraformaldehyde at 4°C. Tissues were dehydrated, embedded in paraffin, sectioned at 6 μm, and mounted on TAPSE-treated glass slides. Tissue sections were pretreated with hyaluronidase (0.5 mg/ml in Tris-buffered saline [TBS] containing 0.01 M Tris-HCl [pH 7.4] and 0.85% NaCl) for 30 minutes at 37°C, rinsed with TBS, pretreated with excess normal serum, and incubated with primary antisem or normal rabbit serum (negative control) overnight at 4°C. The antisem was washed off with TBS containing 0.1% bovine serum albumin and 0.1% Nonidet P-40 in the first wash; this was repeated four times for 5 minutes, and then the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc., Burlingame, Calif.) and avidin-biotin complex detection system (peroxidase) for 1 hour at RT for each reaction.
immunocomplex was localized by incubation with 3-amino-9-ethylcarbazole dissolved in dimethylformamide and 0.02 M acetate buffer (pH 5.2) for 20 minutes at RT. Slides were washed in TBS for 5 minutes, washed in water, counterstained with hematoxylin for 2 minutes, covered with Biomedica Crystal mount (Fisher Scientific), and hardened at 80°C for 20 minutes.

The anti-aFGF polyclonal antisera was originally described and characterized by Jaye et al. This antisera showed limited (<5%) cross-reactivity with bFGF, as previously evaluated by Western blot analysis. The antisera to flg/FGF receptor-1 and bek/FGF receptor-2 were generated with specific peptide sequences located in the COOH-terminal domains derived from their respective DNA coding sequence as previously described. The flg-1A antisera was generated against a synthetic peptide derived from residues 808–822 of the C-terminal tail deduced from the human FGF receptor clone. The polyclonal antisera were purified by peptide affinity and protein A chromatography. Specificity of the antisera to both epitopes was clarified by blocking studies using either intact aFGF protein or the original peptide used to generate the flg/bek antisera. Overnight incubations at 4°C of the peptide or protein with the primary antisera (1:500–2,500) eliminated the cellular staining. Previous studies have indicated that the antisera are specific for their respective receptor subtypes on the basis of Western blot analysis of metabolically labeled cells. Studies with bek-specific antisera were negative and presumed to be due to the lack of detectable expression of this form of the FGF receptor in the myocardium (data not shown).

**Electron Microscopy and Immunogold Localization**

Ventricular tissue was obtained from fetal pups at days 14–18 of gestation and neonatal pups at days 1–21, as previously described. Tissue samples were immersion-fixed in 4% paraformaldehyde containing 0.5% gluteraldehyde for 1 hour at 4°C. Fixed specimens were then processed and embedded in LR White or Lowicryl embedding media according to the manufacturers' directions. The LR White–embedded materials were polymerized for 48–72 hours under ultraviolet light at 4°C. All samples were “thin”-sectioned to gold interference thickness and mounted on formvar-coated zinc 300 mesh grids for subsequent processing. Sections were
FIGURE 2. Photomicrographs showing immunoelectron microscopic localization of acidic fibroblast growth factor in fetal myocardium. Ventricular tissue was obtained from fetal Wistar-Kyoto rats (panels A and C) or spontaneously hypertensive rats (panels B and D–F) at 18 days of gestation, processed, embedded, sectioned, and stained as described in “Materials and Methods.” Nonspecific staining using normal rabbit serum was low to undetectable (panels A and B). Immunoreactivity was readily detected in both strains and was found primarily within the myocytes (panels C–F). The localization of the immunoreactivity was found in both the cytoplasmic (arrowheads), perinuclear (arrows), and occasional nuclear sites. Occasional labeling of the extracellular matrix (asterisk) was also seen (panels C and E). Bar, 0.5 μm.

blocked for nonspecific staining by floating the sections on a drop of 10% goat serum for 3 hours at 4°C. After multiple washes with TBS containing 1% bovine serum albumin, sections were incubated overnight at 4°C with the primary antisera (1:50–500), nonimmune normal rabbit serum (1:25–50), or the immunoglobulin G fraction of nonimmune serum in TBS containing fish gelatin (1%). The following day, sections were washed five to seven times with TBS containing 0.1% Triton X-100 and then twice with TBS alone. The sections were
then incubated for 2 hours at room temperature with a 1:20 dilution of goat anti-rabbit immunoglobulin G that was labeled with 30-nm colloidial gold particles. Sections were then washed five to seven times for 5 minutes each with TBS containing 0.1% Triton X-100 and twice with TBS alone and then counterstained with uranyl acetate (1 minute) and lead citrate (30 seconds). Sections were air-dried under vacuum, carbon-coated, and examined with a Hitachi H 600 electron microscope at 75 kV.

**Results**

**aFGF in the Fetal and Neonatal Myocardium**

Developmental changes in aFGF immunoreactivity are shown in Figure 1. aFGF immunoreactivity was found throughout the ventricle of fetal rat hearts between day 14 of gestation (not shown) and term (day 21), as indicated in Figure 1A. The staining pattern at the light level suggested that the myocytes contained intracellular aFGF immunoreactivity, which was also detected as surrounding immunoprecipitates. As the heart matures, the presence of aFGF immunoreactivity wanes such that at 1 week of age (Figure 1B) the intensity of the staining of the ventricular myocyte begins to diminish, yet most of the myocytes remain moderately immunoreactive. By the second week of age (Figure 1C), the intensity and magnitude of the staining declines markedly, such that by 3-4 weeks of age (Figure 1D) only small pockets of immunoreactive myocytes and possibly some nonmyocytes could be detected throughout the ventricle. The relatively rapid...
change in immunoreactive intensity with ventricular maturation is consistent with a regulatory role for aFGF in both fetal and neonatal heart development.

To better localize the aFGF immunoreactivity to the myocytes and/or their surrounding ECM, immunoelectron microscopy was performed (Figures 2 and 3). In the fetal (day 18 of gestation) myocardium (Figure 2), prominent aFGF immunoreactivity was localized to the intracellular compartment of many myocytes (Figures 2C–2F), with both perinuclear and cytoplasmic regions heavily labeled. No prominent nuclear localization of aFGF immunoreactivity was noted. The labeling of the ECM in the fetal myocardium was noted (Figure 2E), yet it did not represent a major site of immunoreactivity. In the maturing 3-week-old neonatal myocardium (Figure 3), the labeling index of the individual myocytes was less intense (Figures 3C and 3D), far more infrequent, and yet often associated with perinuclear sites of biosynthesis. Extracellular sites of immunolabeling were seen with the ECM in the mature myocardium (Figure 3D).

Because of the temporal patterns determined for aFGF immunoreactivity in the fetal and neonatal heart, we wanted to determine by using in situ hybridization that the peptides detected in the ventricular tissue sections were representative of myocyte-derived RNA. This is necessary, since the nonmyocyte populations predominate in both number and proliferative potential in the neonatal and maturing myocardium and may also contribute to the biosynthesis and deposition of immunoreactive FGFs. The hybridization signal for aFGF (like that for flg, see Figures 6, 7A, and 7B) associated with the fetal cardiomyocyte population was extensive, although not exclusive of the nonmyocyte populations (data not shown). When neonatal (7–14-day-old) ventricular tissue was used, hybridization signals for aFGF were more readily detected in the nonmyocyte populations (particularly the endothelial cells), although residual signals were apparent in the myocytes themselves (see Figure 7D). When the mature myocardium was examined (not shown), the adult myocyte and most nonmyocyte populations were generally devoid of aFGF hybridization signals, yet the continued presence of aFGF immunoreactivity and extractable FGF peptides, as shown by others, was from the mature myocyte suggests that the level of aFGF transcripts may be below the limit of detection when either total RNA or in situ hybridizations are used.

**FGF Receptors in the Fetal and Neonatal Myocardium**

Analysis of the expression of the FGF receptors (flg and bek) in these two rat strains has been performed to determine whether FGF receptor expression pattern coincides with that of the development of the heart and is in any way coordinated with the expression of one of its ligands. The coincidental expression of both peptide growth factor and cognate receptor by the same or adjacent cellular population is a necessary prerequisite for an autocrine or paracrine growth regulatory paradigm to be proposed. In agreement with other studies, our analysis of FGF receptor gene expression indicated that only the flg form is expressed in the heart, with no detectable bek expression (not shown). Our results indicated that relatively abundant levels of flg transcripts were detected in the fetal heart (E14–E20). Shortly before and/or after birth, the level of flg transcripts dropped to low undetectable levels. During the second and third postnatal week of development, ventricular expression for flg returned to readily detectable levels that reached an additional “peak” between the fifth and seventh week of age. In the mature myocardium of the two strains examined, limited to undetectable expression was noted (not shown). There did not appear to be any major variations in transcript abundance when comparing SHR and WKY rats over the ages examined, although further analysis may be warranted during embryonic and fetal stages of development.

Localization of the ventricular cell type responsible for the flg expression pattern previously described was determined by both immunohistochemical and immunoelectron microscopic localization as well as in situ hybridization. Immunohistochemical studies using an antisera directed toward the extracellular COOH-terminal domain of the flg receptor indicated that the embryonic and fetal myocyte was avidly stained (Figures 4A and 4B). In concert with the maturational loss of aFGF immunoreactivity, ventricular flg immunoreactivity also declined rapidly, such that in the maturing myocardium flg immunoreactivity was primarily limited to small muscular arterioles and other nonmuscle components, such as capillary endothelial cells (Figures 4C and 4D). Because the resolution of paraffin-embedded materials is limited, confirmation of the cellular localization of the immunoreactivity was extended by use of immunoelectron microscopy and in situ hybridization.

Figure 5 shows the prominent subcellular immunoreactivity by immunoelectron microscopy of the fetal myocytes (Figures 5A–5D) using the flg antisera. flg immunoreactivity was both perinuclear and membrane bound in its distribution, which would coincide with its biosynthesis and subsequent intracellular localization. In support of the immunohistochemistry, immunogold localization of flg immunoreactivity in the neonate was quite limited and was found primarily in the nonmyocyte population, which appeared to have an endothelial-like morphology (Figures 5E and 5F).

In situ hybridization analysis of flg gene expression is shown in Figures 6 and 7. Fetal expression of flg was easily localized over the entire bulk of the ventricular mass by dark-field microscopy (Figure 6A). The 1-week
FIGURE 5. Photomicrographs showing immunoelectron microscopic localization of flg to cell types in the fetal and neonatal myocardium. Ventricular tissue was obtained from fetal Wistar-Kyoto rats (WKY, panel A) and spontaneously hypertensive rats (SHR, panels B–D) at 18 days of gestation and 3-week-old SHR (panels E and F) or WKY rats (not shown), processed, embedded, sectioned, and stained as described in “Materials and Methods.” Nonspecific staining using normal rabbit serum was low to undetectable (not shown, see Figures 2 and 3). Myocyte morphology and myofilament architecture indicated that the localization was within the myocyte population. As would be expected for a cell-surface receptor, cell membrane–localized (panels A–D) immunoreactivity was readily detected (arrowheads). Subcellular sites of localization were often perinuclear (arrows, panels B and C). Immunolocalization of flg immunoreactivity in the neonatal and mature myocardium (not shown) was limited to the nonmyocyte population, which appeared to be endothelial-like in their morphology (panels E and F). These cells contained perinuclear (arrow, panel F) as well as cytoplasmic/cell membrane (arrowheads, panels E and F) immunoreactive sites. Bar, 0.5 μm.

Old myocardium demonstrated very limited hybridization, with the majority of the signal appearing adjacent to the myocytes and following the endothelial lining of the vasculature (Figure 6B). In the 3-week-old myocardium (Figure 6C), labeling remained primarily on the nonmyocyte populations and was often found at high levels on and around major blood vessels, as previously shown histochemically (Figure 4C). In the mature myocardium (Figure 6D), flg expression was greatly reduced, and very limited expression was occasionally found in the nonmus-
cle components, primarily the vascular endothelial cells surrounding the myocyte bundles. Further analysis of the hybridization signal using bright-field microscopy clearly indicated that the fetal myocytes contained flg transcripts (Figures 7A and 7B). As suggested by the dark-field analysis, flg hybridization in the neonatal myocardium was limited primarily to the nonmuscle cells, with the capillary endothelial cells demonstrating intense labeling indexes (Figures 7C, 7E, and 7F). In concert with this cell-type change in flg expression, limited transcript levels for aFGF could still be localized to the myocytes of the neonatal myocardium (Figure 7D).

Discussion

Coordinate expressions of growth factors and their cognate receptors are essential components of an autocrine/paracrine growth regulatory paradigm. In the present report as well as a previous study, we document the selective temporal expression of aFGF and one of its receptors, flg, in the fetal and neonatal myocardium. We have extended these studies by combined immunological and molecular analyses indicating that the fetal and neonatal myocardium are sites of active aFGF biosynthesis and coordinate flg expression. This report has focused on the developing myocardium and the temporal relation of FGF gene and receptor expression to the processes of cardiomyocyte proliferative growth and ventricular capillary angiogenesis.

FGFs, FGF Receptors, and Fetal Heart Development

A recent study by Parlow et al. has indicated that the chick embryo myocardium is avidly stained when both bFGF and FGF receptor antisera (reported to be flg) are used. As previously demonstrated for both rodent and avian hearts, both aFGF and bFGF are localized to atrial and ventricular myocyte populations at various developmental ages as well as in culture. We have confirmed and extended these studies by colocalization of aFGF and one of its receptors, flg, to various ventricular cellular populations at specific developmental ages.

During embryonic and fetal mouse development, both flg and bek transcripts have been localized to the growing myocardium yet at distinct and somewhat overlapping ventricular regions. bek expression has been reported to be most extensively expressed in the endocardial cushion regions of the fetal mouse myocardi-

um. In addition, Peters et al. also report that flg is found in the developing ventricular chamber myocytes, albeit at lower levels than the endocardial bek levels. In contrast, Orr-Uriotrege et al. indicate by in situ hybridization that bek transcripts may be localized to day-14.5 fetal mouse ventricular myocytes, yet their relation, if any, to heart development is not examined. Of the other two reported FGF receptors identified to date (FGF receptor-3 and -4), there is limited data on FGF receptor-3 expression in the heart at any developmental stage. In contrast, Stark et al. have reported that FGF receptor-4 is not expressed at any developmental stage in cardiac muscle. This indicates that additional studies of FGF receptor gene expression are needed to determine if other FGF receptor family members are involved in any facet of heart development. In agreement with the data and hypothesis presented in this report, Partanen et al. have shown fetal human heart to contain flg transcripts, with no detectable bek, FGF receptor-3, or FGF receptor-4 transcripts identified.

We have documented for the first time that the fetal myocyte subcellular compartment contains both aFGF and FGF receptor flg immunoreactive material by immunoelectron microscopy and companion in situ hybridization studies. The coordinate expression of both ligand (aFGF) and receptor (flg) at relative high levels during fetal heart development suggests that locally produced aFGF may modulate myocyte proliferative growth in a direct manner. Kardami has shown that fetal chick and neonatal rat myocytes in culture respond to exogenous bFGF stimulation by increased cellular proliferation and thymidine incorporation. In agreement with this observation, our studies of the direct effects of multiple growth factors on fetal myocyte growth are underway, and preliminary data indicate that aFGF is the most potent growth-promoting agent for both fetal (day 16 of gestation) and early neonatal (day 2) myocytes in culture (data not shown).

FGFs, FGF Receptors, and Fetal to Neonatal Period of Heart Development

The “transition” period of heart development (between birth and 2 weeks of age) is a unique period of cellular and tissue remodeling, which comprises myocyte proliferative cessation, nonmyocyte/vascular growth, ECM deposition, myocyte maturation, and cellular hypertrophy. The coordinate loss of both aFGF immunoreactivity and flg immunoreactivity and gene expression during the first 2 postnatal weeks of heart development may partially explain the limited proliferative potential of the neonatal myocyte. When coupled with the neonatal increase in ventricular transforming growth factor-β1 and -β2 gene expression (agents with pronounced antiproliferative potential) and a corresponding increase in ECM components, a diminished FGF mitogenic stimulus and loss of appropriate myocyte flg receptors could be important facets of the cellular/biochemical and molecular mechanism(s) regulating myocyte proliferation. Previous studies have clearly indicated that skeletal muscle terminal differentiation and exit from the cell cycle is associated with the loss of FGF receptors. In the chick heart, FGF receptor density has also been shown to be nearly undetectable in the near term hatching when myocyte proliferation is complete.

FGFs, FGF Receptors, and Neonatal Capillary Angiogenesis

aFGF immunoreactivity and flg gene expression persisted beyond the limits of the neonatal heart “transition” period of development. In addition, the cellular populations that appeared to retain flg expression in the mature heart were not the cardiomyocytes. These results suggest that expression of aFGF and flg may play additional roles in heart growth and remodeling that are not directly related to myocyte growth or hypertrophy. Capillary angiogenesis is a major component of neonatal heart growth and ventricular remodeling. Ventricular myocyte-derived aFGF may be “released,” by unknown mechanism(s), into the ECM of the neonatal heart and facilitate the growth of the vasculature and nonmuscle cells of the heart. This is suggested by the demonstration of flg expression in the nonmuscle cells of the maturing myocardium and the long-term pres-
FIGURE 6. Dark-field microscopy showing in situ hybridization analysis of flg transcripts during heart development. Ventricular tissue was isolated from a fetal Wistar-Kyoto rat (WKY, day 14 of gestation, panel A), a 1-week-old neonatal spontaneously hypertensive rat (SHR, panel B), a 3-week-old neonatal WKY rat (panel C), and an adult (>4 months of age) WKY rat (panel D). Hybridization conditions were as described in “Materials and Methods.” Dark-field images of the fetal myocardium (not shown at multiple ages) in both strains showed high transcriptional levels in the myocytes (m) throughout the ventricle. Neonatal and adult myocardium showed very limited hybridizations, with the majority detected in nonmyocyte populations. In the 1-week-old (panel B) and 3-week-old (panel C) samples, the myocytes demonstrated very limited hybridizations, with the majority of the signal generated by small cells adjacent to muscle bundles or in a capillary-like arrangement (white arrowhead, panel B) or larger muscular arterioles (panel C). In the adult, very low levels of near nonspecific labeling was routinely seen (panel D). Bar, 10 μm.
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FIGURE 7. Bright-field microscopy showing in situ hybridization analysis of flg and acidic fibroblast growth factor transcripts during heart development. Ventricular tissue was isolated from a fetal Wistar-Kyoto rat (WKY, panel A) and a spontaneously hypertensive rat (SHR, panel B) at day 14 of gestation, a 1-week-old neonatal WKY rat (panels C and D), a 2-week-old neonatal SHR (panel E), and a 3-week-old neonatal SHR (panel F). Hybridization conditions were as described in "Materials and Methods." Bright-field images of the fetal myocardium of both strains (panels A and B) at multiple ages (not shown) clearly indicated that the myocytes, as well as some nonmyocytes, contained flg transcripts. As shown for ventricular flg expression by Northern blot hybridization, myocyte hybridizations for flg in the 1-week-old neonate were minimal, with the major cell types labeled being the capillary endothelial cells (arrow, panel C). In contrast, the hybridization signal for acidic fibroblast growth factor remained at detectable levels and was found to be on the myocytes (M) as well as on some nonmyocytes (panel D). As the heart matures, flg hybridization remains primarily over the capillary endothelial cells (arrows), and the myocytes remain as a low to undetectable cell type (panels E and F). Bar, 10 μm.

ence of aFGF immunoreactivity in the myocyte and its ECM by both immunohistochemical and immunoelectron microscopic techniques.

The regulated expression pattern of flg in the neonatal and maturing myocardium correlates well with morphological evidence, demonstrating that this is the major period (maximum at 6–7 weeks of age) of active capillary angiogenesis in both the SHR and WKY rat strains.44 The loss of flg hybridization and limited aFGF immunoreactivity in the adult/mature myocardium concurs with previous reports on adult myocyte localization of both aFGF and bFGF22 as well as the limited proliferative growth of the vascular bed in the mature myocardium.46 Using poly-A⁺-enriched RNA, Sullivan and Storch45 have reported that adult rat heart RNA contains transcripts for aFGF. In agreement with our data on flg expression in the maturing myocardium, Speir et al.46 have reported that isolated adult cardiomyocytes are devoid of flg expression yet begin to reexpress flg when placed into culture.
Caveats of Ventricular FGF Receptor Analysis

A confounding variable to the expression patterns detected by both in situ and Northern blot hybridization analyses of FGF receptors by ourselves and others is that multiple alternatively spliced transcripts of the FGF receptor family members have been reported.\(^{41-49}\) Variations in extracellular immunoglobulin-like domains may selectively influence receptor affinity for specific members of the FGF family of heparin-binding growth factors. Possible flg receptor isoform variations may be related to the variability in cell culture responsiveness of the neonatal myocyte to aFGF and bFGF. The best example is the dichotomy of aFGF and bFGF in the modification of cardiomyocyte actin isoform gene expression.\(^{50}\) Preliminary data indicate that fetal cardiomyocytes in culture also respond in a selective manner to aFGF versus bFGF with respect to protein and DNA synthetic responsiveness (data not shown). A recent report by Bernard et al.\(^{51}\) has indicated that mouse heart contains transcripts for both a two and three immunoglobulin-like domain flg receptor. Therefore, further studies are needed to evaluate the splicing variants of flg that may be expressed during heart development, as well as their cellular localization.

Another variable of the cardiac FGF receptor examinations to date is the lack of information concerning the expression of the low-affinity proteoglycan FGF receptor syndecan. Previous studies have indicated that syndecan is not expressed in the adult myocardium\(^{52}\) yet appears to be required/beneficial for FGF binding to high-affinity receptors and signal transduction.\(^{53}\) Preliminary data indicate that transcripts for syndecan are only detected in the fetal myocardium (day 14 of gestation) and at very low to undetectable levels in the two strains examined (data not shown). Nevertheless, recent reports have shown that there are now two additional members of the syndecan family,\(^{54,55}\) with transcripts for one member (N-syndecan) found at detectable levels in the neonatal heart.\(^{56}\) Further studies are warranted to examine the relation, if any, between expression of any member of the syndecan family and heart development.

Conclusions

Although compelling, direct evidence of the hypothesized scheme of ventricular myocyte autocrine/paracrine control of fetal and neonatal myocyte proliferation and subsequent vascular angiogenesis remains to be shown.\(^{5}\) Further in vitro studies are needed to completely document the direct and/or indirect role that FGFs and their receptors may play in heart development, as manifested by myocyte proliferation and associated changes in gene expression. Significant insight into the regulatory influence FGFs may play in the heart await the formation of transgenic animals overexpressing FGF members and/or their cognate receptor variants in the ventricular compartment at distinct developmental periods as well as in response to specific hypertrophic stimuli.

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