Transplanted Skeletal Myoblasts Can Fully Replace the Infarcted Myocardium When They Survive in the Host in Large Numbers

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Background—It is not clear how many skeletal myoblsts (SM) can survive and exert beneficial effects in the host myocardial infarction (MI) area. We assessed the hypothesis that a large number of SM can replace the MI area with reverse left ventricular (LV) remodeling.

Methods and Results—MI was created by left coronary artery ligation in male Lewis rats. Four weeks after ligation, 45 rats had skeletal myoblast transplantation in the MI area. They were randomized into 3 groups according to the number of SM: group I (n=15), 5×10^7; group II (n=15), 5×10^6; and group III (n=15), 5×10^5 cells. Donor SM were obtained from neonatal Lewis rats and directly used without expansion. Another four weeks later, all rats were sacrificed following hemodynamic assessment. All heart sections were stained with anti-fast skeletal myosin heavy chain (FSMHC) antibody to determine the spacial extent of donor myocytes.

Results—Four weeks after transplantation, LV diastolic dimension was decreased, fractional area change was increased, and MI size was decreased maximally in group I. Histological study showed that donor cells positive for FSMHC occupied the MI area with nearly normal wall thickness in group I, in which estimated volume of donor-derived muscle tissue was 40 mm^3. In the other groups, FSMHC-positive cells were found only partly in the MI area.

Conclusions—A large number of freshly isolated neonatal SM can survive in the host and fully replace the infarcted myocardium with reverse LV remodeling in rats with MI. (***Circulation*. 2003;108[suppl II]:II-259-II-263.**)

Key Words: cells ■ myocardial infarction ■ muscle ■ skeletal ■ transplantation/surgical aspects

Cell transplantation has recently emerged as a promising therapeutic strategy for end-stage heart failure.[1–15] Among various types of cell sources, skeletal myoblasts (SM) have drawn our attentions because of their clinical applicability and regenerative capacity.[6–15] To date, several clinical trials of skeletal myoblast transplantation are under way in patients with chronically ischemic hearts that are not suitable for standard myocardial revascularization.[12]

However, the optimal conditions for skeletal myoblast transplantation remain unclear even in experimental models. Since the ultimate goal for cell transplantation is full replacement of diseased myocardium in both dimensional and functional aspects, it would be desirable that transplanted cells maintain their elastic and contractile capacities and survive in the host conditions, which are usually unfavorable for cell survival. Although some authors described that engrafted structures derived from transplanted SM occupied up to 75% of the cryoinjured scar in rabbit hearts,[8–10] the relationship between the number of transplanted SM and their survival extent as muscular tissue or the effects on cardiac remodeling has not been elucidated yet.

Therefore, the purpose of the present study was twofold: (1) to investigate whether the muscle tissue derived from transplanted SM can fully replace the infracted myocardium when they survive in the host in large numbers, and (2) to assess the dimensional and functional improvement in left ventricular (LV) performance after transplantation with relation to the spacial extent of myogenic engraftments, using a rat coronary ligation model.

Methods

Male syngeneic Lewis rats were used as recipients and neonatal Lewis rats as donors in this study. All experimental procedures were performed by experienced surgeons (K.T. and Y.S.) 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.

Skeletal Myoblast Isolation

Isolation of neonatal SM was performed as Li et al described for cardiomyocyte isolation[1] with some modifications. Briefly, skeletal muscles were harvested from four limbs of neonatal rats (2 to 3 days old), and minced into small pieces. For the release of SM, those

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fragments were digested in phosphate-buffered saline solution containing trypsin (0.35%), collagenase (0.07%), and glucose (0.02%) for 30 minutes. After removal of undispersed tissue elements, the cell suspension was purified with 30 minutes of preplating. Then, obtained SM were directly used without expansion. The primary isolates contained 70 to 80% desmin-positive cells (clone D33, DAKO A/S, Glostrup, Denmark), which were then confirmed to be SM. Approximately 50 million SM were recovered from 10 to 15 neonates with this method.

Myocardial Infarction Model

Myocardial infarction was created in rats weighing 250 to 290 g by proximal ligation of the left coronary artery through left thoracotomy, as described in our previous reports.[3–5,16] After coronary ligation, an ST elevation on electrocardiography and a color change in the LV myocardium were recognized in all rats.

Experimental Groups

Four weeks after ligation, 45 rats with moderate-sized MI (infarct size:[17] 20 to 40%) were randomized into 3 groups according to the number of transplanted SM: group I (n=15), 5×10^7; group II (n=15), 5×10^6; and group III (n=15), 5×10^5 cells. In each group, through left thoracotomy, after putting mattress sutures with 6 to 0 polypropylene at injection points to prevent leakage, 100 μL culture medium containing SM were subepicardially injected at 2 opposite sites in the peri-infarct zone with the tip of the needle directed toward the center of the scar using a 27-gauge needle. Bulging over the MI area was confirmed in every case after injection. Anesthetic or surgical procedures are described elsewhere.[3–5]

Echocardiography

Echocardiographic assessment was performed according to the method previously described.[3–5] In brief, LV dimension and function were assessed just before transplantation, and followed-up 2 and 4 weeks later. Images were recorded using a 10 to 12 MHz phased-array transducer (Model 21380A with HP SONOS 5500 imaging system, Agilent Technologies, Andover, MA). LV end-diastolic and end-systolic dimensions (EDD and ESD, respectively) were measured with M-mode tracings from the short-axis view of the left ventricle at the papillary muscle level. Fractional area change (FAC) and the percentage of akinetic endocardial length to the whole LV endocardial circumference (AL) were also calculated from the same short-axis view. All measurements were performed in a blind fashion according to the American Society for Echocardiology, and averaged over 3 consecutive cardiac cycles.

Cardiac Catheterization

After the final echocardiography, the rats underwent cardiac catheterization for more precise assessment of global LV function as previously described.[3,5,16] In brief, under general anesthesia, a 2 F micromanometer-tipped catheter (Millar Instruments Inc, Houston, TX) was inserted via the right carotid artery into the left ventricle, and a 3 F occlusion balloon catheter through the right femoral vein into the inferior vena cava (IVC). LV pressure and its first time-derivative (dP/dt) were continuously monitored using a multiple recording system. LV end-systolic volume was calculated using M-mode echocardiograms by the cube formula. During IVC occlusion with the balloon, pressure waveforms and M-mode tracings were simultaneously recorded. Then, end-systolic elastance (E_{es}) and the time constant of isovolumic relaxation (τ) were derived from the recorded data. In calculating E_{es}, the end-systolic pressure-volume points obtained from echocardiography and cardiac catheterization were subjected to least squared linear regression. All data were acquired under stable conditions.

Histology

After hemodynamic measurements were finished, all 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. Then, in order to detect differentiated myotubes derived from transplanted SM and investigate the spacial extent of myogenic engraftments, immunohistochemistry was performed for fast skeletal myosin heavy chain (FSMHC), which is the component that cardiomyocytes do not have, as follows. After deparaffinization, endogenous peroxidase activity was quenched by incubating with 0.1% NaN_3 and 0.3% H_2O_2 in deionized and distilled water following antigen retrieval with boiled citrate buffer (pH 6.0). Normal rabbit serum [Histofine SAB-PO(M) Kit, Nichirei, Tokyo, Japan] was added followed by endogenous avidin-biotin blocking (Blocking Kit SP-2001, Vector Laboratories Inc, Burlingame, CA). The sections were then incubated with anti-FSMHC antibody (1:400, clone My 32, Sigma-Aldrich Inc, Saint Louis, MO) at 4°C overnight. Biotin-labeled rabbit anti-mouse antibody and peroxidase-labeled streptavidin [Histofine SAB-PO(M) Kit] were sequentially applied. Finally, diaminobenbenzidine (Simple Stain DAB Solution, Nichirei, Tokyo, Japan) was used as a chromagenic substrate. In addition, Masson's trichrome staining was performed for standard light-microscopic examination.

Estimation of Donor-Derived Muscle Volume

After immunohistochemistry for FSMHC, the brown area occupied with FSMHC-positive cells (S, mm_2) was calculated with image analysis software (Scion Image Beta 4.02 Win, Scion Corporation, Frederick, MD) in each section. Then, because the slice interval was 100 μm, donor-derived muscle volume (V, mm^3) was estimated with the following formula in each heart using Simpson's method: $V=0.1\times\Sigma\ (S_1 + S_2 + \cdots + S_n)$ (n=the number of sections that include FSMHC-positive areas).

Data Analysis

All data are expressed as the mean±SEM. Comparisons of echocardiographic data among the groups were performed by 2-way repeated measures analysis of variance (ANOVA) including time, group, and group-by-time interaction terms. Comparisons of cardiac catheterization data and estimated graft volumes among the groups were conducted by one-way factorial ANOVA. If significance was recognized for the group effect or the group-by-time interaction, post hoc comparisons among the groups or among the groups at each time point were performed, and if significance was found for the time effect or the group-by-time interaction, post hoc comparisons among the time points in each group were made, when appropriate, using Fisher's protected least significant difference method. All statistical analyses were performed with using computer software (StatView for Windows version 5.0, SAS Institute Inc, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

The mortality in coronary artery ligation and the size of created MI were similar to our previous report.[5] There was no intraoperative or postoperative death concerning transplantation.

Echocardiography

There were no differences in preoperative data among the 3 groups (Figure 1). No mitral regurgitation was observed in any rats during the experiment period.

In the analyses of EDD and ESD (Figure 1), group and time effects and group-by-time interactions were strongly recognized. EDD in group I was smaller than that in groups II and III at 2 and 4 weeks (2 weeks: 0.93±0.01 versus 0.97±0.01, 1.00±0.01 cm, respectively, $P=0.025$ versus group II, $P=0.0003$ versus group III; 4 weeks: 0.92±0.01 versus 0.95±0.01, 0.99±0.01 cm, respectively, $P=0.045$ versus group II, $P<0.0001$ versus group III). Similarly, group I showed smaller ESD than groups II and III at 2 and 4 weeks (2 weeks: 0.70±0.01 versus 0.74±0.01, 0.76±0.01 cm,

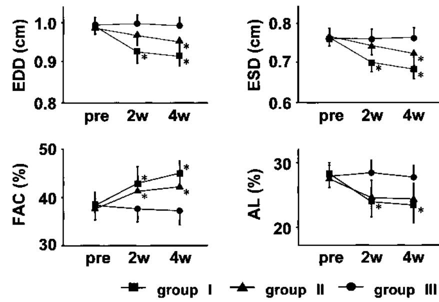

Figure 1. Echocardiographic assessment. Error bars show SEM. EDD indicates end-diastolic dimension; ESD, end-systolic dimension; FAC, fractional area change; and AL, the percentage of akinetic endocardial length to the whole left ventricular endocardial circumference. $^{*}P<0.05$ versus baseline in the same group. The results of group effects are not shown in the graphs.

respectively, $P=0.015$ versus group II, $P=0.0010$ versus group III; 4 weeks: 0.68±0.01 versus 0.72±0.02, 0.76±0.01 cm, respectively, $P=0.045$ versus group II, $P=0.0002$ versus group III). When compared among the time points in each group, in group I, EDD was smaller at 2 and 4 weeks than at baseline, respectively (0.99±0.01 at baseline, $P=0.0006$ versus 2 weeks, $P=0.0001$ versus 4 weeks). Group II also showed smaller EDD at 4 weeks than at baseline (0.99±0.01 at baseline, $P=0.014$). Similar results were also found in ESD measurements.

There were positive group and time effects and group-by-time interactions seen in FAC (Figure 1). FAC in groups I and II were larger than that in group III at 4 weeks (45.0±1.3, 42.2±1.3 versus 37.2±1.4%, respectively, $P=0.0002$ in group I, $P=0.013$ in group II). FAC showed no difference between groups I and II at 4 weeks. When compared among the time points in each group, FAC was larger at 2 and 4 weeks than at baseline in groups I and II, respectively (group I: 42.9±1.7, 45.0±1.3 versus 38.4±1.3%, respectively, $P=0.016$ at 2 weeks, $P=0.0007$ at 4 weeks; group II: 41.2±1.1, 42.2±1.3 versus 37.6±1.2%, respectively, $P=0.014$ at 2 weeks, $P=0.0021$ at 4 weeks).

AL comparison showed positive group and time effects (Figure 1). After transplantation, AL was smaller in groups I and II than in group III (2 weeks: $P=0.025$, $P=0.010$, respectively; 4 weeks: $P=0.041$, $P=0.010$, respectively). Although there seems to be decrease of AL in both groups I and II, post hoc comparisons showed statistically significant AL decrease only in group I (2weeks, 4 weeks: $P=0.0066$, $P=0.0025$ versus baseline, respectively).

Cardiac Catheterization

One-way factorial ANOVA showed a high group effect in both E_{es} and τ (Figure 2). E_{es} in group I was higher than that in groups II and III (0.72±0.04 versus 0.57±0.04, 0.34±0.03 mm Hg/μL, respectively, $P=0.0033$ versus group II, $P<0.0001$ versus group III). E_{es} in group II was also higher than that in group III ($P<0.0001$). τ in group I was lower than that in groups II and III (15.4±0.9 versus 18.2±1.0, 20.6±0.8 ms, respectively, $P=0.0033$ versus group II, $P=0.0002$ versus group III). There was no significant difference in τ between groups II and III.

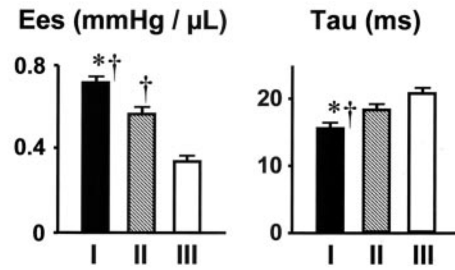

Figure 2. Cardiac catheterization. Error bars show SEM. E_{es} indicates end-systolic elastance; Tau, the time constant of isovolumic relaxation; I, group I; II, group II; and III, group III. $^{*}P<0.01$ versus group II, $^{\dagger}P<0.01$ versus group III.

Histology

Immunohistochemistry for FSMHC revealed that positively stained donor cells were extended over the whole MI area and occupied at least more than half of the infracted area with nearly normal wall thickness in group I (Figure 3A and 3D). In contrast, the cells positive for FSMHC were found along the MI area in group II, but the wall thickness was far from being normalized (Figure 3B). The cells positive for FSMHC were only seen in a small part in group III (Figure 3C). Masson's trichrome staining demonstrated that thickened scar tissue surrounded donor-derived structures in group I (Figure 4A), in which mature myofibers with peripheral nuclei and clear cross-striations were found (Figure 4B). In addition, relatively abundant collagen fibers were observed among myofibers (Figure 4B).

Estimation of Donor-Derived Muscle Volume

Estimated donor-derived muscle volume was by far the largest in group I (group I, II, III: 39.7±5.2, 5.9±0.9, 0.9±0.1 mm^3, $P<0.0001$ versus group II and III, respectively) (Figure 5).

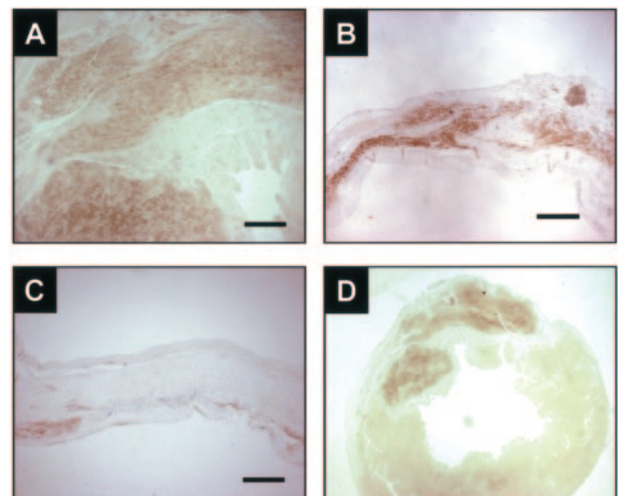

Figure 3. Expression of fast skeletal myosin heavy chain in transplanted cells (brown). A, B, and C are a representative picture from group I, II, and III, respectively (×40). The engrafted wall is by far the thickest in A. To note, the scarred walls had about the same thickness before transplantation. D is a low power field of the same section as A is from (×10). Bars represent distances of 500 μm in A, B, and C.

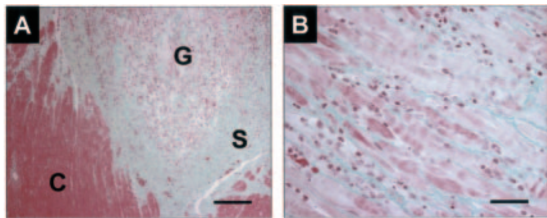

Figure 4. (A) Border region of donor-derived tissue (Masson's trichrome staining, ×100). (B) Donor-derived muscle structure (Masson's trichrome staining, ×400). C indicates myocardium; G, graft; and S, scar. Bars represent distances of 200 μm in A and 50 μm in B.

Discussion

The results in the present study are summarized as follows. The group in which 5×10^7 SM were transplanted to rat infracted hearts demonstrated reverse LV remodeling during the 4 weeks after transplantation, whereas, in the group with 5×10^5 cells transplanted, LV remodeling was inhibited, but not reversed. The effects of transplantation in the group with 5×10^6 myoblasts lied between the two groups. Histologically, 5×10^7 transplanted SM survived and differentiated into myotubes in the host scar tissue, and the engrafted muscle structure occupied most of the MI area with the wall thickness normalized. In contrast, in the groups of 5×10^6 or 5×10^5 SM, myogenic graft cells were found only partly in the MI area, and the scarred wall remained thin. Estimated volume of donor-derived muscle tissue was about 40 mm^3 4 weeks after transplantation of 5×10^7 SM.

A number of studies have documented the improvement in systolic and diastolic LV function after skeletal myoblast transplantation in various animal models and in humans.[8–10,12–15] Pouzet and associates reported that 5-fold difference in the number of transplanted SM (10^6 versus 5×10^6) caused twofold difference in LV ejection fraction.[13] Although we did not find such a remarkable difference in functional improvement by cell numbers, a similar trend was clearly recognized. In echocardiography, we observed an interesting finding that graft contraction in its periphery started at the end-diastolic phase just before the host myocardium began to contract in group I. These graft kinetics may be totally compatible with the concept of *passive contraction* that was described by Taylor and colleagues as 'respond to stretch'.[8,9]

In contrast, there were no papers found which described dimensional improvement of the left ventricle after myoblast transplantation as shown in group I, although attenuation of LV remodeling has been reported.[14,15] Because group I had about 10 times as many cells transplanted as those conventionally applied, we may well ascribe the reverse LV remodeling in that group to the large number of donor SM. Since elastic and/or contractile property of donor-derived structures is important for functional improvement in myoblast transplantation,[8–10] more benefits may be reasonably expected from muscular grafts with larger volume. One may think that it is a matter of course to get more muscle volume with more donor cells. However, we do not agree with the idea, because our experience tells us that it is not the case in cardiomyocyte transplantation, in which donor myocytes do not survive in the center of the scar in most cases.[3,4] Because there is no blood supply to donor cells at least when transplanted, it becomes harder for the cells in the center of a cluster to obtain enough oxygen and nutrients as transplanted cells increase. Therefore, we consider that the ischemia-resistant property of SM[7,18] enabled the cells to remain viable in a large mass without enough blood supply.

As another probable reason for the significant decrease in LV diameters in group I, we should mention the remarkable increase in the wall thickness of the scarred region induced by SM-derived muscle tissue. Because the scarred wall thickness in group I was almost normalized as demonstrated in Figure 3A and 3D, the wall tension applied to the MI area and also the whole left ventricle must have been significantly decreased via LaPlace's law. Consequently, decreased oxygen demand of the LV myocardium may have caused reverse LV remodeling. In addition, we used neonatal SM without expansion in this study, expecting that the viability of cells would not be attenuated during multiple passage procedures. Whether use of freshly isolated SM can enhance the survival rate is unclear, but based on our experience in cardiomyocyte transplantation, the method is unlikely to work against cell viability. Hence, it may also be a possible explanation for the good LV performance in our study.

Another novel aspect of the present study was that the correlation between the volume of SM-derived muscular tissue and the improvement in cardiac performance induced by myoblast transplantation was clearly shown by estimation of donor-derived muscle volume. Given that the left ventricle is considered as a globe with the internal radius 4 mm and the wall thickness 2 mm, the scarred wall can be approximated to the circle with the radius 4 ($\approx 2\pi\times4\times0.3/2$) mm, if infarct size is 30%, and the volume of intact myocardium corresponding to the MI area may be calculated as 100 ($\approx \pi\times4^2\times2$) mm^3. Then, the estimated graft volume shown in group I was about half of the volume that was necessary for complete replacement of the scarred region. Taking into account the histological finding that the scar around the graft was significantly thickened (Figure 4A), the estimated volume may have been enough for full replacement of the MI area in the pathological morphology after cell transplantation. Although, very recently, an experimental trial has been reported to repair the MI area by applying contiguous cell sheets onto the scarred wall to decrease the loss of transplanted cells,[19] this study suggested the possibility that direct cell transplantation may be able to repair the heart completely in certain conditions.

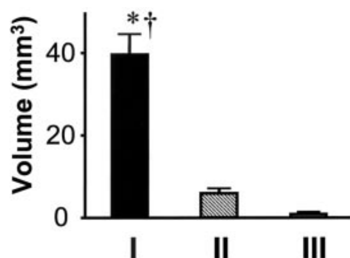

Figure 5. Estimated donor-derived muscle volume. I indicates group I; II, group II; and III, group III. *P<0.0001 versus group II, †P<0.0001 versus group III.

In histology, our findings were almost compatible with those in earlier studies.[7,8] Reinecke and Murry pointed out the risk of tissue overgrowth when SM were transplanted in large numbers.[11] Although some of the hearts in group I had epi- or endocardial distortion and/or distention, dimensional and functional improvement was observed even in distorted hearts. Because SM-derived muscle tissue was confined to the MI area, viable host myocardium may not have significantly affected by the distortion. However, it may always be necessary to pay attention to tissue overgrowth when a large number of SM are transplanted. We found that the scar tissue around engrafted structures was significantly thickened in group I compared with the other 2 groups. It may also be related to the large number of transplanted cells.

There are some limitations to the present study. First, we did not set control or sham-operated rats in the study. Theoretically, it is better to include such groups when designing a comprehensive study. However, we have extensive experiences to use the MI rat model in our laboratory. The technique and outcome were already published in a number of papers,[3–5,16] and the putative results for control groups can be reasonably extrapolated. Therefore, we purposely focused to the effects of myoblast transplantation by differences in cell number. Secondly, it is uncertain that our results can be directly applied to the clinical setting, because we used neonatal SM without expansion in small animals. The next step should be full replacement of the MI area with autologous adult SM in a large animal model. Although, at this stage, it may have some risk to transplant a large number of SM in the clinical setting, temporally multiple transplantation in small cell numbers each time using minimally invasive instruments may solve the issue of tissue overgrowth. Further investigation is warranted.

In conclusion, a large number of transplanted neonatal SM, which are immediately used without culture or expansion, can survive in the host and fully replace the infracted myocardium with reverse LV remodeling in infracted rat hearts. The efficacy in LV performance is correlated to the volume of myogenic structures derived from donor SM.

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