Gene Transfer of Hepatocyte Growth Factor Attenuates Postinfarction Heart Failure

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Background—Despite advances in surgical and percutaneous coronary revascularization, ongoing ischemia that is not amenable to standard revascularization techniques is a major cause of morbidity and mortality. Hepatocyte Growth Factor (HGF) has potent angiogenic and anti-apoptotic activities, and this study evaluated the functional and biochemical effects of HGF gene transfer in a rat model of postinfarction heart failure.

Methods and Results—Lewis rats underwent ligation of the left anterior descending coronary artery with direct intramyocardial injection of replication-deficient recombinant adenovirus encoding HGF (n=10) or empty null virus as control (n=9), and animals were analyzed after six weeks. Pressure-volume conductance catheter measurements demonstrated significantly preserved contractile function in the HGF group compared with Null control animals as measured by maximum developed LV pressure (79±5 versus 56±4 mm Hg, P<0.001) and maximum dP/dt (2890±326 versus 1622±159 mm Hg/sec, P<0.01). Significant preservation of LV geometry was associated with HGF treatment (LV Diameter HGF 13.1±0.54 versus Null 14.4±0.15 mm P<0.01; LV wall thickness 1.73±0.10 versus 1.28±0.07 mm P<0.01). Angiogenesis was significantly enhanced in HGF treated animals as measured by both Von Willebrand’s Factor immunohistochemical staining and a microsphere assay. TUNEL analysis revealed a significant reduction in apoptosis in the HGF group (3.42±0.83% versus 8.36±1.16%, P<0.01), which correlated with increased Bcl-2 and Bcl-xL expression in the HGF animals.

Conclusions—Hepatocyte Growth Factor gene transfer following a large myocardial infarction results in significantly preserved myocardial function and geometry, and is associated with significant angiogenesis and a reduction in apoptosis. This therapy may be useful as an adjunct or alternative to standard revascularization techniques in patients with ischemic heart failure. (Circulation. 2003;108[suppl II]:II-230-II-236.)

Key Words: angiogenesis • heart failure • gene therapy • apoptosis

Ischemic cardiac disease that is not amenable to conventional revascularization poses a significant therapeutic challenge. Despite advances in conventional surgical and percutaneous revascularization techniques, more than 10% of patients referred to tertiary intervention centers have symptomatic coronary artery disease (CAD) that cannot be revascularized.1 Ischemia induces endogenous myocardial angiogenesis, but the result of this process often does not adequately compensate.3 The exogenous induction of angiogenesis in ischemic hearts may provide improved perfusion to support the remaining myocardium. Numerous growth factors have been utilized in attempts to induce therapeutic angiogenesis by gene transfer or as recombinant proteins, with mixed results in clinical trials to date.3-5

Hepatocyte Growth Factor (HGF) is a heterodimeric pluripotent growth factor with an 80-kDa apparent molecular weight that has been shown to have potent angiogenic actions on various cells types.6 In addition, HGF has anti-apoptotic effects,7 blocking the programmed cell death response that is known to contribute significantly to the development of ischemic heart failure.8

Because of its pluripotent effects overexpression of HGF may be particularly effective in treating post-ischemic heart failure. This study was designed to investigate the functional, angiogenic and anti-apoptotic effects of adenoviral-mediated gene transfer of HGF in a rat model of postinfarction heart failure.

Materials and Methods

Animal Care

All animals received humane care in compliance with the “Guide for the Care And Use of Laboratory Animals,” Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, D.C., 1996). The study was conducted in accordance with the animal care and use
Adenoviral Vector Construction
Replication-deficient (E1, E3 deleted) adenoviral vectors containing the rat HGF transgene driven by the human muscle creatine kinase (MCK) promoter were made and obtained from the Institute of Human Gene Therapy, University of Pennsylvania (Adeno-HGF). The MCK promoter was chosen to provide high levels of striated muscle-specific expression. Empty replication-deficient adenovirus containing no transgene was similarly obtained as a control (Adeno-Null).

Animal Surgery
Twenty-nine male Lewis inbred rats (250 to 300 g, Charles River Laboratories) were used for main portion of this study. Lewis rats were chosen due to their consistent infarct size of approximately 35% of the left ventricle and low mortality after ligation of the left anterior descending coronary artery (LAD).§ Rats were anesthetized with intraperitoneal doses of ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated with 0.5% isoflurane at the time of surgery. A left thoracotomy was performed through the fourth interspace, the pericardium was opened, and the proximal LAD artery was encircled with a 7-0 polypropylene suture. Animals were then randomized to 1 of 3 experimental groups: Sham, Adeno-Null (Null), or Adeno-HGF (HGF). In the Sham group (n=10) the suture was removed without tying. In the Null (n=9) and HGF (n=10) groups the suture was tied to create a large anterior left ventricular infarct. Infarction was confirmed by visible blanching of the region at the time of ligation. All animals in the Null group then received direct intramyocardial injections of 5×10⁸ pfu of Adeno-Null virus into the infarction border zone area via a 30 gauge needle. A total volume of 250 μL was injected into 5 separate areas. Animals in the HGF group received similar injections of Adeno-HGF into the border zone. The animals were then closed in three layers and recovered for 6 weeks.

Hemodynamic Measurements
After six weeks the animals were once again anesthetized, intubated, and mechanically ventilated, and a repeat thoracotomy was performed. A 2 French pressure-volume conductance catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle through the apex of the heart. Hemodynamic measurements were recorded and analyzed using the ARIA™ 1 Pressure Volume Analysis software (Millar Instruments, Houston, TX). The heart was then arrested in diastole by injection of 0.1 cc of KCl (1 mMg/ml), and the left ventricular cavity was filled with OCT embedding compound fixative retrograde through the transected aortic root. The catheter was removed and the aortic root was ligated. The distended heart was then placed in a container of OCT embedding compound, bathed in isopentane, frozen in liquid nitrogen, and stored in a −80°C freezer.

Ventricular Geometry
In all groups, four adjacent sections midway between the base and apex spanning the border zone and perpendicular to the longitudinal axis of the ventricle were obtained. Measurements were performed on digitized photomicrographs using Openlab image processing software (Improvision, Lexington, MA) with standards of known length, and were obtained on 2 representative sections for each animal. For chamber size, left ventricular diameter was recorded in both vertical and horizontal axes and averaged. For border-zone wall thickness, measurements were obtained on two separate areas for each section and averaged. A single investigator blinded to the treatment groups performed all measurements. The results are reported for each group as the average chamber diameter and wall thickness in mm±SEM.

Assessment of Angiogenesis by Immunohistochemistry and Microsphere Assay
For immunohistochemistry, frozen heart specimens were obtained from storage in a −80°C freezer warmed to approximately −22°C in the cryostat. Four 10 μm sections were then prepared with a cryostat from the point of LAD ligation to the apex of the heart at 0.25 cm intervals. The sections were subsequently blocked with 5% BSA in PBS, and immunohistochemical staining was performed using a mouse monoclonal antibody to the endothelial cell marker von Willebrand’s Factor (Cedar Lane Laboratories, Hornsby, ON) at a dilution of 1:50. An alkaline phosphatase conjugated secondary antibody was used at a dilution of 1:500. The 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) Liquid Substrate System (Sigma, St. Louis, MO) was used for detection. The specimens were then counterstained with eosin, and quantitative assessment of endothelial cells (ECs) was obtained through four representative border zone high-powered fields (hpf) by a single investigator blinded to the treatment group. The results are reported as the number of endothelial cells per hpf±SEM.

To further quantify the extent of induced angiogenesis, an additional 18 animals were utilized for a microsphere assay (Null n=9, HGF n=9). Animals underwent LAD ligation and virus delivery as described above. Six weeks later the rats underwent repeat thoracotomy and, instead of hemodynamic measurements, the slow injection of 1.5×10⁹ microspheres of 15 μm diameter (Triton Technology, San Diego, CA) was performed directly into the left ventricular cavity. The heart was allowed to beat for approximately 1 minute before treatment with KCl as described above. Biopsy specimens were obtained from the entire borderzone area of the left ventricle and snap frozen in liquid nitrogen. Specimens were removed from liquid nitrogen and processed according to the manufacturer’s specifications. The number of microspheres/gram of tissue was calculated based on spectrophotometric absorbance measurements and comparisons to known standards supplied by the manufacturer. As the spheres lodge in the capillary circulation, the number of spheres obtained is presumed to be proportional to the functional capillary density of the tissue.

TUNEL Assay
Frozen heart specimens were sectioned as described above, the TUNEL assay was performed with a TdT-FragEL™ DNA Fragmentation Detection Kit (Oncogene Research Products, Boston, MA), and tissue slides were subsequently incubated with terminal deoxynucleotidyl transferase biotin-labeled dUTP. Streptavidin-horseradish peroxidase conjugate binds to biotin to produce an insoluble colored precipitate with diaminobenzidine. Counterstaining with methyl green was performed to visualize normal nuclei. The apoptotic index was calculated as: (number of apoptotic nuclei/ number of total nuclei) * 100. Four measurements were obtained from the border zone area of each section by a single investigator blinded to the treatment group. The results are reported for each experimental group as the average apoptotic index±SEM.

Western Blotting
Ventricular biopsy specimens obtained for Western blotting were snap-frozen in liquid nitrogen without OCT fixative. Specimens were sheared with a 25 gauge needle after homogenization in 10 volumes of SDS lysis buffer (100 Mm Tris, pH 8.0, 10% SDS, 10 mmol/L EDTA, 50 mmol/L DTT). After normalizing for protein content, 50 μg of each sample were electrophoresed on a 12.5% SDS-polyacrylamide gel following 10 minutes of denaturation at 100°C. A wet transfer apparatus was then used to transfer proteins to Immobilon-P membranes (Millipore, Bedford, MA) with standards of known molecular weight, and were obtained on 2 representative sections for each animal. For chamber size, left ventricular diameter was recorded in both vertical and horizontal axes and averaged. For border-zone wall thickness, measurements were obtained on two separate areas for each section and averaged. A single investigator blinded to the treatment groups performed all measurements. The results are reported for each group as the average chamber diameter and wall thickness in mm±SEM.

Statistical Analysis
All values are expressed as mean±SEM. The unpaired Student’s t-test was used to calculate the statistical significance between the means of 2 groups. Comparisons between more than 2 groups were analyzed by ANOVA followed by Tukey-Kramer post hoc testing. A probability value of less than 0.05 was considered to be significant.

Results
Viral Delivery and Transgene Expression
Virus delivery and transgene expression using our method of direct intramyocardial injection was confirmed using a replication-deficient E1, E3-deleted adenovirus containing the beta-galactosidase gene. X-gal staining reveals expression throughout the area of injection in the left ventricular free wall. Immunoblotting for the hepatocyte growth factor protein 6 weeks following infarction and virus delivery, with actin staining demonstrating equivalent protein loading between lanes. Null = Adeno-Null virus delivery, HGF = Adeno-HGF virus delivery.

Figure 1. (A) Representative cross-section of a rat heart one week following direct injection of an adenoviral vector containing the beta-galactosidase gene. X-gal staining reveals expression throughout the area of injection in the left ventricular free wall. (B) Immunoblotting for the hepatocyte growth factor protein 6 weeks following infarction and virus delivery, with actin staining demonstrating equivalent protein loading between lanes. Null = Adeno-Null virus delivery, HGF = Adeno-HGF virus delivery.

Hemodynamics and Cardiac Function
The Adeno-HGF group had significant preservation of cardiac contractile function as measured by maximum developed left ventricular pressure and maximum dP/dt compared with the Adeno-Null control group (Table 1). A sensitive measure of the contractile state of the heart is the slope of the maximum dP/dt versus end-diastolic function curve, and significant preservation of contractile function was observed in the HGF group compared with the Null controls (Figure 2). There was no significant difference in cardiac function between sham, uninfarcted animals, and infarcted animals treated with Adeno-HGF.

Ventricular Geometry
The Adeno-HGF group demonstrated significant preservation of normal left ventricular geometry compared with the Adeno-Null controls (Figure 3). The HGF treated animals had reduced chamber dilatation compared with Null controls (HGF 13.1±0.54 versus Null 14.4±0.15 mm, P<0.01; Sham 12.2±0.17 mm). The HGF group also demonstrated significantly less border zone wall thinning when compared with the Null animals (HGF 1.73±0.10 versus Null 1.28±0.07 mm, P<0.01; Sham 1.70±0.11 mm).

Cardiac Function Parameters

<table>
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<th>Group</th>
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<th>Heart Rate (bpm)</th>
<th>Maximum LV Pressure (mm Hg)</th>
<th>Maximum LV dP/dt (mm Hg/sec)</th>
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<td>82.8±2.6</td>
<td>3123±125</td>
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<tr>
<td>Null</td>
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<td>1622±159</td>
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<td>79.4±5.0</td>
<td>2890±326</td>
</tr>
<tr>
<td>p (HGF versus Null)</td>
<td>NS</td>
<td></td>
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</tbody>
</table>

Data were obtained 6 weeks after initial surgery and are presented as mean±SEM.
LV, left ventricular; Sham, sham thoracotomy group; Null, Adeno-Null injected group; HGF, Adeno-HGF injected group; NS, not significant (P>0.05); statistical analysis by ANOVA with Tukey-Kramer post-hoc testing.

Figure 2. Representative cardiac contractility curves for a single animal in each experimental group as measured by a pressure-volume conductance catheter at the time of sacrifice. Contractility is measured as the slope of maximum dP/dt versus end-diastolic volume. Sham = sham thoracotomy, Null-Adeno-Null group, HGF = Adeno-HGF group, RVU = relative volume unit.
Angiogenesis: VWF Immunohistochemistry and Microsphere Assay

Immunohistochemical staining for von Willebrand’s Factor revealed an approximately two-fold increase in endothelial cells per high-powered field in the Adeno-HGF group compared with both Null and Sham control groups (Figure 4). The HGF group had 169±11 ECs/hpf compared with 95±7 in the Null (P<0.001), and 103±16 in the Sham controls. The functional capillary density, as calculated by the microsphere assay, was over 50% greater in the Adeno-HGF group compared with Null control animals (13 624±1436 versus 8829±1477 microsphere/gram tissue, P<0.05) (Figure 5).

TUNEL Analysis

TUNEL assay analysis revealed that the HGF treated animals had a >50% reduction in the percentage of apoptotic cells in the border zone area compared with Null control animals (3.42±0.83% versus 8.36±1.16%, P<0.01) (Figure 6). Sham uninfarcted animals had an apoptosis rate of 0.47±0.07%.
Bcl-2 and Bcl-x₅ Expression
Immunoblotting performed against Bcl-2 and Bcl-x₅ revealed significant overexpression of both proteins in HGF treated animals (Figure 7). The relative expression of Bcl-2 was 3.92 ± 0.54 in the HGF group compared with 1.04 ± 0.25 in the Null group (arbitrary units, P < 0.001). The relative expression of Bcl-x₅ was 3.44 ± 0.56 in the HGF group compared with 1.88 ± 0.27 in the Null group (P < 0.01). Interestingly Bcl-x₅, but not Bcl-2, was elevated in infarcted control animals that received null virus injections. All expression quantification was normalized to the Sham animals, which had relative expression levels of 1.0 for both proteins.

Discussion
This study demonstrates that adenoviral-mediated gene transfer of HGF results in significant preservation of contractile function 6 weeks following a large myocardial infarction in rats. In addition, more favorable geometry was associated with Adeno-HGF administration, with decreased left ventricular dilatation and preserved wall thickness. HGF-treated animals had significant angiogenesis as well as a reduction in apoptosis levels. Importantly, continued overexpression of the HGF protein was demonstrated at the 6-week time point.

Hepatocyte Growth Factor is both a potent angiogenic and anti-apoptotic agent. Previous studies have demonstrated increased levels of the HGF receptor, encoded by the proto-oncogene c-Met, following a large myocardial infarction and in response to hypoxia in animal models of myocardial ischemia. HGF itself may upregulate the c-Met receptor and thus activate an autocrine feedback loop, and blocking endogenous HGF has been shown to worsen cardiac failure and increase mortality in a rat model of ischemic injury. The c-Met receptor is present on both endothelial and vascular smooth muscle cells, and angiogenesis may be stimulated directly and/or indirectly via the induction of VEGF secretion by smooth muscle cells. The post-infarction and ischemic milieu, therefore, provides particularly favorable conditions for HGF to exert its effects via upregulation of the c-Met receptor.

Recently it has been shown that serum levels of HGF are elevated following myocardial infarction and ischemia in human patients. In a study by Watanabe and colleagues in which serum and coronary sinus HGF levels were measured, decreased local HGF production with increased extraction of serum HGF by the heart was seen in patients with CAD versus normal controls. It has also been observed that enhanced secretion of HGF from the infarct zone is associated with decreased ventricular dilatation and improved perfusion following myocardial infarction in human patients. Taken together, these results suggest that while serum HGF levels are increased under conditions of cardiac ischemia, local production of HGF by ischemic and infarcted myocardium is decreased. Increased serum HGF may be a protective response to limit continued poor perfusion following an ischemic insult. In contrast, downregulation of local HGF production appears to be a maladaptive response. Local overexpression of HGF appears to correlate with more favorable geometry and perfusion, making it an ideal therapeutic target.

We propose that the preservation of cardiac function observed in this study is due to the dual angiogenic and anti-apoptotic activities of HGF. Angiogenesis in the border zone may rescue a significant fraction of cardiomyocytes that would otherwise be lost or non-functional due to ongoing

Figure 5. The average number of microspheres per gram of left ventricular border zone tissue for the Null and HGF experimental groups at the study end point. Microspheres were injected at the time of sacrifice and were quantified using spectrophotometric absorbance. N=9 in each group, *P<0.05 versus Null control.

Figure 6. (A) Graph of the average percentage of TUNEL-positive cells in the border zone area surrounding the infarction for each experimental group. (B) Representative TUNEL-stained cross-sections from a single animal in the Null and HGF experimental groups (original magnification ×200). Apoptotic nuclei are stained dark brown (red arrows) and normal nuclei are stained blue-green (Sham n=10, Null n=9, HGF n=10). *P<0.01 versus Null control.
ischemia, and studies have confirmed that late reperfusion after infarction benefits ventricular remodeling and function. Angiogenesis may be particularly effective in the early postinfarction period, when significant areas of borderzone myocardium are viable but hypo- and noncontractile because of ischemia. Improved perfusion would, therefore, both reduce ischemic cell loss and improve global cardiac function.

Ventricular remodeling with chamber dilatation and wall thinning is an important component of post-infarction cardiac failure. The remodeling process has been associated with cardiomyocyte death in and around the region of infarct, Hepatocyte Growth Factor has potent anti-cell death effects, and has specifically been shown to inhibit apoptotic cell death, which is known to contribute significantly to postischemic injury in the heart. In our study we observed upregulation of both Bcl-xL and Bcl-2 with HGF treatment, both of which have anti-apoptotic actions. Interestingly, infarcted Null control animals had an almost 2-fold increase in Bcl-xL expression compared with Sham controls, although no similar increase in Bcl-2 expression was seen. This may be an adaptive response to blunt some of the apoptotic response following myocardial infarction. We hypothesize that blocking the activation of apoptosis following myocardial injury prevents cell loss and preserves myocardial geometry and function, as we have previously demonstrated in a rabbit model of chronic ischemia. Since ventricular volume has been shown to be a positive predictor of mortality in heart failure, the ability of HGF to limit ventricular dilatation is particularly beneficial. In fact, HGF overexpression significantly preserved myocardial function such that Adeno-HGF treated rats with large infarcts did not have significantly different hemodynamic parameters than Sham uninfarcted animals.

The introduction of HGF in a clinical setting may be performed at the time of acute infarction with an open or percutaneous catheter-based method. Direct intramyocardial injection of the adenoviral vector could be easily performed during open or minimally invasive bypass surgery, or during percutaneous interventions. Targeting of the border zone area surrounding the infarct can be accomplished either by direct visualization in the operating room, or by assessment of noncontractile areas in the cardiac catheterization laboratory. We have demonstrated that HGF expression is not necessary at the time of infarction, as adenoviral vectors generally take 2 to 3 days to begin expression. Delivery of the transgene via a viral vector could therefore be done in conjunction with ongoing interventions after an acute infarction.

One limitation of this study is that animals were assessed while viral transgene overexpression was still ongoing. Since adenoviral vectors generally lose expression after 4 to 6 weeks, there may be a substantial decline in beneficial effects once HGF expression is gone. It may be that only early expression of HGF is needed, and that the angiogenic effect confers a lasting benefit via improved long-term perfusion of the borderzone. On the other hand, the anti-apoptotic effects of HGF may be of more importance, and once this is lost the heart may decline into dilated failure. Further studies using long-term endpoints of 12 and 18 weeks are currently in progress to address this issue. If long-term expression is needed for lasting benefit, strategies employing adenoviral vectors will need to be devised.

In conclusion we have demonstrated that Hepatocyte Growth Factor gene transfer following a large myocardial infarction results in preserved cardiac function and geometry, and that this is associated with significant angiogenesis and a reduction in apoptosis. This therapy may be useful as an adjunct or alternative to standard revascularization techniques in patients with ischemic heart failure.

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References


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