Does the Functional Efficacy of Skeletal Myoblast Transplantation Extend to Nonischemic Cardiomyopathy?

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Background—The benefits of skeletal myoblast (SM) transplantation on infarcted myocardium have been investigated extensively; however, little is known about its effects in nonischemic cardiomyopathy models. To address this issue, we tested SM transplantation in CHF147 Syrian hamsters, a strain characterized by a δ-sarcoglycan deficiency that phenotypically features the human setting of primary dilated cardiomyopathy.

Methods and Results—Cell culture techniques were used to prepare \( \approx 5 \times 10^6 \) muscle cells from autologous tibialis anterior muscle, of which 50% were SMs (desmin staining). The cells were injected in 6 sites across the left ventricular wall (n=14). Control animals (n=12) received equivalent volumes of culture medium. Left ventricular systolic function was assessed in a blinded fashion from 2D echocardiographic left ventricular fractional area change, before transplantation, and 4 weeks later. Explanted hearts were processed for the detection of myotubes and quantification of fibrosis. Baseline functional data did not differ between the 2 groups. Four weeks after transplantation, 6 of the 10 surviving grafted hamsters were improved compared with 0 of the 8 survivors of the control group. This translated into a 6% decrease in fractional area change in controls compared with a 24% increase in cell-transplanted hamsters (\( P=0.001 \)). Engrafted myotubes were consistently detected in all SM transplanted hearts by immunohistochemistry, whereas fibrosis was not worsened by cell injections.

Conclusions—These data suggest that the functional benefits of SM transplantation might extend to nonischemic cardiomyopathy. (Circulation. 2004;110:1626-1631.)

Key Words: cardiomyopathy ■ heart failure ■ myoblasts, skeletal ■ tissue therapy

Nonischemic global dilated cardiomyopathy (DCM) accounts for almost one half of new cases of heart failure encountered in clinical practice.1-3 Symptomatic heart failure continues to have a grim prognosis, with 1-year mortality estimated at \( \approx 45\% \).4 Cardiac transplantation is the most effective life-saving therapy of DCM at an advanced stage, but it is associated with serious medical and socioeconomic problems. Another potential strategy is implantation of mechanical assist devices.5 Because these nonpharmacological therapies and cardiac resynchronization6 are invasive and expensive, the search continues for alternative treatment options, among which cell therapy is generating growing interest.

Over the past decade, there has been accumulating experimental evidence that cell transplantation could be a new, effective approach for repairing damaged myocardium.7 The functional benefits of intramyocardially transplanted skeletal myoblasts have been established in various animal models that have involved ischemia as the primary mechanism of injury.8-10 Likewise, only patients with left ventricular (LV) dysfunction have been included in the initial feasibility trials11-13 and in the ongoing randomized phase II efficacy study of autologous skeletal myoblast transplantation. Despite other attractive cell types, skeletal myoblasts have been widely investigated because of their clinically appealing properties, including autologous origin, ease of procurement, and high capacity for large in vitro scale-up.

In contrast, limited preclinical data exist on the effects of cell transplantation in the setting of DCM. Therefore, the present study was designed to address the feasibility and potential efficacy of multiple direct intramyocardial injections of autologous myoblasts in a globally dilated myocardium.

To model the clinical setting of the disease as closely as possible, we used the CHF147 strain of Syrian hamster. This
strain is characterized by a mutation in the δ-sarcoglycan gene that results in a global DCM evolving toward heart failure.14

Methods

All experiments were performed in accordance 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 Resources, Commission on Life Science, National Research Council and published by the National Academy Press (revised 1996).

Animal Model

The operative procedures were performed in the locally maintained animal colony derived from the CHF147 line of Syrian hamsters established by Canadian Hybrid Farms (Halls, Harbor, Nova Scotia, Canada). All animals included in this study underwent a genotyping procedure by a polymerase chain reaction assay to confirm the 27.5-kb homozygous deletion of the δ-sarcoglycan gene.

Cell Culture Methodology

Under general anesthesia with ketamine (75 mg/kg IP), xylazine (15 mg/kg IP), and midazolam (0.75 mg/kg IP), the right and left tibialis anterior muscles were dissected, maintained in a nutrient mixture (MCDB, Hyclone), minced, weighed, and enzymatically dissociated with collagenase (2 mg/mL IA, Sigma) for 1 hour and trypsin-EDTA (0.25%, Gibco) for 20 minutes. The cells were collected by centrifugation (7 minutes at 1200 rpm), and the enzyme reaction was arrested by adding 10% FBS (Hyclone). The cell suspension was filtered through a 100-μm nylon mesh and centrifuged; the supernatant was discarded; and the cells were resuspended in the culture medium composed of MCDB with 20% FBS (Hyclone), 1% penicillin-streptomycin (Gibco), 10−8 mol/L dexamethason (Merck), and 5 ng/mL bFGF (Sigma). Initial plating was performed in 75-cm² culture flasks (Falcon, Becton Dickinson), and cells were grown in humid air supplemented with 5% CO2. One day later, the cells were washed and the growth medium was changed. Before confluence, the cells were split into 2 flasks to avoid fusion. Eight days after initial plating, the cells were harvested by trypsinization and washed, and viability was assessed with trypan blue (0.4%, Gibco). A sample was plated onto 12-well dishes in 0.2-mL culture medium to be counted for viability. The cell suspension was similar to that reported in previously published works.17

Cell Counting

From 18 to 24 hours after plating onto the 12-well dishes, the cells were washed and fixed in methanol cooled at −20°C for 5 minutes. The cells were incubated with desmin mouse anti-human antibody (1 hour, 1/200, DAKO), followed by Cy3-conjugated anti-mouse antibody (1 hour, 1/200, Jackson Immuno Research Laboratories). All nuclei were stained by adding DAPI (0.1 μg/mL in PBS) for 10 minutes. The proportion of myoblasts was calculated by dividing the number of desmin-positive cells by the total number of DAPI-positive nuclei.

Experimental Groups

Twenty-six CHF147 hamsters (22 males, 4 females; age, 6 to 8 months; weight, 110 to 130 g) were included in the study and randomly allocated to receive myoblast transplantation (n=14) or to serve as controls (n=12). On the day of transplantation, the animals were reanesthetized as previously described and tracheally ventilated. The heart was approached through a left lateral thoracotomy, and 30 μL cell suspension was injected in 6 sites (5 μL at each site) with a 32-gauge needle (Hamilton). Control hamsters received an equivalent volume of culture medium according to a similar procedure.

Functional Assessment

Echocardiography

Ten days before and 4 weeks after injection, LV systolic function was studied by 2D echocardiography after anesthesia as previously described. Recordings were performed from left parasternal windows in animals in a supine position with a commercially available 13-MHz linear-array transducer (15L8) connected to a Sequoia 512 system (Acuson Corp), which allows 2D imaging with a 160-Hz maximal frame rate. The heart was imaged in 2D mode, displaying first parasternal LV long-axis views and then short-axis LV views at the midpapillary muscle level that were stored as digital loops. End-systolic (ESA) and end-diastolic LV cavity areas (EDA) were determined by tracing endocardial borders on the stored images. According to the recommendations of the American Society of Echocardiography for 2D echocardiography, the endocardium was traced by covering the innermost edge of that surface. The end-diastolic and end-systolic frames were obtained by moving the loop frame by frame throughout the entire cardiac cycle to catch the largest (EDA) and smallest (ESA) cavity sizes. Systolic function was evaluated as the fractional area change [FAC=(EDA−ESA)/EDA×100], as previously used in murine heart failure models.15,16

For each measurement, 3 consecutive cardiac cycles were traced and averaged by a single experienced examiner in a blinded fashion. The intraobserver variability of measurements was studied on 10 animals analyzed in a random, blinded manner. The means (±SD) of the differences between measurements were 0.012±0.061 cm² for areas, 0.002±0.031 mL for end-diastolic volume, 0.003±0.040 mL for end-systolic volume, and 0.33±3.22% for ejection fraction, similar to that reported in previously published works.17

Histological and Immunohistochemical Assessment

Gross Histology

Animals were killed by an overdose of ketamine, xylazine, and midazolam. Explanted hearts were frozen in isopentane cooled with liquid nitrogen. Serial sections (8 μm thick) were then prepared with the use of a cryostat and subjected to hematoxylin and eosin staining, histomorphometric analyses, and immunohistochemistry.

Histomorphometric Analysis

Hearts were cut into 3 blocks at the level of the apex, mid-LV, and base of the papillary muscles. Each block was sliced into 10 serial slides, each containing four 8-μm-thick heart sections. For each section, the surface of newly formed skeletal muscle tissue was quantified relatively to the LV surface by computerized planimetry. Macroscopic measurements of LV posterior wall and septal thicknesses were based on hematoxylin and eosin staining. Sirius red was used to perform a computerized quantification of fibrosis by an operator blinded to treatment group. Under a light microscope, at ×10 magnification, 3 fields per heart section (apex, midmitral papillary muscle, and base) were captured with a numeric camera connected to the microscope. Quantitative analyses of the pictures were performed using Lucia G software. Results are given as the ratio of specifically colored pixels to the total number of pixels of the 3 fields.

Immunohistochemistry

For immunohistological studies, the slides were rinsed in PBS and fixed with cold methanol for 5 minutes, and the nonspecific labeling was neutralized with the blocking serum. Slides were then incubated with the primary antibody for 1 hour and, after several washes, with the biotinylated goat anti-mouse antibody. The streptavidin-peroxidase complex was incubated for 5 minutes on slides, and the immunoenzymatic reaction was revealed with the Vector Novared kit. The slides were mounted in Faramount (Vector). The transplanted myoblasts were detected with the mouse monoclonal antibody directed against the fast skeletal myosin heavy-chain isoform (1/400, clone My32, Sigma).
Statistical Analysis
Because of the small sample size and the nontheoretical distribution of our data, we used nonparametric statistical methods based on the analysis of ranks. Consequently, data are expressed as median values (minimum and maximum). All analyses were performed with Stata 7 software (Stata Corp). Continuous variables and percent changes from baseline values were compared by a Mann-Whitney U test. A Wilcoxon test was used to compare continuous variables within the same group. A $\chi^2$ test (Fisher exact test) was used to compare the distribution of improved, stable, and worsened animals between the 2 groups. A value of $P<0.05$ was selected as the threshold for significance.

Results
Characterization of the Cell Suspension
Approximately 1000 cells were counted on the well dishes plated on the day of transplantation, among which $\approx50\%$ were desmin positive and therefore considered skeletal muscle cells (Figure 1A through 1C). The amount of myoblasts was $2.8\times10^6\pm7.4\times10^5$, ranging from $1.44\times10^6$ to $3.68\times10^6$ (Table 1). The lowest percentages of myoblasts were obtained from the oldest animals; however, as a whole, we did not observe a statistical difference in yields between animals $>6$ and $<6$ months of age.

Mortality
Of 26 hamsters operated on, 8 died (4 myoblasts, 4 controls; $P=0.7$). Most of the deaths (6 of 8) occurred between 6 and 9 days after the surgical procedure.

Functional Assessment
Echocardiography
Baseline echocardiographic values were not different among the 2 groups (Table 2). Four weeks after transplantation, 6 of the 10 surviving grafted hamsters had improved systolic function compared with 0 in the control group ($P=0.018$; Figure 2). This improvement was reflected by a $24\% (-30\%, 85\%)$ increase in FAC relative to the corresponding baseline value, whereas this parameter decreased by $6\% (-36\%, 3\%)$ in controls ($P=0.019$; Figure 3). The increased FAC seen in transplanted animals was due primarily to a significant decrease in LV ESA [$-0.03\text{ cm}^2 (-0.09\text{ cm}^2, 0.05\text{ cm}^2)$ versus $0.04\text{ cm}^2 (-0.02\text{ cm}^2, 0.14\text{ cm}^2)$ in controls ($P=0.008$)], whereas LV diastolic dimensions were not different between the 2 groups.

Histological and Immunochemical Assessment
Macroscopic measurements did not show any statistical difference between the 2 groups with regard to global and localized

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<th>TABLE 1. Cell Culture Characterization</th>
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<th>TABLE 2. Baseline Echocardiographic Values in the Myoblast and Control Groups</th>
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<td>FAC, %</td>
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BW indicates body weight; HR, heart rate. Data are given as median (minimum, maximum) values.
LV posterior and septal wall thicknesses. At the cellular level, using nucleus area as a surrogate marker of cardiomyocyte hypertrophy, we failed to find differences between transplanted hearts and controls. Multinuclear elongated structures were observed in the different injected areas 4 weeks after transplantation (Figure 4). These structures were seen throughout the LV and were absent from hearts that did not receive the skeletal muscle cells and from noninjected areas such as the interventricular septum and right ventricle. Immunohistochemistry allowed us to characterize these cells as clusters of fast skeletal myosin-expressing cells extending along the needle tracts (Figure 5). Engrafted myotubes and muscle fibers were aligned along either the long or the short axis of the heart. The deposit of these skeletal myoblasts and the needle-induced scar in controls extended from the epicardium to the midcardium.

The surface of the myogenic donor cell–derived graft relative to the LV was 0.9% (0.3%, 3.86%) at the apex, 1.45% (0.7%, 2.61%) at the midpapillary muscle level, and 1.9% (0.51%, 3.75) at the base level of papillary muscles. Averaging individual values of these fast myosin-positive areas across the whole heart yielded a median percentage of engraftment of 1.45% (0.3%, 3.86%).

One month after epicardial injections, interstitial fibrosis was not significantly different between the 2 groups: collagen depo-
sition occupied 7.65% of the heart (5.8%, 13%) in the cell-injected group compared with 9.4% (6.2%, 12%) in controls.

Discussion

The major finding of the present study is that multiple intramyocardial injections of skeletal myoblasts in a nonischemic model of genetically determined global cardiomyopathy result in a consistent engraftment of cells, do not increase disease-associated interstitial fibrosis, and are associated with a better preservation of LV function.

Cellular transplantation is receiving growing interest as a means of replacing lost cardiomyocytes by exogenously supplied contractile cells. Among them, skeletal myoblasts feature attractive characteristics, including ease of procurement, growth potential in vitro, and strong resistance to ischemia. The benefits of myoblast transplantation have been established mainly in settings of ischemic cardiomyopathies and have paved the way for the initial clinical trials in ischemically induced heart failure. Thus, it looked sound to assess whether the ability of cell transplantation to functionally regenerate ischemically damaged segments of myocardium could extend to more diffuse forms of heart disease.

Data on cell transplantation in nonischemic models are scarce but encouraging. Scorsin et al initially reported improved function after fetal cardiomyocyte transplantation in a mouse model of doxorubicin-induced cardiomyopathy. Transplantation of myoblasts was first tested in a canine model of Duchenne muscular dystrophy. Demonstration of dystrophin-positive myogenic cells in the heart of recipient dogs provided the proof of principle that cells could engraft into a nonischemically damaged myocardium, but the functional relevance of this engraftment was not tested in these experiments. Functional studies were developed by Yoo et al, who injected heterologous cultured adult heart cells or autologous smooth muscle cells in a hamster model of genetic cardiomyopathy (Bio53–58) and reported a postransplantation improvement in systolic indexes. Contractility was assessed in this work by ex vivo Langendorff perfusion. Suzuki et al evaluated intracoronary infusion of adult skeletal myoblasts in a doxorubicin-induced model of heart failure in rat and observed a functional improvement, as measured ex vivo, and widely distributed grafted cells throughout the LV. The present results support these findings in that transplantation of skeletal myoblasts in a model mimicking the human setting of DCM allowed LV systolic function to stabilize (3 of 10 cases) or to improve (6 of 10). In contrast, age-matched control animals receiving equivalent volumes of culture medium almost consistently deteriorated (3 of 8) or stabilized (5 of 8) but never improved. This pattern is consistent with the natural history of the disease, which is progressive worsening. Compared with the above-mentioned studies, the distinctive features of our work are as follows. First, we used of a genetic model of global DCM in the δ-sarcoglycan–deficient Syrian hamster (an inbred colony of the CHF147 line). Second, the intramyocardially injected myoblasts were autologous. Although this choice may look paradoxical, it was dictated by 2 considerations: In this strain, skeletal myoblasts are genetically affected but phenotypically spared by the large deletion of the δ-sarcoglycan gene, and the use of autologous cells had the advantage of eliminating the potentially confounding effect of immunosuppressive therapy, which would have been required in the case of allografting. Third, we included in vivo assessment of LV performance.

The myoblast-related improvement involved LV systolic function, as evidenced by a significant increase in echocardiographically determined LV short-axis FAC. In this study, there was a trend for all echocardiographic parameters to improve in the treated group. However, statistical significance was only achieved for short-axis FAC. Additional studies are thus required to fully validate the functional efficacy of skeletal myoblast transplantation in nonischemic heart failure settings.

The improved LV systolic function seen in transplanted hearts correlated with a consistent engraftment of myotubes identified as clusters of fast skeletal myosin-expressing cells along the needle tracts. Although a quantitative estimate of cell survival was not a primary end point of the study, we assessed the surface of the myogenic donor cell–derived graft relative to the LV surface. This analysis yielded a 1.45% (0.3%, 3.86%) engraftment but may underestimate the magnitude of cell engraftment because it was based on the analysis of blocks separated by 560-μm intervals. Because islands of grafted cells may have been harbored in these intermediate portions of myocardium, they were missed by the final calculation of graft volume.

Because a major histological feature of this form of cardiomyopathy is focal fibrosis, a concern raised by multiple injections of cells was that this fibrosis could be worsened by the inflammatory reaction triggered by needle punctures and scavenging of dead grafted cells. However, such was not the case; the quantitative estimate of the collagen content failed to show any difference between the 2 groups.

Although encouraging, the results of the present study need to be tempered by several limitations. The first limitation is inherent in the model itself. The follow-up period was limited to 1 month. Thus, one cannot exclude a delayed graft loss over time, possibly because of an active process intrinsic to the underlying disease. The genetic defect of the injected myoblasts has no phenotypical expression in myoblasts, but their progeny (myotubes and myofibers) might suffer from the lack of δ-sarcoglycan, known to be involved in sarcosomal stabilization and signal transduction.
The second clinically relevant issue not addressed in this study is the potential proarrhythmic risk of myoblast transplantation. This concern has been raised in ischemic settings and related to the possible set-up of reentry circuits resulting from the differences in electrical membrane properties between skeletal muscle and cardiac muscle cells. Whether such a concern is equally relevant to a nonischemic myocardial substrate remains to be determined.

Furthermore, our experiments do not provide a mechanistic explanation for the improvement yielded by myoblast transplantation. The lack of between-group differences in diastolic dimensions does not favor a scaffolding effect of the grafted cells. Likewise, that connexin-43 is not expressed on grafted myoblasts/myotubes precludes the achievement of a functional syncitium and electromechanical coupling between donor and recipient cells. Thus, the beneficial effects of myoblasts might be due instead to changes in extracellular matrix composition, direct contraction of engrafted myotubes in response to gap junction–independent electronic currents fired by neighboring cardiomyocytes, or secretion of growth factors, leading to a paracrine mobilization of cardiac stem cells.

In conclusion, the positive results of this study should first be taken as an additional proof of principle that transplanted myoblasts can engraft into a nonischemically diseased myocardium and contribute to improved LV performance. Should these data be confirmed by additional experiments, the consistency and robustness of myoblast engraftment in these globally failing hearts could provide new, interesting perspectives in the treatment of DCM in heart failure patients who are not suitable candidates for transplantation or mechanical assist devices. Because of the diffuse nature of the disease, cell delivery would then be optimally achieved by catheter-based techniques, allowing widespread distribution of cells in a minimally invasive fashion.

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