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

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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 × 10⁶) were transplanted in the MI area with a gelatin sheet incorporating 40 μg (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 ≈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.)

Key Words: apoptosis • cells • muscles • myocardial infarction • transplantation

For the last decade, substantial evidence has been accumulated for the efficacy of skeletal myoblast (SM) transplantation (Tx) to infarcted hearts.¹⁻⁷ Since the first human application was performed in June of 2000,³ several clinical trials are underway with favorable results in some.⁴⁻⁵ Although the mechanisms by which transplanted SMs improve cardiac function remain unclear, it has been demonstrated that there is a positive correlation between the magnitude of improvement and the donor cell number or donor-derived muscle volume.⁶⁻⁷ Therefore, increasing graft volume may be a reasonable strategy to maximize the efficacy of SM Tx.

Hepatocyte growth factor (HGF) is a heterodimeric growth factor, which is known to exert pluripotent effects, such as mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types.⁸ When applied to failing hearts, it was reported that HGF reduced myocardial infarct size and improved cardiac function by its antiapoptotic action in ischemic or infarcted models.⁹,¹⁰ In addition, HGF is a unique growth factor with angiogenic and antifibrotic functions, and we and others showed that those effects worked favorably in the treatment of dilated cardiomyopathy.¹¹⁻¹²

Taken together, when combined with SM Tx, exogenous HGF administration to the implantation site may exert beneficial effects to infarcted hearts by facilitating the development of a large muscular graft through its antiapoptotic and antifibrotic effects and by inducing angiogenesis in and around the donor-derived tissue.

However, when injected in free form, HGF does not stay in situ for a certain period long enough to exert its functions. To solve this problem, we developed an HGF-incorporating biodegradable hydrogel sheet composed of acidic gelatin to enable HGF to be released at the site of action for an extended period of time.¹²⁻¹³ In the present study, by applying the peptide formulation method, we investigated whether simultaneous ad-
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 law of Guide for the Care and Use of Laboratory Animals in Japan.

**SM Preparation**

Isolation of neonatal SMs was performed as previously described. 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 5x10^6/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)2 (Nitta Gelatin Co.). Gelatin hydrogel sheets were made using the process described previously. 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-incorporating gelatin hydrogel sheets. The prepared sheets were used for experiment immediately after preparation.

**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. In brief, a 2 F micrometerometer-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 (r) 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% NaNO2 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. 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) are 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.7 In brief, the area occupied with FSMHC-positive cells ($S$, mm$^2$) was calculated in each section with image analysis software (Scion Image Beta 4.02 Win), and the graft volume ($V$, mm$^3$) was estimated with the following formula using Simpson’s method:

$$V = \frac{0.1 \times \Sigma (S_1 + S_2 + \ldots + S_n)}{n}$$

($n$=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.

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 I, 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 Ees 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 τ 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/mm$^2$, 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/mm$^2$, 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 lower.

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 $^{125}$I-labeled HGF ($n$=3 at each time point) as described elsewhere.12–14 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.
higher in Group I than in Group II (79.3 ± 5.3 versus 28.2 ± 2.7 mm²; 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).

TABLE 1. Echocardiographic Data

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDD (cm)</td>
<td>0.99 ± 0.01</td>
<td>0.97 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.99 ± 0.01</td>
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<tr>
<td>ESD (cm)</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.01</td>
<td>0.76 ± 0.02</td>
<td>0.77 ± 0.02</td>
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<tr>
<td>FAC (%)</td>
<td>34.2 ± 2.2</td>
<td>35.9 ± 1.8</td>
<td>34.8 ± 2.0</td>
<td>35.9 ± 2.5</td>
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<tr>
<td>AL (%)</td>
<td>29.0 ± 1.1</td>
<td>28.1 ± 1.3</td>
<td>27.6 ± 1.1</td>
<td>28.8 ± 0.9</td>
</tr>
<tr>
<td>After treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDD (cm)</td>
<td>0.95 ± 0.01†</td>
<td>0.97 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.01 ± 0.01</td>
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<td>ESD (cm)</td>
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<td>0.76 ± 0.02</td>
<td>0.78 ± 0.02</td>
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<tr>
<td>FAC (%)</td>
<td>43.3 ± 2.2‡</td>
<td>37.9 ± 2.3</td>
<td>34.4 ± 1.9</td>
<td>33.6 ± 2.4</td>
</tr>
<tr>
<td>AL (%)</td>
<td>23.8 ± 1.1‡</td>
<td>25.7 ± 1.3</td>
<td>28.0 ± 1.2</td>
<td>29.3 ± 1.2</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± SEM.
†P < 0.01 vs Group IV.
‡P < 0.05 vs Group III.
‡P < 0.05 vs Group IV.

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 mm³; 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
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