Survival, Integration, and Differentiation of Cardiomyocyte Grafts

A Study in Normal and Injured Rat Hearts

Hans Reinecke, PhD; Ming Zhang, MD, MS; Trudy Bartosek, BS; Charles E. Murry, MD, PhD

Background—Cardiomyocyte grafting augments myocyte numbers in the heart. We investigated (1) how developmental stage influences graft survival; (2) whether acutely necrotic or healing cardiac lesions support grafts; and (3) the differentiation and integration of cardiomyocyte grafts in injured hearts.

Methods and Results—Cardiomyocytes from fetal, neonatal, or adult inbred rats were grafted into normal myocardium, acutely cryoinjured myocardium, or granulation tissue (6 days after injury). Adult cardiomyocytes did not survive under any conditions. In contrast, fetal and neonatal cardiomyocytes formed viable grafts under all conditions. Time-course studies with neonatal cardiomyocytes showed that the grafts recapitulated many aspects of normal development. The adherens junction protein N-cadherin was distributed circumferentially at day 1 but began to organize into intercalated disk–like structures by day 6. The gap junction protein connexin43 followed a similar but delayed pattern relative to N-cadherin. From 2 to 8 weeks, there was progressive hypertrophy and the formation of mature intercalated disks. In some hearts, graft cells formed adherens and gap junctions with host cardiomyocytes, suggesting electromechanical coupling. More commonly, however, grafts were separated from the host myocardium by scar tissue. Gap and adherens junctions formed between neonatal and adult cardiomyocytes in coculture, as evidenced by dye transfer and localization of cadherin and connexin43 at intercellular junctions.

Conclusions—Grafted fetal and neonatal cardiomyocytes form new, mature myocardium with the capacity to couple with injured host myocardium. Optimal repair, however, may require reducing the isolation of the graft by the intervening scar tissue. **(*Circulation*. 1999;100:193-202.)**

Key Words: myocardial infarction ■ cell transplantation ■ gap junctions ■ cadherins ■ connexin43

The heart cannot regenerate functional muscle after injury. Thus, our group and others have explored whether cardiomyocyte transplantation can repair damaged myocardium. Initial studies of cell grafting in normal hearts were encouraging. Soonpaa et al[1] and Koh et al[2] showed that fetal cardiomyocytes could be grafted into normal mouse or dog hearts, in which they formed intercalated disks with host cardiomyocytes. The behavior of grafted cardiomyocytes in injured hearts, in contrast, is less well understood. Several groups have shown that cardiomyocytes can be grafted successfully into myocardial infarcts or cardiac cryoinjuries.[3–6] In contrast, a recent article by Watanabe et al[7] reported that fetal and neonatal pig cardiomyocytes and the cardiac-derived cell line HL-1 did not survive after grafting into pig infarcts.

Among studies that show graft survival, discrepancies still exist regarding the amount of differentiation that occurs in the grafts. For example, Leor et al[3] reported that embryonic human cardiomyocytes grafted into injured rat hearts did not differentiate into an adult phenotype, even when studied several months after grafting. Similarly, in the study by Watanabe et al,[7] the grafted fetal cardiomyocytes seemed to maintain their fetal phenotype in the normal host heart for the 5 weeks of the study. In contrast, Connold et al[5] reported that embryonic rat cardiomyocytes formed organized gap junctions with other graft cells when grafted into injured rat hearts, suggesting that some differentiation could occur. Importantly, no data demonstrate electromechanical coupling between graft and host myocardium after injury.

Many of the above discrepancies may result from differences in the type of donor cells used or the status of the host myocardium at the time of grafting. In the current study, we systematically evaluated several key variables that may influence the success of cardiomyocyte grafting. In particular, we asked the following questions. (1) How does the developmental stage of the donor cell (fetal, neonatal, or adult) influence the success of the graft? (2) Can grafts survive equally well in normal myocardium, acutely necrotic tissue, and granulation (wound repair) tissue? (3) Will grafted cells retain an immature phenotype

Received January 13, 1999; revision received March 16, 1999; accepted March 31, 1999.

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or differentiate toward an adult phenotype? (4) Will grafted cells integrate, ie, form electrical and mechanical junctions, with host cardiomyocytes?

Methods

Fetal and Neonatal Rat Cardiomyocytes

These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. The syngeneic rat strain Fischer 344 (Simonsen Labs, Gilroy, Calif) was used to avoid rejection. Fetal and neonatal cardiomyocytes were tagged in utero for later identification in the graft by giving bromodeoxyuridine (BrdU; 50 mg tablet every 24 hours SC) to pregnant rats on each of the 2 days before the removal of the fetuses or before normal delivery. Cardiomyocytes were isolated from day-15 fetuses and from 1- to 2-day-old newborn rats. If neonates were used at day 2, they were given an additional BrdU injection on day 1 (50 μL of a 10 mg/mL solution). Cardiomyocytes were isolated according to a protocol modified from that of Iwaki et al.[8] Pregnant rats were anesthetized with ketamine-xylazine (68 and 4.4 mg/kg IP, respectively). Fetal and neonatal rats were killed by decapitation, and their hearts were rapidly removed and placed into an ice-cold buffer (in mmol/L: NaCl 116.4, HEPES 20, NaH_2PO_4 1, glucose 5.5, KCl 5.4, $MgSO_4$ 0.8; pH 7.4). The atria and great vessels were trimmed and discarded. The ventricles were cut in 2 to 3 mm^3 pieces and incubated (37°C, 25 minutes) repeatedly (5 to 6 times) in buffer supplemented with collagenase type II (95 U/mL; Worthington) and pancreatin (0.6 mg/mL; Gibco BRL). After each round of digestion, the supernatant was centrifuged (600*g*, 5 minutes) and the resulting cell pellet was resuspended in DMEM/M199 (4:1) supplemented with 10% horse serum (ICN Flow), 50% fetal bovine serum (HyClone), penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (Gibco). Cells were preplated for 30 minutes to reduce the number of contaminating nonmyocytes. Cardiomyocytes were pooled, counted, and incubated in medium until further processing. The cell yield was $\approx 3\times10^5$ per embryonic heart and $\approx 2\times10^6$ per neonatal heart, and the preparations averaged $\geq$90% cardiomyocytes, as determined by MF-20 antibody staining for sarcomeric myosin heavy chain. A total of 15% to 20% of the cardiomyocytes were positive for BrdU (data not shown). The cells were washed twice in serum-free DMEM and resuspended in 70 μL of serum-free medium immediately before the injection.

Adult Rat Cardiomyocytes

Adult rat cardiomyocytes were isolated as described previously.[9] Briefly, hearts were removed from ketamine-xylazine–anesthetized adult male Fischer 344 rats (weight, 300 to 400 g), retrogradely perfused with Ca^{2+}-free buffer, and then perfused with collagenase/hyaluronidase (Boehringer Mannheim). Cells were resuspended in M199 (Sigma) and counted. For later identification in the graft, cells were infected for 4 hours with a β-galactosidase adenovirus (1000 pfu/cell)[10]; in some experiments, the adenovirus was omitted. Nonattached cells were washed and resuspended in 70 μL of serum-free M199 immediately before injection. Pilot experiments showed that adult cardiomyocytes rounded up relatively quickly when stored at high density. We determined that cells should be kept on ice for as short a duration as possible (<10 minutes) for optimal viability. The cell suspensions used for the injections contained $\approx$60% rod-shaped cardiomyocytes under these optimized conditions. Aliquots of the cell suspensions remaining after injection were plated to verify viability.

Cell Grafting Experiments

We investigated the survival of fetal, neonatal, and adult cardiomyocytes under the following different grafting conditions: normal heart, acutely injured heart (immediately after freeze-thaw), and granulation tissue (6-day-old injury). Adult male Fischer 344 rats were subjected to cardiac cryoinjury, as recently described.[10,11] A total of 5×10^5 adult, 2×10^6 fetal, or 4×10^6 neonatal cardiomyocytes suspended in 70 μL of serum-free medium were injected into the center of the injured region immediately after injury, into the granulation tissue of 6-day-old injuries, or into normal hearts using a 27-gauge needle. The chest was closed, and the rats were allowed to recover for 6 days (n=2 to 4 per time point). Rats receiving adult cardiomyocytes were also studied 1 to 2 days after grafting.

Rats were killed with a pentobarbital overdose, and their hearts were excised. The aorta was cannulated, and the hearts were perfusion-fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% glacial acetic acid), transversely sectioned, and embedded in paraffin by routine methods. If adult cardiomyocytes were grafted, hearts were perfusion-fixed with 4% paraformaldehyde and immersed in paraformaldehyde for 2 hours at room temperature. The hearts were then perfused with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining solution (5 mmol/L $K_4Fe(CN)_6$, 5 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$, and 1 mg/mL X-Gal in PBS; pH 7.4) and incubated at 37°C for an additional 3 hours. Then, hearts were processed for histologic examination as described above.

On the basis of the survival study, we chose neonatal cardiomyocytes and the acute injury model to investigate the time course of graft cardiomyocyte proliferation, differentiation, and integration. A total of 4×10^6 neonatal cardiomyocytes were injected (as described above), and wounds were allowed to heal for 1, 3, or 6 days or 2, 4, or 8 weeks (n=5 for days 1, 2, and 3 and week 2; n=8 for weeks 4 and 8). Hearts were then excised and processed for immunocytochemical examination as described above.

Dye Transfer Between Cultured Neonatal and Adult Cardiomyocytes

The neonatal and the adult cardiomyocytes were isolated on the same day. Adult cardiomyocytes were plated onto laminin-coated 60-mm dishes or glass slides at a density of 2×10^5/dish and incubated for $\approx$4 hours (37°C, 5% CO_2). The plates were then washed twice to remove cell debris and unattached cells. The resulting cell population consisted of $\geq$80% rod-shaped cardiomyocytes. Approximately 7×10^5 neonatal cardiomyocytes were added per 60-mm dish, and cells were cocultured for a total of 20 hours. Microinjection was performed using an inverted phase-contrast microscope (Zeiss Axiovert, Zeiss) equipped with an automated microinjection system (Micromanipulator 5170, Microinjector 5242, Eppendorf). Sterile micropipettes (Femtotips, Eppendorf) were loaded with 1 μL of fluorescent dye solution, which was composed of 10% lucifer yellow (gap junction transferable) and 10% tetramethylrhodamine-dextran (nontransferable; Molecular Probes) in sterile, deionized, distilled H_2O. Beating neonatal cardiomyocytes in close approximation to adult cardiomyocytes were pressure-injected (time setting, 0.3 s; pressure setting, 18 kPa). Dye transfer was evaluated under a fluorescence microscope (Olympus BH-2, Olympus).

Confocal Microscopy

For confocal microscopy, neonatal and adult cardiomyocytes were cocultured for 24 or 48 hours on laminin-coated glass slides. Then, cells were fixed for 2 minutes in a solution consisting of 3% paraformaldehyde in PBS, 0.2% Triton X-100, and 5 mmol/L EGTA (pH 7.2). This was followed by fixation with 3% paraformaldehyde in PBS for 20 minutes. Slides were incubated with pan-cadherin or connexin43 antibodies for 60 minutes. Primary antibodies were detected with a rabbit anti-mouse fluorescein isothiocyanate–conjugated secondary antibody (DAKO). Counterstaining for f-actin was performed using rhodamine phalloidin (Molecular Probes). Vectashield (Vector) was used as a mounting medium. Slides were evaluated using a Bio-Rad MRC 600 confocal microscope equipped with a krypton-argon laser.

Antibodies and Immunocytochemistry

Immunostaining on deparaffinized sections was performed using immunoperoxidase and immunofluorescent methods, as described in detail elsewhere.[10,11] In double-staining reactions for α-myosin heavy chain and proliferating cell nuclear antigen (PCNA), diaminobenzidine (DAB, Sigma) was used as the first chromogen and true blue (KPL, Gaithersburg, MD) as the second chromogen. Antibodies

TABLE 1. Antibodies Used for Immunocytochemistry

Antibody	Antigen Recognized	Dilution	Source	Reference
CH-19	Pan-cadherin	1:2000	Sigma, St. Louis, Mo	12
		1:200 IF		
MAB3068	Connexin43	1:200 LM and IF	Chemicon, Temecula, Calif	13
G3G4	BrdU	1:50 (Hyb Sup)	Developmental Studies Hybridoma Bank*	14
BA-G5	Cardiac myosin heavy chain-α	1:50 (Hyb Sup)	ATCC, Rockville, Md	15
MF-20	Sarcomeric myosin heavy chains	(Hyb Sup)	ATCC, Rockville, Md	16
		1:100 LM		
		1:1 IF		
PC10	PCNA	1:1000	DAKO, Carpinteria, Calif	17
D33	Anti-human desmin	1:5	DAKO, Carpinteria, Calif	18

All antibodies are mouse monoclonal IgGs. All dilutions refer to light microscopy (LM) unless otherwise noted. IF indicates immunofluorescence; Hyb Sup, hybridoma supernatant; ATCC, American Type Culture Collection; and MAb, monoclonal antibody.

*Monoclonal antibody G3G4 was developed in the laboratory of Dr S.J. Kaufman, and it was obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, Department of Biological Sciences, Iowa City, under contract NO1-HD-7-3263, National Institute of Child Health and Human Development.

used for immunostaining and their respective dilutions are given in Table 1.

Results

Characterization and Identification of the Graft Cell Population

Neonatal graft cells were tagged in utero with BrdU for at least 2 days before isolation, allowing us to identify the location of the graft unambiguously. The isolated cells consisted of ≥90% cardiomyocytes, as determined by immunostaining with the MF-20 anti-sarcomeric myosin antibody.[16] Serial sections of each heart and time point were stained with a monoclonal anti-BrdU antibody (see below). Graft cells were detected by BrdU staining throughout the time course.

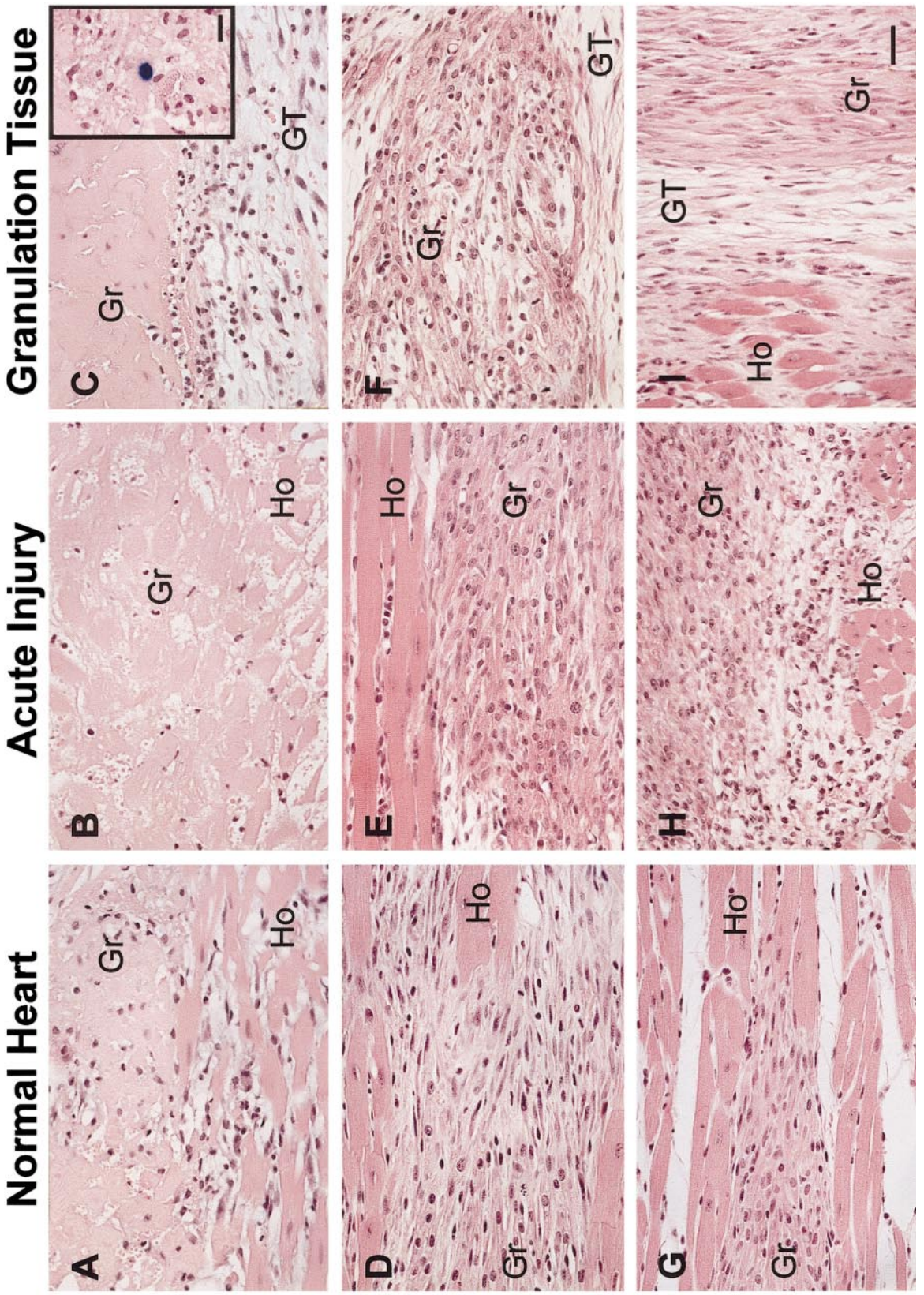
Survival of Grafted Cardiomyocytes

Figure 1 summarizes the survival pattern of fetal, neonatal, and adult cardiomyocytes after grafting into normal myocardium, acutely injured myocardium, and granulation tissue (6-day-old injury). Adult cardiomyocyte grafts showed no survival at day 6 and hence were studied at day 1. Even at day 1 (Figures 1A through 1C), the vast majority of adult cardiomyocytes had features typical of coagulation necrosis, including loss of nuclei and more intensely eosinophilic cytoplasm. A few viable adult cardiomyocytes were found at day 1 after grafting into granulation tissue (Figure 1C, inset), but none were seen in any other group. Because the adult cardiomyocytes that remained after injection survived in vitro and showed rod-shaped morphology after 24 hours in culture, it is clear that they were viable at the time of injection. Furthermore, pilot experiments in which adult cardiomyocytes were not adenovirally tagged also failed to yield viable grafts, indicating that viral infection did not cause their death. In contrast, fetal and neonatal cardiomyocytes (Figures 1D through 1I) formed viable grafts after transplantation under all conditions. Because the cell yield from neonatal hearts was ≈7 times higher than that from embryonic hearts and no major differences in viability after grafting were observed, we chose to use neonatal cardiomyocytes for the time-course study.

Histology and Differentiation Patterns of Neonatal Graft Cardiomyocytes

At day 1 after grafting into acutely injured myocardium, the neonatal cardiomyocytes appeared small, round, and undifferentiated. The graft area was surrounded by necrotic host myocardium containing numerous inflammatory cells and interstitial hemorrhage (Figure 2A). It should be noted that dead neonatal graft cells were also identified at day 1 by BrdU-positive cell fragments (Figure 2M, inset). By day 3, wound healing was underway, characterized by phagocytosis of necrotic host cardiomyocytes, concomitant with the ingrowth of fibroblasts and capillaries to form granulation tissue. The graft cardiomyocytes had elongated and frequently were in close approximation to intact host cardiomyocytes (Figure 2B). By day 6, almost all of the necrotic host myocardium was removed. The graft cardiomyocytes appeared more differentiated in terms of cell elongation and the formation of sarcomeres (Figures 2C and 3). At 2, 4 (data not shown), and 8 weeks, there was progressive elongation into a rod-shaped morphology and sarcomeres became increasingly well defined (Figures 2D and 3).

At all time points, consecutive heart sections were stained for the BrdU tag to identify grafted cells reliably. Figure 2 (M through P) shows that BrdU-positive cells were detected throughout the time course. Furthermore, BrdU-positive cardiomyocytes were readily demonstrated at 8 weeks, and they had typical rod-shaped morphology and well-defined sarcomeres (Figure 2D, inset). Development of sarcomeres in grafted cardiomyocytes was further studied by desmin immunofluorescence staining (Figure 3). Rudimentary sarcomeres were detected as early as day 6 after grafting. By 2 and 8 weeks, virtually all grafted cardiomyocytes had well-developed sarcomeres (Figure 3).


Normal Heart
Acute Injury
Granulation Tissue
Adult
Fetal
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A
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C
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E
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The diameter of the graft cardiomyocytes progressively increased over time, indicating significant hypertrophy. Figure 4 shows that graft cell diameter increased by ≈20% by day 6, ≈60% by week 2, ≈100% by week 4, and 160% by week 8, as compared with day 1. However, despite the progressive hypertrophy, the diameter of grafted cardiomyocytes at 8 weeks was still 34% smaller than that of host cardiomyocytes (Figure 4). Taken together, these data show that grafted neonatal cardiomyocytes progress toward the morphologic phenotype of adult cardiomyocytes.

N-Cadherin and Connexin43 in Neonatal Graft Cardiomyocytes

To investigate the electromechanical integration of grafted neonatal cardiomyocytes and host myocardium, immunostaining was performed for major components of the intercalated disk. Note that although a pan-cadherin antibody was used for immunostaining, N-cadherin is the only isoform expressed by cardiomyocytes. At day 1, the graft cardiomyocytes expressed cadherin in a mostly circumferential or scattered fashion (Figure 2E). In contrast, the connexin43 antibody did not stain the graft cardiomyocytes at day 1 (Figure 2I). By 3 days, when the graft cardiomyocytes had elongated, cadherin was partially concentrated at the cell ends (Figure 2F). At this time point, however, the vast majority of the graft cardiomyocytes still appeared negative for connexin43 (Figure 2J). In contrast, in adult host cardiomyocytes, cadherin and connexin43 were concentrated and colocalized in the intercalated disk (Figures 2F and 2J). By day 6, there was progressive concentration of cadherin staining at the graft cell ends, indicating the organization of newly formed intercalated disks (Figure 2G). Interestingly, by day 6, most of the graft cells expressed connexin43 in a scattered, punctate fashion (Figure 2K). However, occasional graft cardiomyocytes showed partial concentration of connexin43 molecules in the forming intercalated disk (Figure 2K).

Between days 1 and 6, neonatal graft cardiomyocytes were observed in close apposition to host myocardium (Table 2). Adult host myocytes at the interface often extended cell processes toward the graft myocytes (Figure 5A). Furthermore, using confocal microscopy, we observed gap junctions between graft and host cardiomyocytes, suggesting electromechanical integration of the graft (Figure 5B). In a minority of hearts at this time, however, graft cells were separated from host myocardium by the intervening wound tissue (Table 2). Over the ensuing weeks, there was progressive concentration of cadherin and connexin43 in the intercalated disk while the graft cardiomyocytes developed a more rod-shaped (adult-like) appearance. By 8 weeks, cadherin and connexin43 were restricted to intercalated disks that were virtually indistinguishable from the host myocardium (Figures 2H and 2L). At the later time points, we observed less frequent contact between graft and host (≈40% of successful grafts; Table 2). Furthermore, in the hearts with contact, the interaction was restricted to the edges of the grafts, with most of the graft being separated from the host by intervening scar tissue (Figure 5C).

Figure 1. Facing page. Survival of cardiomyocyte grafts. Fetal, neonatal, or adult rat cardiomyocytes were grafted into normal myocardium, acutely cryoinjured myocardium, or granulation tissue (6 days after injury). Fetal and neonatal grafts are shown at day 6, and adult cardiomyocyte grafts are shown at day 1 after transplantation. A, B, and C, Adult cardiomyocytes did not survive to a significant extent under any grafting condition. Inset in C shows rarely observed X-GAL–positive adult cardiomyocyte at day 1 (Bar=25 μm). In contrast, fetal (D, E, and F) and neonatal (G, H, and I) cardiomyocytes formed viable grafts in all 3 kinds of host tissue. Close approximation to host cardiomyocytes was frequently observed in normal myocardium. In injured myocardium, however, graft and host were often separated by intervening wound tissue. All panels show hematoxylin and eosin staining. Bar=25 μm. Gr indicates graft cells; Ho, host myocardium; and GT, granulation tissue.

Proliferation of Grafted Neonatal Cardiomyocytes

To determine whether neonatal cardiomyocytes might proliferate after grafting, we performed double-staining for PCNA and α-cardiac myosin heavy chain. The identity of graft cells in the myocardium was confirmed by matching BrdU stains of serial sections. PCNA-positive graft cardiomyocytes were readily observed from 3 days to 2 weeks, with a peak frequency of 2% double-positive cells occurring at 6 days (Figure 2C, inset). These data suggest DNA synthesis for up to 2 weeks after grafting. However, PCNA cannot distinguish endoreplication of DNA from replication followed by cytokinesis.

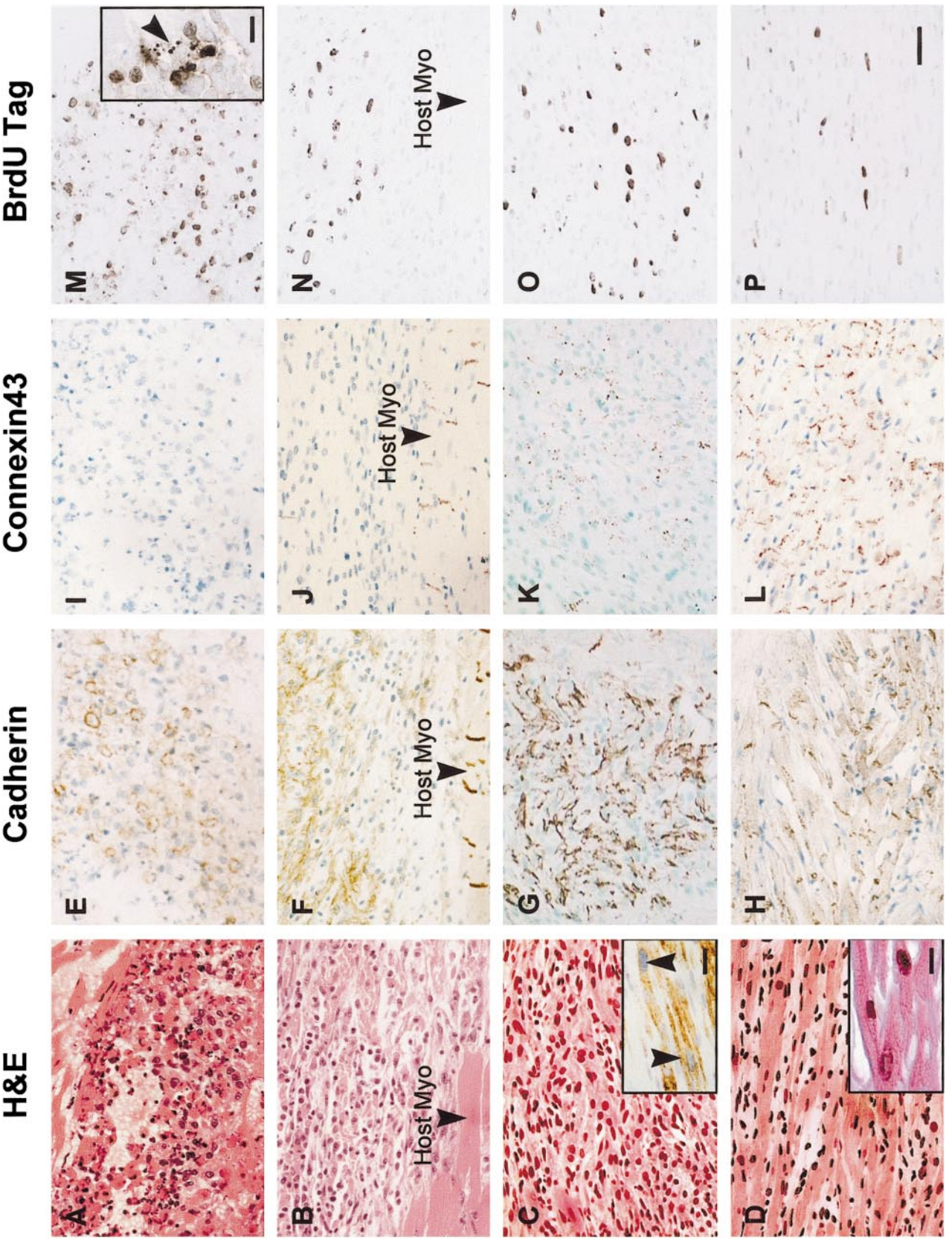
Electromechanical Coupling of Neonatal and Adult Cardiomyocytes In Vitro

Cocultures of adult and neonatal cardiomyocytes were studied to determine the capacity of the 2 cell populations to form electromechanical junctions. When the cells were placed in coculture, they formed a synchronously beating network, indicating electrical coupling. Confocal microscopy showed that cadherin (Figure 5D) and connexin43 (Figure 5E) localized to contact sites between neonatal and adult cardiomyocytes. To test directly whether the 2 cell types were connected by gap junctions, we performed microinjection studies. Beating neonatal cardiomyocytes were microinjected with a combination of lucifer yellow (≈250 Da), which readily passes through gap junctions,[19] and rhodamine-dextran (≈10 000 Da), which is not transferable through gap junctions. Figure 5 (F, G, and H) shows dye transfer from a neonatal cardiomyocyte (donor) to adjacent neonatal cardiomyocytes and, most importantly, to an adult cardiomyocyte (which was identified by its typical rod shape) (Figure 5H). These results clearly demonstrate that neonatal cardiomyocytes can form gap junctions with adult cardiomyocytes in coculture.

Discussion

Developmental Influences on Graft Survival

We isolated cardiomyocytes from fetal, neonatal, and adult donors to investigate whether these cells might differ in their survival capacity. To a first approximation, both fetal and neonatal cardiomyocytes formed comparable grafts in the normal heart, acutely injured myocardium, and granulation tissue. In contrast, we found that adult cardiomyocytes did not survive under any of the grafting conditions. We observed some cell death at day 1 in the neonatal cardiomyocytes, but it was clearly not of the magnitude


H&E
Cadherin
Connexin43
BrdU Tag
1 d
3 d
6 d
8 w
A
B
C
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M
N
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Host Myo
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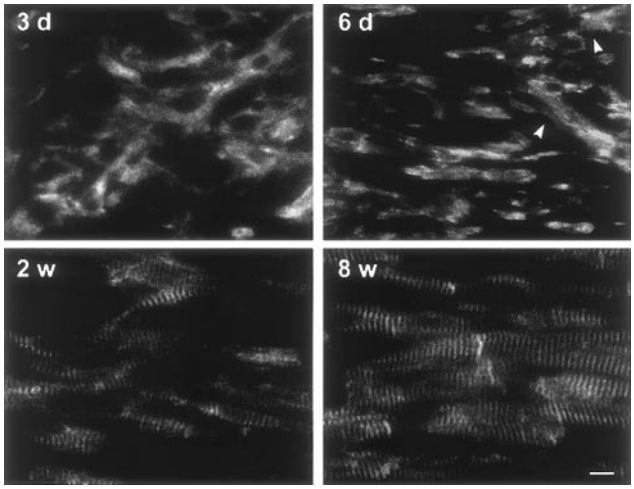


Figure 3. Desmin immunostaining in cardiomyocyte grafts. Top left, Desmin was initially diffusely distributed in cytoplasm (3 days). Top right, Rudimentary sarcomeres were detected at day 6 (arrowheads). Bottom left, By 2 weeks, virtually all grafted cardiomyocytes had well-developed sarcomeres. Note progressive hypertrophy between 2 and 8 weeks (bottom, right). Bar=10 μm.

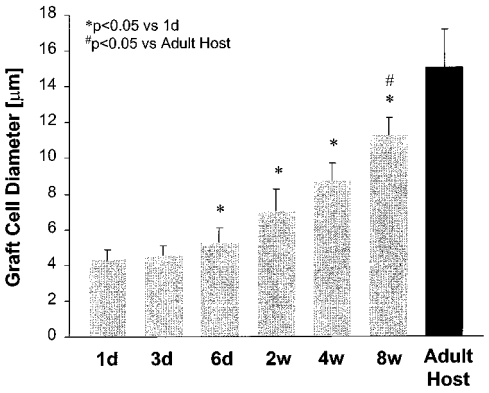


Figure 4. Hypertrophy of grafted neonatal cardiomyocytes over time. Cardiomyocyte diameter was measured in longitudinal sections containing central nuclei using calibrated microscope scale. Significant hypertrophy of graft cardiomyocytes was observed from day 6 onward. Despite this, graft cardiomyocytes did not quite reach diameter of host cardiomyocytes by 8 weeks. Measurements were performed on hematoxylin-and-eosin–stained sections from 3 animals in each group (30 cardiomyocytes measured per animal; n=90 per group). Graft area was verified on consecutive BrdU-stained sections. Values are mean±SD. ANOVA was followed by Student-Newman-Keuls test; *$P<0.05$ vs day 1 after grafting; #$P<0.05$ vs adult host.

Figure 2. Facing page. Differentiation of neonatal cardiomyocyte grafts. Host myocardium is shown only in day-3 panels (B, F, J, and N). Each time point shows serial sections from same heart. Bar=35 μm. Hematoxylin and eosin staining: A, At day 1, graft cardiomyocytes appeared small, round, and undifferentiated. Graft area was mostly surrounded by necrotic host myocardium and interstitial hemorrhage. B, At day 3, progressive wound healing was observed and graft cardiomyocytes began to elongate. C, At day 6, all necrotic host myocardium was removed. Graft cardiomyocytes began to enlarge and had early sarcomere formation (see also Figure 3). Inset in C, Potential proliferation of graft neonatal cardiomyocytes was studied by double-staining for PCNA (blue) and α-cardiac myosin heavy chain (brown). PCNA-positive graft neonatal cardiomyocytes were observed from 3 days to 2 weeks, with peak frequency of 2% double-positive cells occurring at 6 days (as shown). Bar=10 μm. D, By 8 weeks, graft cardiomyocytes had undergone progressive hypertrophy, resulting in adult-like, rod-shaped phenotype with well-developed sarcomeres. Inset in D, Anti-BrdU and eosin staining of graft area 8 weeks after grafting. BrdU-positive graft neonatal cardiomyocytes were identified by typical cross-striations, as demonstrated by eosin counterstaining. Bar=10 μm. Cadherin: E, At day 1, graft cardiomyocytes were stained in mostly circumferential or scattered fashion. F, By day 3, graft cardiomyocytes appeared more elongated, and cadherin was partially concentrated at cell ends and sides. Note that in adult host cardiomyocytes (arrowhead), cadherin was exclusively concentrated in intercalated disks. G, At day 6, cadherin was progressively concentrated at ends of graft cells, indicating organization of newly formed intercalated disks. H, By 8 weeks, cadherin was almost exclusively concentrated in newly formed intercalated disks. Connexin43: At days 1 (I) and 3 (J), graft cardiomyocytes were negative for connexin43. Note strong staining for connexin43 at intercalated disks of adult host cardiomyocytes (J, arrowhead). K, By day 6, graft cardiomyocytes expressed connexin43 in scattered, punctate fashion. L, By 8 weeks, connexin43 was mostly expressed in newly formed intercalated disks. BrdU: Graft cells were labeled with BrdU in utero before isolation, and BrdU staining was later used to track them in adult recipients. Graft cells were detected throughout time course (M through P). Inset in M (Bar=10 μm) shows nuclear fragmentation in some grafted neonatal cardiomyocytes (arrowhead), indicating cell death after transplantation. Host Myo indicates host cardiomyocytes.

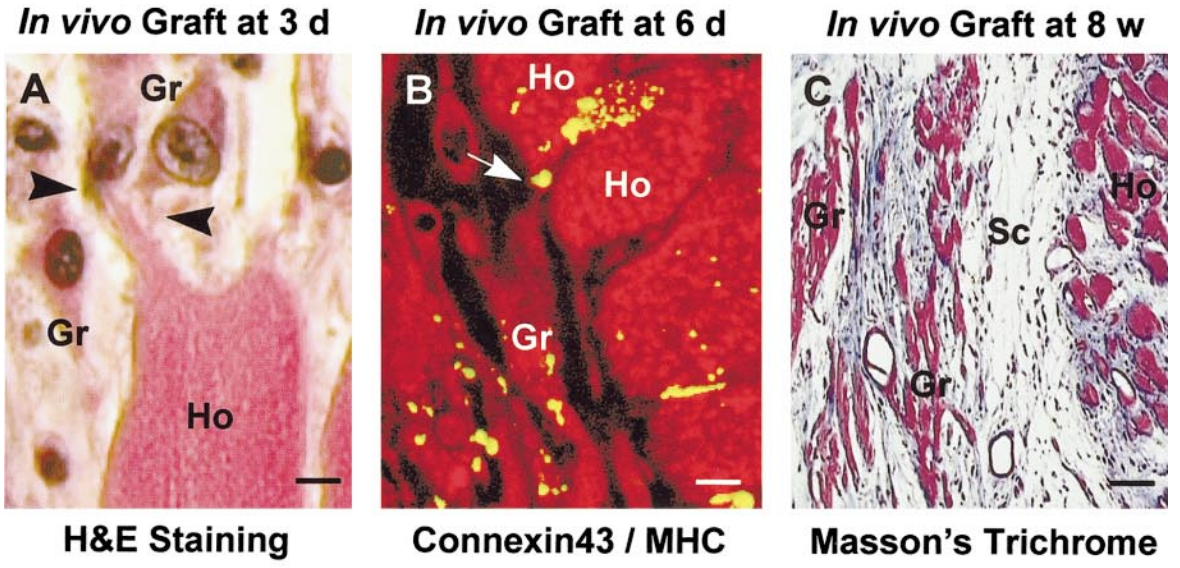
seen with adult grafts. Possible mechanisms of death in graft cells include ischemic injury or activation of apoptotic pathways, eg, by loss of matrix attachments (anoikis)[20] or inflammatory cytokines.

Our results contrast somewhat with data recently reported by Watanabe et al.[7] The authors grafted fetal and neonatal pig cardiomyocytes and cardiac-derived mouse HL-1 cells (a subclone of AT-1 cardiomyocytes) into normal and infarcted pig hearts. Whereas the fetal and HL-1 cells survived in normal myocardium, none of the 3 cell types survived in the infarct. Moreover, neonatal cardiomyocytes also died after grafting into normal myocardium.[7] We speculate that neonatal pig cardiomyocytes might be further developed than neonatal rat cardiomyocytes, which, in turn, could affect survival after grafting. The state of differentiation would not explain the death of the HL-1 cells, which are a relatively undifferentiated, transformed tumor cell line. An obvious difference is that the pig infarct is many times larger than our cryoinjuries. One would predict that diffusion of oxygen, other nutrients, and waste products could enhance graft survival in a smaller lesion.

TABLE 2. Survival and Integration of Neonatal Cardiomyocyte Grafts

Time After Grafting	No. of Grafts	No. of Successful Grafts	No. of Grafts With Contact to Host
1 d	5	4	3
3 d	5	5	5
6 d	5	5	3
2 wk	5	3	2*
4 wk	8	3	1*
8 wk	8	5	2*

*Graft and host were mostly separated by developing scar tissue, and contact sites were restricted to graft edges.


In vivo Graft at 3 d
In vivo Graft at 6 d
In vivo Graft at 8 w
A
Gr
Gr
Ho
B
Ho
Ho
Gr
C
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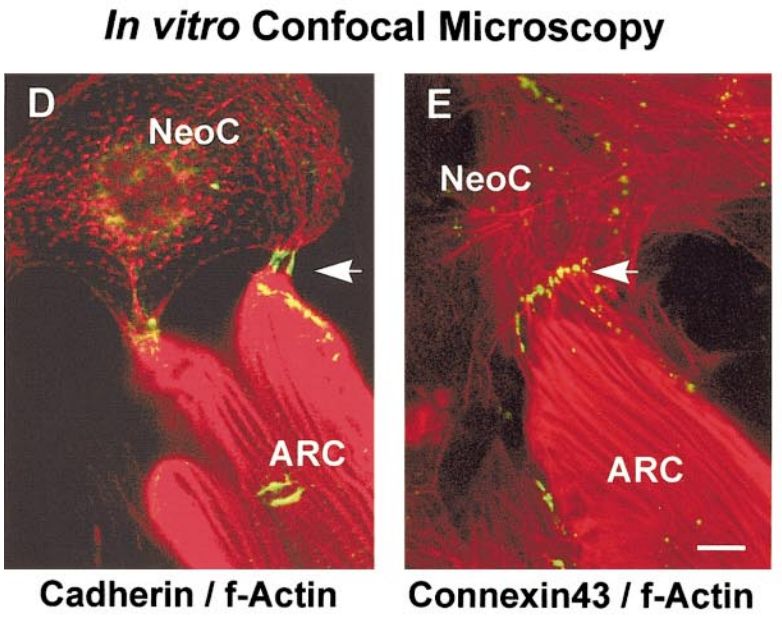
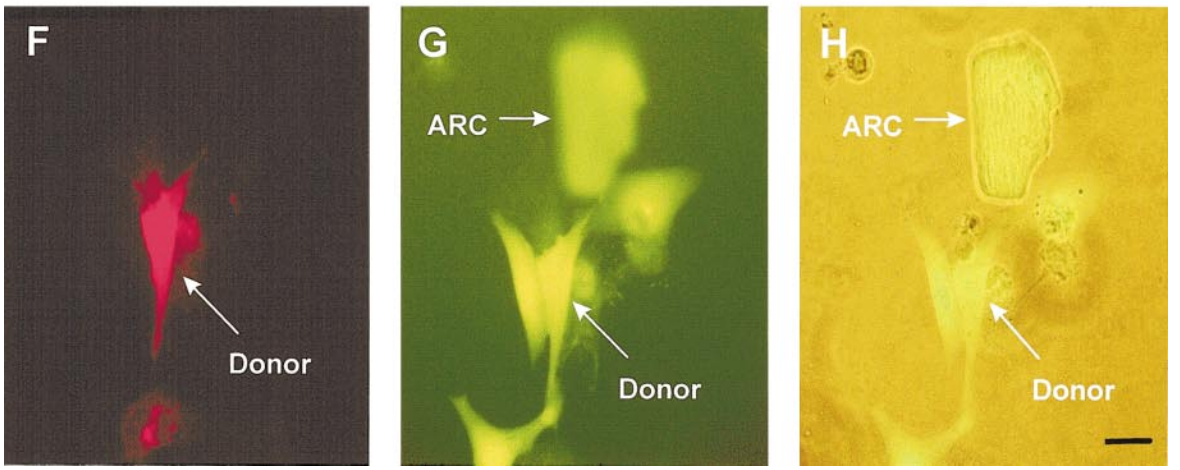
Sc
Ho
Gr
H&E Staining
Connexin43 / MHC
Masson's Trichrome


In vitro Confocal Microscopy
D
NeoC
ARC
E
NeoC
ARC
Cadherin / f-Actin
Connexin43 / f-Actin


In vitro Dye Transfer
F
Donor
G
ARC
Donor
H
ARC
Donor

Differentiation of Cardiomyocyte Grafts

For the grafts to function as a syncytium, it is important that they express N-cadherin and connexin43 in a proper topological fashion.[21–23] We found that cadherin was expressed in the graft cells throughout the time studied. Cadherin expression was initially circumferential and, with ongoing cell elongation toward the adult phenotype, progressively restricted to the intercalated disk. By 8 weeks, virtually all of the cadherin was concentrated in intercalated disks. Connexin43 expression followed a similar spatial pattern, but it was delayed relative to cadherin. Connold et al[5] recently reported a similar pattern of connexin43 expression in grafted fragments of fetal myocardium. These data strongly suggest that neonatal graft cardiomyocytes have the capacity to form with each other the electromechanical junctions necessary to provide improvement of cardiac function. Note that the expression patterns of both N-cadherin and connexin43 in grafts closely parallel normal cardiac development. In the fetal heart, cadherin expression precedes that of connexin43, and both molecules are initially expressed circumferentially, followed by concentration at the intercalated disk.[24–26]

The cardiomyocyte grafts showed progressive hypertrophy over the 2-month study period. Hirakow et al[27] reported that in normal rat development, left ventricular myocyte diameter increased by 100% between birth and 4 weeks and by 140% by 8 weeks; our graft cell diameters increased by 100% and 160% at these time points, respectively. Thus, for hypertrophy and gene expression patterns, our neonatal grafts appeared to recapitulate the normal cardiac developmental program. Quantitative data on hypertrophy are not available from other grafting studies, but Soonpaa et al[1] reported that fetal mouse cardiomyocytes reached adult size by 2 months after grafting into normal hearts.

Integration of Grafts With Host Myocardium

Coculture experiments clearly demonstrated that neonatal and adult cardiomyocytes could form electromechanical junctions. The 2 cell populations formed synchronously beating networks, expressed N-cadherin and connexin43 at their junctions, and permitted dye transfer after microinjection. Similarly, at early time points after grafting, it was easy to demonstrate close spatial approximation of graft and host cardiomyocytes in vivo and the expression of connexin43, suggesting electromechanical coupling (Figures 5A and 5B and Table 2). On the other hand, from 2 to 8 weeks after grafting, most grafts were separated from the host myocardium by granulation or scar tissue. Contact sites to host myocardium were present only at the edges of the grafts in 40% of hearts, whereas in 60%, no integration was detectable (Table 2). Our interpretation is that growth of scar tissue at later times separates the graft from the host myocardium. Other variables, such as injection quality or survival, migration, or proliferation of graft cells after injection, may also be involved.

A key question for cell transplantation into the injured heart is whether this approach will improve cardiac function. The results of our cardiomyocyte grafting experiments and those of others[1,3–6] suggest that the principal requirements for functional improvement (integration of the graft cardiomyocytes by electromechanical junctions, potential proliferation to some extent, and differentiation toward the adult cardiomyocyte phenotype) might be achieved. Indeed, a recent study by Li et al[6] reported that cardiomyocyte grafting into cryoinjured rat hearts improved global cardiac function. Because the grafts were completely encased in scar tissue, they may not have contributed actively to systolic function. The authors speculated that grafting may have improved passive mechanical properties of the scar or cardiac remodeling.[6]

Limitations of BrdU Tagging

The use of BrdU tagging in utero resulted in the labeling of 15% to 20% of cardiomyocytes after isolation. We chose this approach because we are interested in developing this rat model for physiological analyses that cannot be done in genetically tagged mouse hearts. Proliferating cells will progressively dilute their BrdU label after transplantation, and we do not know whether the modest replication we detected was enough to alter staining thresholds. In any case, it is clear that some graft cardiomyocytes cannot be detected by BrdU staining. Graft size, therefore, will be underestimated by BrdU staining, although the BrdU label probably outlines the general boundaries of the graft. More importantly, it is not possible to say definitively whether a given BrdU-negative cell came from the graft or the host. For the most part, the graft and host cardiomyocytes had such different morphologies that distinguishing the 2 was not a problem. Because of this limitation, however, one must be

Figure 5. Facing page. A, Hematoxylin and eosin staining of graft cells at day 3. Cell processes of host cardiomyocyte (Ho) appear to be in contact with graft cardiomyocytes (Gr) (arrowheads). Bar=5 μm. B, Confocal microscopy of day-6 graft. Connexin43 staining is in green, and myosin heavy chain (MHC) staining (MF-20 antibody) is in red fluorescence. Graft-host interface is shown. Graft cardiomyocyte appears to form gap junction (arrow) with host cardiomyocyte. Optical section=100 nm thick; bar=5 μm. C, Masson's trichrome staining of 8-week graft. At time points >2 weeks, host and graft cells were often separated by scar tissue (Sc). Bar=40 μm. D and E, Confocal microscopy of cocultured neonatal and adult rat cardiomyocytes. D, Cadherin (green)-mediated adherens junctions (arrow) were readily observed after 24 hours of coculture. E, Connexin43 (green)-mediated gap junctions (arrow) are shown at 48 hours. Frequency of gap junction formation and intensity of connexin43 immunofluorescence seemed to increase with coculture time. f-Actin was used as counterstain (red). Note that sarcomeres in adult cardiomyocytes have been obscured by need to overexpose image to detect f-actin in neonatal cells. Yellow indicates areas where f-actin and junctional proteins were too close to resolve in 0.1-μm image plane. NeoC indicates neonatal cardiomyocyte and ARC, adult rat cardiomyocyte. Bar=8 μm. F, G, and H, Dye transfer between neonatal and adult cardiomyocytes in vitro. After 20 hours of coculture, beating neonatal cardiomyocyte (donor) was microinjected with lucifer yellow (transferable) and rhodamine-dextran (nontransferable). F (rhodamine filter) shows microinjected donor cell. G (lucifer yellow filter) identifies cells to which lucifer yellow has been transferred via gap junctions. Among these cells are adjacent neonatal cardiomyocytes and adult rat cardiomyocyte, which is identified by its typical morphology in H by bright field exposure. Bar=20 μm.

conservative when interpreting coupling between graft and host cells in this study. A final consideration is that fibroblasts included in the grafts also were labeled by BrdU. Because fibroblasts do not express significant amounts of cadherin or connexin43, these cells would not interfere with any conclusions from this experiment.

In summary, we have shown that neonatal cardiomyocytes seem to be well suited for cell grafting into the injured heart with regard to integration, potential proliferation, and differentiation. On the basis of in vivo immunostaining and in vitro demonstration of electromechanical coupling between neonatal and adult cardiomyocytes, we hypothesize that grafted cardiomyocytes do indeed form electromechanical junctions with host cardiomyocytes in some hearts. In other hearts, however, scar tissue appeared to isolate the graft from the host myocardium. Overall, these results encourage additional studies of cardiomyocyte transplantation as an approach for cardiac repair after infarction. It clearly remains to be investigated whether cardiomyocyte grafting will improve cardiac function and, if so, how many cardiomyocytes are required.

Acknowledgments

The authors are indebted to Drs John Angello and Stephen D. Hauschka for assistance with the microinjection system. This study was supported in part by a Grant-in-Aid from the National Center of the American Heart Association, grants from the National Institutes of Health (K08 HL-03094 and P01 HL-3174), and a fellowship grant in support of cardiac research from Collateral Therapeutics, Inc. Dr Murry is the recipient of a Burroughs Wellcome Career Award in the Biomedical Sciences.

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