CARDIOPULMONARY BYPASS, MYOCARDIAL MANAGEMENT, AND SUPPORT TECHNIQUES

MOLECULAR CARDIOMYOPLASTY: POTENTIAL CARDIAC GENE THERAPY FOR CHRONIC HEART FAILURE

In this study, we evaluated the feasibility of converting cardiac fibroblasts into skeletal muscle cells by forced expression of the MyoD gene, one of the basic helix-loop-helix myogenic factors. Primary cardiac fibroblasts, isolated from newborn rats, were infected with retrovirus-carrying sense or antisense MyoD gene. Ten days after infection, expression of MyoD protein was demonstrated in 95% of cells infected with sense MyoD virus by intense nuclear immunostaining with a MyoD polyclonal antibody. In contrast, none of the cells infected with antisense MyoD virus showed staining. On withdrawal of serum, 95% of MyoD positive cells became elongated and, in the presence of appropriate cell density, fused to form multinucleated myotubes, morphologically similar to striated muscle cell. Expression of downstream myogenic differentiation markers, myosin heavy chain and myocyte-specific enhancer factor 2, in 95% of these myotubes were detected by intense cytoplasmic and nuclear immunostaining, respectively, with specific antibodies. In contrast, no detectable staining was noted in MyoD negative cells. Spontaneous contractile movements were noted in a few clusters of myotubes. In summary, cardiac fibroblasts were able to be converted into bonafide potentially functional skeletal myocytes as shown by definitive morphologic and biochemical changes. Further studies with in vivo models are needed to explore this unique molecular strategy to treat patients with chronic heart failure. (*J THORAC CARDIOVASC SURG* 1995;109:918-24)

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Cardiomyoplasty is an operation consisting of wrapping synchronously paced transformed fatigue-resistant skeletal muscle, commonly the latissimus dorsi, around the heart. The operation offers a potential therapy for chronic heart failure, by using the power derived from skeletal muscle.^{1,2} Another strategy to treat patients with heart failure is to provide additional endogenous power, that is, more cardiac myocytes. However, mammalian car-

diac myocytes become terminally differentiated and lose their ability to undergo mitosis soon after the newborn period.^{3,4} The mechanism responsible for this cell cycle arrest is largely unknown. As a result, we have few clues as to how to induce cardiac myocytes to reenter the cell cycle. In addition, because possible cardiac myogenic factors have not been identified, conversion of cardiac nonmuscle cells to cardiac myocytes is currently impossible.

In skeletal muscle, myogenic transcription factors, the MyoD family of basic helix-loop-helix proteins, are known to function as master genes for induction of the skeletal muscle differentiation program.⁵ Expression of transfected MyoD gene has been shown to mediate the conversion of mouse C3H10T1/2 fibroblast cell line into stable myoblast, which could be induced to undergo terminal differentiation into multinucleated myotubes, morphologically similar to skeletal muscle cells.⁶ Biochemically, in addition

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Read at the Seventy-fourth Annual Meeting of The American Association for Thoracic Surgery, New York, N.Y., April 24-27, 1994.

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0022-5223/95 \$3.00 + 0 12/6/61883

to myosin heavy chain (MHC), MyoD is known to induce the expression of other downstream myogenic differentiation markers, like myocyte-specific enhancer factor 2 (MEF2).⁷ Because essentially the same structural contractile apparatus is shared by both cardiac and skeletal myocytes, conversion of cardiac fibroblasts, for example, those in a scar after myocardial infarction, into potentially functional skeletal myocytes may be of benefit for the treatment of heart failure. In this study, we evaluated the feasibility of converting cardiac fibroblasts into skeletal muscle cells by forced expression of the MyoD gene in primary cultured cardiac fibroblasts.

Materials and methods

Isolation of cardiac fibroblasts. Primary cardiac fibroblasts were prepared from newborn rat ventricles. Hearts were removed from 1-day-old newborn Wistar rats. The ventricles were washed with 4° C balanced salt solution (MgSO₄ · 7H₂O 200 mg/L, NaCl 68 gm/L, KCl 400 mg/L, NaH₂PO₄ · H₂O 1.5 gm/L, glucose 1 gm/L, and HEPES buffer, pH 7.5, 4.76 gm/L). They were then minced into fine fragments and again washed with 4° C balanced salt solution. The ventricular tissue was transferred into a 50 ml tube containing 3 to 5 ml balanced salt solution, with 0.01% collagenase II and 0.5% pancreatin, and incubated in 37° C with gentle agitation for 10 to 15 minutes. The supernatant was added to 1.5 ml of fetal calf serum (FCS). After 5 to 6 cycles of digestion, the supernatant was pooled and centrifuged at 500 to 800 rpm for 5 minutes. The pellet was resuspended in growth medium (20% FCS in Dulbecco's modified Eagle's medium [DMEM]) and plated at 100 cells/mm² in each 100 × 20 mm uncoated plastic Petri dishes. The supernatant was aspirated and discarded after 15 to 20 minutes of incubation at 37° C to get rid of unattached cells. The fibroblasts were then maintained in growth medium.

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Viral propagation and cell infection. Amphotropic retrovirus-producing cell lines generated with PA317 cells were obtained from Dr. D. Miller.⁸ These cell lines produced retroviruses that carry neomycin G418 resistance gene and sense or antisense MyoD gene. The virus-producing cells were maintained in growth medium (20% FCS DMEM). Viral infection of fibroblasts was performed as described by Weintraub and associates.⁸ After 3 days, infected cells were selected by adding neomycin G418 in a concentration of 500 µg/ml (Gibco, Grand Island, N.Y.) to the medium. Seven days after neomycin G418 selection, serum was withdrawn by changing the medium to 1% FCS DMEM. After 1 to 2 weeks, the cells were examined for morphologic changes by phase

contrast microscopy by means of Zeiss objectives (Carl Zeiss, Inc., Thornwood, N.Y.).

Immunofluorescence. Cells were fixed by direct immersion in 3.7% paraformaldehyde in phosphate-buffered saline for 10 minutes. Fixed cells were then made permeable by immersion in 0.3% Triton X100 octyphenoxy polyethoxyethanol in phosphate-buffered saline for 5 minutes, washed three times with phosphate-buffered saline, and incubated with specific antibodies. Primary antibodies used for immunofluorescence were MF₂₀ monoclonal anti-MHC antibody, rabbit polyclonal anti-MEF2, and anti-MyoD antibodies. The secondary antibodies used were rhodamine-conjugated goat antimouse immunoglobulin G and fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G (Boehringer, Ingelheim Corp., Indianapolis, Ind.). Cells were examined by immunofluorescent microscopy with a Zeiss 63× objective.

Results

Morphologic changes. Between 1 and 2 weeks after the withdrawal of serum, the cell structure was examined by phase contrast microscopy. As shown in Fig. 1, *A*, 95% of the fibroblasts infected with sense MyoD virus had become elongated and, in the presence of appropriate cell density, fused to form multinucleated cells (myotubes). In contrast, all cells infected with antisense MyoD virus remained round and unchanged, as shown in Fig. 1, *B*. The differences in morphologic details were demonstrated in higher magnification (63×) as shown in Fig. 2, *A* and *B*. The fused myotube (Fig. 2, *A*) with its elongated cell body and multiple nuclei is morphologically similar to striated muscle fibers, whereas the control cells (Fig. 2, *B*), infected with antisense MyoD virus, remained unchanged.

A few clusters of myotubes were observed to have spontaneous contractions under the culture conditions described herein, suggesting that these converted myotubes had intact contractile machinery. Further studies with physiologic conditions and stimuli are needed.

Biochemical changes. Ten days after infection, expression of MyoD protein was demonstrated in 95% of fibroblasts infected with sense MyoD virus by intense immunostaining with anti-MyoD antibody. In contrast, none of the cells infected with antisense MyoD virus showed staining (data not shown). Double immunostaining for the presence of known downstream MyoD-inducible myogenic differentiation markers, MHC and MEF2,⁷ was performed 1 to 2 weeks after serum withdrawal (i.e., 2 to 3 weeks after infection), at the time when there were marked morphologic changes with the sense

Fig. 1. Morphologic analysis of primary cardiac fibroblasts infected with sense or antisense MyoD virus by phase contrast microscopy. This analysis was performed 1 to 2 weeks after the withdrawal of serum. **A,** Magnification ($\times 40$) of cardiac fibroblasts, which were converted into myotubes after sense MyoD virus infection. **B,** Magnification ($\times 40$) of cardiac fibroblasts, which remained unchanged after antisense MyoD virus infection.

MyoD virus-infected cells. As depicted in Fig. 2, C, the elongated sense MyoD virus-infected cell (the same frame as in Fig. 2, A) expressed abundant downstream myogenic differentiation markers, MHC (red) in its cytoplasm and MEF2 (green) in its nuclei. The pattern of cytoplasmic MHC staining suggested the presence of striation. These definitive

biochemical changes demonstrated that the myogenic differentiation program has been induced. In contrast, the antisense MyoD virus-infected cells (Fig. 2, D, same frame as in Fig. 2, B) failed to turn on the myogenic differentiation program by having no staining, indicating the absence of MHC and MEF2 protein.

Fig. 2. Combined morphologic and immunofluorescent analysis of primary cardiac fibroblast infected with sense or antisense MyoD virus. This analysis was performed 1 to 2 weeks after the withdrawal of serum. **A**, Phase contrast microscopy at 63 $\times$ magnification of fibroblasts that were converted to myotube by sense MyoD virus infection. Note the cell shape and multiple nuclei. **B**, Phase contrast microscopy at 63 $\times$ magnification of fibroblast that remained unchanged by antisense MyoD virus infection.

Discussion

Chronic congestive heart failure has emerged as an important public health problem. According to the resources of the Rochester Epidemiology Project⁹ and the current population estimate of 260 million in the United States,¹⁰ the annual incidence of chronic congestive heart failure is about 300,000,

with the overall prevalence being about 650,000. The overall prognosis is poor and has shown minimal improvements, with 80% survival at 3 months after diagnosis and 66% at 1 year,¹¹ despite proven efficacy of newer therapies like vasodilators.¹² Surgical therapy for chronic heart failure includes organ transplantation and dynamic cardiomyoplasty. Or-

Fig. 2. Cont'd. C, Immunofluorescent microscopy at 63 $\times$ magnification of the same fibroblasts as in **A**, which were converted to multinucleated myotube by sense MyoD virus infection. Primary antibodies used included specific anti-MEF2 and anti-MHC antibodies. Because rhodamine-conjugated goat antimouse secondary antibody was used, the presence of MHC, a cytoplasmic protein, will appear red. Where fluorescein isothiocyanate-conjugated goat antirabbit secondary antibody was used, the presence of MEF2, a nuclear transcription factor, will appear green. Note the hint of red cytoplasmic striation. **D,** Immunofluorescent microscopy at 63 $\times$ magnification of the same fibroblasts as in **B**. No staining was noted, indicating the absence of both MHC and MEF2 protein.

gan transplantation is currently plagued by lack of donor availability, immunosuppression and rejection, and graft atherosclerosis. Early clinical hemodynamic results with dynamic cardiomyoplasty were encouraging, but long-term survival studies are lack-

ing.^{13, 14} More recently, cell transplantation with mouse fetal cardiac myocytes, in an attempt to restore cardiac function, indicated that donor cells were stably incorporated into the recipient heart and that there appeared to be nascent intercalated

disks between donor and recipient myocytes.¹⁵ However, problems with fetal cell availability make this approach impractical. A more applicable strategy to restore cardiac function is to convert cardiac fibroblasts into contracting muscle cells. However, cardiac myogenic factors that are needed to induce "cardiac regeneration" have not been identified. In contrast, skeletal myogenic factors, the MyoD family of basic helix-loop-helix proteins, have been well described.⁵ Because essentially the same structural contractile apparatus is shared by both cardiac and skeletal myocytes, conversion of cardiac fibroblasts, for example, those in a scar after myocardial infarction, into potentially functional skeletal myocytes may be of benefit for the treatment of heart failure. This study shows that freshly cultured primary cardiac fibroblasts can be converted into skeletal myocytes as indicated by definitive morphologic and biochemical parameters and represents the first step in evaluating this new molecular strategy to restore cardiac function. The potential that these converted myocytes may be functional was suggested by the presence of spontaneous contractions in a few clusters of these cells. In vitro study to elucidate conditions needed for paced contractions of these converted cells is currently being evaluated. In vivo models will be used to further evaluate the feasibility and the efficiency of converting fibroblasts into myocytes and its physiologic consequences. In addition, studies to develop safe and efficient technique of gene delivery into the myocardium are ongoing.

We thank Dr. D. Miller, University of Washington, Seattle, for providing the virus-producing cell lines.

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Discussion

Dr. George J. Magovern (Pittsburgh, Pa.). In 1989 we submitted an abstract to The Society of Thoracic Surgeons forum (unfortunately not accepted) that described a different approach to the same idea. We were able to isolate satellite cells from a skeletal muscle fiber. We grew those satellite cells in culture and labeled them with thymidine. We then performed a cryoinjury to the heart, injected these cells, and were able to obtain biopsy tissue from that area 6 weeks after implantation.

It appeared to us that indeed the thymidine-labeled satellite cell, which is embedded in cardiac muscle fiber, appears to be growing into intercalated disk. We thought that perhaps the skeletal muscle satellite cell was actually forming cardiac cells, myoblasts. In other words, in the milieu of the heart with the electrical stimulus over a period of time, a satellite cell of the skeletal muscle, being

a stem cell, might grow into a cardiac cell rather than a skeletal muscle cell.

Dr. Tam. Certainly the ultimate goal is to regenerate the cardiac muscle cell itself. I do not know of any evidence or suggestion that the environment of the heart can induce skeletal muscle cell transformation into cardiac muscle cells.

As I am sure you are aware, an article just published in *Science*¹⁵ described fetal cardiac muscle cell transplantation. In that case the authors thought they saw intercalated disk formed between the transplanted cells and the native cells. In that case they need more study to see if these cells are actually functional.

Dr. Magovern. We did show intercalated disk in this area, and subsequent work by Dr. Gaugh confirmed that the gene, the *lax* gene, was present in these cells. We think it is possible that a skeletal muscle satellite cell in the heart can develop into a cardiac myocyte.

Dr. Thoralf M. Sundt (Harefield, England). It is my understanding that one of the difficulties with gene therapy is the stability of the transferred gene. You have demonstrated evidence of expression of protein products in the transfected cells, but do you have any direct evidence regarding the long-term stability of the transferred genes themselves in those cells? Of course, stability of that gene may not be necessary, because once the

differentiation is initiated the signal proteins may not be required further. That would be a significant advantage to your approach. Do you have any comments or data regarding this?

Dr. Tam. Yes, in fact, you are right. The MyoD gene is actually a gene that only needed to be expressed for a short time. In fact, once it turned on the muscle differentiation program, the cell continues to express muscle differentiated phenotype. In this instance, long-term expression of the gene is not critical.

Mr. Magdi H. Yacoub (London, England). Are you optimistic to apply similar techniques to myocardium knowing that the myocardial cell is quite different from the skeletal cell with regard to the genetic composition? This is particularly true in the total absence of a transcription factor like MyoD, which would govern the transformation of the muscle.

Dr. Tam. It certainly is not an ideal approach. The reason we took this approach is that we have not yet identified cardiac type myogenic factors.

There is no evidence at this time that these skeletal muscle cells will be functional in the heart in terms of contraction. One notion is that the presence of skeletal muscle cells may impede further dilation of the heart, and that may be another way that this process may help. I agree with you that we are far from regenerating a heart.