Myocardial Regeneration Therapy for Heart Failure
Hepatocyte Growth Factor Enhances the Effect of Cellular Cardiomyoplasty

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Background—We hypothesized that transfection of the gene for human hepatocyte growth factor (hHGF) combined with cellular cardiomyoplasty might regenerate the impaired myocardium.

Methods and Results—We used a ligation model of proximal left anterior descending coronary artery (LAD) of Lewis rats. Two weeks after LAD ligation, 3 different treatments were conducted: (1) neonatal rat cardiomyocytes group (10^6 cells, T group, n=11), (2) HVJ-liposomes bearing the hHGF gene group (H group, n=10), and (3) combined (T-H group, n=10). The injection site was the scar area of myocardial infarction. For control, culture medium was injected (C group, n=13). Echocardiography demonstrated that cardiac performance was significantly ameliorated in the T-H group 4 and 8 weeks after injection. Contrast echocardiography also showed a marked increase in myocardial perfusion in the T-H group but not in the other groups. In the T-H group, neovascularization and a marked reduction of fibrosis were observed histologically. In an immunohistochemical study, strong staining for β1-integrin, α1- and β-1-dystroglycan were found principally in the basement membrane of myocytes in the T-H group 8 weeks after transplantation, although there was weak immunoreactivity in the T group.

Conclusions—HGF gene transfection enhanced the cellular cardiomyoplasty possibly by stimulating angiogenesis, restoring the impaired ECM, and promoting the integration of the dissociated grafted myocytes. The combined effects might have lead to the improved cardiac performance. Thus, combined therapy may be a promising strategy for the treatment of heart failure caused by myocardial infarction. (Circulation. 2002;105:2556-2561.)

Key Words: cells ■ heart failure ■ angiogenesis ■ cell adhesion molecules ■ transplantation

Despite the recent remarkable progress in medical and surgical treatments for heart failure, end-stage heart failure, mostly of ischemic origin, has been still a major cause of death worldwide. Cardiac transplantation and mechanical support using left ventricular assist system (LVAS) implantation have been well accepted as the ultimate lifesaving means of supporting these patients. However, these treatments have limitations such as donor shortages, rejection, infection for cardiac transplantation, and durability for LVAS. In these circumstances, alternative treatments with new concept have been developed.

The recent development of cellular cardiomyoplasty has offered a new approach to restore impaired heart function. There are, however, several remaining problems with this treatment. Even when the regenerating myocytes are introduced by needle injection, cell transplantation cannot recreate the necessary microenvironment around the impaired myocardium. One problem related to this is blood perfusion in the scar and another issue is cell-cell attachment in grafted cells. Hepatocyte growth factor (HGF) is an angiogenic factor and has an antifibrotic activity both through the activation of a matrix degradation pathway by autocrine and paracrine secretion. In addition, HGF enhances cell-cell and cell-extracellular matrix (ECM) interactions through the modulation of β1-integrin, laminin, and fibronectin.

Given this body of evidence, we hypothesized that HGF administration may have an important adjuvant effect in cellular cardiomyoplasty for chronic heart failure caused by ischemic injury.

In this study, we analyzed the functional and histological regeneration of the damaged myocardium after combined therapy with cellular cardiomyoplasty and HGF gene transfection.

Methods

Myocardial Infarction Model
Forty-four Lewis strain male rats (300 g, 8 weeks old; Seac Yoshitomi Ltd, Fukuoka) were used for this study. Humane animal care was used, in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resource and published by the National Institutes of Health (NIH Publication...
No. 86-23, revised 1985). Acute myocardial infarction was induced as described elsewhere.\textsuperscript{11}

Cardiomyocyte Isolation and Culture
Cardiomyocytes were isolated from neonatal Lewis strain rats according to a modified protocol.\textsuperscript{12}

Transfection of the Human HGF Gene Into the Heart
The human HGF cDNA was inserted into the NotI site of the pUC-SRα expression vector.\textsuperscript{13} The preparation of the liposome complex with hemagglutinating virus of Japan (HVJ) is described elsewhere.\textsuperscript{14} Approximately 0.2 mL of the HVJ-liposome-plasmid complex (including 15 μg of the human HGF cDNA) was injected into the infarcted area. For the control group, empty vector was transfected into the infarcted myocardium. The concentration of human HGF in cardiac tissue was measured by enzyme-linked immunosorbent assay (ELISA) using an anti-human HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan).\textsuperscript{15}

Cardiomyocyte Transplantation
The recipient rats were anesthetized and the thorax was opened at the fifth left intercostal space to expose the heart. The rats were subclassified into 4 groups according to the material administered into the infarcted area: T group, neonatal rat cardiomyocyte suspension (in serum-free culture medium, 1×10\textsuperscript{6} cells/0.2 mL; n = 11); H group, HVJ-liposome-plasmid complex including human HGF cDNA (n = 10); T-H group, a combination of neonatal rat cardiomyocyte suspension and HVJ-liposome-plasmid complex including human HGF cDNA (n = 10); and C group, culture medium alone (n = 13). In all cases, these materials were injected into the infarcted area 2 weeks after LAD ligation using a 30G tuberculin syringe.

Measurement of Rat Heart Cardiac Function
Rats were anesthetized with sodium pentobarbital, and diethyl ether (Wako, Japan) was used as a supplement to maintain mild anesthesia. Cardiac ultrasonography was performed with a commercially available echocardiograph, SONOS 5500 (Agilent Technologies, USA). A 12-MHz annular array transducer was used. The heart was first imaged in the 2-dimensional mode in short-axis views at the level of the largest left ventricle (LV) diameter. The systolic and diastolic LV area was determined at the same time. The calculation of the LV volume was based on the LV short-axis area.\textsuperscript{16} This view was used to position the M-mode cursor perpendicular to the LV anterior and posterior wall. Diastolic measurements were made at the time of the apparent maximal LV diastolic dimension. The LV end-systolic dimension was measured at the time of the most anterior systolic excursion of the LV posterior wall. The LV dimension at end-diastole (LVDd), LV dimension at end-systole (LVDs), and LV anterior wall thickness (AWTh) were determined. Dimension and area data are presented as the average of measurements of 3 selected beats. The ejection fraction (EF) of the LV was calculated as follows:

\[
\text{LVEF} = \frac{\text{LVd} - \text{LVDs}}{\text{LVd}} \times 100
\]

The LV percent fractional shortening (FS) was calculated as follows:

\[
\text{LV%FS} = \frac{\text{LVd} - \text{LVDs}}{\text{LVd}} \times 100
\]

Myocardial Contrast Echocardiography
After the rats were anesthetized with sodium pentobarbital and positive-pressure respiration was applied through an endotracheal tube, a 21 Fr catheter was inserted into the right femoral vein to inject a contrast agent, Levobist (Schering Corporation, Japan). Echocardiographic studies were performed with a SONOS 5500. Images of the LV were obtained in a short-axis view as described previously. The myocardial scar tissue and nonischemic region were evaluated as the regions of interest. Regional myocardial perfusion was

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Figure 1. Detection of human HGF by ELISA in recipient hearts. Human HGF protein was detected in recipient hearts 3 days after transfection with the hemagglutinating virus of Japan (HVJ)-liposome method. *P<0.05.

The contrast agent was prepared at 250 mg/mL according to the manufacturer’s instructions. The contrast agent was injected into the infarcted heart via the right femoral vein at approximately 0.3 mL/kg. End-systolic frames were loaded into the computer, and a time-intensity curve was calculated for each region of interest. To correct for background, the echo intensity was determined by subtracting the baseline value from the raw value. Hearts with a time-intensity curve that showed a peak of blood flow were defined as having good myocardial perfusion; in contrast, hearts with a time-intensity curve that showed flat blood flow had poor myocardial perfusion.

Histological Analyses
LV myocardium specimens were obtained 8 weeks after the cardiac myocyte transplantation. Each specimen was fixed with 10% buffered formalin and embedded in paraffin. A few serial sections were prepared from each specimen and stained with hematoxylin and eosin for histological examination or with Masson’s trichrome stain to assess the collagen content.

To label vascular endothelial cells so that the blood vessels could be counted, immunohistochemical staining of factor VIII–related antigen was performed according to a modified protocol. We used EPOS-conjugated antibody against factor VIII–related antigen coupled with HRP (Dako EPOS Anti-Human Von Willebrand Factor/HRP, Dako) as primary antibodies. The stained vascular endothelial cells were counted under a light microscope at ×200 magnification. The result was expressed as the number of blood vessels/mm\textsuperscript{2}.

The following antibodies against several adhesion molecules were used to evaluate the interactions of grafted cells with the extracellular matrix or another grafted cell. Primary antibodies: a rabbit polyclonal antibody to laminin (ICN Biomedicals, USA), a hamster monoclonal antibody to β\textsubscript{1}-integrin (Sumitomo Electric, Japan), a mouse monoclonal antibody to α\textsubscript{-dystroglycan (Upstate Biotechnology, Lake Placid, NY), and a mouse monoclonal antibody to β\textsubscript{-Dystroglycan (Castra Novo, UK); secondary antibodies: biotinylated anti-rabbit immunoglobulin (Dako, Denmark), biotinylated anti-hamster immunoglobulin (Vector laboratories, USA), and biotinylated anti-mouse immunoglobulin (Amersham Pharmacia Biotech, England).

Data Analyses
Data are expressed as mean±SD. To assess the significance of the differences between individual groups, statistical evaluation was performed with an unpaired t test. Differences in cardiac function data were assessed using a 2-way repeated-measures analysis of variance. If a significant F ratio was obtained, further analysis was carried out with a post hoc test. Statistical significance was determined as having a value of P<0.05.

Results
In Vivo HGF Gene Transfection of the Heart
Three days after transfecting hearts with human HGF, we measured the HGF protein content of the transected hearts
using an ELISA. Hearts transfected with the HGF gene contained human HGF protein at levels as high as 7 ± 1.2 ng/g tissue on the third day after transfection. In contrast, human HGF was not detected in cardiac tissues obtained from hearts transfected with an empty vector (Figure 1).

Functional Assessment of the Infarcted Myocardium

The EF, FS, left ventricle end-systolic area (LVESA), and AWTh scores at baseline were not significantly different between the 4 groups.

Four weeks after the injection, 2-dimensional echocardiography showed significant improvement of the EF (Figure 2) and FS (Figure 3) in the T-H group compared with the other 3 groups. These functional improvements were preserved 8 weeks after injection. The LVESA was significantly smaller in the T-H group than in the other groups 4 and 8 weeks after the injection, and the enlargement of the LVESA was decelerated in the T-H group compared with the other groups (Figure 4). The reduced AWTh after LAD ligation was significantly recovered in the T-H group, whereas no recovery of AWTh was seen in the other groups 4 weeks after the injection, and this anterior wall recovery was preserved until 8 weeks after the injection (Figure 5).

Histological Assessment

The T-H group showed a significant increase in LV wall thickness and decrease in the cross-sectional LV area compared with the other groups. In the microscopic examination, we found that newly formed cardiac tissue supplemented the infarcted area of the LV wall in the T and T-H groups. However, the thickness of the newly formed LV wall was markedly enhanced in the T-H group compared with the T group. The proportion of the fibrosis-occupying area (percent fibrosis) at an area that was remote from the infarction was significantly reduced in the T-H group compared with the other groups (T-H versus T versus H versus C: 13.5 ± 3.5 versus 24.5 ± 6.8 versus 18.5 ± 12.0 versus 36.7 ± 8.2%; \( P < 0.05 \)) (Figure 7).

Immunohistochemical studies showed the highest expression of both \( \beta_1 \)-integrin and \( \beta \)-dystroglycan on the surface of cardiomyocytes in the T-H group 8 weeks after transplantation. In the T group, the expression of both \( \beta_1 \)-integrin and \( \beta \)-dystroglycan was quite strong, although noticeably lower than in the T-H group. The expression of \( \alpha \)-dystroglycan and laminin was detected in the basement membrane beneath the cardiomyocytes in the T-H group. Their expression was much weaker in the T group 8 weeks after transplantation. No positive immunoreactions of \( \alpha \)-dystroglycan and laminin were found in the H group and the Control group (Figure 6).
Figure 6. Histological findings of the heart after combined therapy or cellular cardiomyoplasty. A and B, Short-axis area of the LV is small, and the anterior wall has significantly recovered. Moreover, engrafted cardiomyocytes with well-generated sarcomeres extend linearly along the scar and are surrounded by well-developed neovascularization (C). In contrast, T group (D, E, and F) and C group (G and H) show a dilated LV and the anterior wall is thinner than in T-H group. At 8 weeks after transplantation, T-H group (K and M) showed a greater progressive concentration of $\beta_1$-integrin and $\beta$-dystroglycan in the basement membrane of myocytes than did T group (L and N). $\alpha$-Dystroglycan was weakly enhanced in the basement membrane of myocytes in the T-H group (I), although a small amount was seen in T group (J) 8 weeks after transplantation. A, C, D, F, G, and H, hematoxylin and eosin stain; B and E, Masson’s trichrome stain; I through N, immunostain: I, $\alpha$-dystroglycan (T-H group); J, $\alpha$-dystroglycan (T group); K, $\beta$-dystroglycan (T-H group); L, $\beta$-dystroglycan (T group); M, $\beta_1$-integrin (T-H group); and N, $\beta_1$-integrin (T group).
Vascular density in the myocardial scar tissues was evaluated by counting the number of Factor VIII–related antigen-positive cells. In the combined therapy group, vascular density was found to be significantly higher than in the other groups (T-H versus T versus H versus C: 14.2 ± 0.7 versus 4.9 ± 0.6 versus 1.5 ± 0.7 × 10^3/mm^2; P < 0.05) (Figure 8).

**Improvement of Myocardial Perfusion in the Scar**

By contrast echocardiography, the time-intensity curve for the T-H group showed a peak-shaped flow, indicating good myocardial perfusion in the scar. On the other hand, the H, T, and C groups showed a flat time-intensity curve, indicating poor myocardial perfusion.

**Discussion**

Over the past several years, increasing awareness of the shortcomings of heart transplantation and left ventricular assist system implantation has led cardiovascular surgeons to consider alternative means of treating end-stage heart failure. Transfection of the genes for several growth factors into the damaged heart has been shown to promote angiogenesis and lead to improvement in cardiac function. However, this functional improvement is due to angiogenesis alone and angiogenic therapy is inadequate because it does not target myocardial scar thinning and expansion. On the other hand, cellular cardiomyoplasty has been proposed to help prevent scar thinning and expansion, but it does not prevent ischemia or interstitial fibrosis in the ischemic myocardium. We therefore hypothesized that combining cellular cardiomyoplasty with gene therapy using an angiogenic factor might overcome the shortcomings of each therapy and facilitate superior myocardial regeneration.

To examine the effects of the combined therapy, we analyzed cardiac function and performed a histological assessment of the infarcted heart in a rat model. Combined therapy induced angiogenesis, reduction of fibrosis, and a significant increase in the anterior wall thickness. The anterior wall contained a newly formed area with well-generated myocytes and capillaries and enhanced remodeling of cellular interactions, which resulted in significant attenuation of LV remodeling and excellent improvement in cardiac performance. These data proved our hypothesis that gene transfection of hHGF combined with cellular cardiomyoplasty might support myocardial regeneration in the infarcted heart.

One key to successful cellular cardiomyoplasty may be angiogenesis. Taylor et al. observed very few blood vessels near the grafted cells and discussed how poor blood supply might limit the growth of the implanted cells or decrease their survival after successful differentiation. Moreover, Reinlib and Field emphasized that transplanted cells are highly sensitive to ischemic injury, and that therapeutic cellular cardiomyoplasty will ultimately require additional interventions to give vascular support to the new tissues. In the present study, newly formed tissues had the benefit of good blood supply resulting in appropriate cellular interactions for the cardiomyocytes and well-generated sarcomeres. Therefore, adequate angiogenesis to the new tissues is important to maintain the viability of the grafted cells for successful cellular cardiomyoplasty.

Many angiogenic factors have been reported, and vascular endothelial growth factor (VEGF) is the most potent factor known. However, excessive VEGF induces a large number of immature blood vessels, resulting in marked bleeding and edema. HGF is also a potent angiogenic factor, and Morishita et al. demonstrated that HGF induces enough mature blood vessels to, in principle, cure patients with arteriosclerosis obliterans. Furthermore, HGF is not only an angiogenic factor but also has a variety of other functions including antifibrotic and cardiotoxic activities. Therefore, we believed HGF might be effective in combination with cellular cardiomyoplasty and that it might exert its beneficial effects on both the infarcted heart and new tissues through autocrine and paracrine secretion.

Another key to successful cellular cardiomyoplasty may be cell-cell and cell-matrix adhesion for which integrin is the most important molecule for cell-matrix adhesion. Without cell-matrix interaction through integrin, cells of every type, except blood cells, fall into apoptosis called anoikis. In addition to survival, cells exert various functions through cell-matrix interactions that are mediated by integrin, including cell proliferation, cell differentiation, cell motility, and protein production. In the present study, we found the highest expression of β1-integrin on cardiomyocytes in the combined therapy group. This result indicates that cardiomyocytes could function the most effectively on the basis of adhesion to the extracellular matrix. Besides integrin molecules, myogenic cells require another molecule, dystroglycan. β-Dystroglycan is known to be expressed on the surface of skeletal muscle cells and is intracellu-
early bound to another molecule called dystrophin, which is connected to actin filaments. Extracellularly, β-dystroglycan is bound to α-dystroglycan, which is connected with laminin in the basement membrane of skeletal muscles. β-Dystroglycan is associated with a sarcoglycan complex composed of α, β, γ, and δ subunits on the cell membrane. α- and β-dystroglycan form a complex together with the sarcoglycan complex. This complex, alone with dystrophin, plays a key role in skeletal muscle functions: point mutations of the molecules in this system cause several types of muscular dystrophy. Recently, this adhesion system was reported to be important in heart muscle function as well, and mutations in these molecules may cause cardiomyopathies.18 Our results of the strongest expression of both α- and β-dystroglycan in combined therapy group showed that cardiomyoplasty combined with HGF gene transfection induced sufficient expression levels of another important cell adhesion molecules for cardiomyocytes. Laminin is one of the best-described molecules comprising the extracellular matrix proteins and is the main component of the basement membrane. Laminin receptors include some integrins and α- and β-dystroglycan complexes on myogenic cells. Our finding that the combined therapy group had the highest expression of laminin in the basement membrane suggests not only that the transplanted cardiomyocytes could adhere to the laminin on the basement membrane most sufficiently, but also that the combined therapy could induce the most appropriate microenvironment through ECM remodeling, although the mechanism remains to be elucidated. Furthermore, HGF might not be as effective in injured cardiomyocytes as in freshly transplanted ones because the highest expressions of both adhesion molecules and matrix proteins were observed in the combined therapy group.

In the chronic phase of myocardial infarction, the progression of cardiac remodeling with reduced cardiac function is responsive to interstitial fibrosis as well as for damage to the cardiomyocytes.19 In particular, fibrosis remote from the infarcted area is considered to be “the major cause of ventricular remodeling” in ischemic cardiomyopathy.20 Thus, an additional strategy to target interstitial fibrosis has been needed for the regeneration of infarcted myocardium, even with cellular cardiomyoplasty.

Some of the molecular contributors to fibrosis during cardiac remodeling have been identified. Transforming growth factor-β and angiotensin II are believed to play an important role in the pathogenesis of fibrosis.21 Interestingly, these molecules are strong negative regulators of local HGF production in various cell types.22 Furthermore, increased local HGF expression prevents myocardial fibrosis, not only through its antifibrotic action but also through inhibition of angiotensin II production.23 In our study, HGF appeared to significantly prevent fibrosis, even in an area that was remote from the infarct, probably through these mechanisms.

Thus, this combined therapy conferred good blood perfusion to both transplanted myocytes and the infarcted myocardium and helped to prevent fibrosis.

In conclusion, we have demonstrated that HGF gene transfection combined with cellular cardiomyoplasty produced histologically and functionally prevented the dilatation of the impaired myocardial wall. Combined therapy using cellular cardiomyoplasty and human HGF gene transfection may be one of the armamentarium of regenerative therapy for chronic heart failure caused by myocardial infarction.

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