Original Article

Enhanced Myocardial Vascularity and Contractility by Novel FGF-1 Transgene in a Porcine Model of Chronic Coronary Occlusion

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Received 29 May 2008; Accepted 30 September 2008

Abstract

Background: *Angiogenesis gene therapy has long been sought as a novel alternative treatment for restoring the blood flow and improving the contractile function of the ischemic heart in selected clinical settings. Angiogenic fibroblast growth factor-1 (FGF-1) is a promising candidate for developing a promising gene therapy protocol due to its multipotent ability to stimulate endothelial cell (EC) growth, migration, and tube formation. Despite these advantages, however, FGF gene therapy has suffered setbacks mainly due to the inefficient delivery rate of the growth factor in vivo. Given the potent angiogenic effect of FGF-1, we reasoned that constitutively synthesized minute quantities of this polypeptide hormone, when empowered with the ability to escape the cellular constraint, could freely act in a paracrine/autocrine fashion on nearby existing capillary plexuses and lead to neovascularization and restoration of the blood flow to ischemic tissues for reparative purpose.*

Methods: *We report the direct gene transfer of a retroviral-based mammalian expression vector encoding a secreted form of FGF-1 (sp-FGF-1) for the purpose of therapeutic angiogenesis into the porcine myocardium subjected to the surgical placement of an ameroid occluder to induce the chronic coronary occlusion of the left circumflex coronary artery (LCx) and regional myocardial ischemia. Coronary angiography, performed 3 weeks after surgery, confirmed the interruption of the blood flow in the LCx distal to the site of ameroid placement.*

Results: *Immunohistochemical analysis using antibody specific to von Willebrand factor (vWF), an endothelial marker, showed a significant increase ($p<0.05$) in myocardial vascularity in the sp-FGF-1 hearts compared to the control (vector alone).*

Importantly, an assessment of the cardiac function by echocardiography, performed 3 weeks after surgery, demonstrated improved cardiac contractility due to increased left ventricular free wall contraction in the sp-FGF-1-treated animals only.

Conclusion: *These results suggest that the intramyocardial delivery of our chimeric secretory FGF-1 gene can enhance vascularity and improve cardiac contractility in a chronic ischemic heart. This protocol may serve useful for developing reparative angiogenesis strategies aimed at improving the pumping function of the ischemic hearts in human patients.*

J Teh Univ Heart Ctr 4 (2008) 197-204

Keywords: *Angiogenesis inducing agents • Fibroblast growth factor 1 • Ischemia • Heart • Gene therapy • Transfection*

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Introduction

Angiogenesis gene therapy is viewed as an alternative treatment for patients with myocardial and peripheral ischemia in whom conventional therapies including antianginal medications, angioplasty, and bypass surgery have failed. The goal of angiogenesis gene therapy is to improve the blood flow to ischemic tissues through the induction of neovascularization by angiogenic agents introduced by gene transfer. Highly promising angiogenic factor candidates are members of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) families.[1-6] However, a major limitation to progress in developing useful angiogenesis gene therapy protocols using FGF and VEGF has been the inefficient delivery of the growth factors to ischemic target sites in animal and human subjects.[7] Efficient synthesis and release of transgene in target tissues is largely determined by the ability of host cells to express the transgene, choice of vector, and route of delivery. To circumvent problems associated with poor in vivo transfection, we developed strategies for therapeutic angiogenesis in an ischemia-induced pig heart model using a novel secreted form of FGF-1 gene construct. For this study, we employed a chimeric version of fibroblast growth factor-1 (sp-FGF-1), composed of a secretory signal peptide (sp) attached to the 5'-end of human FGF-1 gene construct, which was previously shown to exhibit very strong angiogenic properties both in cultured endothelial cells and in the developing chick embryo.[8,9]

In the current study, we set out to test our hypothesis that the direct in vivo gene transfer of sp-FGF-1 into ischemic myocardium leads to increased synthesis and secretion of recombinant FGF-1 protein, acting in autocrine/paracrine modes to induce successful neovascularization and improved cardiac pumping function. The ischemic pig heart model used in this study is a progressive chronic coronary artery occlusion (CCO) model,[10-15] whereby an ameroid occluder is surgically placed around the left circumflex coronary artery (LCx) of a pig followed by direct intramyocardial injections of sp-FGF-1 plasmid DNA into the myocardium residing just distal to the occluder. This model of CCO for sp-FGF-1 gene therapy was selected because porcine and human hearts exhibit many similarities in coronary and collateral physiology/anatomy.[16] For example, normal pigs and humans possess minimal innate coronary collaterals; a sudden blockade of a major coronary artery will typically induce infarction and possibly death.[17] Although progressive chronic coronary artery stenosis/occlusion will enhance the collateral development to ischemic myocardium-at-risk, collateral development remains limited and long-term phenotypic and physiologic changes are observed in pigs that mimic those of human patients with coronary artery disease and CCO. In light of the foregoing discussions, we evaluated the potential application of a novel secreted angiogenic factor FGF-1 (sp-FGF-1) for the design of new angiogenic gene therapies aimed at repairing damaged ischemia-induced porcine heart tissues.

Methods

We have previously described the construction and angiogenic characteristics of sp-FGF-1.[8-9] Briefly, the human FGF-1 open reading frame (ORF) was isolated from a human cDNA library using polymerase chain reaction technique (PCR) and subcloned into the unique SalI and EcoRI DNA restriction enzyme sites of the mammalian expression vector pMEXneo. Subsequently, a 78-base pair oligo (encoding 22 hydrophobic amino acids) corresponding to the published signal-peptide sequence of one of the secretory members of the FGF family known as FGF-4[18,19] was joined in-frame to 5' end of the FGF-1 ORF in the eukaryotic expression vector pMEXneo. We chose the FGF-4 signal peptide sequence in our construct because in its native form, FGF-4 is a member of the FGF family and encodes a growth factor of 30-40% homology to FGF-1, which is secreted outside the cell. In addition, the PCR was used to add a Kozak sequence (CCACCATGG) to the final construct for improving the efficiency of the sp-FGF-1 protein synthesis.[20] pMEXneo is a retroviral-based vector driven by mouse sarcoma virus promoter (MSV-LTR). It stably integrates into the host chromosome. In addition, pMEXneo contains a gene encoding for neomycin phosphotransferase, which allows the isolation of stable transfectants.[21] sp-FGF-1 final gene construct is approximately 500 bp in length.

Yucatan miniature swine (25-45kg) were surgically instrumented with an ameroid occluder on the proximal LCx via a left lateral thoracotomy. Immediately following occluder placements, two groups of pigs were used to investigate the potential effects of sp-FGF-1 gene on angiogenesis. One group (n=4) received myocardial injections of pMexNeo vector alone, and the other group (n=5) received injections of sp-FGF-1/pMexNeo plasmid-each injection contained 25 µg of DNA in an equal volume of TransIT in vivo transfection solution. There were four total injection sites (100 µg total plasmid DNA/heart was injected). The injection sites were (in relation to the right edge of the occluder): 1 cm down, 1 cm distal and 1 cm down, 2 cm down, and 1 cm distal and 2 cm down.[22] Two additional sham-occluded pigs received no occluder and no injections. Each pig was allowed 3 weeks to recover, during which time the gradual closure of the LCx occurred. Echocardiography was performed on the sp-FGF-1 (n=3), vector-control (n=1), and sham (n=1) animals prior to surgery and 3 weeks after surgery in order to compare the cardiac function before and after the occlusion and also to compare the control and FGF-treated pigs. The animals were then euthanized, and

the myocardial tissue was sampled at injection sites as well as the non-occluded, non-ischemic regions of each heart. The samples were subdivided for freezing and immediate histological sectioning for vessel analysis and future biochemical/molecular studies. Additional tissues, including liver, spleen, eye, and skin, were harvested for potential angiogenic side-effects.

All the echocardiographic images were obtained with a General Electric System Five echocardiograph using a 3.5 MHz transducer with harmonic imaging. The images were recorded on optical disk and stored for later offline analysis (EchoPac™ work station). Short-axis and long-axis images were obtained with the transducer placed in contact with the right hemithorax, positioned to optimize the standard tomographic planes. A digital loop consisting of at least three cardiac cycles were recorded in the short axis at the level of the papillary muscles (midventricle) and in the long axis to obtain the maximal left ventricular dimension and allow visualization of the mitral valve. Fractional shortening, wall thickening percent, and endocardial excursion were measured or calculated from m-mode recordings obtained from the short-axis image. All the echocardiographic analyses were performed by an independent observer blinded to the treatment which the animals received. Segmental contractions were compared in all the segments at all times using the animal as its own control.

The pigs were anesthetized with and maintained on isoflurane via endotracheal intubation. A carotid artery was isolated, and a French soft-tipped coronary guiding catheter was advanced into the aorta and then selectively into the right and then left coronary ostium. Fluoroscopy contrast was injected into the proximal coronary artery to visualize the coronary vasculature and potential collateral arteries. The injections were recorded on video and radiographic film. Immediately upon the completion of the angiography (approx. 45-60 min.), the animal was sacrificed and the heart removed.

Total RNA was extracted from frozen heart tissues containing the plasmid injected sites using Totally RNA Kit ™ (Ambion Inc., Austin, TX) according to the manufacturer's recommendations. The aliquots of each RNA sample were subjected to Agilent bioanalyzer (Agilent Technologies, New Castle, DE) to determine the integrity and quantity of the isolated RNA.

For reverse transcription (RT), 1.0 µg of total RNA was incubated with 0.25 µg of a random primers in the presence of M-MLV enzyme (Invitrogen, Carlsbad, CA) at 37°C for 1h. For PCR, we designed the following sense and antisense oligonucleotides corresponding to the sp-FGF-1 coding frame: (sense primer) 5'-GTCCTGCTGGCCTTGCTG-3' and (antisense primer) 5'-AAACAAGATTGCTTTCTG-GCCAT-3'; for internal control, Oligonucleotide primers corresponding to the regions of the house keeping gene glyceraldehydes-6-phosphate dehydrogenase (GAPDH): (sense primer) 5'-TTGTCAGCATGCCTCCTGCACC-3' and (antisense primer) 5'-AACTGGTCCTCAGTGTAGC-CTAG-3' were used. All the primers for this study were synthesized by Integrated DNA Technologies, Inc., Coralville, IA. We successfully amplified sp-FGF-1 transgene and internal control GAPDH using the following PCR condition: cycle one was 94°C for 1', cycle 2 was 55°C for 2', and cycle 3 was 72°C for 3'; thereafter, cycles 1 to 3 were repeated 34 times. An additional 7' extension at 72°C was added at the end of the last cycle of the PCR. The thermostable enzyme DyNAzyme EXT™ (MJ Research, Inc.; Waltham, MA) was used for the PCR step. The PCR products were resolved on a 1% agarose gel, ethidium bromide stained, and photographed.

For factor VIII staining of endothelial cells, we used the streptavidin-biotin/horseradish peroxidase method (Vectastain Elite ABC Kit; Vector Laboratories Inc.; Burlingame, CA) with 3,3'-diaminobenzidine (Histomark DAB chromogen kit; KPL Inc., Gaithersburg, MD) as a chromogen. The sections were deparaffinized in xylene and rehydrated in graded alcohols, incubated in proteinase K (DAKO Corp., Carpinteria, CA) for 3 minutes for antigen retrieval. The sections were treated with 3% hydrogen peroxide for 15 minutes in order to block their endogenous peroxidase activity. Subsequently, the sections were incubated with Universal Block (KPL Inc.), washed, and incubated with normal serum blocking reagent using R.T.U. Vectastain Elite ABC Kit (Vector Laboratories Inc.) to block the nonspecific sites. Endothelial layers were identified by a rabbit polyclonal antibody (Ab) to von Willebrand Factor (vWF) (Cat#A0082; DAKO) (1:800). Negative controls included the substitution of an unrelated Ab for vWF Ab. Immunohistochemical staining of the blood vessels was completed using the ABC Elite Kit according to the manufacturer's recommendations of biotinylated secondary Ab and ABC label reagent. Gill No.3 hematoxylin (Sigma Inc.; St. Louis, MO) was used to counterstain the sections.

For the quantitation of vascularization, digital images were taken at 40X per random fields, and vWF-positive stains were counted using Version 6.0 Metamorph Software (Universal Imaging Corp., Downingtown, PA). Picture taking and quantitation of vascularization were performed by an independent observer blinded to the treatment which the animals received. Differences between the sp-FGF-1 and control groups were analyzed using an unpaired two-tailed t-test at $p<0.05$ using GraphPad PRISM Version 4.0 (GraphPad Software, San Diego, CA).

Results

An evaluation at 21 days post-surgery by coronary angiography confirmed the formation of coronary stenosis and the interruption of the blood flow, at and distal to the occlu-

sion site of the LCx, respectively (Figure 1). A comparison of the intensities of the contrast dye within the LCx at sites proximal and distal to the ameroid occluder demonstrates the shutdown of the blood flow downstream to the occlusion/stenosis (Figure 1). This is in agreement with the previous observation that complete ameroid occlusion occurs around 17-20 days post-implantation in the porcine CCO model and precedes the formation of ischemia within the left ventricular tissue.[23]

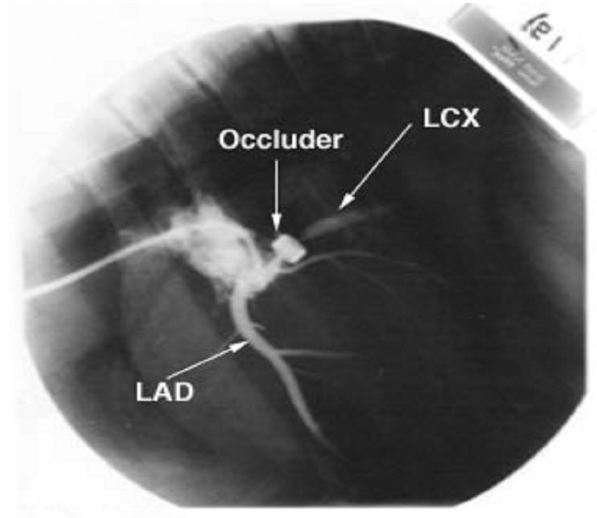

Figure 1. Representative angiogram of a treated pig. Note the contrast dye intensities distal and proximal to the occluder, demonstrating a successful interruption of blood flow downstream the occluder device

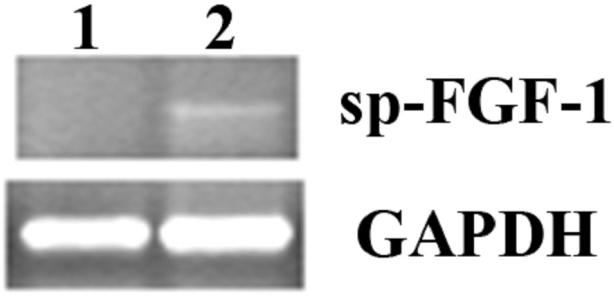

Figure 2. sp-FGF-1 transgene expression in pig heart demonstrated by reverse transcription (RT)-polymerase chain reaction technique (PCR) technique. Total RNA (1 μg) was extracted from the vector control (lane 1) or sp-FGF-1 (lane 2) transfected heart tissues. RT products were PCR-amplified (30 cycles) using a pair of specific primers to sp-FGF-1 (upper panel) and the house keeping gene glyceraldehydes-6-phosphate dehydrogenase (GAPDH) (lower panel). PCR-amplified materials were resolved on an agarose gel, stained with ethidium bromide, and photographed

Confirmation of sp-FGF-1 gene expression was also determined in the transfected porcine hearts. As described above, immediately following the occluder placement, either 100 μg of the sp-FGF-1/pMEXneo or pMEXneo plasmid alone was directly transfected into myocardia using lipid agents.

The plasmids were introduced into the exact same tissue sites relative to the occluder position for each pig in order to reduce variability in tissues that were later retrieved from the sp-FGF-1 and control pigs for comparison of their vascularity and the transgene expression. Sp-FGF-1 transgene expression was confirmed by RT-PCR, in which the forward primer corresponded to the portion of the gene encoding the signal peptide sequence and the reverse primer corresponded to the DNA region encompassing the carboxyl terminus of the FGF-1 (Figure 2). These primers were designed to specifically recognize the sp-FGF-1 chimera but not theendogenous porcine FGF-1 in the heart tissues.

These results document that 21 days following the proposed gene therapy: 1) the ameroid occluder successfully blocked the blood flow through the LCx to the myocardial regions within the left ventricle and 2) direct intramyocardially transfected sp-FGF-1 gene was expressed within the tissues at the sites of the transgene injection.

Similar geographic sites were injected with the plasmid constructs for two reasons. First, this reduces variability in the innate vascularity, which may exist from region to region of porcine hearts. Second, the injected sites were carefully selected to encompass the border regions of the ischemic zones in the left ventricle. This region is known to exhibit active angiogenesis and collaterization in ischemic conditions.[17]

sp-FGF-1/pMEXneo and pMEXneo vector-alone plasmids were separately mixed with Trans IT In Vivo (Pan Vera Inc.), a cationic liposome transfection reagent, and the mixture was immediately used to directly transfect the exposed myocardium.

Immunostaining of the transfected heart tissues for vWF, an endothelial cell specific marker, followed by the quantitation of vWF-positive stains at day 21 clearly demonstrated a higher degree of angiogenesis in the sp-FGF-1 compared to vector-alone-transfected animals at the injected sites. In this analysis, the number of vessels (vWF-positive by immunostaining) in the plasmid injected sites using a 'point counting' method confirmed the difference to be statistically significant (sp-FGF-1: 45.79±2.056, n=5; vector control: 37.42±1.71, n=4; $p<0.05$, unpaired two-tailed t-test with Welch Correction). It revealed a 19% increase in the vascularity of the myocardium of the sp-FGF-1 -treated hearts compared to the vector control (Figure 3). These data indicate that a bolus intramyocardial injection of 100 μg of the secreted form of FGF-1 DNA induces an elevated number of blood vessels 21 days later at the injected site in an ischemia-induced pig heart model.

Echocardiography was used to assess the cardiac function in the sp-FGF-1 and control plasmid-treated porcine hearts. Echocardiography using m-mode was performed (n=3, sp-FGF-1/pMEXneo; n=2, pMEXneo); and n=1, sham) at baseline (prior to surgery) and at 3 weeks after surgery in

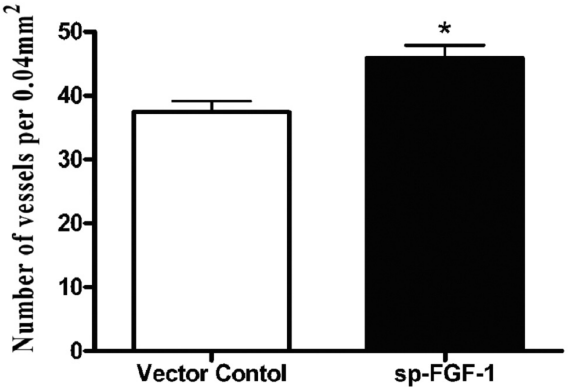

Figure 3. Angiogenic effects of sp-FGF-1 gene construct (n=5) compared to control vector-alone pigs (n=4) at the injected sites. Direct plasmid gene delivery was accomplished. After 21 days, hearts were removed, sectioned, immunostained using antibody specific to von Willebrand Factor, and their blood vessel cross-sections (a total of 48 digital images collected from randomly selected sites per treatment) were counted in a blind manner. The asterisk indicates statistical difference (P<0.05)

order to evaluate the cardiac pumping function in the vector-alone (control) and sp-FGF-treated pigs. Interestingly, there were no significant baseline (prior to surgery) variations based on echocardiographic analyses between the pigs. In other words, it would be acceptable to calculate an average baseline value by the echocardiographic analysis of only a few randomly selected pigs prior to surgery and use the obtained data in a generalized manner to represent the baseline value for each individual animal in the study. In addition, during the echocardiographic analysis, each porcine heart was stressed by exposure to progressively increasing heart rates using the dobutamine stress test (similar to that used in human CAD testing). During the stress test, increasing dobutamine doses of 5 µg/kg/min were administered for five-minute periods increasing to 40 µg/kg/min. Contractile function was assessed at each increased level of heart rate. Interestingly, sp-FGF-1 had improved the cardiac function compared to the control pigs both in resting and stressed states. In fact, during the stress test when the highest heart rate had been achieved, the sp-FGF-1-injected pigs had no obvious ventricular dysfunction upon echocardiogram (percent thickening=100%). The control showed a dramatic decrease in free wall contraction and shortening (percent thickening=50%), which indicates profound regional myocardial systolic dysfunction (Figure 4). In summary, a significant aspect of this observation is that the improvement in the left ventricular function exhibited by the sp-FGF-1-treated porcines still was measurable in the absence of the dobutamine stress test, indicating a more profound stimulating effect of sp-FGF-1 on the ventricular pumping function.

Discussion

The objective of the current study was to assess a

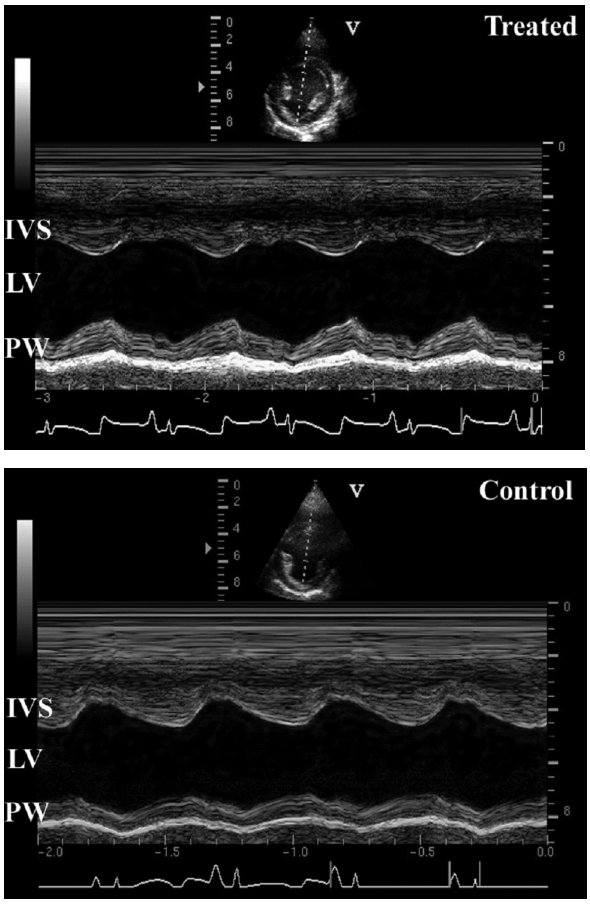

Figure 4. Representative echocardiograms of sp-FGF-1 (Treated) and vector-alone (Control) pigs. The final echocardiograms were performed in order to determine if there was any left ventricular dysfunction. The m-mode images show a two-dimensional view of the lumen of the left ventricle. As the heart contracts, the walls of the interventricular septum (IVS) and the posterior free wall (PW) of the left ventricle (LV) contract and thicken to eject blood from the ventricle. The amount of contraction and thickening, known as the percent thickening value, is a measure of regional cardiac function. The sp-FGF-1-injected pigs (upper panel) had no obvious ventricular dysfunction upon echocardiogram (percent thickening=100%; please note the PW thickness). The control (lower panel) showed a dramatic decrease in free wall contraction and shortening (percent thickening=50%; please note the PW thickness), which indicates profound regional myocardial systolic dysfunction

potentially new angiogenic gene therapy protocol for the improvement of the cardiac function in the ischemic porcine heart. We demonstrated that a direct intramyocardial administration of a signal peptide-containing FGF-1 in a porcine heart model of chronic coronary occlusion and ischemia resulted in an approximately 19% increase in the density of coronary vascularization when compared to the control vector-alone injected hearts. Importantly, this increased vascularity appears associated with improved contractile function. Furthermore, our sp-FGF-1 gene therapy in ischemic porcine heart appears to exert no overt detrimental side effects in remote organs examined for the presence of pathological signs.

Several previous investigators have attempted to stimulate therapeutic angiogenesis using members of FGF family,[24] delivered either as recombinant protein or DNA to the ischemic tissues of end-stage cardiac patients or those of animal models of chronic ischemia.[25-29] Despite the fact that other FGF-1 or FGF-2 angiogenesis therapy protocols appear safe, a major challenge to their use has been an unequivocal demonstration of a clinical improvement in the magnitude of the cardiac function following FGF therapy.[30]

We sought to improve on the FGF therapy protocol by making the FGF-1 gene construct a more potent angiogenic factor through the addition of a signal peptide for the secretion to its amino terminus. Although in vivo transfection efficiency is generally poor, we reasoned that the secreted FGF, albeit inefficient transfection, could readily promote angiogenesis due to its autocrine/paracrine functions in nearby sites. We employed naked plasmid DNA as the vector of choice for introducing sp-FGF-1 gene into porcine hearts rather than the use of a more efficient adenoviral vector. This choice was made because adenoviral vectors sustain short-term expression of the foreign gene and also stimulate a vigorous immune response in the host, which results in a progressive loss of the recombinant virus.[31] However, in order to improve transfection efficiency, we chose to introduce the transgene via a direct intramyocardial route. This selection was based on the observations of others that the administration of a set of selected foreign genes through either intravenous (intracoronary), pericardial, or intramyocardial injection into the porcine hearts, yielded the highest efficiency of gene transfection and expression when the myocardium was directly targeted (Laham RJ, Rezaee M, Garcia L, Post M, Sellke FW, Baim DS, Simons M. Tissue and myocardial distribution of intracoronary, intravenous, intrapericardial, and intramyocardial 125I-labeled basic fibroblast growth factor (bFGF) favor intramyocardial delivery. J AM Coll Cardiol 1999;35:10A). Indeed, our RT-PCR analysis using a set of primers corresponding to different exons of the sp-FGF-1 chimera (sense primer encoding the signal peptide and the antisense primer encoding the C-termius of the FGF-1) demonstrated efficient and sustained sp-FGF-1 gene expression at 21 days post-transfection.

An important observation of this study was that the sp-FGF-1-injected pigs had no obvious ventricular dysfunction upon echocardiogram (percent thickening=100%). In contrast, the control heart (vector alone) showed a dramatic decrease in free wall contraction and shortening (percent thickening=50%), which indicates profound regional myocardial systolic dysfunction. Furthermore, the performance of the sp-FGF-1-injected pigs during the dobutamine stress test was improved relative to the pigs in the control group.

Conclusion

Although coronary artery bypass graft surgery and coronary angioplasty are often highly effective in restoring coronary artery function, many conditions are not treatable in this way and some patients are not candidates for surgery. Such refractory conditions may be responsive to newly developed and novel angiogenic therapies. Our findings suggest the value of further consideration of the selective therapeutic use of sp-FGF-1, wherein the angiogenic effects of sp-FGF-1 could allow reparative revascularization of ischemic regions of the myocardium-at-risk downstream to a coronary artery stenosis/occlusion, as well as protection and/or recovery from ischemia-induced systolic contractile dysfunction in the setting of chronic coronary artery disease.

Acknowledgement

This work was supported by The Texas A&M University System HSC Funds (RF) and NIH grant HL64931 (JLP).

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