Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts

Farah Sheikh, David P. Sontag, Robert R. Fandrich, Elissavet Kardami, and Peter A. Cattini

Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts. Am J Physiol Heart Circ Physiol 280: H1039–H1050, 2001.—We generated transgenic (TG) mice overexpressing fibroblast growth factor (FGF)-2 protein (22- to 34-fold) in the heart. Chronic FGF-2 overexpression revealed no significant effect on heart weight-to-body weight ratio or expression of cardiac differentiation markers. There was, however, a significant 20% increase in capillary density. Although there was no change in FGF-receptor-1 expression, relative levels of phosphorylated c-Jun NH2-terminal kinase and p38 kinase as well as of membrane-associated protein kinase C (PKC)-α and total PKC-ε were increased in FGF-2-TG mouse hearts. An isolated mouse heart model of ischemia-reperfusion injury was used to assess the potential of increased endogenous FGF-2 for cardioprotection. A significant 34–45% increase in myocyte viability, reflected in a decrease in lactate dehydrogenase released into the perfusate, was observed in FGF-2 overexpressing mice and non-TG mice treated exogenously with FGF-2. In conclusion, FGF-2 overexpression causes augmentation of signal transduction pathways and increased resistance to ischemic injury. Thus, stimulation of endogenous FGF-2 expression offers a potential mechanism to enhance cardioprotection.

FGF-2 and FGFR-1 play vital roles in the early stages of growth and development of the heart and vasculature (4, 19). In addition, ablating the endogenous FGF-2 gene in a genetic mouse model resulted in a hypotensive phenotype with decreased cardiac vascular tone (9, 47). There is also growing evidence that FGF-2 may play an important role in the response to cardiac injury. FGF-2 is reported to induce hypertrophy in neonatal rat cardiac myocytes in vitro (35), but the situation in vivo is less clear because there are no gain-of-function models to directly assess the effects of increased FGF-2 in the heart. There is significant evidence, however, implicating FGF-2 with cardioprotection. Addition of FGF-2 to neonatal rat cardiac myocyte cultures treated with hydrogen peroxide or starved for serum resulted in improved cell survival (18). Exogenous FGF-2 addition before ischemic injury in various heart ischemia-reperfusion models resulted in an increase in functional recovery in the rat heart (6, 33, 34). Increasing FGF-2 levels also stimulated myocardial function in ischemic porcine, canine, and human hearts through increased angiogenesis and systolic function (11, 22, 43, 45). It is not known, however, if FGF-2 affects myocyte viability, specifically, in the mouse heart. This information is essential given the importance that genetically altered mice now play in studies of heart function. Furthermore, this information would facilitate the use of genetic approaches in assessing whether stimulating endogenous FGF-2 levels would allow prolonged and increased FGF-2 release from intracellular pools during contractions. This may be important for maintaining healthy myocardium or limiting the extent of injury.

In this study, we generated two transgenic mouse lines by using the FGF-2 cDNA under the control of the Rous sarcoma virus (RSV) promoter as a transgene. We confirmed the overexpression of FGF-2 in the heart. These lines were used to assess the effect of chronic FGF-2 production on cardiac growth, expression of genes [atrial natriuretic factor (ANF) and myosin heavy chain (MHC) isoforms] coding for markers of cardiac differentiation, FGFR-1 levels, kinases that are downstream targets for FGF-2 signaling, and blood vessel density. We also established an isolated mouse heart preparation (Langendorff) and used it to assess the possible cardioprotective effects of increased “endogenous” FGF-2 production on global ischemia-reperfusion injury. Effects on both contractile recovery and...
muscle cell damage were investigated. These results are discussed in terms of the exposure of the myocardium to FGF-2 and the mode of FGF-2 delivery, specifically, endogenous versus exogenous.

**MATERIALS AND METHODS**

**Animals.** Two homozygous transgenic (TG) mouse lines (no. 5318 and no. 5323) were generated by pronuclear injection of CD-1 mouse eggs with a modified rat FGF-2 cDNA coding specifically for 18-kDa FGF-2 (RSVp.metFGF linearized with SacI) (36, 37). Age-matched CD-1 mice were used as non-TG controls. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care.

**RNA and protein blotting.** RNA and protein blotting were performed as described previously (16, 40). FGF-2 (3), FGFR-1 (40), ANF cDNAs (30), as well as α-MHC and β-MHC (39) oligonucleotides (5′-CTCTGGAGAGGTTAT-TCTCTG-3′ and 5′-TGCAAGCTCAGTCTGAGGC-3′, respectively) were used to probe RNA (50 ng). Immunodetection of FGF-2 in cardiac heparin-binding protein was performed by using mouse monoclonal antibodies (1 µg/ml, Upstate Biotechnology; Lake Placid, NY) followed by horseradish peroxidase-conjugated anti-mouse Ig (Bio-Rad Laboratories; Hercules, CA). Cytosolic and membrane fractions from hearts were extracted as described previously (33). Immunodetection of the 54-kDa c-Jun NH2-terminal kinase (JNK) and 38-kDa p38 kinase was performed in cardiac cytosolic fractions by using rabbit polyclonal antibodies to 1) phospho-stress-activated protein kinase (SAPK)/JNK (Thr^183/Tyr^185), which detects the dually phosphorylated isoforms of all three SAPK/JNKs (1:1,000, New England Biolabs; Mississauga, ON, Canada); 2) SAPK/JNK, which detects total SAPK/JNK levels (1:1,000, New England Biolabs); 3) phospho-p38 mitogen-activated protein (MAP) kinase (Thr^180/Tyr^182), which detects the dually phosphorylated isoform of p38 (1:1,000, New England Biolabs); and 4) p38 MAP kinase, which detects total p38 MAP kinase (phosphorylation-state independent) levels (1:1,000, New England Biolabs). Immunodetection of the 82-kDa protein kinase C (PKC)-α and the 90-kDa PKC-ε in cardiac cytosolic and membrane fractions was performed by using rabbit polyclonal antibodies to the carboxyl terminus of PKC-α (1:200, Santa Cruz Biotechnology; Santa Cruz, CA) or the carboxyl terminus of PKC-ε (1:200, Santa Cruz Biotechnology). All rabbit polyclonal antibodies were followed by horseradish peroxidase-conjugated anti-rabbit Ig (Bio-Rad Laboratories). Results were visualized by using enhanced chemiluminesence (Pierce; Rockford, IL). Autoradiographs from RNA and protein blots were assessed by densitometry.

**Immunofluorescence microscopy.** Mouse hearts were excised, blocked dry to remove blood, placed in TissueTek OCT compound (Miles Laboratories; Elkhart, IN), immediately frozen on dry ice, and then cut into 7-µm thin cryosections. Sections were fixed in 1% paraformaldehyde-PBS for 15 min at 4°C. To detect FGF-2, sections were incubated overnight at 4°C in 1% BSA-PBS containing specific and well-characterized rabbit polyclonal FGF-2 antibodies (16, 17, 1:1,000) and counterstained with either mouse α-actin (1:400, Sigma), mouse α-smooth muscle actin (1:200, Sigma), and goat collagen IV (1:40, Southern Biotechnology Associates; Birmingham, AL) antibodies to detect muscle, smooth muscle-containing blood vessels, and extracellular matrix. To detect endothelial cells (capillaries), sections were incubated overnight at 4°C in 1% BSA-PBS containing rabbit human von Willebrand factor antibodies (1:100, Sigma). Normal rabbit or mouse Ig were substituted for primary antibodies at equivalent dilutions as controls. Sections were then incubated with biotinylated donkey rabbit Ig (1:50, Amersham; Arlington Heights, IL) antibodies in 1% BSA-PBS for 1.5 h at room temperature. Subsequently, sections were incubated overnight at 4°C with FITC-streptavidin conjugate (1:20, Amersham) and Texas Red conjugated donkey anti-mouse Ig (1:20, Amersham) or Texas Red conjugated donkey anti-goat Ig (1:20, Jackson Immunoresearch Laboratories; Westgrove, PA) antibodies in 1% BSA-PBS. For counterstaining of nuclei, sections were incubated for 5 min with 0.0125% Hoechst-33342 in PBS and then mounted in mounting medium (Crystal/Mount, Biomedia; Foster City, CA) and examined by epifluorescence.

**Langendorff perfusion apparatus.** Adult mice were euthanized by cervical dislocation, and their hearts were excised and perfused by using a retrograde Langendorff method (31). The ascending aorta was cannulated by using a 21-gauge needle and tied with a 6-0 silk suture and perfused within 5 min of excision. The perfusate, consisting of a Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, 10 glucose, 24 NaHCO3, and 3% BSA (Roche Molecular Biochemicals; Laval, QC, Canada), was bubbled with 95% O2-5% CO2 (pH 7.4, 37°C) under nonrecirculating conditions at a constant pressure of 60 mmHg. The atria were removed and a KH-filled latex balloon was inserted into the left ventricle through the mitral valve. This allows monitoring of systolic left ventricular pressure, defined as developed pressure, and left ventricular end-diastolic pressure (EDP) using a Digimed Heart Performance Analyzer (Micro-Med; Louisville, KY). In addition, a thermocouple was inserted into the right ventricle to monitor the temperature of the KH in the heart, which was kept at 37°C. All hearts were electrically paced by using platinum electrodes placed on the top of the right ventricle with 1-ms pulses at 6 Hz and 3 volts throughout the experiment. Preload in all hearts was adjusted to achieve maximal developed pressure while maintaining a positive EDP (2–5 mmHg) to monitor for balloon integrity. Only hearts demonstrating a minimal developed pressure of 70 mmHg and stable EDP were utilized for experimentation.

The experimental protocol to assess injury in the mouse model was adopted from a previously established rat-heart Langendorff preparation (33). After an equilibration period of 30 min, hearts were subjected to 30 min of global ischemia by turning off flow of perfusion medium to the heart. Perfusion was restored after 30 min of ischemia, and continued for 60 min. The volume of perfusate during 1-min periods was collected from TG and non-TG adult mouse hearts at various time points during the period of preischemia and ischemia-reperfusion. Time points include preischemia (30-min equilibration time) and reperfusion time points of 1, 5, 10, 15, 30, 45, and 60 min. The coronary flow rate was determined by measuring the volume of perfusate collected during 1-min periods before ischemia (30-min equilibration time) and during reperfusion (1, 5, 10, 15, 30, 45, and 60 min) and by normalizing these values to heart weight (in ml·min⁻¹·g⁻¹). For exogenous FGF-2 studies, hearts were equilibrated for 25 min with KH and then supplemented with either vehicle (20 mM Tris·HCl pH 7.9, 0.5 M NaCl, 10% glycerol, 260 mM imidazol, 5 mM β-mercaptoethanol, and 1 mM EDTA) or 10 µg of recombinant rat FGF-2 (33, 34) dissolved in 1 ml KH for 2 min followed by KH solution for 3 min, before 30-min global ischemia and 60-min reperfusion.

To extract FGF-2 from the extracellular matrix using the Langendorff preparation, hearts were equilibrated for 20 min with KH and then perfused for 5 min with a high salt buffer...
(1.6 M NaCl, 10 mM Tris pH 7.0) followed by KH solution for 5 min. Hearts were then sectioned and processed for immunofluorescence microscopy as described above.

**Lactate dehydrogenase assay.** Perfusates from mouse hearts were collected on ice for 1 min at various time points before and during ischemia-reperfusion. The time points include: before ischemia (30-min equilibration) and postischemic times of 1, 5, 10, 15, 30, 45, and 60 min. Quantitative kinetic determination of lactate dehydrogenase (LDH) activity in perfusates was assessed according to the kit manufacturer's instructions (LDH Optimized, Sigma). LDH activity was normalized for coronary flow rate and heart weight (in g heart wt -1).

**FGF-2 ELISA.** Perfusates from mouse hearts were collected for 1 min at various times during the equilibration period (before ischemia). Blood was collected from euthanized adult mice and was allowed to clot for 30 min before centrifugation at 10,000 g for 30 min to collect serum. Quantitative determination of FGF-2 in perfusates and serum was assessed by using a Quantikine HS human FGF basic immunoassay (R&D Systems; Minneapolis, MN) as described previously (40). FGF-2 in perfusates was normalized for coronary flow rate and heart weight (in g heart wt -1).

**Statistical analysis.** Data are presented as means ± SE. Student t- (parametric) or Mann-Whitney and alternate Welch t- (nonparametric) tests were used for statistical analysis. A P < 0.05 was considered significant.

### RESULTS

The FGF-2 transgene is expressed in striated muscle. To assess the level and range of transgene expression, RNA (50 μg) was isolated from various tissues of FGF-2 TG mice and examined by RNA blot analysis. A 1.3-kb transcript, consistent with expression of the FGF-2 transgene, was detected in cardiac and skeletal muscle tissue, but not in lung, brain, kidney, spleen, or liver (closed arrowhead, Fig. 1A). Transcripts from the FGF-2 transgene were observed in both cardiac atria and ventricles. Although endogenous FGF-2 mRNA was too low to be detectable in cardiac tissue, it was detected in the lung, brain, and liver as indicated by a 6.1-kb transcript (open arrowhead, Fig. 1A). Overexpression of FGF-2 in the cardiac (ventricle) muscle of both FGF-2 TG lines was confirmed by protein blot analysis using specific monoclonal FGF-2 antibodies. On the basis of densitometry, levels of FGF-2 protein were increased about 22- and 34-fold (n = 6–9) in the no. 5318 and no. 5323 lines, respectively (Fig. 1B). An assessment of serum FGF-2 levels in the no. 5318 (1.75 ± 0.8 ng/ml, n = 4) and no. 5323 (2.23 ± 0.52 ng/ml, n = 5) lines was not significantly different from non-TG mouse values (1.35 ± 0.32 ng/ml, n = 5) in 3-mo-old adults.

Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG and TG mouse hearts using specific polyclonal antibodies to FGF-2 (Fig. 2; 15, 16). Ventricular tissue was triple labeled for FGF-2, DNA, and either α-actinin or α-smooth muscle actin, to specifically identify myocytes or smooth muscle cells (blood vessels) staining for FGF-2. In the case of non-TG mice, nuclei and cytoplasm of cardiac myocytes were stained specifically for FGF-2 at levels clearly above the background observed with control Ig (Fig. 2, A and B). No FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 2B). In contrast, cardiac myocytes from TG mice were stained uniformly and more intensely for FGF-2. We also observed the accumulation of specific FGF-2...
staining surrounding the cardiac myocytes (Fig. 2, A and B). Again, no FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 2B).

Heart weight-to-body weight ratio is unchanged in the FGF-2 TG mouse. Adult mice (9–12 wk) and their excised hearts, with atria removed, were weighed to determine heart weight-to-body weight ratios (μg/g). There was no significant difference between the heart weight-to-body weight ratios for FGF-2-treated TG lines no. 5323 (5.75 ± 0.51 μg/g, n = 4) and no. 5318 (4.97 ± 0.27 μg/g, n = 5) and non-TG (5.14 ± 0.18 μg/g, n = 10) mice. In addition, we used RNA blotting to compare the expression of the cardiac differentiation markers ANF, α-MHC, and β-MHC in FGF-2 TG (no. 5323, highest FGF-2 expressing line) and non-TG mouse ventricles. Expected transcript sizes of 0.9 and 6.0 kb for ANF and α-MHC, respectively, were observed (Fig. 3). On the basis of densitometry (n = 4), there were no significant differences in ANF and α-MHC RNA levels in TG versus non-TG mouse ventricles. Although β-MHC transcripts (6.0 kb) were detected in embryonic mouse heart RNA (data not shown), no expression, and thus difference, was detected in either FGF-2 TG or non-TG adult mouse ventricles due, presumably, to low abundance. FGF-2 transgene expression was also confirmed in these RNA samples by detection of the 1.3-kb FGF-2 (transgene) transcript (Fig. 3). An assessment of the 4.3- and 4.1-kb FGFR-1 RNA levels showed no difference in FGF-2 TG versus non-TG mouse hearts (Fig. 3).

Capillary density is increased in the FGF-2 TG mouse heart. The density of blood vessels was estimated in cardiac ventricular sections from FGF-2 TG versus non-TG hearts (n = 4) by staining for α-smooth muscle actin or for capillaries with von Willebrand factor. For smooth muscle-containing blood vessels, four fields (1.1 mm² by using ×20 objective) from three sections from each of four FGF-2 TG and four non-TG mice were counted. Similar values of 19.5 ± 0.4 blood vessels/mm² and 20.7 ± 1.4 blood vessels/mm² were
obtained for FGF-2 TG and non-TG mice, respectively. To assess capillary density, 40 fields (0.02 mm² by using ×40 objective) from four sections from four FGF-2 TG and four non-TG mice were counted. The value for capillary density was increased significantly (about 1.2-fold) from 1,866 ± 169 capillaries/mm² in non-TG to 2,297 ± 52 capillaries/mm² in TG mouse hearts (P < 0.05).

Relative levels of JNK, p38 kinase, and PKC are increased in FGF-2 TG mouse hearts. Stress-activated MAP kinases (JNK and p38) and PKC isoforms are known downstream targets of FGF-2 signaling (11, 22, 24, 26, 31). The relative levels of these kinases in FGF-2 TG (line no. 5323) versus non-TG mouse hearts (n = 3) were assessed in membrane and/or cytosolic fractions by protein blotting. For JNK and p38 kinase, antibodies to both phosphorylated (active) and phosphorylation-state independent (active + inactive) forms were used to probe cytosolic protein (Fig. 4A). On the basis of densitometry, levels of phosphorylated JNK and p38 were increased about 14- and 42-fold, respectively, in TG mouse hearts (P < 0.05, n = 3). There was no significant difference, however, in the "total" levels of JNK and p38 kinase in TG versus non-TG mouse hearts. For PKCs, relative levels of membrane-associated PKC-α were significantly increased about 15-fold in TG mouse hearts (P < 0.05, n = 3), but cytosolic levels were unchanged (Fig. 4B). In contrast, cytosolic levels of PKC-ε were increased significantly about twofold in TG mouse hearts (P <
0.05, n = 3), however, membrane-associated levels were unchanged (Fig. 4B).

Characterization of the isolated mouse heart (Langendorff) preparation. The stability of our mouse Langendorff preparation was determined in isolated non-TG mouse hearts throughout a 2-h period. Both contractile force and percent cell damage as measured by developed pressure and LDH activity, respectively, were assessed. Representative profiles are shown for two non-TG mice and reveal stable developed pressures. 70 mmHg (Fig. 5A). Perfusates taken from these same non-TG mouse hearts showed no significant changes in LDH release from baseline at various time points throughout the 2-h period (Fig. 5B). On the basis of the stability of our preparation during a 2-h period and existing protocols for the rat Langendorff preparation, an experimental protocol for myocardial injury was devised (Fig. 5C).

Distribution of FGF-2 in isolated and perfused TG versus non-TG mouse hearts. Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG and TG-perfused mouse hearts after 30-min equilibration (Fig. 6). Cardiac myocytes from “equilibrated” non-TG mouse hearts displayed the same predominantly nuclear and weaker cytoplasmic FGF-2-staining (Fig. 6A) as seen in “freshly” isolated non-TG mouse hearts (Fig. 6A). In the case of FGF-2 TG mouse hearts, FGF-2 was localized predominantly around cardiac myocytes, and intracellular staining for FGF-2 was more intense than seen in non-TG cardiac myocytes (Fig. 6A). No FGF-2 staining of smooth muscle cells/blood vessels was observed in either non-TG or TG mouse hearts (Fig. 6B).

Ventricular tissue sections from “equilibrated” FGF-2 TG mouse hearts were triple labeled for FGF-2, DNA, and collagen IV as a marker for the basal lamina and extracellular matrix. In addition to nuclear staining (arrows), we observed the accumulation of specific FGF-2 staining surrounding the cardiac myocytes, which colocated with collagen IV staining (Fig. 7A). Immunofluorescence microscopy of ventricular sections from “equilibrated” FGF-2 TG mouse hearts perfused with a high salt buffer resulted in the loss of FGF-2 staining from the extracellular matrix (Fig. 7B). In contrast, nuclear FGF-2 staining was still evident (Fig. 7B, arrows).

Given the accumulation of FGF-2 in the extracellular matrix, the effect of FGF-2 overexpression on release of FGF-2 from isolated TG mouse hearts was assessed. The level of FGF-2 release from non-TG and FGF-2 TG mouse line no. 5323 was measured by ELISA of perfusates collected at various time points. A decrease in FGF-2 release over the 30-min equilibration time was detected for both non-TG and FGF-2 TG mouse hearts (Fig. 8). The level of FGF-2 release, however, was consistently (about 2.5-fold) higher from FGF-2 TG hearts throughout the equilibration period (Fig. 8).

Decreased myocyte damage is observed in FGF-2 TG mouse hearts after injury. To examine resistance to injury, isolated non-TG and FGF-2 TG mouse hearts from both lines were subjected to global ischemia-reperfusion injury (Fig. 5C). Myocardial performance
of both FGF-2 TG and non-TG mouse hearts were measured as percent left ventricular contractile recovery in developed pressure after reperfusion. Absolute values obtained for developed pressure just before ischemia were used to represent maximal recovery and arbitrarily set to 100% (see legend of Fig. 9). FGF-2-treated TG mouse hearts displayed no significant difference in contractile recovery after 30, 45, and 60 min of reperfusion compared with non-TG hearts (Fig. 9, A and B). In contrast, a significant decrease in perfusate LDH activity and thus increase in cardiac myocyte viability, was observed at the 30-, 45-, and 60-min reperfusion time points for the FGF-2 TG line no. 5318 as well as 1-min and 60-min time points for the no. 5323 line (Fig. 9, C and D). When the total LDH release/activity was assessed throughout 60 min of reperfusion, the decreases (and thus increases in cell viability) were highly significant for both the no. 5318 (38%, \( P < 0.0001, n = 28-41 \)) and no. 5323 (45%, \( P < 0.0001, n = 28 \)) FGF-2 TG mouse lines.

**Exogenous addition of FGF-2 increases contractile function and myocyte viability in the mouse heart.** Exogenous FGF-2 addition increases both contractile recovery and myocyte viability in the isolated rat heart after injury (33, 34). Thus it was possible that the lack of improved cardiac function in the isolated FGF-2 TG mouse hearts after injury may reflect a difference between endogenous (transgenic) and exogenous delivery of FGF-2 or a species (mouse versus rat)-related effect. To address this, we determined the effect of exogenous FGF-2 (10 \( \mu \)g) or vehicle on isolated non-TG mouse hearts subjected to global ischemia-reperfusion injury (Fig. 5C). Myocardial performance of both FGF-2- and vehicle-treated hearts was measured as percent left ventricular contractile recovery in developed pressure at 30, 45, and 60 min of reperfusion. The absolute values obtained for developed pressure in FGF-2- and vehicle-treated hearts before ischemia were 88.6 \( \pm \) 5.3 (\( n = 4 \)) and 90.4 \( \pm \) 2.1 mmHg (\( n = 4 \)), respectively. These absolute values were used to represent maximal recovery and arbitrarily set to 100%. The contractile recovery increased from 34.3 \( \pm \) 3.9, 43.5 \( \pm \) 4.3, and

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**Fig. 6.** Subcellular distribution of FGF-2 in isolated non-TG and FGF-2 TG mouse hearts after 30-min equilibration by double immunostaining for FGF-2 and muscle proteins as well as cytochemical staining for DNA. Ventricle sections were triple stained for FGF-2, \( \alpha \)-actinin, and DNA (A) or FGF-2, \( \alpha \)-sm actin, and DNA (B). Low levels of endogenous FGF-2 staining are observed in nuclei of cardiac myocytes (white arrows, A). FGF-2 TG mouse hearts show strong FGF-2 staining in the cytoplasm and extracellular spaces of cardiac myocytes (A and B). FGF-2 staining of smooth muscle cells was not observed in FGF-2 TG mouse hearts. The bar in A is equivalent to 75 \( \mu \)m.

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**Fig. 7.** Subcellular distribution of FGF-2 in isolated FGF-2 TG mouse hearts after 30-min equilibration by double immunostaining for FGF-2 and an extracellular matrix protein as well as cytochemical staining for DNA. Ventricle sections were triple stained for FGF-2, collagen IV, and DNA in the absence (A) or presence (B) of 5-min high salt perfusion. Intense FGF-2 staining in the extracellular spaces of cardiac myocytes was observed (A). FGF-2 TG hearts perfused with high salt resulted in a loss of FGF-2 staining in areas surrounding cardiac myocytes or extracellular matrix (B). Examples of nuclei stained for FGF-2 are indicated with arrows. The bar is equivalent to 75 \( \mu \)m.
Cardiac cellular damage in FGF-2- and vehicle-treated hearts was assessed by measuring LDH release in perfusates at various time points during ischemia-reperfusion (Fig. 10A). Mouse hearts treated with FGF-2 showed a significant (34%) reduction in LDH release at 15, 30, 45, and 60 min of reperfusion compared with vehicle-treated hearts (Fig. 10B).

Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG mouse hearts treated with exogenous FGF-2 (Fig. 11). FGF-2 was localized to the nuclei and cytoplasm of cardiac myocytes and extracellular spaces (Fig. 11). Unlike FGF-2 TG hearts (Fig. 6), intense FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 11).

DISCUSSION

We generated TG mice overexpressing 18-kDa FGF-2 and established an isolated mouse heart Langendorff preparation to assess the potential cardioprotective effect of increased “endogenous” FGF-2. Novel findings presented in this paper are that the stimulation of “endogenous” production of FGF-2 as achieved in the FGF-2 TG mouse hearts resulted in increased:

- FGF-2 release
- FGF-2 in apparent association with the extracellular matrix (basement membrane), capillary density, activity of downstream kinases (JNK, p38, PKC-α)
- Resistance of the myocardium to ischemia-reperfusion injury.

We suggest that myocardial protection in FGF-2 TG mice is, at least in part, a reflection of a direct effect of FGF-2 on cardiomyocytes, including the activation of stress MAP kinases and PKC-α. Our data support the notion that stimulation of endogenous
FGF-2 expression could provide a strategy for improving cardiac resistance to injury.

To generate FGF-2 TG mice, we used the RSV promoter resulting in preferential overexpression in striated muscles (Figs. 1 and 2), which is consistent with previous reports (5, 13). The effects of overexpression would be expected to manifest locally at the tissue level and not systemically, and this was confirmed by the absence of significant changes in serum FGF-2 levels. It is widely accepted that FGF-2 released from cells is retained by the extracellular matrix and its specialized component, the basement membrane. The basement membrane has been proposed to act as a dynamic reservoir for FGF-2 and the extent of FGF-2 diffusion from the membrane is dependent on FGF-2 concentration, whereas physical damage of the basement membrane results in massive release of FGF-2 (7, 10). Binding and release of FGF-2 from the basement membrane is rapid and may not absolutely require matrix degradation to “free” FGF-2 (32). Two lines of evidence presented here support that FGF-2 overexpression was also accompanied by increased FGF-2 release (and thus increased FGF-2 potentially available to cell FGF receptors) in FGF-2 TG mice. This includes the increased anti-FGF-2 immunostaining in apparent association with the basement membrane (Fig. 7) and the increased levels of FGF-2 in the effluent of FGF-2 TG mouse hearts during the 30-min equilibration before ischemia (Fig. 8). Furthermore, the increased intracellular FGF-2 levels, indicated by immunostaining (Fig. 2) and inferred by the 22- and 34-fold increase in total FGF-2 extracted from FGF-2 TG hearts, would be expected to result in increased levels of FGF-2 release both chronically and acutely. In the absence of a signal peptide, endogenous FGF-2 appears to be released from cardiac myocytes on a beat-to-beat basis through contraction-induced transient remodeling of the myocyte plasma membrane under normal physiological conditions.

Fig. 10. Effect of exogenous addition of FGF-2 in mouse hearts on contractile recovery and cellular damage after ischemia-reperfusion injury. A: functional measurement defined as developed pressure for each heart after either vehicle or FGF-2 treatment at time points during reperfusion. Values are expressed as percentage of the corresponding values of the same heart obtained before ischemia (equil). The absolute values measured for developed pressure after 30-min equilibration (before ischemia) in vehicle and FGF-2-treated hearts were 90.4 ± 2.1 (n = 4) and 88.6 ± 5.3 mmHg (n = 4), respectively. B: LDH levels in perfusates from vehicle and FGF-2-treated mouse hearts before ischemia and during reperfusion. All values are presented as means ± SE (n = 4). Asterisks in panels indicate statistically significant differences between the FGF-2-treated hearts compared with vehicle-treated values (*P < 0.05, **P < 0.01).

Fig. 11. Subcellular distribution of FGF-2 in isolated FGF-2-treated mouse hearts after 30-min equilibration by double immunostaining for FGF-2 and muscle proteins as well as cytochemical staining for DNA. Ventricular sections were triple stained for FGF-2, α-actinin, and DNA (A) or FGF-2, α-smooth muscle actin and DNA (B). Intense FGF-2 staining in the cytoplasm and extracellular spaces of cardiac myocytes (A and B), as well as smooth muscle cells was observed (arrowheads, B). The bar in A is equivalent to 75 µm.
conditions (4, 19). Additional FGF-2 is released with increased heart rate and force of contraction (4), and on damage to the cell membrane resulting in the liberation of intracellular stores (19). Thus our data indicate that “loading” cardiac myocytes with FGF-2 through endogenous overexpression does translate into increased levels of release.

Hearts from adult FGF-2 TG and non-TG mice displayed no gross differences as reflected by similar heart weight-to-body weight ratios and density of smooth muscle-containing blood vessels, and expression of cardiac differentiation markers (Fig. 3). These data, therefore, do not provide evidence for a hypertrophic effect on the heart. There was, however, a significant 20% increase in capillary density in FGF-2 overexpressing hearts. It is likely that FGF-2 overexpression resulted in chronically elevated basal levels of local FGF-2 release that would result in increased capillary density in view of the angiogenic properties of FGF-2 (42, 45). It is also possible that FGF-2 overexpression may have caused increased capillary density indirectly, perhaps by inducing expression/release of other angiogenic factors. Whatever the mechanism of capillary induction, an increase in capillary density might be expected to contribute to the increase in myocardial viability during ischemia-reperfusion injury by increasing tissue perfusion in areas of cell damage. However, an increase in capillary density is not required for a positive effect on myocardial viability because similar results were obtained through short-term exposure to FGF-2 via exogenous addition (Fig. 10). Studies in the rat heart indicated that the acute cardioprotective effects of exogenous FGF-2 were not dependent on effects on the vasculature leading to flow modulation (33). Rather, a direct effect of FGF-2 on the adult cardiac myocytes was implied (33). Thus the increase in myocardial viability caused by the increase in endogenous FGF-2 may reflect partly a direct protective effect on the adult myocytes.

Although expression of the high-affinity receptor for FGF-2, FGR-1, was unchanged in TG mouse hearts (Fig. 3), baseline activity levels of downstream targets of FGF-2 signaling such as stress-activated MAP kinases (JNK and p38) and membrane-associated (presumably active) PKC-α were augmented (Fig. 4). The upregulation of active JNK, p38, and PKC-α have all been implicated in ischemic preconditioning, thus suggesting that FGF-2 TG hearts may be in a “preconditioned” and thus “protected” state before injury (20, 29, 38, 46), irrespective of “freshly” released FGF-2. This is consistent with the observed increase in cytosolic PKC-ε, which has also been implicated in FGF-2-induced cardioprotection and ischemic preconditioning and thus may reflect a potential source available for cardioprotection (33, 46).

Despite the increase in FGF-2 levels and resistance of myocytes to injury, there was no significant difference between the contractile recovery seen with FGF-2 TG and non-TG mouse hearts after myocardial injury (Fig. 9, A and B). The lack of improved cardiac function was not expected given the positive effect of FGF-2 overexpression on mouse myocyte viability (Fig. 9, C and D) as well as the enhanced contractile recovery and cell integrity reported for exogenous FGF-2 treatment of isolated rat heart preparations (33, 34). This raised the question of whether the effect of FGF-2 on systolic function might be species related. We showed that this was not the case by using mouse hearts perfused with exogenous FGF-2. There was an increase in contractile recovery (Fig. 10A) and significantly less damage to the myocardium as reflected in decreased LDH levels (Fig. 10B). The level of contractile recovery in the mouse heart (average improvement from 40.9 ± 1.9 to 64.2 ± 1.1%) was less than previously reported (improvement from 34.1 ± 5.1 to 76.4 ± 4.1%) for the effect of FGF-2 in a similar isolated rat heart preparation (31). This may reflect differences in the extent of the damage seen with these species because the mouse myocardium is reported to be more sensitive to changes in calcium concentration than the rat myocardium (2).

Several factors may have contributed to the differences in cardioprotection between exogenously administered and overexpressed endogenous FGF-2. These include the amount of FGF-2 available to the receptors of cardiomyocytes and other cardiac cells and the mode of delivery. Total FGF-2 released from FGF-2 TG and non-TG mouse hearts during the 30-min period before ischemia was about 3 and 1 ng (based on the data presented in Fig. 8), respectively. Although FGF-2 TG cardiomyocytes could be considered exposed to at least three times as much FGF-2 as non-TG cardiomyocytes, the absolute levels may have been insufficient for increased contractile recovery. Infusion of 10 μg FGF-2 in the non-TG perfused hearts on the other hand may have resulted in higher overall levels of exposure to FGF-2, at least for the duration of the experiment. Certainly there is evidence that cardioprotective and angiogenic properties of FGF-2 are dose dependent (11, 22, 26, 34). In terms of mode of delivery, FGF-2 added exogenously was distributed via the blood vessels to the cardiac myocytes, whereas FGF-2 released as a consequence of endogenous overexpression was released by cardiac myocytes into the vessels. Blood vessels were intensely stained for FGF-2 (indicating local retention of this factor) in exogenously treated, but not FGF-2 TG or non-TG mouse hearts (Fig. 6B vs. 11B), a finding consistent with the mode of delivery. It is possible that exogenous administration of FGF-2 resulted in higher exposure and, therefore, protection from injury of a wider range of cells, particularly smooth muscle and endothelial cells of blood vessels, compared with local FGF-2 release from cardiac myocytes.

Our data, in combination with previous reports on the cardioprotective effect of exogenous FGF-2, suggest that FGF-2 expression and release from cardiomyocytes could be viewed as part of a normal process for maintaining a healthy myocardium and part of the response to injury. In this context, α-adrenergic regulation has been proposed to serve as a reserve mechanism and provide a compensatory role in maintaining cardiac responsiveness to catecholamines under patho-
logical conditions such as myocardial infarction (44). We showed recently that FGF-2 promoter activity was increased by α-adrenergic stimulation (8) and implicated the stress-related early growth response-1 protein in this synthetic event (15).

In summary, our data demonstrate that overexpression of FGF-2 in vivo has significant phenotypic effects on the adult heart that might influence its response to injury. Chronic FGF-2 overexpression (associated with increased angiogenesis/capillary density and augmentation of kinases linked with ischemic preconditioning and cardioprotection) and increased acute FGF-2 release are likely contributing to increased cardiac myocyte viability seen after ischemia-reperfusion injury. Therefore, stimulation of endogenous expression of genes implicated with cardioprotection, such as FGF-2, may provide an additional strategy for improving cardiac resistance to injury.

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