Intracoronary Infusion of Skeletal Myoblasts Improves Cardiac Function in Doxorubicin-Induced Heart Failure

Ken Suzuki, MD, PhD; Bari Murtuza, MA, FRCS; Noriko Suzuki, MD; Ryszard T. Smolenski, MD, PhD; Magdi H. Yacoub, FRS

Background—Skeletal myoblast transplantation is promising for the treatment of end-stage heart failure. Direct intramyocardial injection is useful for local cell delivery but may not be effective in global dissemination of cells into the heart, which would be advantageous in treating generalized cardiac dysfunction as in dilated cardiomyopathy. We hypothesized that intracoronary infusion of myoblasts would disseminate cells more effectively, leading to functional improvement in global heart failure.

Methods and Results—Heart failure was induced by the intraperitoneal administration of doxorubicin (total dose 15 mg/kg) in rat. One million primary skeletal myoblasts were then infused via the coronary arteries of an excised, failing doxorubicin-treated heart. After incubation under increased intracoronary pressure, the hearts were subsequently transplanted into syngeneic recipients. For the control group, doxorubicin-treated hearts were infused with medium only and transplanted. Four weeks after transplantation, Langendorff perfusion demonstrated that both maximum dP/dt (2797.6±103.3 versus 2326.9±133.1 mm Hg/s, \( P = 0.01 \)) and minimum dP/dt (−1718.8±91.3 mm Hg/s, \( P = 0.02 \)) were improved in myoblast-transplanted hearts compared with medium-infused hearts. This was associated with a sharper slope of the left ventricular developed pressure-volume curve and a reduced slope of the end-diastolic pressure-volume relation in the myoblast-transplanted hearts. Immunohistochemistry for skeletal myosin heavy chain showed that globally disseminated myoblasts had survived and differentiated into multinucleated myotubes that had aligned with the cardiac fiber axis within host myocardium. No significant myocardial infarction was observed.

Conclusions—We demonstrated the feasibility and efficiency of skeletal myoblast transplantation via the intracoronary route as a promising strategy for improving cardiac function in global heart failure. (Circulation. 2001;104[suppl I]:I-213-I-217.)

Key Words: cells ♦ transplantation ♦ heart failure ♦ cardiomyopathy ♦ arteries

It has been shown that skeletal myoblasts survive and retain the capacity to differentiate into functional muscle when transplanted into myocardium in various species, including mice, rats, rabbits, and dogs. Further, the grafted myoblasts have been reported to improve cardiac function in experimental heart failure models. Skeletal myoblast transplantation is thus promising for the treatment of end-stage heart failure. Direct intramyocardial injection is a popular method for cell delivery into the heart and would be advantageous in the treatment of ischemic cardiomyopathy due to its ability to deliver cells selectively into the myocardial tissue, into either normal or infarcted areas. However, cells infused via this method usually produce islet-like formations, resulting in possible limitation of the cell-to-cell interaction between grafted cells and native cardiomyocytes. We speculate that this might limit integration of the grafted cells into native myocardium, restricting the efficiency of this strategy. In addition, direct intramyocardial injection may not be beneficial in disseminating cells globally into the heart, which might be advantageous in treating globally damaged hearts as in dilated cardiomyopathy.

Recently, we reported the feasibility of intracoronary transplantation of skeletal myoblasts into the intact rat heart. In this protocol, 1 million L6 cells (a rat skeletal myoblast–derived cell line) expressing LacZ were infused into explanted intact rat hearts via the coronary arteries, followed by heterotopic heart transplantation. Consequently, grafted myoblasts, observed throughout the cardiac layers of both left and right coronary territories, had proliferated, differentiated, and integrated into host myocardium, forming gap junctions with native cardiomyocytes, without reduction in cardiac function or coronary embolism. In the present study, we investigated the efficiency of this intracoronary transplantation method in improving cardiac function of doxorubicin-induced global heart failure.
Methods

Isolation of Rat Primary Skeletal Myoblasts
All studies were performed with the approval of the institutional ethics committee. The investigation conforms to 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 published by National Institutes of Health (NIH publication No. 85-23, 1996).

Primary skeletal myoblasts were isolated using the single-fiber culture system.8 Briefly, the extensor digitorum longus of an 80-g male Lewis rat was removed carefully and incubated in 0.2% type I collagenase (Sigma Chemical Co) in DMEM at 35°C for 1.5 hours. Single muscle fibers were liberated by titration, and individual, viable muscle fibers were placed in each well of 24-well tissue culture plates in 10% horse serum (HS) and 0.5% chicken embryo extract (GIBCO), 2 mmol/L glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin in DMEM. Putative skeletal myoblasts (satellite cells) dissociated from the fiber within 1 day after plating and proliferated. The fiber was then removed, and the medium was switched to proliferation medium, consisting of 20% FCS, 10% HS, 0.5% chicken embryo extract, 2 mmol/L glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin in DMEM. The cells were fed every other day, and the culture was passaged before reaching 75% confluence to maintain the undifferentiated state. To induce myogenic differentiation, myoblasts were grown to subconfluence in proliferation medium, and then the medium was switched to differentiation medium: DMEM supplemented with 2 mmol/L glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 2% HS.8 To confirm purity of the culture, cells were seeded onto 4-well chamber slides (Labteks), incubated to subconfluence, and fixed with −20°C methanol/acetone. After blocking, the samples were incubated in a 1:5 dilution of anti-α-sarcosomic actin monoclonal antibody (DAKO) at 4°C overnight and then in a 1:200 dilution of FITC-conjugated secondary antibody (DAKO). Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI).

Generation of Doxorubicin-Induced Cardiomyopathy and Intracoronary Infusion of Myoblasts
Doxorubicin-induced heart failure was generated as described before.9,10 Doxorubicin hydrochloride (Sigma Chemical Co) was administered in 6 equal injections (each containing 2.5 mg/kg in saline IP) to male Lewis rats (200 g) over a period of 2 weeks for a total dose of 15 mg/kg. Rats were observed for 4 weeks after the final injection for their general appearance, behavior, and mortality. At the end of the 4-week posttreatment period, surviving animals were treated by intramyocardial transplantation via the coronary artery as follows.7 The doxorubicin-treated failing hearts were arrested with cold cardioplegia and removed under anesthesia with sodium pentobarbital (50 mg/kg IP) and anticoagulation with heparin (200 USP units IV). The aortic arch was preserved with major branches ligated to make the following steps of Langendorff perfusion easy. Just before perfusion, the syngeneic myoblasts (although not autografts) were harvested from culture flasks using trypsin, filtered through a 20-μm membrane filter (Millipore), and adjusted to a concentration of 1×10^6 cells/mL in DMEM. Subsequently, the hearts were infused with 1×10^6 myoblasts via the coronary arteries, with the venae cavae and pulmonary arteries and veins ligated. For the control group, 1 mL DMEM was infused. After incubation under increased intracoronary pressure (40 to 80 mm Hg) on ice for 10 minutes, the hearts were transplanted into the abdomens of syngeneic recipient rats (225 g).7,8 The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava of recipient rats, respectively. After confirmation of full recovery, the rats were returned to the cages. All surgical procedures were performed aseptically.

Functional Assessment of the Heart
At 4 weeks after cell transplantation, recipient rats were anticoagulated by intravenous heparin injection under anesthesia as described earlier (n=8 in each group). The transplanted hearts were quickly excised from the abdomen and perfused at a pressure of 100 cm H2O with modified Krebs-Henseleit buffer containing (in mmol/L) NaCl 120.0, KC1 4.5, NaHCO3 20.0, KH2PO4 1.2, MgCl2 1.2, CaCl2 1.25, and glucose 10.0, gassed with 95% O2 and 5% CO2 at 37°C, using a Langendorff apparatus.7,11 After 20 minutes of stabilization, parameters were measured using a thin-walled balloon with left ventricular (LV) end-diastolic pressure (LVEDP) stabilized at 10 mm Hg. After completion of measurement at LVEDP of 10 mm Hg, the balloon was deflated to 0 μL volume and its size was increased by the addition of water in 25-μL increments. The LV systolic and diastolic pressures were recorded at each balloon volume, and developed pressure was calculated using a computer-associated recorder (Linton).

Histological Assessment
At 4 weeks of cell transplantation, grafted hearts for histological assessment (n=4 in each group) were removed after Langendorff perfusion, and the LV was cut into 4 segments to be frozen in an embedding medium. A 6-μm section was cut from each segment and fixed in −20°C methanol. The sections were dried, incubated with 0.6% H2O2 in methanol, and then immersed in 0.1% Triton X-100 and 1% BSA. The sections were incubated with a 1:40 dilution of anti–skeletal myosin heavy chain (MHC) antibody (Zymed Laboratories) at 4°C overnight. This antibody reacts with skeletal MHC but not to cardiac or smooth muscle myosin. It was followed by a 1-hour incubation with a 1:100 dilution of biotinylated secondary antibody (DAKO). The sections were colored with a DAKO streptABCComplex/HRP Duet kit according to the company’s instructions and counterstained with hematoxylin and eosin.

Statistical Analysis
All values are expressed as mean±SEM. Statistical comparison of the data for pressure-volume curves was performed using ANOVA for repeated measures followed by Bonferroni’s test for individual significant difference. The differences in other data were determined with Student’s t test. P<0.05 was considered statistically significant.

Results

Characteristics of Skeletal Myoblasts
Primary skeletal myoblasts were isolated using the single-fiber culture system. Putative skeletal myoblasts dissociated from their fiber (0.3 to 1.0 cm long, ~20 μm in diameter) were cultured for 4 weeks after plating (Figures 1A and 1B) and proliferated to subconfluence, the myoblasts fused and differentiated into multinucleated myotubes within 1 week after switching medium (Figure 1D).

Mortality Rate
The mortality rate caused by doxorubicin administration was 30.0% during the 6-week incubation time from the first injection of doxorubicin until cell transplantation. During the 4-week period after skeletal myoblast transplantation or medium injection, the total operative mortality rate of the recipient rat was 6.7% and 7.7%, respectively.

Functional Improvement After Skeletal Myoblast Transplantation
Failing, doxorubicin-administered hearts were treated by intracoronary infusion of a skeletal myoblast suspension or...
At 4 weeks after cell transplantation, both maximum and minimum dP/dt values at 10 mm Hg LVEDP were significantly improved in skeletal myoblast–transplanted hearts compared with the medium-injected, control hearts (Figures 2C and 2D). Coronary flow in the skeletal myoblast-transplanted hearts was also significantly higher than that in the control hearts (Figure 2B), whereas the difference in heart rate was not significant (Figure 2A). In addition, according to the pressure-volume curves, a sharper slope of the developed pressure-volume relation (Figure 3A) and a reduced slope of LVEDP-volume relation (Figure 3B) were observed in skeletal myoblast–transplanted hearts compared with the control hearts.

**Histological Findings After Skeletal Myoblast Transplantation**

At 4 weeks after skeletal myoblast transplantation via coronary arteries, hearts were collected to investigate the behavior of grafted myoblasts. Under a low-power magnification, discrete loci positively stained for skeletal MHC were observed in all samples from cell-transplanted hearts (Figure 4A), whereas no cells stained in the medium-injected, control hearts. Because the antibody reacts with rat skeletal MHC but not with cardiac or smooth muscle MHC, surviving myoblasts had differentiated into multinucleated myotubes and aligned with cardiac fiber axis within native myocardium (D). Scale bar: 100 μm for A through C, 25 μm for D.
muscle myosin, these positively stained cells were presumed to be of skeletal myoblast origin. At these loci, it was observed that surviving myoblasts had completely differentiated into multinucleated myotubes in the native myocardium and aligned with cardiac fiber axis (Figure 4D). In addition, histological evidence of intracoronary thrombosis or myocardial infarction was not found.

Discussion

We demonstrated that intracoronary infusion can successfully disseminate primary skeletal myoblasts into a doxorubicin-induced rat heart failure model. Transplanted myoblasts differentiated into multinucleated myotubes that aligned with cardiac fiber axis within native myocardium, and this was associated with significant improvement in cardiac function of the doxorubicin-induced cardiomyopathy. Intracoronary infusion of cells provides theoretical merits over direct intramuscular injection, such as producing less myocardial damage during engraftment, in addition to disseminating cells globally into the myocardium. Although it has already been reported that direct intramyocardial injection of neonatal cardiomyocytes can improve the cardiac function of doxorubicin-induced cardiomyopathy in mice, one might expect that intracoronary global dissemination would be more advantageous for treating global cardiac failure as in dilated cardiomyopathy. The possibility of the intracoronary transplantation route for cellular cardiomyoplasty has been proposed and investigated, but the present study is the first report to provide data on the efficacy of this method in improving the cardiac function of damaged hearts. The present data are consistent with our previous study, in which we used intracoronary infusion of β-galactosidase–expressing skeletal myoblasts and showed that myocardial infarction (coronary embolism) is not found following our strategy with no embolism observed in other organs such as liver, kidney, spleen, or lung of the recipient rats.

Previous studies have shown that not only diastolic but also systolic function of hearts locally damaged by cryoinjury or infarction is improved after direct intramyocardial injection of skeletal myoblasts. We have shown that the doxorubicin-treated failing heart that underwent intracoronary skeletal myoblast grafting (followed by heterotopic transplantation) demonstrates higher maximum dP/dt and a sharper slope of the LV developed pressure-volume relation as well as improved minimum dP/dt with a reduced slope of the LVEDP-volume curve in comparison with control-treated hearts that were subjected to exactly the same condition of heterotopic transplantation. This suggests that the intracoronary myoblast grafting strategy is able to enhance systolic and diastolic function of failing, doxorubicin-treated hearts. However, a comparison of cardiac function after this cell transplantation with that of nontransplanted hearts such as doxorubicin-treated, non–cell-transplanted, or normal hearts would not be possible in this study, because these cell-treated hearts were subjected to a 28-day period of mechanical unloading after heterotopic transplantation. Further study is needed to investigate this issue.

The mechanism of improved cardiac function by a relatively small mass of grafted skeletal myoblasts/myotubes remains unclear. A possible explanation is that function might be improved by contractile properties of grafted myotubes in addition to release of endocrine or paracrine factors by grafted cells resulting in attenuation of adverse remodeling, improved angiogenesis, and enhanced contractile ability of cardiomyocytes. Regarding diastolic function, we speculate that physical characteristics of doxorubicin-treated failing hearts and atrophy after heterotopic transplantation might be attenuated by grafted skeletal myoblasts/myotubes, which are likely to be less stiff than infarct scar tissue. Further study, including measurement of chamber size with echocardiography, is needed to clarify this issue.

In our previous study in which we used this protocol for intracoronary skeletal myoblast transplantation, we found that some engrafted L6 skeletal myoblasts expressing β-galactosidase differentiated into multinucleated myotubes and integrated into the myocardium with formation of gap junctions with surrounding host cardiomyocytes, whereas others formed undifferentiated tumor-like colonies. It is still unclear why some remained in the undifferentiated state. However, such undifferentiated colonies were not observed in the present study using primary skeletal myoblasts without genetic modification. In this study, we extrapolated the protocol to investigate the efficiency of this strategy in a situation closer to the clinical setting by using primary myoblasts and avoiding the use of prior dye staining and genetic manipulation. Furthermore, we used myoblasts derived from extensor digitorum longus (a fast-twitch muscle) because it has been demonstrated that myoblasts of this muscle proliferate less actively but fuse into myotubes more efficiently than those of a slow-twitch muscle like soleus in rats. These findings might suggest that the differentiation ability of the primary myoblasts of extensor digitorum longus is better compared with the L6 cell line that had been genetically engineered to express β-galactosidase, when transplanted into the myocardium in vivo. It might be interesting to further investigate the difference in the results of intracoronary cell transplantation when using skeletal myoblasts derived from a slow-twitch muscle.

In skeletal myoblast transplantation, the purity of skeletal myoblast culture is an important factor that could affect the efficiency in improving cardiac function, because contaminating nonmyogenic cells, mostly fibroblasts, are unlikely to significantly contribute to contractile function. With traditional methods for skeletal myoblast isolation, the myogenic cells are usually inundated by such nonmyogenic cells, and thus additional procedures are needed to enrich the myogenic cell fraction, such as preplating, differential centrifugation, or sedimentation in a Percoll density gradient. These procedures, however, only marginally increase purity, often at the expense of a substantial loss of myogenic cells. In this study, we used the "single-fiber culture system," from which we can obtain virtually pure cultures of skeletal myoblasts without additional hazardous processes for purification, because the cells that originate from single fibers are all myogenic. This isolation method would therefore be advantageous for skeletal myoblast transplantation.

It is important to clarify the optimal graft cell number and mass of surviving graft required to achieve the best improvement in cardiac function after skeletal myoblast transplantation, even though the overall functional result may be affected by other factors, such as species, graft survival, differentiation capacity, ability to form gap junctions with host myocardium, and condi-
tion of host myocardium. Taylor’s group demonstrated that direct intramuscular injection of $10^5$ skeletal myoblasts improves function of cryoinjured rabbit hearts,\textsuperscript{4,5} whereas Scorsin et al\textsuperscript{6} showed that 5 million myoblasts improve postinfarction function in rats. In this study, we demonstrated that only 1 million primary skeletal myoblasts infused via coronary arteries could significantly improve cardiac function of doxorubicin-induced heart failure in rats. The optimal mass of grafted myoblasts/ myotubes required to improve cardiac function of diseased hearts is still unclear. According to our previous data, 1 million L6 myoblasts expressing β-galactosidase infused via coronary arteries increased in number to $\approx 5 \times 10^6$ on day 28 after grafting.\textsuperscript{7} Although grafted cell survival would be different in intact and doxorubicin-treated hearts to some extent, these data could be useful to estimate the skeletal myoblast mass surviving in doxorubicin-treated hearts. Regarding the possible number of cells for grafting, intracoronary transplantation has an apparent disadvantage over intramuscular injection because of the risk of coronary embolism. When $1 \times 10^7$ or $10^8$ myoblasts were infused into the heart using the same system in our previous study, the engrafted heart developed a large myocardial infarction just after reperfusion and stopped beating immediately with concurrent extreme swelling and hemorrhage. It has been shown, however, that $\approx 1$ million myoblasts can safely be transplanted into rat hearts using this protocol, without forming significant coronary embolism. For the purpose of overcoming this limitation of cell number, methods to increase cell survival after grafting, such as prior heat shock treatment,\textsuperscript{21} would be useful. This treatment can double the graft survival after intracoronary skeletal myoblast transplantation.

Skeletal myoblast transplantation is also promising as a tool for cell-mediated gene therapy, providing extended, local expression of useful proteins in the heart.\textsuperscript{22,23} So far, only direct intramyocardial injection has been reported for the cell-delivery method, applying skeletal myoblasts that express tissue growth factor-β,\textsuperscript{22} or vascular endothelial growth factor.\textsuperscript{23} For the purpose of angiogenesis induction, direct intramyocardial injection of myoblasts expressing a certain growth factor into an ischemic region would be advantageous due to their ability for sustained, selective, regional protein delivery. One might, however, consider that a more global and uniform protein expression within the whole heart would be more beneficial in some cases, for example, gene therapy for generalized cardiac failure using myoblasts expressing proteins capable of improving cardiomyocyte contraction. We speculate that the ability of intracoronary infusion to disseminate cells into myocardium would be of significance in these cases.

In conclusion, we have demonstrated the feasibility and efficiency of intracoronary skeletal myoblast infusion as a promising strategy for improving cardiac function of global heart failure. Although death from heart disease has shown a steady decline for the past decade, the prevalence of congestive heart failure continues to increase. At present, the effect of drug treatment is limited, whereas heart transplantation has some serious disadvantages, such as complications from immunosuppressive agents and high cost.\textsuperscript{24} The present method, which is applicable to the clinical setting in cardiac surgery, could be useful as an alternative strategy for the treatment of global heart failure.

Acknowledgments

We would like to thank Prof Tereza A. Partridge, Dr Louise Haslop, and Dr Jennifer E. Morgan (Muscle Cell Biology Group, Hammer-smith Hospital, UK) for their expert support in establishing the rat skeletal myoblast isolation technique.

References

Intracoronary Infusion of Skeletal Myoblasts Improves Cardiac Function in Doxorubicin-Induced Heart Failure
Ken Suzuki, Bari Murtuza, Noriko Suzuki, Ryszard T. Smolenski and Magdi H. Yacoub

Circulation. 2001;104:I-213-I-217
doi: 10.1161/hc37t1.094929

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/104/suppl_1/I-213

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/