Administration of Control-Released Hepatocyte Growth Factor Enhances the Efficacy of Skeletal Myoblast Transplantation in Rat Infarcted Hearts by Greatly Increasing Both Quantity and Quality of the Graft

Keiichi Tambara, MD; Goditha U. Premaratne, MD; Genichi Sakaguchi, MD; Naoki Kanemitsu, MD; Xue Lin, MD; Hiroyuki Nakajima, MD; Yutaka Sakakibara, MD; Yu Kimura, BS; Masaya Yamamoto, PhD; Yasuhiro Tabata, PhD, DMedSci, Dpharm; Tadashi Ikeda, MD, PhD; Masashi Komeda, MD, PhD

Background—We investigated whether simultaneous administration of control-released hepatocyte growth factor (HGF) enhances the efficacy of skeletal myoblast (SM) transplantation (Tx) through its antiapoptotic, angiogenic, and antifibrotic effects in myocardial infarction (MI).

Methods and Results—Forty-eight Lewis rats with chronic MI were divided into 4 groups. In Group I (n=14), neonatal SMs \(5\times 10^6\) were transplanted in the MI area with a gelatin sheet incorporating 40 \(\mu\)g (1 g/L) of HGF applied. Group II (n=14) had SM Tx and placement of a saline sheet. Groups III (n=10) and IV (n=10) had culture medium injection plus HGF and saline sheet application, respectively. Four rats each from Groups I and II were sacrificed at day 1 for TUNEL assay on donor SMs. The percentage of TUNEL-positive donor cells was much lower in Group I than in Group II \((p<0.05)\). At 4 weeks, in Group I, left ventricular diastolic dimension was smallest in echocardiography, end-systolic elastance was highest, and \(r\) was the lowest (both \(p<0.0005\) in ANOVA) in cardiac catheterization. Vascular density inside the graft was higher in Group I than in Group II \((p<0.0001)\). The percentage of fibrotic area inside the graft was smaller in Group I than in Group II \((p<0.001)\). The graft volume as estimated by fast skeletal myosin heavy chain-positive areas was \(\approx 7\)-fold larger in Group I than in Group II \((p<0.0001)\).

Conclusions—In SM Tx, HGF can greatly increase the graft volume and vascularity and reduce fibrosis inside the graft, which enhances the efficacy of SM Tx to infarcted hearts. (Circulation. 2005;112[suppl I]:I-129–I-134.)

KeyWords: apoptosis • cells • muscles • myocardial infarction • transplantation
ministration of control-released HGF enhances the efficacy of SM Tx using a rat myocardial infarction (MI) model.

**Methods**

Male syngeneic Lewis rats were used as recipients, and neonatal Lewis rats (2 to 3 days old) were used as donors. Cell processing and surgical procedures were performed by one researcher (G.U.P.) and by one experienced surgeon (K.T.), respectively, in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to the laws of Care and Use of Laboratory Animals in Japan.

**SM Preparation**

Isolation of neonatal SMs was performed as previously described.7 The cell suspension was purified with 30 minutes of preplating after enzymatic digestion, and obtained SMs were plated at 6 to 10 million cells/dish for expansion. Forty-eight hours later, cultures were trypsinized immediately before Tx and suspended at a concentration of 5×10^6/mL. The cell suspension contained 70% to 80% desmin-positive cells (clone D33, DAKO A/S), which were confirmed to be SM.

**Preparation of HGF-Incorporating Gelatin Hydrogel Sheets**

Gelatin with an isoelectric point of 5.0 was isolated from bovine bone collagen by an alkaline process using Ca(OH), (Nitta Gelatin Co.). Gelatin hydrogel sheets were made using the process described previously.13 Sheets were freeze dried and cut in a rectangular shape. Then, they were impregnated with PBS containing human recombinant HGF (deleted form; courtesy of Prof Nakamura at Osaka University) at room temperature for 1 hour to obtain HGF-nant HGF (deleted form; courtesy of Prof Nakamura at Osaka University). Then, they were impregnated with PBS containing human recombinant HGF (deleted form; courtesy of Prof Nakamura at Osaka University). Then, they were impregnated with PBS containing human recombinant HGF (deleted form; courtesy of Prof Nakamura at Osaka University).

**MI Model**

MI was created in rats weighing 250 to 290 g by proximal ligation of the left coronary artery through left thoracotomy, as described previously.14

**Experimental Groups**

Four weeks after ligation, 48 rats with moderate-sized MI (infarct size, 20% to 40%) were divided into 4 groups. In Group I (n=14), neonatal SMs (5×10^6) were transplanted in the scar, and a gelatin sheet (20×20 mm) incorporating 40 μg (1 g/L) of HGF was placed to cover the MI area. Group II (n=14) had SM Tx and placement of a gelatin sheet with saline. Group III (n=10) had culture medium injection and application of an HGF-incorporating gelatin sheet. Group IV (n=10) had culture medium injection plus saline sheet application, which was served as control.

In each group, through left thoracotomy, 100-μL cell suspension or culture medium were subepicardially administered with a single injection into the scar. Then, a rectangular gelatin sheet was stabilized to the LV wall with 6-0 polypropylene sutures to cover the SM implantation region and MI area completely. Anesthetic procedures were described elsewhere.14

**Assessment of Apoptotic Cells**

Four rats each from Groups I and II were transplanted with PKH26-labeled SMs (PKH26 Red Fluorescent Cell Linker Mini Kit, Sigma Chemical Co.). They were sacrificed at day 1, and the heart specimens were cryopreserved. TUNEL assay was performed for transplanted cells as described elsewhere after delineating the extent of donor cells by red fluorescence of PKH26. Individual nuclei were visualized at ×400 magnification, and the percentage of apoptotic nuclei (apoptotic nuclei/total nuclei) was calculated in 3 randomly chosen fields per slide in a blinded manner and averaged for statistical analysis.

**Echocardiography**

The remaining 40 rats were hemodynamics assessed. Echocardiographic assessment was performed according to the method described previously.14 In brief, left ventricular (LV) dimension and function were assessed just before and 4 weeks after treatment. Images were recorded using a 10 to 12 MHz phased-array transducer (Model 21380A with HP SONOS 5500 imaging system, Agilent Technologies). LV end-diastolic and end-systolic dimensions (EDD and ESD, respectively), fractional area change (FAC), and the percentage of akinetic endocardial length to the whole LV endocardial circumference (AL) were measured from the short-axis view of the left ventricle at the papillary muscle level.

**Cardiac Catheterization**

After the final echocardiography, animals underwent cardiac catheterization for a more precise assessment of global LV function as described previously.14 In brief, a 2 F micromanometer-tipped catheter (Millar Instruments Inc.) was inserted via the right carotid artery into the LV, and a 3 F occlusion balloon catheter was inserted through the right femoral vein into the inferior vena cava (IVC). LV pressure and its first time-derivative (dP/dt) were continuously monitored. LV end-systolic volume was calculated using M-mode echocardiograms by the cube formula. During IVC balloon occlusion, pressure waveforms and M-mode tracings were simultaneously recorded. Then, end-systolic elastance (Ees) and the time constant of isovolumic relaxation (τ) were derived from the recorded data. In calculating Ees, the end-systolic pressure-volume points obtained from echocardiography and cardiac catheterization were subjected to least-squared linear regression. All of the data were acquired under stable conditions.

**Histological Staining**

After hemodynamic measurements were finished, all of the rats were sacrificed for histological study. The hearts were removed and fixed with 4% buffered paraformaldehyde. The specimens were paraffin embedded, and the whole hearts were sectioned in 3-μm thickness at 100-μm intervals along the short axis. Immunohistochemistry for von Willebrand factor was performed using a sensitive peroxidase-labeled avidin-biotin system as follows. After deparaffinization, endogenous peroxidase activity was quenched by incubating with 0.1% NaO3 and 0.3% H2O2 in deionized and distilled water. After incubation with proteinase K, sections were incubated with anti-von Willebrand factor antibody (U0034, Dako A/S) at 4°C overnight. Immunohistochemistry for fast skeletal myosin heavy chain (FSMHC) was also conducted using the avidin-biotin-complex method as described previously.7 Diaminobenzidine (Simple Stain DAB Solution, Nichirei) was used as a chromogenic substrate. In addition, Masson’s trichrome staining was performed for light-microscopic examination.

**Analysis of Vascular Density**

The MI area was divided into 5 parts (top left panel in Figure 1), and the central part (central-MI) and the lateral 2 parts (peri-MI) were analyzed. In the central-MI and peri-MI areas and inside the graft, the number of vessels was counted in each heart using immunohistochemistry for von Willebrand factor. The central-MI, peri-MI, and engrafted zones in the sections were determined by referring adjacent preparations for Masson’s trichrome staining and FSMHC immunohistochemistry. In the specimens from Groups I and II, analysis was conducted outside the graft. The vessels per 1 mm² in the pertinent zone were counted in 3 randomly chosen fields per slide in a blinded manner and averaged for statistical analysis.

**Analysis of Fibrotic Area Inside the Graft**

The percentage of fibrotic area inside the graft was calculated in each heart using image analysis software (Scion Image Beta 4.02 Win, Scion Corporation,) in a representative preparation for Masson’s trichrome staining, with the blue areas regarded as fibrotic.
Estimation of Donor-Derived Muscle Volume

After immunohistochemistry for FSMHC, donor-derived muscle volume was estimated in each heart by the method described previously. In brief, the area occupied with FSMHC-positive cells (S, mm²) was calculated in each section with image analysis software (Scion Image Beta 4.02 Win), and the graft volume (V, mm³) was estimated with the following formula using Simpson’s method:

\[ V = 0.1 \times \sum (S_i + S_{i+1} + \ldots + S_n) \]  

where \( n \) is the number of sections that include FSMHC-positive areas. Note that the specimens were prepared with sectioning at 100-μm intervals.

Data Analysis

All of the data are expressed as the mean±SEM. Comparisons of echocardiographic data among the groups were performed by 2-way repeated measures ANOVA. Comparisons of cardiac catheterization data, percent apoptosis, vascular densities, percent fibrosis, and estimated graft volumes among the groups were conducted by the unpaired Student t test or 1-way factorial ANOVA, as appropriate. In ANOVA, if a significant F ratio was obtained, post hoc analysis was carried out with the Scheffe test. All of the statistical analyses were performed using computer software (StatView for Windows version 5.0, SAS Institute Inc.). \( P<0.05 \) was considered statistically significant.

Results

The mortality in coronary artery ligation was 11%. There was no intraoperative or postoperative death concerning treatment procedures.

Tissue HGF Concentration

In advance of the present study, we followed up on tissue concentrations of HGF up to 4 weeks after epicardial application of an HGF-incorporating gelatin hydrogel sheet to the MI area using \(^{125}\text{I}\)-labeled HGF (n=3 at each time point) as described elsewhere. The HGF levels remaining in the infarct tissue were 101±8 and 21±2 ng/g tissue at 1 week and 2 weeks, respectively, but were less than detectable levels at 4 weeks.

Ratio of Apoptotic Cells

Whereas 8.5±1.7% of the donor cells were positive for TUNEL staining in Group II, only 2.7±1.7% were TUNEL-positive in Group I (\( P<0.05 \)) (Figure 2).

Echocardiography

There were no differences in baseline data among the 4 groups. No mitral regurgitation was observed in any rats. After treatment, EDD in Group I was smaller than that in Groups III and IV (\( P<0.05 \) and \( P<0.01 \), respectively), and ESD in Group I was also smaller than that in Group IV (\( P<0.05 \)). In addition, only in Group, FAC was larger and AL was smaller than in Group IV (both \( P<0.05 \)). Those data were summarized in Table 1.

Cardiac Catheterization

In Group I, the E\( \text{es} \) was highest among the 4 groups (Groups I, II, III, IV: 1.00±0.10, 0.59±0.07, 0.37±0.05, and 0.34±0.03 mm Hg/μL, respectively, \( P<0.01 \), versus Groups II, III, and IV). Also, the \( \tau \) was lowest in Group I among them (13.8±1.9, 15.6±1.1, 20.7±0.7, and 21.0±0.6 ms, \( P<0.01 \), versus Groups III and IV).

Vascular Density

The vascular density in the central-MI area was higher in Groups I and III than in the others (Groups I, II, III, IV: 34.5±3.2, 13.2±0.9, 28.5±2.4, and 14.5±1.3/mm², respectively; \( P<0.0001 \) in ANOVA). Similar results were obtained in the peri-MI area (Groups I, II, III, IV: 112.6±7.5, 53.1±2.9, 110.5±5.8, and 48.9±3.0/mm², respectively; \( P<0.0001 \) in ANOVA), although the vascular density was 3-fold higher than in the central-MI area. There were no differences in the number of vessels between Groups I and III and between Groups II and IV, in the MI and peri-MI areas, respectively. The vascular density inside the graft was much...
higher in Group I than in Group II (79.3 ± 5.3 versus 28.2 ± 2.7/\text{mm}^2; \ P < 0.0001). Representative pictures are shown in Figure 3. Results of post hoc analysis were shown with symbols in Figure 1.

### Fibrotic Area Inside the Graft

Although mature myofibers with peripheral nuclei and clear cross-striations were recognized in the graft in both Groups I and II, interstitial collagen fibers were more abundantly found in Group II (Figure 4). The percentage of fibrotic area inside the graft was 15.5 ± 1.3% in Group I, whereas it was 21.3 ± 0.8% in Group II (\ P < 0.001).

#### Donor-Derived Muscle Volume

The estimated donor-derived muscle volume was ≈7-fold larger in Group I than in Group II (34.5 ± 2.0 versus 5.0 ± 0.7 mm^3; \ P < 0.0001) (Figure 5).

### Discussion

The findings in the present study are summarized as follows. SM Tx with HGF administration demonstrated the best...
cardiac performance in both systolic and diastolic functions among the 4 treatments, which was followed by SM Tx with saline. HGF greatly increased the graft volume and vascularity, and the fibrotic area inside the graft was lower with HGF than without. In addition, HGF induced angiogenesis in the central-MI and peri-MI areas, but by itself failed to improve cardiac function in the chronic MI model.

In SM Tx, it has been reported that the more cells are implanted, the more benefits are provided, whereas the risk of tissue overgrowth was pointed out when myoblasts are transplanted in large numbers. Therefore, it may be a reasonable strategy of cell delivery to provide a relatively small number of SMs initially followed by controlled proliferation of the cells in vivo later. On the other hand, because it has been observed that most of the transplanted SMs die soon after implantation, it is necessary for improvement in the efficacy of SM Tx to keep as many donor cells viable in situ as possible.

In this context, HGF has a desirable characteristic to increase cell survival or proliferation in vivo through its antiapoptotic effect, and the present study is the first, to our knowledge, to show that exogenous HGF was effective for the prevention of donor cell apoptosis in SM Tx to the heart. Before this study, we followed up on the apoptosis of transplanted SMs for 1 week (n=1 per day) with TUNEL assay. Then, we found that donor apoptosis was maximum at day 1, which we selected as the timing for TUNEL assay in this study. Similar findings were also reported in cardiomyocyte Tx.

In addition, although various growth factors are considered to play a role in different stages of muscle differentiation, only HGF has been shown to promote the entry of quiescent SMs into the cell cycle. Furthermore, it has been demonstrated that exogenous HGF increases SM number and inhibits muscle differentiation. In this study, because the tissue concentration of HGF was less than detectable levels at 4 weeks, it was likely that the inhibitory effect of HGF on SM differentiation diminished, and the regenerative process of donor-derived muscular tissue recovered enough before collecting specimens. Then, the proliferative effect on SMs may have been actualized as the greatly increased graft volume.

According to our findings, it is speculated that angiogenesis around the graft in Group I was mainly induced by HGF released from gelatin sheets rather than by angiogenic factors secreted from transplanted SMs. HGF also induced neovascularization inside the graft in this group. Although not investigated in this study, an increase in vascular density inside the graft may favorably affect viability and, in turn, function of transplanted SMs.

The decrease in the fibrotic area inside the graft was another beneficial aspect in simultaneous HGF administration. Because the elastic property of the graft and the maintenance of wall integrity are among the likely working mechanisms of SM Tx, a lower percent of fibrosis inside the graft should be advantageous for the treatment.

Although this study showed that simultaneous administration of HGF greatly increased both the quantity and quality of the skeletal muscle graft, sustained release of HGF was essential, because the biological halftime of HGF is too short for its beneficial effects to be exerted enough. At the same time, we have to consider that persistent or prolonged activity could be less effective or sometimes even harmful. In fact, if the tissue HGF concentration had been maintained much longer in this study, the inhibitory effect on SM differentiation might have offset the promoting effect on SM proliferation. In our method, the period of action can be adjusted as required by changing the water content of gelatin hydrogel.

In the present study, HGF alone did not demonstrate a favorable effect on cardiac function, although there were some reports in which HGF was effective in ischemic or infarcted hearts. However, those studies were performed in the early phase after the induction of ischemia or MI. Because...
we used a chronic MI model in which acute inflammatory reaction was completely finished, this discrepancy may be ascribed to the difference in the timing of HGF administration.

There are some limitations to the present study. First, it is uncertain how far epicardially administered HGF is directly infiltrated into tissue. Because the HGF level in the septum was very low, if the same drug delivery system as used in this study is applied in human hearts, tissue HGF concentrations in the endocardial side may not be high enough for HGF to exert its activities. Secondly, we used neonatal SMs for this study expecting a more proliferative ability of the cells. However, some researchers pointed out the possibility that neonate-derived SMs are more sensitive for the expansion procedure. Finally, the time course of HGF activities on SMs was not investigated. These issues are to be addressed before the results of the present study are clinically applied.

In conclusion, simultaneous administration of control-released HGF can greatly increase the graft volume and vascularity and reduce fibrosis inside the graft, which enhances the efficacy of SM Tx to infarcted hearts. HGF alone may not improve cardiac function in chronic MI.

Acknowledgment

We thank Kataoka for her meticulous work in histological studies.

References

Administration of Control-Released Hepatocyte Growth Factor Enhances the Efficacy of Skeletal Myoblast Transplantation in Rat Infarcted Hearts by Greatly Increasing Both Quantity and Quality of the Graft


_Circulation_. 2005;112:I-129-I-134
doi: 10.1161/CIRCULATIONAHA.104.526293

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/9_suppl/I-129

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/