Differentiation and Long-Term Survival of C2C12 Myoblast Grafts in Heart

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Abstract

We have assessed the ability of skeletal myoblasts to form long-term, differentiated grafts in ventricular myocardium. C2C12 myoblasts were grafted directly into the heart of syngeneic mice. Viable grafts were observed as long as 3 mo after implantation. Immunohistological analyses revealed the presence of differentiated myotubes that stably expressed the skeletal myosin heavy chain isoform. Thymidine uptake studies indicated that virtually all of the grafted skeletal myocytes were withdrawn from the cell cycle by 14 d after grafting. Graft myocytes exhibited ultrastructural characteristics typical of differentiated myotubes. Graft formation and the associated myocardial remodeling did not induce overt cardiac arrhythmia. This study indicates that the myocardium can serve as a stable platform for skeletal myoblast grafts. The long-term survival, differentiated phenotype, and absence of sustained proliferative activity observed in myoblast grafts raise the possibility that similar grafting approaches may be used to replace diseased myocardium. Furthermore, the genetic tractability of myoblasts could provide a useful means for the local delivery of recombinant molecules to the heart. (*J. Clin. Invest.* 1993. 92:1548–1554.) Key words: skeletal myoblasts • intracardiac grafts • cell transplantation • gene therapy • myocardial repair

Introduction

It is now well established that the regenerative capacity of skeletal muscle resides in the satellite cells first described by Mauro in 1961 (1). Trauma induces satellite cell proliferation and subsequent differentiation into new (or regenerated) muscle fibers. Satellite cell (myoblast) lines that retain the capacity to differentiate into myotubes in culture have been isolated from a number of species, including mouse and human. Several groups have shown that myoblasts have the capacity to fuse to preexisting fibers when introduced directly into the skeletal muscle of a syngeneic host (2, 3). Thus, skeletal muscle regeneration can be engendered via heterokaryon formation between host myotubes and histocompatible donor myoblasts. This observation has been exploited in studies with mdx mice (4) and Duchenne's patients (5), where the introduction of myoblasts carrying a normal dystrophin gene resulted in the formation of chimeric myotubes expressing wild-type dystrophin.

In contrast to skeletal muscle, the myocardium lacks a regenerative stem cell system. Furthermore, ventricular cardiomyocytes in the adult mammalian heart are permanently withdrawn from the cell cycle (6, 7). Myofiber loss due to trauma or disease is consequently irreversible. These observations prompted several groups to determine if targeted oncogene expression could induce cardiomyocyte proliferation. Expression of the SV40 large T antigen oncogene in the hearts of transgenic mice (8–10) or in virally transfected rat cardiomyocytes (11) resulted in a sustained proliferative response. Elevated expression of c-myc during embryogenesis increased developmental cardiomyocyte hyperplasia in transgenic mice (12). Cardiomyocyte proliferation was also observed following retroviral transduction of ras or myc in embryonic rat (13) or avian (14) cardiomyocytes. Thus targeted oncogene expression, at least in some instances, can bypass the cues that usher cell cycle withdrawal during normal cardiomyocyte development. Presently it is not clear if targeted oncogene expression can induce proliferation in adult cardiomyocytes.

Given the absence of significant regenerative potential in mammalian myocardium, we have previously conducted a series of experiments to determine if cardiomyocytes manipulated in vitro could form intracardiac grafts (15). These experiments utilized AT-1 cardiomyocytes, which were derived from transgenic mice expressing the SV40 large T antigen oncogene in the atrium (8, 16). AT-1 cardiomyocytes retain the capacity for proliferation in culture while displaying a differentiated phenotype (17, 18). Our results showed that AT-1 cardiomyocytes formed long-term, differentiated intracardiac grafts without having a deleterious effect on cardiac function. However, the unchecked proliferation of grafted AT-1 cells limits the potential value of these grafts for both myocardial repair and long-term delivery of recombinant molecules.

The successful use of skeletal myoblasts in the grafting experiments mentioned above prompted us to test their capacity to generate intracardiac grafts. In this study we addressed three issues. First, we determined if C2C12 myoblasts could form stable grafts in a syngeneic myocardium. Second, we ascertained the proliferative and differentiated status of grafted cells. Third, we determined if the presence of C2C12 grafts had a deleterious effect on host cardiac function. Our results indicate that C2C12 myoblasts differentiate into myotubes when grafted into the heart. The absence of tritiated thymidine uptake indicates that the grafted cells are withdrawn from the cell cycle by 14 days after implantation. There was no overt effect on cardiac function in animals bearing differentiated myotube grafts. The implication of these results with respect to the potential replacement of myocardial tissue and to the long-term delivery of recombinant proteins to the heart is discussed.

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Methods

C2C12 cell culture and myocardial grafting protocol. C2C12 myoblasts were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in the undifferentiated state by culturing at low density in high glucose DME supplemented with 20% FBS, 1% chicken embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin. For some studies, myogenic differentiation was induced by culturing in DME supplemented with 2% horse serum and antibiotics. Immediately before injection, myoblasts were harvested with trypsin, washed three times with serum-free DME, and directly injected into the ventricular myocardium of adult syngeneic C3Heb/FeJ mice (Jackson Laboratories, Bar Harbor, ME) under open heart surgery as described (15, 19). 4–10 × 10^4 cells were injected in a volume of 2–3 μl using a plastic syringe fitted with a 30-gauge needle.

Histology. Hearts were removed following cervical dislocation and cryoprotected in 30% sucrose, embedded and sectioned at 10 μm with a cryomicrotome as described (20). For hematoxylin and eosin (H and E)[1] staining, sections were postfixed in acetone/methanol (1:1) and stained according to manufacturer's specifications (Sigma Diagnostics, St. Louis, MO). For immunohistology, methanol-fixed sections (−20°C, 10 min) were reacted with the monoclonal anti-skeletal myosin heavy chain antibody (MY-32; Sigma Chemical Corp., St. Louis, MO) followed by rhodamine-conjugated sheep anti-mouse IgG $F(ab')_2$ fragment (Boehringer Mannheim, Indianapolis, IN), and visualized by epifluorescence. For [^{3}H]thymidine incorporation, mice were given a single bolus injection of isotope (400 μCi at 28 Ci/mM, Amersham, Arlington Heights, IL) and 18 h later killed by cervical dislocation. The heart was removed, cryoprotected in 30% sucrose, embedded, and sectioned with a cryomicrotome. Sections were postfixed in methanol/acetone (1:1), stained with H and E, and coated with a thin layer of photographic emulsion (Ilford L.4; Polysciences, Inc., Warrington, PA) diluted 1:1 with distilled water. Sections were exposed for 5–7 d at 4°C, and developed in Kodak D-19 (Eastman-Kodak, Rochester, NY) at 20°C for 4 min, washed with distilled water for 1 min, fixed in 30% sodium thiosulfate for 10 min, and washed in distilled water.

Electron microscopy. Tissue blocks were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), 0.1 M sucrose and postfixed in 2% osmium tetroxide (Stevens Metallurgical Corp., New York). All other electron microscopy (EM) chemicals were obtained from Ladd Research Industries, Inc. (Burlington, VT). Tissue was stained en bloc with 2% uranyl acetate in pH 5.2 maleate buffer (0.05 M), dehydrated, and embedded in Ladd LX-112. Grafts were located using 1 μm sections stained with toluidine blue. After trimming, the block was thin sectioned and stained with uranyl acetate and lead citrate. Specimens were viewed on a transmission electron microscope (model 400; Philips Electronic Instruments, Mahwah, NJ).

Electrocardiogram analyses. For surface electrocardiogram (ECG) records, mice were anesthetized (2.5% Avertin, 0.015 ml/g body wt, i.p., (Fluka Chemicals, Lake Ronkomkoma, NY), surface electrodes were placed in the standard lead 1 position, and ECG was recorded with a high gain amplifier (Narco Bio-Systems, Inc., Houston, TX) coupled to an analog/digital converter (Coulbourn Instruments, Inc., Lehigh Valley, PA).

Plasma enzyme assay. For lactate dehydrogenase (LDH) isoform assay, plasma was isolated by retro-orbital sinus bleeds under anesthesia (2.5% Avertin, 0.015 ml/g body wt, i.p.). Plasma was fractionated on 1% agarose gels (creatine kinase [CK] isoenzyme electrophoresis system, CIBA-Corning Diagnostics, Corning NY) and the LDH isoforms were visualized by a tetranitroblue tetrazolium-formazan histochemical assay (LDH assay kit; Sigma Diagnostics, St. Louis, MO).

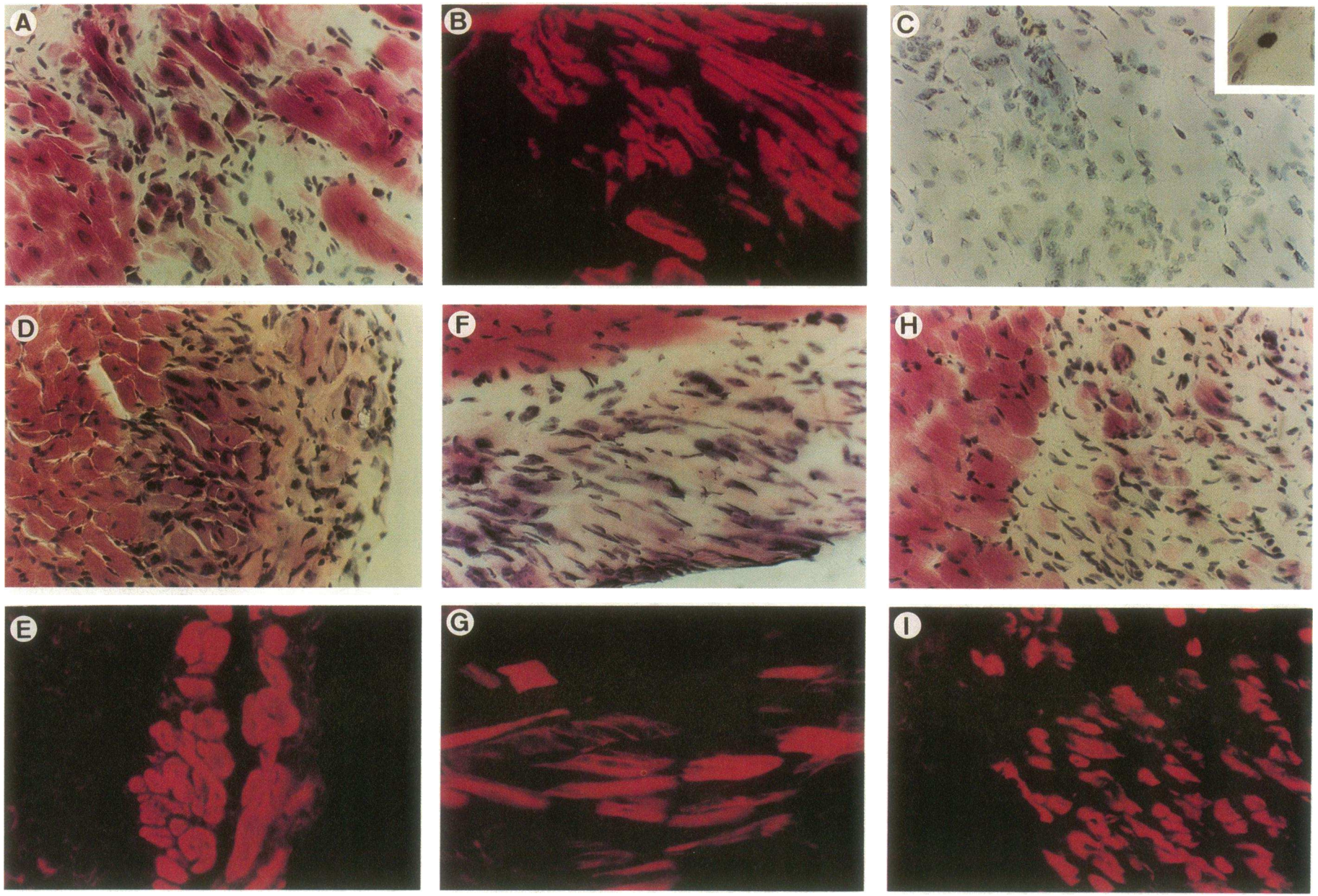
1. *Abbreviations used in this paper:* ECG, electrocardiogram; EM, electron microscopy; H and E, hematoxylin and eosin; LDH, lactate dehydrogenase.

Results

Several myoblast cell lines have been described that, as exemplified by C2C12 cells, have the capacity to differentiate into myotubes in culture (21). C2C12 myoblasts were derived from cultured explants of injured thigh muscle of C3H mice. When maintained in serum-rich media, the myoblasts proliferate rapidly and retain an undifferentiated phenotype. However, when cultured in serum-poor media, myogenic differentiation is induced. The C2C12 cells withdraw from the cell cycle and fuse, thereby forming multinucleated myotubes. Myogenic differentiation is also induced, as evidenced by the appearance of numerous muscle-specific gene products. Thus, in this model, proliferation and myogenic differentiation are mutually exclusive (22). Myoblast differentiation in vitro is thought to mimic satellite cell mediated myofiber regeneration in vivo.

We have used C2C12 myoblasts to generate long-term grafts in the adult heart. Myoblasts were injected directly into the myocardium of syngeneic C3Heb/FeJ mice and the viability of the grafted material was assessed. 100% (13/13) of the mice receiving intracardiac implants of C2C12 myoblasts developed grafts in the heart. Viable grafts were observed as long as 3 mo after implantation, the latest time point assayed to date. In all instances, the grafted material was not encapsulated (Fig. 1, *A, D, F,* and *H*). The differentiated status of the grafted C2C12 cells was determined by immunohistological assay with an anti-myosin heavy chain antibody (MY-32). This antibody does not react with myoblasts (M. G. Klug, unpublished result) nor with cardiac myosin heavy chain. Although differentiated C2C12 cells were observed in every heart receiving myoblast injections (Fig. 1, *B, E, G,* and *I*), the grafting efficiency of individual cells was not determined. As an additional control, hearts bearing AT-1 intracardiac grafts (15) were examined with the MY-32 antibody. No staining was observed (M. G. Klug and G. Y. Koh, unpublished results), thereby ruling out the possibility that the signal seen in the C2C12 grafts was due to skeletal myosin heavy chain induction in host cardiomyocytes. Although unlikely, it is a formal possibility that a portion of the MY-32 immunostaining observed in the intracardiac grafts may be due to C2C12 myoblast-mediated induction of myogenic differentiation in host cardiac fibroblasts. Intracardiac grafts with retrovirally tagged C2C12 cells will directly address this caveat.

We have previously shown that AT-1 cardiomyocytes also form stable grafts in syngeneic myocardium (15). However, the observation that these cells retained the capacity for proliferation in vivo raised the possibility that sustained cell division might be required for successful intracardiac grafting. We therefore examined the proliferative status of the C2C12 grafts. Virtually no DNA synthesis (as assessed by tritiated thymidine incorporation, Fig. 1 *C*) was observed, indicating that the majority of the grafted C2C12 cells had indeed withdrawn from the cell cycle. Examination of serial sections indicated that less than 0.1% of the cells in or near the grafts were synthesizing DNA. This result most likely reflects fibroblast proliferation during the remodeling process, although we cannot rule out the possibility of very low levels of C2C12 proliferation. As with our AT-1 grafts, immunohistological analyses of C2C12 grafts failed to detect macrophage, inflammatory leukocyte, or lymphocyte infiltration at 2 mo after implantation (G. Y. Koh, unpublished results), indicating the absence of chronic graft rejection by the syngeneic hosts.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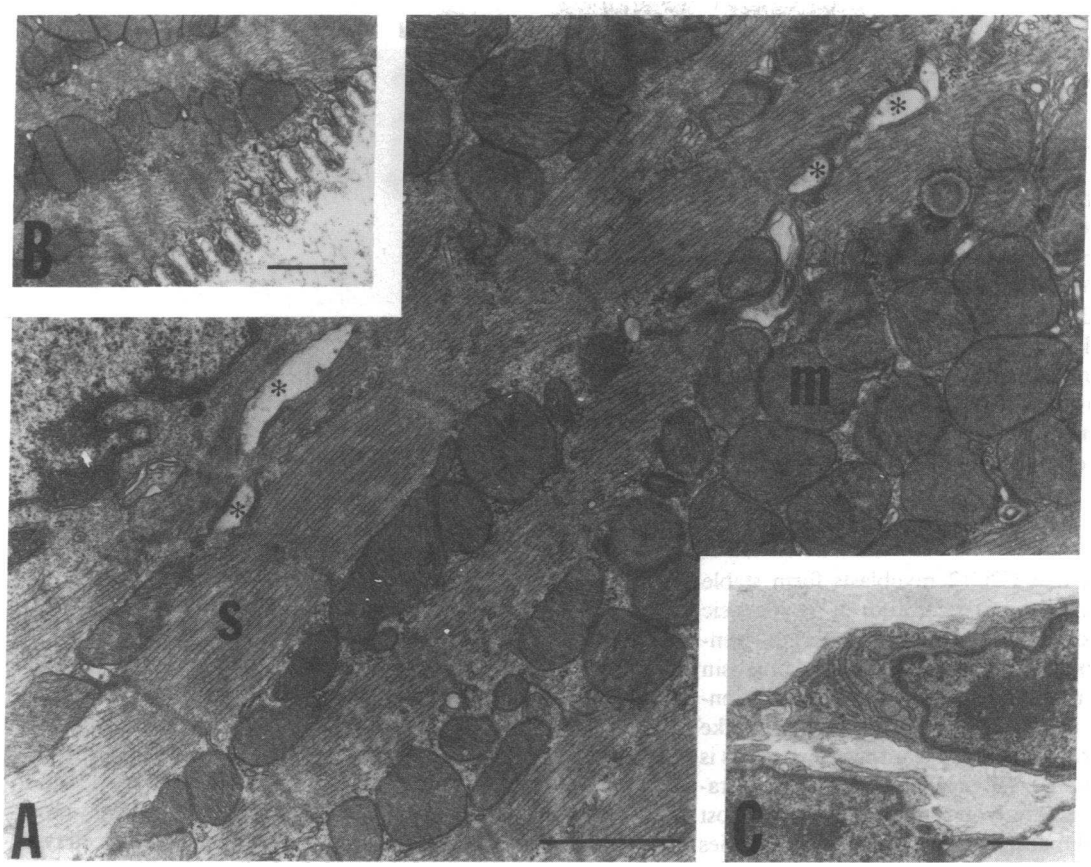

Figure 2. Electron micrographs of C2C12 intracardiac grafts. (*A*) Graft myocyte with complete, well-defined sarcomeres (s) separated by numerous mitochondria (m), and enlarged t tubules (*). Bar = 1 μm. (*B*) Ruffled edge of graft myocyte. Bar = 1 μm. (*C*) Blast cells in graft area. Note chromatin pattern and mitochondrial content typical of satellite cells. Bar = 1 μm.

At the level of light microscopy, the C2C12 intracardiac grafts exhibited cellular heterogeneity with both H and E and MY-32 immunofluorescence staining. EM analyses were employed in an effort to further characterize the cellular makeup of the C2C12 grafts. Toluidine-stained 1-μm sections were surveyed at 100-μm intervals to locate graft sites for EM analysis. Once localized, thin sections were prepared from the block. Cells with morphology typical of skeletal myocytes were observed throughout the graft (Fig. 2 *A*). Abundant mitochondria localized between well-developed sarcomeres were readily detected. Prominent Z bands and thick and thin filaments were observed. Occasionally, expanded t tubules and ruffled cell membranes were detected in the grafted myocytes (Fig. 2, *A* and *B*). In addition to well-developed myocytes, a second less differentiated cell type was observed in C2C12 grafts (Fig. 2 *C*). Most notably, these cells exhibited a large nucleus-to-cytoplasm ratio, with a prominent band of heterochomatin at the nuclear periphery. Moderate amounts of centrally located heterochromatin were also detected. Limited rough endoplasmic reticulum and few mitochondria were observed in these cells. Similar ultrastructural characteristics have been ascribed to satellite cells in vivo and in culture (23, 24).

Two studies were initiated in order to assess any deleterious effects of C2C12 intracardiac grafts on host heart function. In the first study, surface electrocardiograms failed to detect any appreciable differences between records from control and ex-

Figure 1. Histological analysis of mice carrying C2C12 myocardial grafts. (*A*) C2C12 cardiac graft harvested at 22 d after implantation, H and E. Note the juxtaposition of graft myotubes with cardiomyocytes. (*B*) Immunofluorescence assay for skeletal myosin heavy chain (antibody MY-32) with a rhodamine-conjugated secondary antibody. Section shows the same graft depicted in *A;* red fluorescence marks the location of graft myotubes. Note that cardiac myocytes do not react with MY-32 (compare *A* and *B*). (*C*) Autoradiograph of the same graft shown in *A*. Note the absence of silver grains over the graft, indicating cell cycle withdrawal. For a positive control, the inset shows DNA synthesis (as evidenced by the presence of silver grains over the nucleus) in a cardiac fibroblast from the same section. (*D* and *E*) H and E and immunofluorescence analysis, respectively, of a C2C12 graft that traversed the subpericardium and myocardium of the host heart, harvested 14 d after implantation. Note that the C2C12 graft (the myocytes are shown in cross-section) is totally devoid of encapsulation. (*F* and *G*) H and E and immunofluorescence analysis, respectively, of a C2C12 graft localized to the subpericardial region of the host heart harvested 30 d after implantation. Cross-striations in the skeletal myotubes can be seen in *G*. (*H* and *I*) H and E and immunofluorescence analysis, respectively, of a C2C12 graft localized in the left ventricular free wall harvested 63 d after implantation. Note heterogeneous H and E staining, and heterogeneous MY-32 immunoreactivity in the graft. All panels were photographed at ×400.

perimental mice (Fig. 3). All animals examined had normal P-QRS coupling and exhibited normal sinus rhythm with an anesthetized heart rate of approximately 400 beats per min. These data indicate that the intracardiac myoblast grafts did not induce overt cardiac arrhythmias. In the second study, plasma LDH levels were monitored in graft-bearing animals. The presence of the cardiac LDH isoform in the circulation is a well-established hallmark of myocardial infarction. The cardiac-specific LDH isoforms (isoforms 1, 2, and 3) were not observed in plasma before grafting (Fig. 4). Immediately after grafting, an increase in the cardiac isoforms was observed in plasma, which most likely reflected damage to the host myocardium. A transient increase in the plasma skeletal LDH isoform (isoform 5) was also observed, presumably reflecting damage caused by the trans-thoracic incision. Plasma LDH profiles returned to normal by 7 d after implantation.

Discussion

This study demonstrates that C2C12 myoblasts form stable grafts when introduced into the myocardium of a syngeneic host. Grafted C2C12 myoblasts underwent myogenic differentiation as evidenced by both the induction of skeletal myosin heavy chain expression and the presence of highly differentiated ultrastructure. The absence of tritiated thymidine uptake demonstrates that the formation of stable intracardiac grafts is not dependent upon sustained cell proliferation. C2C12 intracardiac grafts did not have overtly negative effects on the host heart, as evidenced by the absence of rhythm abnormalities and the presence of normal plasma LDH. Given the long-term survival, differentiated phenotype, absence of sustained proliferative activity, and absence of deleterious effects on host heart function, several applications for intracardiac grafting can be envisioned. Specifically, intracardiac grafting approaches may be used to replace diseased myocardium, or alternatively to provide a means for the local delivery of recombinant molecules to the heart.

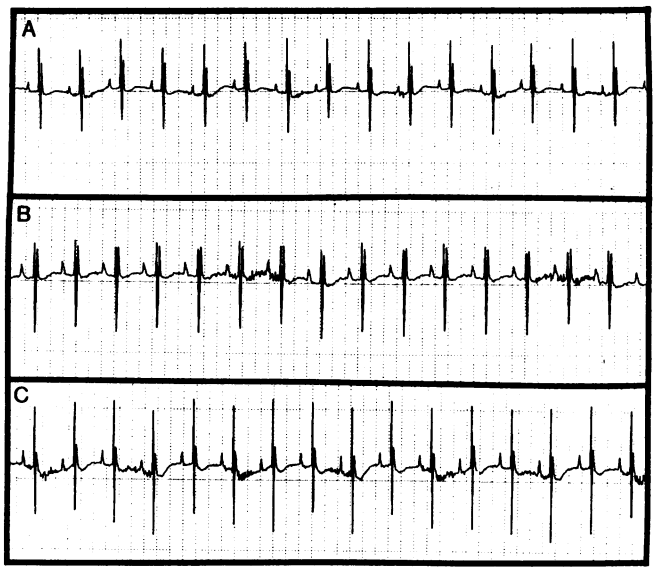

Figure 3. Representative surface ECG recordings from mice harboring C2C12 myocardial grafts. Records from sham (*A*) and graft bearing (*B, C*) mice are shown.

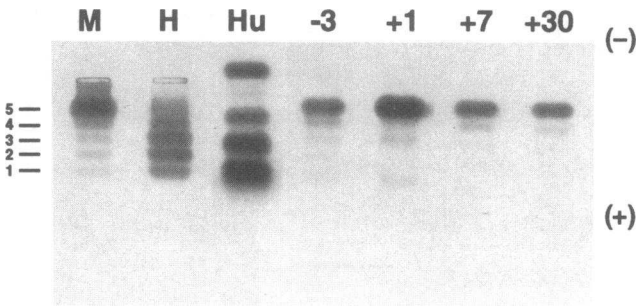

Figure 4. Plasma LDH profile from a mouse harboring a C2C12 myocardial graft. Plasma harvested at 3 d before implantation (−3) and at +1, +7, and +30 d after implantation was subjected to histochemical analysis for lactate dehydrogenase isoform content as described (18). Controls for skeletal (M) and cardiac (H) muscle, as well as from human plasma (Hu) are shown. Note transient appearance of the cardiac LDH isoforms in plasma from mice one day after implantation. A similar pattern of plasma LDH profiles was observed for all grafted animals examined.

With regard to the potential use of intracardiac grafting as a method for recombinant protein delivery to the myocardium, it should be noted that the use of cellular grafts as ectopic platforms for the expression of recombinant molecules is emerging as a powerful approach for ex vivo gene therapy (reviewed in 25–28). Typically, cells are amplified in vitro, genetically modified, and then reintroduced into the host. A variety of cell types have been used toward this end, including fibroblasts, hepatocytes, endothelial cells, bone marrow progenitors, and lymphocytes. The ability of skeletal myoblasts to fuse to preexisting fibers has been exploited independently by Leiden (29) and Blau (30) to effect therapeutic delivery of a recombinant molecule in experimental animals. Myoblasts transfected with a growth hormone expression vector produced stable grafts that elevated plasma hormone levels when introduced into mice.

Targeted expression of recombinant molecules in intracardiac grafts could induce a beneficial response in the myocardium. For example, grafts expressing angiogenic factors (as exemplified by basic and acidic fibroblast growth factor, TGFβ, vascular endothelial growth factor, and hepatocyte growth factor, see 31–34) might induce neovascularization. Similarly, grafts expressing neurotrophic agents near an infarcted region might ameliorate the arrhythmogenesis associated with the border zone (35). In support of the notion that ectopic neurotroph expression could alter host myocardium, recent studies have shown that expression of nerve growth factor in the myocardium of transgenic mice significantly alters sympathetic innervation of the heart. (Steinhelper, M. E., A. Hassankhani, M. H. Soonpaa, E. B. Katz, D. A. Taylor, L. J. Field, and H. J. Federoff. Over expression of nerve growth factor in the hearts of transgenic mice leads to cardiac pathology and death. Manuscript submitted for publication.)

A more exciting application of intracardiac myoblast grafting lies in the potential replacement of diseased myocardium. It is widely accepted that ventricular cardiomyocytes in the adult mammal have no capacity to reenter the cell cycle (7). Cardiomyocyte loss due to infarct or other disease processes is consequently irreversible. The results presented here demonstrate that grafted myoblasts can undergo myogenic differentiation in the heart. This finding raises the possibility that dis-

eased myocardium could be replaced by differentiating myotubes. However, several criteria, some of which may prove to be formidable obstacles, must be met in order to realize this goal. First and foremost, the nascent myotubes must be electrically coupled to the host conduction system. Although the grafted C2C12 myocytes were clearly juxtaposed with host cardiomyocytes, we currently do not know to what extent, if any, electrical coupling occurs. In the event that no coupling occurs, gene transfer approaches could be employed. For example, targeted expression of the major cardiac gap junction protein (connexin43, see reference 36) as well as the cell adhesion molecules expressed in the developing heart (reviewed in 37, 38) might facilitate coupling between host cardiomyocytes and nascent myotubes. These issues are readily addressed in co-culture experiments.

In addition to being coupled to the host myocardium, grafted myotubes must be oriented such that their contractile activity will contribute to cardiac function. In this regard, it is of interest to note that the C2C12 intracardiac grafts generated to date appear to have a somewhat organized structure. Although this observation is phenomenological at present, we have noted that adjacent myotubes within a graft tend to align themselves in similar orientations (Fig. 2). Cardiomyocytes cultured under conditions of oscillating tension tend to align themselves parallel to the direction of stretch (39). Although it is clear that force generation per se can influence the orientation of fibers, the extent to which preexisting myocardial extracellular matrix exerts an effect upon myotube orientation is not known. Further studies are clearly needed to determine this relationship.

The C2C12 grafts described in this report appear to satisfy several other criteria for successful myocardial repair. For example, proliferation of graft myocytes would obviously limit the usefulness of this approach. The absence of tritiated thymidine incorporation suggests that withdrawal from the cell cycle has occurred in the intracardiac grafts. In addition, no evidence of tumorigenesis was found in syngeneic intraskeletal muscle grafts that utilized either C2C12 (30) or primary (40, 41) myoblasts. In contrast, tumors of C2 origin were observed following grafting into muscle of athymic (nu/nu) mice (42). Although it is possible that the immunodeficiency in the athymic host contributed to this discrepancy, it is important to note that the use of primary myoblasts could virtually eliminate the tumorigenic potential of grafted cells. To this end, it will be informative to determine the ability of such cells to form stable intracardiac grafts.

The absence of overt graft-induced arrhythmias is also an important prerequisite that appears to be met by the C2C12 intracardiac grafts. The normal sinus rhythm observed in graft-bearing animals is noteworthy, as the border zone between healthy myocardium and scar tissue frequently gives rise to circus loops, resulting in clinically significant arrhythmias in patients suffering from myocardial infarct (43, 44). This observation, as well as the absence of long-term changes in the plasma enzyme profile, suggests that intracardiac grafts do not have an overtly negative effect on the host heart. Although the size of the mouse heart precludes exhaustive assessment of mechanical performance, recent studies have successfully measured cardiac output and coronary blood flow in intact, conscious mice (45), as well as Starling functional curves in in vitro working mouse heart preparations (46). Additionally, the ability to generate primary myoblast cells from other species raises the possibility of extending these studies to animals more suitable for cardiovascular functional studies.

It is clear that stable intracardiac grafts can be established with cultured myoblasts (this report) and with transformed cardiomyocytes (15). The potential utility of these grafts has not yet been exploited. Given the successful results obtained with other transplantation schemes, there is little doubt that myoblasts can be used as a stable platform for expression of recombinant molecules in the heart. Although likely, it remains to be proven that regional myocardial expression of a given compound is superior to systemic delivery. As outlined above, several formidable obstacles must be overcome in order to realize successful myocardial replacement. Further experimentation with myoblast grafts, as well as grafts originating from other cell types, may enable us to circumvent these obstacles.

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References

1. Mauro, A. 1961. Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9:493–495.

2. Morgan, J. E., D. J. Watt, J. C. Sloper, and T. A. Partridge. 1988. Partial correction of an inherited biochemical defect of skeletal muscle by grafts of normal muscle precursor cells. *J. Neurosci.* 86:137–147.

3. Watt, D. J., J. E. Morgan, and T. A. Partridge. 1984. Use of mononuclear precursor cells to insert allogeneic genes into growing mouse muscles. *Muscle & Nerve.* 7:741–750.

4. Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature (Lond.).* 337:176–179.

5. Gussoni, E., G. K. Pavlath, A. M. Lanctot, K. R. Sharma, R. G. Miller, L. Steinman, and H. M. Blau. 1992. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. *Nature (Lond.).* 356:435–438.

6. Rumyantsev, P. P. 1977. Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. *Int. Rev. Cytol.* 51:186–273.

7. Rumyantsev, P. P. 1991. Reproduction of cardiac myocytes developing in vivo and its relationship to processes of differentiation. *In* Growth and Hyperplasia of Cardiac Muscle Cells. P. P. Rumyantsev, editor. Harwood Academic Press, New York 70–159.

8. Field, L. J. 1988. Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. *Science (Wash. DC).* 239:1029–1033.

9. Behringer, R. R., J. J. Peschon, A. Messing, C. L. Gartside, S. D. Hauschka, R. D. Palmiter, and R. L. Brinster. 1988. Heart and bone tumors in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 85:2648–2652.

10. Katz, E., M. E. Steinhelper, A. Daud, J. B. Delcarpio, W. C. Claycomb, and L. J. Field. 1992. Ventricular cardiomyocyte proliferation in transgenic mice expressing α-Cardiac Myosin Heavy Chain-SV40 T antigen fusion genes. *Am. J. Physiol.* 262 (Heart Circ. Physiol. 31):H1867–1876.

11. Sen, A., P. Dunnmon, S. A. Henderson, R. D. Gerard, and K. R. Chien. 1988. Terminally differentiated neonatal rat myocardial cells proliferate and maintain specific differentiated functions following expression of SV40 large T antigen. *J. Biol. Chem.* 263:19132–19136.

12. Jackson, T., M. F. Allard, C. M. Sreenan, L. K. Doss, S. P. Bishop, and J. L. Swain. 1990. The c-myc proto-oncogene regulates cardiac development in transgenic mice. *Mol. Cell. Biol.* 10:3709–3716.

13. Engelmann, G. L., M. C. Birchenall-Roberts, F. W. Ruscetti, and A. M. Samarel. 1991. Cardiac myocyte cell line formation. *J. Cell. Biochem. Suppl.* 15C:161. (Abst.)

14. Saule, S., J. P. Merigaud, A. E. Al-Moustafa, F. Ferre, P. M. Rong, P.

Amouyel, B. Quatannens, D. Stehelin, and F. Dieterlen-Lievre. 1987. Heart tumors specifically induced in young avian embryos by the v-myc oncogene. *Proc. Natl. Acad. Sci. USA.* 84:7982–7986.

15. Koh, G. Y., M. H. Soonpaa, M. G. Klug, and L. J. Field. 1993. Long-term survival of AT-1 cardiomyocyte grafts in syngeneic myocardium. *Am. J. Physiol.* 264 (Heart Circ. Physiol. 33), H1727–H1733.

16. Steinhelper, M. E., N. Lanson, K. R. Dresdner, J. B. Delcarpio, A. L. Wit, W. C. Claycomb, and L. J. Field. 1990. Proliferation in vivo and in culture of differentiated adult atrial cardiomyocytes from transgenic mice. *Am. J. Physiol.* 259 (Heart and Circ. Physiol. 28):H1826–H1834.

17. Delcarpio, J. B., N. A. Lanson, Jr., L. J. Field, and W. C. Claycomb. 1991. Morphological characterization of cardiomyocytes isolated from a transplantable cardiac tumor derived from transgenic mouse atria (AT-1 cells). *Circ. Res.* 69:1591–1600.

18. Lanson, N. A., Jr., C. C. Glembotski, M. E. Steinhelper, L. J. Field, and W. C. Claycomb. 1992. Gene expression and ANF processing and secretion in cultured AT-1 cardiac myocytes. *Circulation.* 85:1835–1841.

19. Rockman, H. A., R. S. Ross, A. N. Harris, K. U. Knowlton, M. E. Steinhelper, L. J. Field, J. Ross, Jr., and K. R. Chien. 1991. Segregation of atrial-specific and inducible expression of an ANF transgene in an in vivo murine model of cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA.* 88:8277–8281.

20. Bullock, G. R., and P. Petrusz. 1983. Techniques in Immunocytochemistry, Vol. II. Academic Press, New York.

21. Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270:725–727.

22. Nadal-Ginard, B. 1978. Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell.* 15:855–864.

23. Bruni, C. 1979. Mitotic activity of muscle satellite cells during the early stages of rhabdomyosarcomas induction with nickel subsulfide. *In* Muscle Regeneration. A. Mauro, editor. Raven Press, New York.

24. Rubin, L. L., C. E. Keller, and S. M. Schuetze. 1979. Satellite cells in isolated adult muscle fibers in tissue culture. *In* Muscle Regeneration. A. Mauro, editor. Raven Press, New York.

25. Behara, A. M., A. J. Wescott, and P. L. Chang. 1992. Intrathymic implants of genetically modified fibroblasts. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:2853–2858.

26. Gage, F. H., M. D. Kawaja, and L. J. Fisher. 1991. Genetically modified cells: applications for cerebral grafting. *Trends Neurosci.* 14:328–333.

27. Jiao, S., and J. A. Wolff. 1992. Long-term survival of autologous muscle grafts in rat brain. *Neurosci. Lett.* 137:207–210.

28. Zwiebel, J. A., S. M. Freeman, K. Newman, D. Dichek, U. S. Ryan, and W. F. Anderson. 1991. Drug delivery by genetically engineered cell implants. *Ann. NY Acad. Sci.* 618:394–404.

29. Barr, E., and J. M. Leiden. 1991. Systemic delivery of recombinant proteins by genetically modified myoblasts. *Science (Wash. DC).* 254:1507–1509.

30. Dhawan, J., L. C. Pan, G. K. Pavlath, M. A. Travis, A. M. Lanctot, and H. M. Blau. 1991. Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. *Science (Wash. DC).* 254:1509–1512.

31. Bussolino, F., M. F. DiRenzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, and P. M. Comoglio. 1992. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.* 119:629–641.

32. Folkman, J., and M. Klagsburn. 1987. Angiogenic factors. *Science (Wash. DC).* 235:442–447.

33. Plate, K. H., G. Breier, H. A. Weich, and W. Risau. 1992. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature (Lond.).* 359:845–848.

34. Shweiki, D., A. Itin, D. Soffer, and E. Keshet. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature (Lond.).* 359:843–845.

35. Zipes, D. P. 1990. Influence of myocardial ischemia and infarction on autonomic innervation of heart. *Circulation.* 82:1095–1105.

36. Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 105:2621–2629.

37. Markwald, R. R., C. H. Mjaatvedt, E. L. Krug, and A. R. Sinning. 1990. Inductive interactions in heart development. Role of cardiac adherons in cushion tissue formation. *Ann. NY Acad. Sci.* 588:13–25.

38. Hoffman, S., K. L. Crossin, E. A. Prediger, B. A. Cunningham, and G. M. Edelman. 1990. Expression and function of cell adhesion molecules during the early development of the heart. *Ann. NY Acad. Sci.* 588:73–86.

39. Samuel, J.-L., and H. H. Vandenburgh. 1990. Mechanically induced orientation of adult rat cardiac myocytes in vitro. *In Vitro Cell. & Dev. Biol.* 26:905–914.

40. Karpati, G., Y. Pouliot, E. Zubrzycka-Gaarn, S. Carpenter, P. N. Ray, R. G. Worton, and P. Holland. 1989. Dystrophin is expressed in mdx skeletal muscle fibers after normal myoblast implantation. *Am. J. Pathol.* 135:27–32.

41. Morgan, J. E., E. P. Hoffman, and T. A. Partridge. 1990. Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. *J. Cell Biol.* 111:2437–2449.

42. Partridge, T. A., J. E. Morgan, S. E. Moore, and F. S. Walsh. 1988. Myogenesis in vivo from the mouse C2 muscle cell line. *J. Cell. Biochem. Suppl.* 12C:331. (Abstr.)

43. Janse, M. J., J. Cinca, H. Morena, J. W. Fiolet, A. G. Kleber, G. P. deVries, A. E. Becker, and D. Durrer. 1979. The "border zone" in myocardial ischemia. An electrophysiological, metabolic, and histochemical correlation in the pig heart. *Circ. Res.* 44:576–588.

44. Spear, J. F., E. L. Michelson, and E. N. Moore. 1983. Cellular electrophysiologic characteristics of chronically infarcted myocardium in dogs susceptible to sustained ventricular tachyarrhythmias. *J. Am. Coll. Cardiol.* 4:1099–1110.

45. Barbee, R. W., B. D. Perry, R. N. Re, and J. P. Murgo. 1992. Microsphere and dilution techniques for the determination of blood flows and volumes in conscious mice. *Am. J. Physiol.* 263:R728–R733.

46. Ng, W. A., I. L. Grupp, A. Subramaniam, and J. Robbins. 1991. Cardiac myosin heavy chain mRNA expression and myocardial function in the mouse heart. *Circ. Res.* 68:1742–1750.