Muscle Differentiation during Repair of Myocardial Necrosis in Rats via Gene Transfer with MyoD

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Abstract

Myocardial infarcts heal by scar formation because there are no stem cells in myocardium, and because adult myocytes cannot divide and repopulate the wound. We sought to redirect the heart to form skeletal muscle instead of scar by transferring the myogenic determination gene, MyoD, into cardiac granulation (wound repair) tissue. A replication-defective adenovirus was constructed containing MyoD under transcriptional control of the Rous sarcoma virus long terminal repeat. The virus converted cultured cardiac fibroblasts to skeletal muscle, indicated by expression of myogenin and skeletal myosin heavy chains (MHCs). To determine if MyoD could induce muscle differentiation in vivo, we injected 2×10^9 or 10^{10} pfu of either the MyoD or a control β-galactosidase adenovirus into healing rat hearts, injured 1 wk previously by freeze-thaw. After receiving the lower viral dose, cardiac granulation tissue expressed MyoD mRNA and protein, but did not express myogenin or skeletal MHC. When the higher dose of virus was administered, double immunostaining showed that cells in reparative tissue expressed both myogenin and embryonic skeletal MHC. No muscle differentiation occurred after β-galactosidase transfection. Thus, MyoD gene transfer can induce skeletal muscle differentiation in healing heart lesions. Modifications of this strategy might eventually provide new contractile tissue to repair myocardial infarcts. (*J. Clin. Invest.* 1996. 98:2209–2217.) Key words: myocardial infarction • myogenic determination factor • MyoD • regeneration • myocardial infarct repair

Introduction

It is well known that myocardial necrosis results in scar formation rather than muscle regeneration (1). The heart cannot regenerate because cardiac myocytes cannot replicate significantly in vivo after injury (2), and because there are apparently no muscle stem cells in myocardium. As a result, patients who suffer myocardial infarctions have significantly impaired cardiac function. Thus, therapy to induce muscle regeneration after myocardial infarction might be of clinical benefit. One possible route to muscle regeneration is forcing the fibroblasts in healing heart lesions to differentiate into muscle. There is not enough known at present about cardiac muscle determination and differentiation to induce cells to form myocardium. However, much more is known about skeletal muscle differentiation. Thus, the goal of this study was to determine whether the cells in healing heart lesions can be converted into skeletal muscle.

Developing skeletal muscle expresses a family of regulatory genes termed myogenic determination factors. The prototype family member, MyoD, was cloned based on its ability to induce cultured fibroblasts to convert into skeletal muscle (3–5). Three other family members, myogenin, Myf-5, and MRF-4/Myf-6/herculin, were subsequently cloned (for reviews see references 4, 6, 7). Gene deletion studies have shown that MyoD and Myf-5 act somewhat redundantly to commit cells to the muscle lineage (8), while myogenin is required for normal muscle differentiation (9, 10). All myogenic determination factor family members can convert many different cell types into skeletal muscle in vitro. Importantly, MyoD activates both its own transcription and that of myogenin (11). Myogenin, in turn, is also autoactivating and capable of *trans*-activating MyoD transcription. The autoactivation creates a positive feedback loop, suggesting that transient expression of exogenous MyoD should permanently activate transcription of endogenous MyoD and myogenin, creating a stable muscle lineage. In this study, we hypothesized that forced expression of MyoD could induce cells of cardiac granulation tissue (the fibroblast-rich tissue of wound repair) to differentiate into skeletal muscle in vivo.

Methods

Construction and propagation of a MyoD adenovirus

This project was approved by the institutional Recombinant DNA Committee and Biohazard Committee. A replication-deficient adenovirus was constructed according to the general protocol of Graham and Prevec (12) (Fig. 1). In brief, a 2.0-kb EcoRI fragment of murine MyoD cDNA (3) was cloned into the expression vector pAd1.RSV (13) by blunt ligation. This vector, derived from a parent vector pXCX2, contains 16% of the 5′ region of the adenoviral genome cloned into pBR322. The critical E1A region has been deleted and replaced by the Rous sarcoma virus long terminal repeat, a multicloning site, and the bovine growth hormone polyadenylation sequence.

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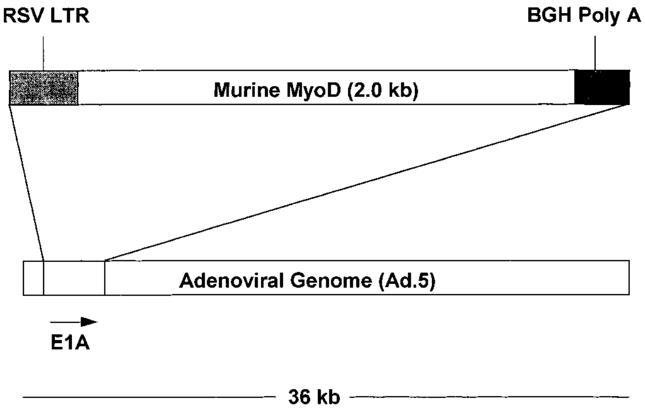

Figure 1. Schematic diagram of MyoD adenovirus. A replication-defective adenovirus was derived from the parent strain Ad5 as described in Methods. The virus contained a 2.0-kb murine MyoD cDNA insert under transcriptional control of the Rous sarcoma virus long terminal repeat (*RSV LTR*), inserted in place of the critical E1A region. A bovine growth hormone polyadenylation sequence (BGH Poly A) was present at the 3′ end of the cloning site.

The MyoD-containing plasmid was cotransfected into 293 cells with a second plasmid containing the majority of the adenoviral genome, termed pJM17 (14), using standard calcium phosphate transfection methods. After recombination in vivo, plaques resulting from viral cytopathic effects were selected and expanded in 293 cells. 293 cells have been transformed with adenoviral E1A, and therefore provide this viral transcription factor in *trans*. The crude virus-containing media from 293 cells were tested for the ability to induce myotube formation in C3H10T1/2 mouse embryonic fibroblasts. One myogenic adenoviral clone was chosen and termed AdMyoD. The MyoD adenovirus and a control adenovirus encoding nuclear-targeted β-galactosidase (15) were propagated in 293 cells as described (12). The virus preparation was purified and concentrated from cell lysates by two rounds of ultracentrifugation over a CsCl gradient, followed by dialysis in 10 mmol/liter Tris (pH 7.5)/1.0 mmol/liter $MgCl_2$/10% glycerol. The viral suspensions were aliquoted and stored at −70°C. Viral titer was determined by optical density at 260 nm and by counting plaque-forming units in 293 cells. After purification, viral titers typically ranged from 1–5 $\times 10^{11}$ pfu/ml.

Rat cardiac injury models

Animal experiments were approved by our institutional Animal Care Committee and were conducted in accordance with federal guidelines. Male Sprague-Dawley rats (Zivic Miller Laboratories, Inc., Allison Park, PA) weighing 350–500 grams were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. Under aseptic conditions a thoracotomy was performed through the left fifth intercostal space and the heart was exposed. Because coronary occlusion caused excessive mortality (~ 80%) due to congestive heart failure, a freeze-thaw injury model was used to induce myocardial necrosis (16, 17). After opening the chest, a 1 cm diameter aluminum rod, precooled with liquid nitrogen, was placed in direct contact with the anterior wall of the left ventricle for 15 s. The chest was then closed and the animals were allowed to recover. Freeze-thaw reproducibly caused a disc-shaped region of coagulation necrosis, ~ 1 cm in diameter, extending 2–3 mm into the myocardium. It should be noted that while infarcts typically have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, freeze-thaw lesions consist of confluent necrosis in the subepicardium with viable myocardium in the subendocardium. Because they are not transmural, freeze-thaw lesions do not cause cardiac aneurysm formation. Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation, and scarring after freeze-thaw injury are indistinguishable from myocardial infarction (16, 18, 19), making it a suitable model to study myocardial repair. Mortality after freeze-thaw injury was $< 10\%$. After a 1- or 2-wk period of recovery, the rats were reanesthetized and a thoracotomy was repeated. The heart was exposed, and a 100-μl suspension containing either 2×10^9 or 10^{10} pfu of MyoD or β-galactosidase adenoviral particles was injected into the wound under direct visualization, using a 27-gauge needle. The injection typically raised a bleb on the surface of the wound. The chest was closed and the animals were allowed to recover for a specified interval thereafter.

Cell culture studies

Cardiac fibroblasts. Cardiac fibroblasts were a gift from Dr. Francisco Villarreal (University of California, San Diego) (20). The fibroblasts were obtained from the hearts of adult male Sprague-Dawley rats by explant culture and were grown in high glucose DMEM with 10% fetal bovine serum. All experiments were carried out using cells with no more than four passages. For immunostaining experiments, fibroblasts were plated in four chamber glass slides at 5,000 cells/cm^2. All of the cells stained with antibodies to vimentin and smooth muscle α-actin, typical for cultured fibroblasts. None of the cells stained with antibodies to cardiac myosin heavy chain (MHC)-α[1] or to von Willebrand factor, indicating that the cultures were free of contaminating cardiocytes and endothelial cells. Adenovirus suspensions were diluted with tissue culture medium and added directly to the chambers 24 h after plating. For studies of myogenic differentiation, the medium was withdrawn after 24 h, the cells were rinsed with phosphate-buffered saline, and a differentiation medium was added consisting of DMEM with 1.5% horse serum and 6 μg/ml insulin. Cells were maintained in differentiation medium for 72 h and then fixed in methanol for immunocytochemistry.

Wound fibroblast culture. Rats were subjected to freeze-thaw injury and the wounds were allowed to heal for 1 wk. MyoD or β-gal adenovirus suspensions (100 μl containing 2×10^9 pfu viral particles) were injected directly into the injured tissue, and the animals were killed 1 wk later. The hearts were excised and the area of injury was dissected from the surrounding normal myocardium under direct visualization. The wounds were finely minced and plated into 24-well gelatinized tissue culture plates. The explants were maintained in DMEM with 10% fetal bovine serum for 1 wk, when a halo of cells had grown out from most tissue fragments. The outgrowing cells were morphologically consistent with fibroblasts. The medium was then changed to DMEM with 1.5% horse serum and 6 μg/ml insulin to promote muscle differentiation. After 3 d in differentiation medium, the cells were fixed and processed for immunocytochemistry or β-galactosidase histochemistry.

Immunocytochemistry

The following mouse monoclonal antibodies were used in this study: (*a*) MF-20, which recognizes all forms of sarcomeric MHC in heart and adult skeletal muscle (21); a hybridoma supernatant was used at a dilution of 1:50; (*b*) MY-32 (Sigma Immunochemicals, St. Louis, MO), which is specific for the skeletal isoform of adult fast MHC (22); mouse ascites fluid was used at a dilution of 1:2,000; (*c*) BA-G5 (American Type Culture Collection, Bethesda, MD), which is specific for cardiac MHC-α (23); hybridoma supernatant was used at a dilution of 1:5; (*d*) BF-G6 (a gift from Regeneron Pharmaceuticals, New Brunswick, NJ), which recognizes embryonic skeletal MHC (24, 25); a hybridoma supernatant was used at a dilution of 1:5; (*e*) 5.8A (a gift from Drs. Peter Houghton and Peter Dias, St. Jude Children's Hospital, Memphis, TN), which is specific for MyoD (26); purified IgG was used at a concentration of 20 μg/ml; and (*f*) F5D (a gift from Dr. Marshall Horwitz, University of Washington), which recognizes myogenin (27); hybridoma supernatant was used at full strength.

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[1] *Abbreviation used in this paper:* MHC, myosin heavy chain.

After time of killing, the hearts were sectioned transversely and frozen in OCT (Miles Diagnostics, Kankakee, IL). 6-μm frozen sections were cut and fixed briefly in methanol, except for those used for MyoD or β-galactosidase staining, which were fixed in 1 and 4% paraformaldehyde, respectively. Immunostaining with one antibody was performed using diaminobenzidine as chromagen or by immunofluorescence as described (17). For double immunostaining with antibodies to myogenin and embryonic MHC, tissue sections were incubated overnight with the antimyogenin antibody, which was visualized routinely with diaminobenzidine. The slides were then blocked for 1 h and incubated with the antibody to embryonic MHC. The embryonic MHC antibody was visualized by an alkaline phosphatase staining kit (Vector Labs, Burlingame, CA), using Vector Red as substrate. β-Galactosidase staining in tissue sections and cultured cells was performed as described by Lee et al. (28).

RNA isolation and Northern blotting

Hearts were removed quickly after time of killing, and the injured tissue was carefully trimmed of grossly evident normal myocardium and frozen in liquid nitrogen. Normal myocardium remote from the area of injury was also obtained and frozen. Frozen samples were pulverized and RNA extracted using the guanidinium isothiocyanate-acid phenol chloroform method of Chomczynski et al. (29), as previously described (17). 20-μg samples of total RNA were electrophoresed through a 1.2% agarose gel containing formaldehyde and transferred to Hybond N nylon membranes (Amersham, Arlington Heights, IL). The RNA was cross-linked to the membrane using ultraviolet light. The Northern blots were probed with random primed ^{32}P cDNA probes made from full-length murine MyoD or myogenin cDNA templates, as described previously (17). The blots were washed at high stringency, and autoradiography was performed. After exposure the blots were stripped, reprobed with a ^{32}P–end-labeled oligonucleotide to the 28S ribosomal RNA subunit, and reexposed, to verify that the lanes were equally loaded (17).

Results

Adenoviral gene transfer in cultured cardiac fibroblasts. The MyoD adenovirus was tested for its ability to promote muscle differentiation in cultured adult rat cardiac fibroblasts, since these were the intended target cells in healing wounds in vivo. 1 d after receiving the MyoD virus, cultures were switched to a differentiation-promoting medium containing 1.5% horse serum and maintained for 3 d. Cultures exposed to 2,000 pfu/cell of the MyoD virus expressed MHC, indicating myogenic differentiation (Fig. 2 *A*). Cells infected with the control β-galactosidase virus were mononucleated with the usual cobblestone morphology and did not express any isoforms of MHC (Fig. 2 *B*).

The MyoD virus gave a dose-dependent increase in gene transfer (Fig. 3 *A*). After transfection with 20 pfu/cell, an average of 30% of cells showed nuclear staining with a MyoD antibody. Frequency of transfection increased steadily with increasing dose of virus, plateauing at 1,000 pfu/cell, where 80% of cells stained with the MyoD antibody. Transfection with the β-galactosidase virus gave a similar dose–response curve, except that 100% of the cells in culture had β-galactosidase nuclear staining at 1,000 pfu/cell (not shown). Both viruses induced cytotoxicity at 12,000 pfu/cell. The difference in plateau (80% staining for MyoD versus 100% for β-galactosidase) most likely resulted from a greater sensitivity in the β-galactosidase assay, which detects enzymatic activity rather than the presence of an antigen.

The dose–response curve for induction of muscle differentiation (Fig. 3 *B*) exhibited two significant differences from the gene transfer curve. First, myogenic conversion required a substantially higher multiplicity of infection compared with gene transfer. Although 60% of the cells expressed MyoD protein after a viral dose of 200 pfu/cell, only 2% of the cells was converted into skeletal muscle as determined by sarcomeric myosin staining. Second, at the highest doses of virus an average of only 14% of fibroblasts expressed sarcomeric myosin, although 80% expressed MyoD protein. The frequency of multinucleated myotubes increased with increasing dosage of virus, although myotube formation was less frequent with cardiac fi-

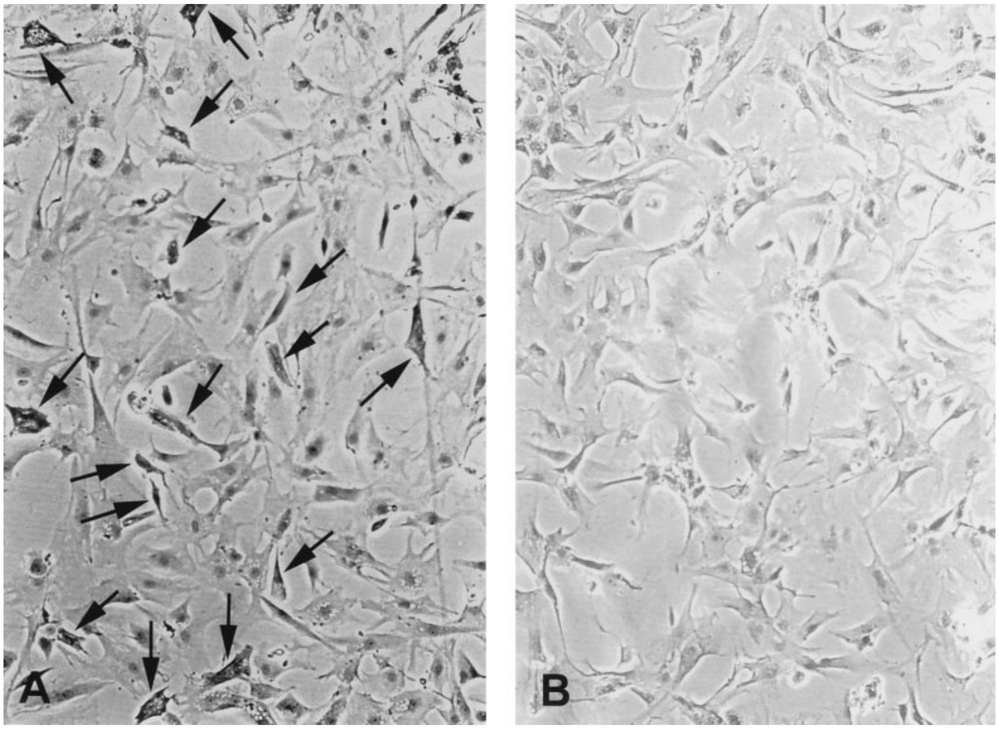


Figure 2. (*A*) Adult cardiac fibroblasts after receiving 2,000 pfu/cell of MyoD adenovirus. Immunostaining with an antibody to sarcomeric myosin (MF-20) demonstrates numerous cells expressing MHC (dark gray cells, *arrows*). Hematoxylin counterstain. ×270. (*B*) Adult cardiac fibroblasts after receiving 2,000 pfu/cell of control adenovirus encoding nuclear targeted β-galactosidase. None of the cells stain positively for MHC. Hematoxylin counterstain. ×270.

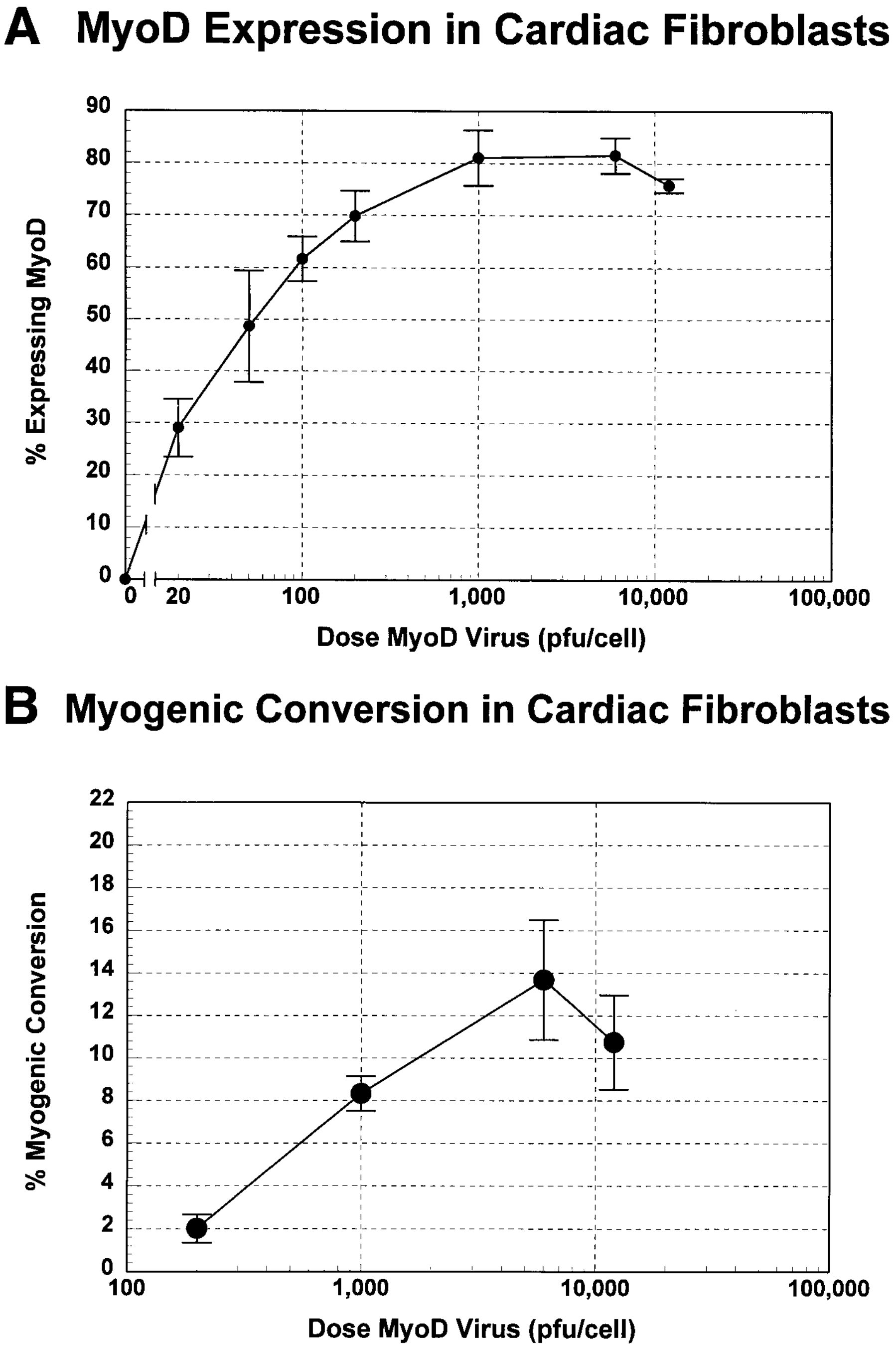


Figure 3. (*A*) Dose–response curve for MyoD gene transfer into cultured adult rat cardiac fibroblasts. There was a dose-dependent increase in MyoD immunostaining after exposure to the MyoD adenovirus. The percentage expressing MyoD plateaued at 80% with a dose of 1,000 pfu/cell. At 12,000 pfu/cell viral cytotoxicity was observed. Data represent means±SEM for three experiments. For each experiment a minimum of 500 cells was counted. (*B*) Dose–response curve for conversion of cultured cardiac fibroblasts into skeletal muscle cells after MyoD gene transfer. Muscle differentiation was not observed at viral doses below 200 pfu/cell. Muscle differentiation increased with increasing dose of virus, reaching a maximum at 6,000 pfu/cell, where an average of 14% of cells expressed MHC. Note that muscle differentiation required a higher multiplicity of infection and achieved a lower maximal frequency compared with the gene transfer curve. Data represent the fraction of total nuclei appearing in myosin-positive cells, expressed as means± SEM for three experiments. For each experiment a minimum of 500 nuclei was counted.

broblasts than with 10T1/2 cells. Thus, these experiments showed that the MyoD adenovirus induced cultured cardiac fibroblasts to differentiate into skeletal muscle when a relatively high multiplicity of infection was used.

Adenoviral gene transfer in injured rat hearts in vivo. Next, a series of in vivo studies was undertaken using injured rat hearts. Because coronary occlusion caused excessive mortality (~ 80%) due to congestive heart failure, a freeze-thaw injury model was used to induce myocardial necrosis (16, 17). Mortality after freeze-thaw injury was < 10%. The initial protocol was to induce cardiac injury, allow the lesions to heal for 1 wk, and then inject 2 × 10^9 pfu virus in 100 μl tissue culture media directly into the healing wound. 1 wk after injection the rats

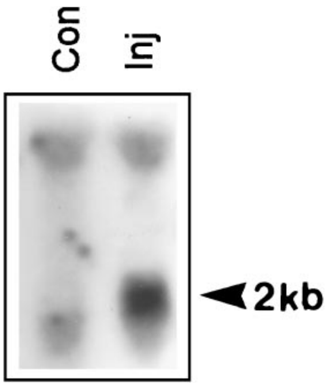
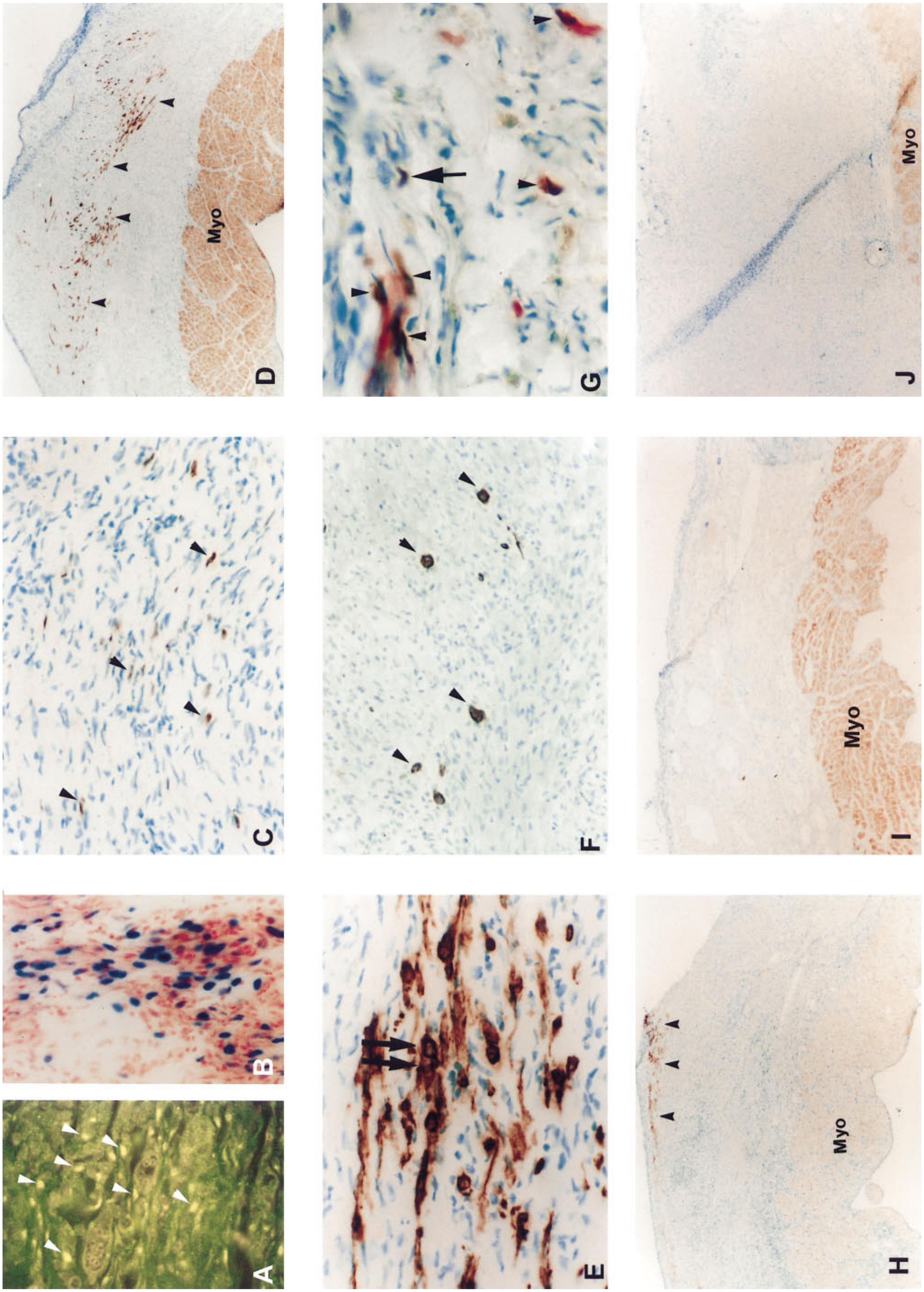


Figure 4. Northern blot analysis from control and injured regions of one heart receiving an injection of 10^9 pfu MyoD adenovirus 1 wk after freeze-thaw injury. Tissue was obtained 1 wk after adenoviral infection. A 2.0-kb MyoD message was detected in the injured RNA sample (*Inj*), but not in the control sample (*Con*).


A
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were killed and we assayed for myogenic conversion in the injured region. We reasoned that granulation tissue, a highly cellular tissue with a provisional matrix, would be a better target for myogenic conversion than either inflamed, necrotic tissue or dense scar tissue. At 1 wk after injury most of the necrotic myocardium has been replaced by granulation tissue, and scar tissue formation is minimal (16). A dose of 2×10^9 pfu was chosen based on studies from Kass-Eisler et al. (30) showing that higher doses caused viral cytotoxicity in normal hearts.

Northern blotting demonstrated MyoD mRNA in the injured regions of hearts receiving the MyoD virus, but not in hearts receiving the β-galactosidase virus (Fig. 4). Myogenin, a second myogenic determination factor which is induced by MyoD in vitro (11), was not detected by Northern blotting (not shown). By immunostaining, many cells in the granulation tissue from MyoD-treated hearts contained MyoD protein in their nuclei (Fig. 5 *A*). Most of the stained cells were in the interstitium, consistent with their being either fibroblasts or inflammatory cells. Some cells in the adventitia and media of blood vessels also stained. No staining was observed in tissue that did not receive the MyoD virus (not shown). Comparable nuclear staining patterns for β-galactosidase were observed in granulation tissue of hearts receiving the control virus (Fig. 5 *B*). Despite expression of MyoD mRNA and protein after viral treatment, none of the hearts reacted with antibodies to skeletal muscle–specific myosins (not shown). Thus, skeletal muscle differentiation was not detected.

To determine if lesions with more advanced healing were better suited for myogenic conversion, 2×10^9 pfu of MyoD or β-galactosidase adenovirus ($n = 4$ rats/group) was administered 2 wk after freeze-thaw injury, and hearts were studied after a 1-wk incubation period. None of the hearts expressed skeletal muscle MHCs. To determine if a longer incubation period was required for myogenic conversion, virus was administered 1 wk after injury and the hearts ($n = 4$ rats/group) were studied 3 wk later; cyclosporine A (8 mg/kg/d subcutaneously) was given beginning 4 d before virus administration and continued until time of killing to suppress the immune response to adenovirus. Again, no myogenic conversion was detected. Two experiments were then performed to test whether factors in the healing wound inhibited muscle differentiation (31–34). First, wound fibroblasts were cultured from hearts 1 wk after MyoD or β-galactosidase virus administration. Despite expression of MyoD and β-galactosidase in the explanted fibroblasts, no muscle differentiation took place in vitro. Next, skeletal myoblasts were isolated from the limbs of inbred Fischer rat pups (35) and transplanted into healing freeze-thaw lesions in adult Fischer rats. The engrafted myoblasts fused to form multinucleated myotubes and expressed skeletal MHC isoforms. Thus, the environment of the healing wound was not responsible for inhibiting myogenic differentiation.

We next considered the possibility that the fibroblasts in vivo were not receiving a sufficient dose of the MyoD virus. This possibility was supported by the in vitro data, which showed that a high multiplicity of infection was required to achieve differentiation (Fig. 3). To test this hypothesis, 10^{10} pfu of MyoD or β-galactosidase virus was administered to hearts ($n = 10$ and 9/group, respectively) 1 wk after injury. To suppress the immune response, rats received cyclosporine A (8 mg/kg/d subcutaneously) beginning 4 d before viral treatment and continuing until time of killing. In contrast to the previous experiments, 8 of 10 hearts receiving high-dose MyoD adenovirus showed positive immunostaining for myogenin (Fig. 5 *C*), and 7 of 10 hearts showed expression of embryonic MHC (Fig. 5, *D–F*). Double immunolabeling showed coexpression of myogenin and embryonic MHC in the same cells, providing strong evidence that these were new muscle cells induced by the MyoD adenovirus (Fig. 5 *G*). Only rare myofibers stained with an antibody to adult fast MHC (Fig. 5 *H*), indicating that most of the new myofibers had an embryonic phenotype. The embryonic myosin-positive cells in the injured regions did not react with an antibody to cardiac MHC-α (Fig. 5 *I*), indicating they were not simply residual cardiocytes that survived the injury. In several hearts, we observed cells that appeared to have either two or three nuclei (Fig. 5 *E*), suggestive of multinucleated myotubes. However, by light microscopy, we could not rule out the possibility that these were closely apposed mononucleated cells. None of the nine hearts receiving the control

Figure 5. (*A*) Immunofluorescent localization of MyoD protein in the granulation tissue of a 2-wk-old myocardial injury. The MyoD virus was injected into the healing lesion 1 wk after injury and the rat was killed 1 wk after injection. MyoD fluorescence shows a punctate yellow-green staining pattern (*arrowheads*), consistent with the known nuclear localization of this transcription factor. Numerous thin walled blood vessels containing erythrocytes are present. ×400. (*B*) β-Galactosidase histochemistry in the granulation tissue of a heart receiving the control β-galactosidase adenovirus. Numerous cells within the granulation tissue show characteristic blue nuclear staining. Nuclear fast red counterstain. ×430. (*C*) Immunoperoxidase staining for myogenin in an injured heart receiving 10^{10} pfu of MyoD adenovirus 1 wk after cryoinjury, killed 1 wk after viral treatment. Multiple myogenin-positive nuclei are present in the granulation tissue (*arrowheads*). Methyl green counterstain. ×450. (*D*) Myogenic differentiation in an injured heart receiving 10^{10} pfu of MyoD adenovirus. Immunostaining with an antibody to embryonic MHC demonstrates numerous myosin-positive cells in the granulation tissue of the injured region (*arrowheads*). Uninjured myocardium (*Myo*) can be seen in the lower portion of the photograph, due to cross-reaction of the antibody. This heart showed the largest number of MHC-positive cells in the injured region. Methyl green counterstain. ×90. (*E*) Higher magnification of embryonic MHC-stained section from *D*, demonstrating elongated, fusiform cells, consistent with MyoD-induced myofibers. Arrows indicate two nuclei which may be located within the same cell. Methyl green counterstain. ×450. (*F*) Embryonic MHC immunostaining from a heart with relatively few myofibers within the injured region (*arrowheads*). Methyl green counterstain. ×225. (*G*) Double immunostaining for myogenin and embryonic MHC. Multiple cells in the field show colocalization of myogenin (brown nuclei) and embryonic MHC (red cytoplasm), as indicated by the arrowheads. Note the myogenin-positive cell which does not stain for embryonic MHC (*arrow*). Methyl green counterstain. ×1,100. (*H*) Immunostaining with an antibody to adult fast MHC in the same heart as in *D*. Only rare cells in the MyoD-treated heart are stained (*arrowheads*), indicating that most of the new myofibers exhibit an embryonic phenotype. Methyl green counterstain. ×90. (*I*) Cardiac MHC-α immunostaining in the same heart shown in *D*. Myocardium which survived the cryoinjury (*Myo*) stains with the antibody, but the cells in the injured region in the top half of the figure do not stain, indicating they are not residual cardiocytes. Methyl green counterstain. ×90. (*J*) Immunostaining for embryonic MHC in a control heart receiving 10^{10} pfu of the control β-galactosidase virus. None of the cells in the injured region stain, indicating that embryonic MHC expression was specific to MyoD treatment. Residual myocardium (*Myo*) is present at the lower right. Methyl green counterstain. ×90.

β-galactosidase adenovirus contained cells expressing myogenin or embryonic skeletal MHC (Fig. 5 *J*). The density of myosin-positive cells after MyoD treatment was lower than the muscle density after myoblast transplantation. On the other hand, the density of transfected cells (including myosin-negative) after MyoD treatment was comparable with the density of muscle grafts.

Hearts receiving the high-dose virus had extensive inflammatory cell infiltrates, consistent with macrophages and lymphocytes by standard pathological criteria (not shown). Substantially smaller inflammatory infiltrates were also present in hearts receiving the lower dose of adenovirus. Additionally, both the high-dose MyoD- and β-galactosidase–treated hearts had unresorbed necrotic tissue in the central regions of the wounds, a finding not seen with any of our previous protocols. It is not clear whether this necrotic tissue is the result of delayed healing (possibly due to cyclosporine treatment) or to additional cell death induced by the high dose of adenovirus.

Discussion

The principal findings of this study are: (*a*) cultured cardiac fibroblasts can be converted to skeletal muscle by an adenovirus encoding MyoD; (*b*) myogenic conversion in vitro requires a high multiplicity of infection; (*c*) the adenovirus can infect cells in cardiac granulation tissue in vivo, resulting in MyoD mRNA and protein synthesis; and (*d*) cells in cardiac granulation tissue can be induced to differentiate into skeletal muscle after transfection with the MyoD adenovirus, when a relatively high dose of virus is administered.

We considered the possibility that the myosin-positive cells in the injured region could be residual cardiocytes which survived the injury, since the antibody used to detect skeletal muscle embryonic myosin expression also cross-reacted with cardiac myosin. Several lines of evidence argue that the embryonic myosin-positive cells within the injured region are not residual cardiocytes, but are truly new skeletal muscle fibers induced by MyoD. First, we have shown coexpression of myogenin, a myogenic determination factor induced by MyoD, and embryonic myosin within the same cells. Second, the embryonic myosin-positive cells do not express cardiac MHC-α, a specific marker for cardiocytes. Third, no embryonic myosin-positive cells were present in control hearts receiving the β-galactosidase virus. Finally, our cryoinjury model induces confluent necrosis and does not spare cells within the center of the lesion, unlike myocardial infarcts which can have interdigitating viable and necrotic tissue. These facts provide strong evidence that MyoD induced the formation of new skeletal muscle fibers within the healing hearts.

We are aware of one other group also attempting to use MyoD to treat myocardial infarction. In a preliminary report from Prentice et al. (36), a retroviral vector was used to introduce MyoD into infarcted dog hearts. They reported rare cells expressing the adult skeletal muscle–specific MHC-fast in infarct granulation tissue. No cell fusion was identified, possibly because the frequency of myosin-positive cells was too low. Their results appear qualitatively similar to our own. In the current study, we also noted occasional cells expressing MHC-fast (Fig. 5 *H*), although in most hearts only embryonic myosin expression was observed.

There is relatively little information on the effects of overexpressing MyoD in vivo in other systems. In transgenic mouse studies, constitutive expression of MyoD driven by the β-actin promoter was found to be lethal. Although the affected embryos showed ectopic activation of myogenin and myosin light chain-2, there was not evidence of widespread muscle formation (37). In a related study, MyoD was expressed in the hearts of transgenic mice beginning at about embryonic day 13, by linking it to the muscle creatine kinase promoter (38). MyoD expression was embryonically lethal due to abnormal heart development. Although some skeletal muscle–specific mRNAs were expressed, MyoD did not override the cardiac muscle phenotype. Transgenic mice have also been generated where the murine sarcoma virus promoter was used to target expression of *myf*5, another myogenic determination gene, to the brain and heart (39, 40). These mice showed ectopic skeletal muscle formation in brain, and in the hearts there was synthesis of several skeletal muscle transcripts, including myogenin and embryonic MHC. The hearts also exhibited cardiocyte loss and fibrosis, which again suggests apparent incompatibility of skeletal and cardiac muscle programs within the same cell. Other studies have shown that injection of MyoD mRNA into *Xenopus* oocytes transiently induced muscle-specific gene expression (41) and apparently recruited nonsomitic cells into the skeletal muscle lineage (42). Thus, although ectopic expression of MyoD can induce skeletal muscle differentiation in some circumstances in vivo, in other circumstances there is only partial activation of the skeletal muscle program.

MyoD gene expression versus myogenic conversion. The MyoD adenovirus was an efficient vector for gene transfer both in vitro (Fig. 3 *A*) and in vivo (Fig. 5 *A*). Despite this, only a subset of MyoD-expressing cells differentiated into skeletal muscle. The fact that resistance to myogenic conversion could be overcome partially by increasing the dose of virus argues that multiple copies of MyoD per cell were required to achieve muscle differentiation. On the other hand, the fact that many cells never differentiated, despite near-cytotoxic doses of adenovirus, is evidence that some fibroblasts are not readily converted into muscle by MyoD. Similarly, studies from other laboratories have demonstrated that although a wide range of cell types can be induced to form muscle by MyoD, other cells are resistant to myogenic conversion (43, 44). The factors which permit or prevent myogenic conversion are not well understood. Cell fusion studies suggest that some resistant cells are missing necessary cofactors, while others express myogenic inhibitors (45). Molecules known to antagonize MyoD include c-*jun* (46, 47), c-*fos* (43), and cyclin D1 (48). On the other hand, the cyclin-dependent kinase inhibitors p21 and p16 (48, 49) and myocyte enhancer factor-2 family members (50, 51) have been shown to enhance myogenic conversion after MyoD transfection. It might be possible to manipulate these pathways to increase muscle differentiation in injured hearts in vivo.

Could MyoD-induced muscle repair infarcts? In addition to increasing the frequency of myogenic conversion, several other challenges must be overcome to improve contractile function after cardiac injury. For example, the muscle cells must differentiate into mature myofibers and persist within the healing lesion. In this study the MyoD-induced muscle cells exhibited an embryonic phenotype 1 wk after viral treatment. Although it seems likely that the new muscle fibers would differentiate further into adult fibers given more time, long-term studies will depend on reducing the immune response to adenoviral vectors, which are currently highly immunogenic (52, 53). Furthermore, the skeletal muscle should undergo electrical and mechanical coupling to cardiac muscle if it is to provide coordinated mechanical support. Studies of skeletal myoblast engraftment suggested that skeletal muscle cells do not couple with cardiac muscle (54). Skeletal muscle fibers do not synthesize gap junction proteins, nor do they make the principal adhesion proteins of the intercalated disk, N-cadherin or desmoplakin (55, 56). Thus, it may be necessary to engineer new skeletal muscle to make these junctional proteins, or to provide alternative routes of electrical activation such as pacing or innervation.

Several electrophysiological differences between the muscle types should be mentioned. In comparison with cardiac muscle, skeletal muscle has no automaticity, a very short action potential duration and refractory period (and can therefore contract tetanically), and a 10-fold higher conduction velocity. Acetylcholine triggers depolarization in skeletal muscle, while in cardiac muscle acetylcholine influences the rate and force of contraction, without inducing depolarization directly (57). These electrical differences may be important, because in order to repair the heart, the new muscle cells must enhance regional contractile function without generating arrhythmias. A final consideration is that muscular repair might change the passive mechanical properties of the infarct. It is possible, for example, that skeletal muscle might not form ventricular aneurysms or cause as much chamber dilation as scar tissue.

Thus, although there are considerable challenges remaining, the finding that it is possible to induce skeletal muscle differentiation in the injured heart by MyoD gene transfer suggests that it may be possible to regenerate functional muscle after myocardial infarction.

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