Cellular Cardiomyoplasty of Cardiac Fibroblasts by Adenoviral Delivery of MyoD Ex Vivo: An Unlimited Source of Cells for Myocardial Repair

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**Background**—The muscle-specific MyoD family of transcription factors function as master genes that are able to prompt myogenesis in a variety of cells. The purpose of our study was to determine whether MyoD could induce primary cardiac fibroblasts, isolated from infarcted myocardium or pericardium, to undergo myogenic conversion in a clinically relevant approach.

**Methods and Results**—Primary rat fibroblasts from 7-day-old infarcted myocardium or normal pericardium were transfected by an E1/E3-deleted adenoviral vector carrying both a human MyoD cDNA driven by a CMV promoter and a green fluorescent protein (GFP) reporter gene driven by a second CMV promoter. Expression of MyoD caused myogenic differentiation of cultured fibroblasts, as defined by elongation and fusion into multinucleated myotubes, typical cross striation as identified by electron microscopy, and positive immunostaining for sarcomeric actin, fast myosin heavy chain (MHC), and actinin. The myogenic cells (1.5×10^6) were transplanted into the infarcted myocardium 7 days after coronary artery occlusion. By 1 month after transplantation, the converted fibroblasts gave rise to a cluster of myogenic cells that in a few hearts occupied a large part of the scar with positive immunostaining for the myogenic proteins fast-MHC and sarcomeric actin. A few cells expressed the gap junction protein connexin 43 in a disorganized manner. There was no positive staining in the control hearts treated with injections of untreated fibroblasts or culture medium.

**Conclusions**—Our work shows that it is possible to exploit the unique capacity of MyoD to activate myogenesis in fibroblasts ex vivo and to create a vast source of autologous myogenic cells for transplantation. (Circulation. 2002; 106[suppl I]:I-125-I-130.)

**Key Words:** cells ■ gene therapy ■ myocytes ■ transplantation

Cellular cardiomyoplasty by cell transplantation is a promising strategy that offers the creation of new functional tissue to replace lost or failing myocardium.1,2 A major barrier for the clinical application of cell transplantation is obtaining sufficiently large quantities of suitable cells.1 Several cell sources have been tested, although each of them entails considerable limitations.1,3

The muscle-specific MyoD family of transcription factors function as unique master genes that are able to prompt myogenesis in a variety of cells including fibroblasts.4 Transformation of cardiac fibroblasts in situ into skeletal myocytes in myocardial infarct scar tissue could add to postinfarction adaptation. However, attempts to convert the infarcted myocardium into contractile tissue in situ by direct injection of viral vectors encoding the MyoD required a high dose of recombinant adenovirus, and yielded disappointing results.2,5

Ex vivo cellular cardiomyoplasty is an alternative myocardial repair strategy that combines the advances in genetic and tissue engineering. This approach may provide an alternative source of autologous, transplantable, myogenic cells by ex vivo genetic manipulation of cardiac fibroblasts. The aim of our study was, therefore, to address the issue of whether the phenomenon of myogenic conversion of primary fibroblasts with MyoDH could be exploited in practical terms to provide an alternative source of myogenic cells for transplantation.

**Materials and Methods**

Our study was performed in accordance with the guidelines of The Animal Care and Use Committee of the Tel-Aviv University, Tel Aviv, Israel, which conforms to the policies of the American Heart Association.

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Isolation of Primary Cardiac Fibroblasts
Primary fibroblasts were obtained from 2 sources: In the first group, Sprague-Dawley rats were subjected to permanent left coronary artery occlusion and the animals were euthanized 1 week later. The hearts were excised and the scar (granulation tissue) was dissected from the surrounding normal myocardium under direct visualization into 3 to 4 pieces of 1 mm³. In the second group, the pericardium of normal Sprague-Dawley rats was removed and the pericardial tissue was dissected into 3 to 4 tissue segments (1 mm³). Tissue fragments were plated into a 60-mm dish for 2 hours. After 2 hours of adhesion, we added a few drops of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) covering each fragment. The next day, we added a few drops of culture medium to each piece of tissue and 2 days later we covered the tissue fragments with culture medium. After 4 to 5 days, fibroblasts began to proliferate from the fragment margin (“halo of cells”) and created a monolayer. The outgrowing cells were morphologically consistent with fibroblasts by their characteristic spindle-shaped appearance. All experiments were carried out with cells derived from up to 4 passages. For in vitro immunostaining experiments, fibroblasts (100,000 cells) were plated in 2 chamber glass slides. For myogenic conversion and cell transplantation studies, fibroblasts were plated in a 25-mm² flask and each flask contained 1.5×10⁶ cells.

Construction and Propagation of MyoD/GFP Adenoviral Vector
MyoD/GFP adenoviral vector was created in 2 steps. First, MyoD cDNA was inserted into a transfer vector. Next, it was transferred into the adenoviral genome by homologous recombination in Escherichia coli. Recombinants were selected with antibiotic resistance. This two-step method minimizes manipulation of viral DNA and allows for convenient selection of recombinants. Both transfer and adenoviral vectors were gifts from Dr. Nori Kasahara (Institute of Genetic Medicine, University of Southern California, Los Angeles, Calif., USA).

The first step was to clone MyoD into transfer vector: MyoD cDNA containing a Flag peptide epitope was excised from pCDNA3–MyoD with XbaI and HindIII. The 5' overhang of the XbaI site was filled in with Klenow fragment. The resulting MyoD cDNA was inserted into the HindIII, EcoR V restriction sites of pAdTrack CMV transfer vector.

The second step was transfer to adenoviral vector by homologous recombination: pAdTrack CMV–MyoD was linearized with Pmel. Linearized plasmid, isolated by gel electrophoresis, was co-transformed with pAdEasy1 adenoviral vector (Ad5ΔE1/ΔE3) into E. coli by electroporation. A particular strain of E. coli, BJ5183 (Genotype: endA sbcBC recBC dgal galk met thi-1 bioT hsdR (Strr)), was used for its recombination capabilities. On homologous recombination between the 2 co-transformed plasmids, CMV–MyoD and CMV–GFP and a kanamycin resistance gene were inserted into the E1 region of the adenoviral genome. Potential recombinants carrying MyoD/GFP were screened by selecting for kanamycin resistance. Subsequently, viral genome was confirmed by restriction analysis. The virus was grown, amplified, and purified as previously described and was frozen to -70°C. Virus concentration was calculated as 10¹⁰ viral particles per ml.

Ex Vivo Myogenic Conversion of Fibroblasts
Seven days before transplantation, primary fibroblasts (1.5×10⁶ per flask) were incubated with 100 µL of adenoviral vector in concentration of 130 to 150 of multiplicity of infection (MOI–viral particles/cell), in serum-free DMEM medium for 3 hours at 37°C. The cells were washed 3 times to remove any remaining virus. The transfected cells were incubated for 40 hours in medium DMEM with 10% FCS, then, shifted to differentiation (low serum) medium (DMEM medium supplemented with 2% FCS) for 5 days. Transfection efficiency was determined 24 hours after transfection by the extent of the expression of GFP reporter gene with a fluorescent inverted microscope. By day 6, representative samples of converted fibroblasts were fixed, immunolabelled with skeletal antibodies, and counterstained with hematoxylin. Other plates were prepared for in vivo transplantation by trypsinization.

Rat Model of Myocardial Infarction
Our MI model has been previously described. Male Sprague-Dawley rats (~250 g) were anesthetized with a combination of 40 mg/kg ketamine and 10 mg/kg xylazine, intubated, and mechanically ventilated. The chest was opened by left thoracotomy, the pericardium was removed, and the proximal left coronary artery was permanently occluded with an intramural stitch.

Cell Transplantation
Cell transplantsations were performed 7 days after MI, as previously described. To simplify the fibroblast isolation protocol, the transplant experiments were carried out using primary pericardial fibroblasts. After 6 to 7 days in culture, converted fibroblasts were selected microscopically: Only plates in which >50% of the cells were multinucleated myotubes were selected for transplantation. Cells were prepared for transplantation by trypsinization with 0.25% trypsin-EDTA and then resuspended in serum free DMEM medium. The final cell density for implantation was 1.5×10⁶ cells/150 µL. Rats were anesthetized and under sterile technique the chest was opened. The infarcted area was identified visually on the basis of surface scar and wall motion abnormality. All injections were made into the center of the scar. Rats were randomly allocated into 3 groups of injections: (1) 1.5×10⁶ myogenic converted fibroblasts transfected with Ad–MyoD/GFP, (2) 1.5×10⁶ control fibroblasts transfected with Ad-GFP, and (3) serum-free medium. The site of injection was marked with a small suture placed apical to the injection site. After injections into the scar, air was expelled from the chest and surgical incision was sutured closed.

Histology and Immunohistochemistry
Seven days after transfection the converted fibroblasts were fixed in cold ethanol 70% for 15 minutes and stained for the specific skeletal proteins α-sarcromeric actin (Zymed), fast myosin heavy chain (MHC) (Zymed), and actinin (Sigma), the gap junction marker connexin 43 (Zymed), and counterstained with hematoxylin.

In a subgroup of animals (n=3), hearts were harvested 7 days after cell transplantation, washed in ice-cold saline, and cut into 4 slices (3 to 4 mm thick) parallel to the atrioventricular groove. The slices were immediately frozen, sectioned, and evaluation of GFP transgene expression was performed with a fluorescent microscope.

Other animals were killed with an overdose of pentobarbital 4 weeks after cell transplantation. Hearts were harvested and processed for histological and immunohistochemical examination. Adjacent blocks were embedded in paraffin, sectioned into 5-µm slices, and stained with hematoxylin and eosin. Serial sections were immunolabelled with the skeletal specific antibodies α-sarcromeric actin, fast-MHC, and actinin or the gap junction marker connexin 43 and counterstained with hematoxylin.

Electron Microscopy
For transmission electron microscopy, the MyoD-treated cells were immersion fixed for at least 4 hours in a buffered glutaraldehyde and paraformaldehyde solution (Karnovsky fixative) at 4°C. This was followed by 1-hour post fixation in buffered 1% osmium tetroxide at 4°C. After 12 hours in 0.5% uranyl acetate, the tissues were dehydrated and embedded in Medcast resin (Ted Pella, Redding, Calif.). After being sectioned and stained with uranyl acetate and lead citrate, the specimens were examined by electron microscope.

Results
Overall, 21 infarcted rats were included in the in vivo study. Animals were treated by implantation of MyoD converted fibroblasts (n=6), by genetically marked fibroblasts with GFP gene (first control group; n=6) and by serum-free medium (second control group; n=6). Another group, treated with MyoD-treated cells (n=2) or serum-free medium (n=1),
These morphologic and myogenic changes were absent in control fibroblasts that were stained negative for fast-MHC and sarcomeric actin (Figure 1C). To assess the degree of differentiation of the treated cells, immunostaining against fast-MHC isoform, sarcomeric actin, actinin, and connexin 43 was used. Myotubes were stained positive for fast-MHC, α-sarcomeric actin (Figure 1B, 1D, and 1E), and actinin (data not shown), indicating myogenic differentiation. Many cells developed typical cross striation with early sarcomeric organization (Figure 1E). The myogenic conversion efficiency induced by forced expression of MyoD was nearly 50%, with similar efficiency for fibroblasts prepared from the infarcted or pericardial tissue. The gap junction protein connexin 43 was undetectable.

Ultrastructural Analysis
Transmission electron microscopy of the multinucleated myotubes revealed parallel arrays of myofibrillar bundles oriented in an irregular manner in some cells, whereas more mature sarcomeric organization was apparent in other cells (Figure 1F). The degree of myofibrillar organization varied within different areas of the same cell. Some cells showed an immature phenotype manifested by disorganized myofibrillar stacks whereas others showed a more organized sarcomeric structure. In some foci, the formation of early and more developed Z bands could be observed (Figure 1F). We were unable to identify intercalated discs, cellular structures that characteristically appear during in vivo cardiomyocyte differentiation.

Cell Transplantation Into the Heart
We investigated whether the converted fibroblasts could be transplanted into the infarcted myocardium and survive for at least 4 weeks. To identify the transplanted fibroblasts in the infarcted zone, fluorescent localization using expression of GFP transgene was determined 1 week after transplantation in 3 animals under fluorescent microscopy. Both transplanted groups (Ad-MyoD/GFP and Ad-GFP) expressed the GFP following transplantation (Figure 2A). After 4 weeks, both MyoD-converted and control fibroblasts formed a viable graft that was, in some cases, separated from the host myocardium by scar tissue (Figure 2B).

Immunohistochemical staining of Ad-MyoD/GFP treated-rats revealed elongated cells fused into multinucleated myotubes (Figure 2C). Expression of myogenic proteins, fast-MHC, and sarcomeric actin in the grafted cells confirmed the survival and differentiation of the MyoD-converted fibroblasts in the infarcted myocardium 4 weeks after transplantation (Figure 2C, 2D, and 2E). No staining for fast-MHC was observed in the host myocardium or scar tissue either in the hearts treated with Ad-GFP fibroblasts or those treated with culture medium (Figure 2B). No staining for sarcomeric actin was observed in the scar tissue of rats treated with either Ad-GFP fibroblasts or culture medium. In several hearts, the cell implant occupied >50% of the scar (Figure 2B) whereas in others only a few cells were detected.

Connexin 43, a known component of the gap junction, appears to be expressed in a few grafted cells in a disorganized fashion (Figure 2F). There was no positive immunostaining for connexin 43 in the hearts treated with GFP-transfected fibroblasts or in the culture medium-injected hearts.
Discussion

In the present study, we have shown for the first time that cardiac fibroblasts converted to myogenesis by forced expression of \textit{MyoD} can be transplanted into the infarcted myocardium, forming a viable graft and expressing skeletal and gap junction proteins. Our findings suggest that \textit{MyoD} conversion of cardiac fibroblasts ex vivo may provide an alternative, attractive source of transplantable myogenic cells in a clinically relevant manner.

Cellular Cardiomyoplasty

Cellular cardiomyoplasty has been introduced as an innovative approach to repair the injured myocardium.\cite{1,2,10} Recent interest has focused on autologous cells: growing replacement tissues for damaged heart from a patient’s own cells that would avoid rejection of transplanted tissues.\cite{19} Controversy data have shown that skeletal myoblasts engrafted into cryoinjured or infarcted hearts successfully seeded the lesion and formed myotubes in rats, dogs, and rabbits.\cite{11,12,13,14} After transplantation into the heart, satellite myogenic skeletal muscle cells might undergo transdifferentiation into functioning cardiac-like myocytes.\cite{11,12}

However, the use of autologous skeletal myoblasts could be limited in elderly patients by the low recovery of satellite cells from muscle biopsies,\cite{15,16} and the reduced capacity to create functional myofibers.\cite{17} Furthermore, this strategy might be compromised by the metabolic and structural abnormalities of skeletal muscle cells obtained from heart failure patients.\cite{18} Therefore, in the majority of the heart failure population (eg, elderly patients) there may be difficulty in obtaining reasonable numbers of appropriate healthy cells. In these patients an alternative autologous cell source might be needed.

\textbf{MyoD-Based Therapy}

Transformation of cardiac fibroblasts in the scar tissue into muscle could reduce left ventricular (LV) remodeling and improve LV function. It was shown that forced expression of the \textit{MyoD} gene in cultured non-muscle cells could initiate the process of myogenesis.\cite{4,19} However, the results of in vivo studies attempting to convert the scar into muscle by direct injection of high dose of recombinant adenovirus were disappointing,\cite{2,5} probably because the necrotic myocardium is resistant to direct gene transfer.\cite{5,7,20}

An alternative strategy that combines the advances in genetic and tissue engineering is ex vivo cellular cardiomyoplasty, which is defined as ex vivo genetic manipulation of cardiac fibroblasts by adenoviral vector encoding \textit{MyoD} and creation of alternative sources of transplantable myogenic cells. Previous reports have shown the feasibility of fibroblast transplantation into the infarcted myocardium.\cite{21,22} One major advantage of the ex vivo approach is that transient forced expression of the \textit{MyoD} transgene activates endogenous \textit{MyoD} and irreversibly assigns cells to myogenesis.\cite*{5,19} Furthermore, the conversion efficiency of the ex vivo approach is significantly better than the direct injection approach in the setting of MI.\cite{7} The clear advantage of genetically modified fibroblasts is that fibroblasts are autologous, abundant, easily expandable, and simple to harvest from clinically assessable sites such as skin. We preferentially used pericardial rather than skin fibroblasts because we hoped that pericardial fibroblasts might have a higher tendency for transdifferentiation into cardiomyocytes after transplantation.\cite{5} However, the
present study did not provide evidence of transdifferentiation of the implanted cells into cardiomyocytes. Thus, skin fibroblasts, which are more easily assessed, are an attractive and better alternative.

Lattanzi et al demonstrated that genetic manipulation of fetal fibroblasts by adenoviral vector encoding MyoD could provide a source of transplantable myogenic cells in a mouse model of skeletal injury. The myotubes originating from converted fibroblasts cannot be distinguished from those derived from primary myoblasts. After transplantation into the injured skeletal muscle, the MyoD-converted fibroblasts formed muscle fibers indistinguishable from those originating from primary myogenic cells. Our findings support and extend those findings in the setting of MI and heart failure and indicate that the MyoD-converted fibroblasts may participate in muscle regeneration and provide a feasible alternative strategy for myocardial repair by cell transplantation and ex vivo gene therapy.

Some limitations of the study need to be mentioned. The percentage of myogenic converted cells was ~50%. We used a relatively low viral particles/cell (MOI) to convert the cells, a dose that may be clinically relevant. It is likely that by increasing the virus titer to that used by Murry et al or Lattanzi et al (up to 2000 MOI) we would increase the efficiency of myogenic conversion and the number of myogenic transplantable cells.

The outgrowing cultured cells were morphologically consistent with fibroblasts. However, more extensive characterization of the cultured cells would aid in identifying the precise nature of our cells. Moreover, a higher purity of the cell fraction used could be achieved by employment of a sorting method such as fluorescent activated cell sorting.

Gap junction formation between grafted myoblasts and host cardiomyocytes is important and controversial issue concerning skeletal myoblast transplantation and function. Although our converted fibroblasts did not express connexin 43 in vitro, we identified a few myogenic cells that were stained positive for the gap junction protein connexin 43, in a disorganized fashion, 1 month after transplantation. Still, the presence of connexin 43 in the engrafted region could represent persistence of some host cardiomyocytes in the infarcted region. The capacity to produce gap junction proteins is critical for the engrafted cells to create connections and integrate with the host myocardium. However, in our previous study using fetal cardiomyocytes, we showed that isolated cell grafts might prevent LV remodeling and dysfunction, independent of differentiation and integration, in a rat model of MI.

We have proposed that our strategy might be relevant to elderly people in whom the availability of myoblasts or adult stem cells is limited. Thus, an animal model that mimics the biology of elderly patients, with fewer myoblasts or stem cells, will be needed to strengthen our findings. To prove our concept, we used recombinant adenovector for transgene delivery. Application of this approach in clinical practice may be limited because of a potential immune response against remains of viral antigens. Future approaches will involve the development of new vectors that do not provoke an immune response. An additional limitation is that the area of the cell graft was not measured and the percentage of the surviving cells is unknown. Because not all the engineered fibroblasts were myogenic, the total number of cells required for transplantation may be higher than other myogenic cells derived from other sources.

Finally, the effect of the implanted myogenic cells on LV remodeling and function was not tested in the present study. Direct contribution of the implanted myogenic cells to contractility is unlikely based on our histologic findings. However, the beneficial effects of the donor cells seems to be independent of differentiation and integration. The engrafted genetically engineered fibroblasts might have the capacity to inhibit host matrix degradation, to improve diastolic function, to enhance regional hypertrophy and neovascularization, and, by increasing scar thickness, will reduce wall stress and prevent infarct expansion, LV dilatation, and deterioration of function.

Implications and Future Research

Our work shows that it is possible to exploit the unique capacity of MyoD to activate myogenesis in cardiac fibroblasts ex vivo and to create a vast source of autologous myogenic cells for transplantation. Fibroblasts can be obtained easily from clinically and easily accessible sites, such as the skin, expanded, converted to myogenesis by transient expression of MyoD, and then implanted into the infarcted myocardium of the same patient recovered from MI. The MyoD-converted cells can be utilized to repair the infarcted myocardium by direct injection into the scar, as done in our study, or by seeding into 3D scaffold and creation of biografts. Furthermore, the genetically modified cells could be used to deliver therapeutic genes into the infarcted myocardium.

This clinically relevant, combined strategy of genetic and tissue engineering could be of importance in treating elderly patients with massive myocardial damage, patients whose normal myogenic cells have been depleted or are inadequate in their growth potential, to prevent LV deterioration and heart failure. The relative place of this strategy compared with other approaches for cardiac tissue engineering and myocardial regeneration needs further investigation and includes functional studies and direct comparison with alternative cell sources such as bone marrow stem cells and skeletal myoblasts.

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