Comparison of Intracardiac Cell Transplantation: Autologous Skeletal Myoblasts Versus Bone Marrow Cells

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Background—Multiple cell types are being proposed for cardiac repair, but side-by-side comparisons are lacking. We tested the hypothesis that intracardiac transplantation of autologous bone marrow- or skeletal muscle-derived progenitor cells improve regional heart function to a similar degree.

Methods and Results—Thirty-nine New Zealand White rabbits underwent cryoinjury of the left ventricle and simultaneous hind limb bone marrow aspiration or soleus muscle biopsy. Both muscle and bone marrow cells were expanded in vitro. After 2 weeks, 10^8 skeletal muscle (SM group) or bone marrow-derived progenitor cells (BM group) were injected into the cryoinjured region (SM: n=12; BM: n=8). Medium alone was injected into the remaining animals (Control: n=16). Regional systolic function was measured using micromanometry and sonomicrometry at baseline, before, and 4 weeks after cell injection. Cell treatment resulted in a similar degree of improvement in a derivative of stroke work in the SM and BM groups ($P=0.0026$ and $P=0.0085$ versus Control, respectively). No significant difference was seen between BM and SM groups ($P=0.9$). On histology, engrafted cells were found in all of the cell treated animals. Injected myoblasts formed myotubes or muscle cells throughout the scar that expressed slow and fast myosin heavy chain. A subset of bone marrow cells differentiated toward a myogenic phenotype, as indicated by expression of desmin and α-sarcomeric actin in the engrafted areas.

Conclusion—Transplantation and myogenic differentiation of bone marrow-derived progenitor cells increased regional systolic heart function after myocardial injury to a similar degree as skeletal myoblasts. **(*Circulation*. 2003;108[suppl II]:II-264-II-271.)**

Key Words: cellular cardiomyoplasty ■ myoblasts ■ cell transplantation ■ stem cells ■ infarction

Cellular therapy for heart failure has risen to the forefront of cardiac research. Several preclinical and clinical studies indicate that both skeletal muscle and bone marrow-derived progenitor cells can successfully repopulate injured myocardium and improve heart function.[1–5] Increasing clinical use has created interest in evaluating the particular capability of each cell type to repair the injured heart.

Two of the most widely used cell types for cardiac repair today are skeletal muscle-derived progenitors, or myoblasts, and bone marrow-derived progenitors. Both cell types share advantages over other cells proposed for cardiac repair in that they are readily available, autologous, and easily expanded in vitro.

Myoblasts engraft,[6] differentiate toward a phenotype adapted to cardiac workload,[7] and improve heart function.[1,8] Bone marrow progenitor cells are reported to have the capacity to differentiate into cardiomyocytes both in vitro[9] and in vivo,[5] and improve global heart function preclinically.[4,10] However, to date, no study has been performed to directly compare these 2 cell types with respect to their improvement of cardiac function.

In this study, we transplanted equal numbers of myoblasts or bone marrow-derived progenitor cells into the center of cryoinjured rabbit hearts and compared the improvement in regional systolic function using micromanometry and sonomicrometry.

Materials and Methods

All of the experiments were conducted in accordance with guidelines published in *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 86 to 23, revised 1985), and under the protocols approved by the Institutional Animal Care and Use Committee at Duke University.

Surgical Preparation

New Zealand White rabbits (n=39), weighing 3.5–4.5 kg, were premedicated with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated. Anesthesia was maintained with 2% isoflurane.

A soleus muscle biopsy or femoral bone marrow aspirate was then performed from the left hind limb. A left thoracotomy through the fourth intercostal space was performed, as described previously,[11] and the heart was exposed. A cryoprobe (diameter 1.4 cm) cooled to −70°C (Frigitronics) was applied to the epicardial surface of the

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anterolateral left ventricular (LV) wall for 3 minutes to create a transmural cryoinjury of ≈1.5 cm in diameter. Fourteen days after cryoinjury, the animals underwent a second thoracotomy for initial data acquisition using sonomicrometry and micromanometry, and injection of SM (n=12), BM (n=8), or medium (Control; n=16) in the infarct area. The injections were performed in 3 parallel tracts of 0.33 mL in each.

Four weeks after cell injection, a third thoracotomy was performed, and sonomicrometry and micromanometry measurements were repeated. After recording final data, animals were euthanized, and hearts were excised for histologic analysis.

A separate subset of animals (n=3) had identical surgical procedures but were transplanted with lysed 4′,6-diamidino-2-phenylindole (DAPI) -labeled myoblasts. These animals were sacrificed 7 days after cell injection for analyses of artifactual DAPI reuptake into surrounding cells.

Expansion of Myoblasts

Tissue from a 500–700 mg soleus biopsy was mechanically dissected and washed in PBS. The tissue was then plated in growth medium, consisting of DMEM with 20% horse serum and 0.5% gentamicin (10 mg/mL). The tissue fragments were triturated after 3 days. After another 3 days, the tissue was removed, and the myoblasts were fed with growth medium. Cells were fed every other day and passaged when reaching ~70% confluency, resulting in 2–3 passages before injection. The day before injection, cells were labeled overnight with the nuclear stain DAPI (Sigma; 10 μg/mL). For injection, cells were washed with PBS to remove all DAPI, trypsinized, washed twice more with PBS, counted, and resuspended at 10^8 cells/ml of DMEM.

Expansion of Bone Marrow Cells

Bone marrow aspirates were plated in growth medium consisting of DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 0.5% gentamicin. After 3 days, the flasks were washed twice with PBS to remove nonadherent cells. The remaining cells were fed every third day. Bone marrow cultures were maintained at <50% confluence and passaged 3–5 times before the time of injection. The day before injection, cells were labeled with DAPI (10 μg/mL). For injection, bone marrow cells were washed with PBS to remove excess DAPI, trypsinized, washed twice with PBS, counted, and resuspended at 10^8 cells/mL of DMEM.

Characterization of the Injected Cell Population

For fluorescence activated cell sorting (FACS) analysis, bone marrow-derived progenitors were trypsinized and blocked for 15 minutes with blocking buffer, consisting of Hank's Balanced Salt Solution supplemented with 3% FBS, 2 mmol/L EDTA, and 10 mmol/L HEPES buffer. Cells were stained with a rabbit specific polyclonal FITC-labeled CD45 antibody (Serotec) for 30 minutes and resuspended in blocking buffer for analysis using a FACS Vantage SE flow cytometer (BD Biosciences).

To assess the number of myogenic cells in the injected cell population, ≈10^4 myoblasts or bone marrow-derived progenitors were plated on coverslips, grown overnight, and fixed in 5% paraformaldehyde for 10 minutes. Cells were permeabilized in 0.2% NP-40 and 0.2% Tween in PBS for 10 minutes. Blocking was performed for 1 hour in PBS with 5% horse serum and 5% FBS. Cells were then incubated in primary monoclonal anti-desmin antibody (1:200) for 90 minutes followed by a Phycoerythrin (PE) -conjugated anti-mouse secondary antibody (1:200) in blocking buffer. Staining was analyzed on a Nikon TE 200 Eclipse inverted microscope, and the number of desmin-positive cells was counted and expressed as a percentage of the total cell number.

Physiological Data Acquisition

Micromanometry and sonomicrometry were performed 14 days after cryoinfarction (baseline) and 4 weeks after cell injection as follows. A pair of ultrasonic dimension transducers (Sonometrics Corp) were sutured to the epicardial surface, within 2 mm of the border of the cryoinjured region in the plane of the LV minor axis. A 3.0F micromanometer (Millar Instruments) was inserted into the LV cavity via the left atrium. Data were then collected using a sonomicrometry acquisition system (Sonometrics Corp).

Hemodynamic data that were collected, both at baseline and after 4 weeks, included heart rate, LV end-diastolic pressure, and dP/dt_{max}.

Data Analysis

Physiological data were analyzed using Cardiosoft software (Sonometrics Corp). The cardiac cycle was defined using the first derivative of LV pressure (dP/dt) as detailed previously.[12]

A regional systolic performance index (SW_A) analogous to Stroke Work was defined as the area within a plot of pressure versus dimension and calculated as the integral of ventricular pressure (P) with respect to the epicardial segment length (L):

$$SW_A = \int P dL$$

LV end-diastolic and peak pressure were obtained from the LV pressure waveform. Maximal dP/dt was obtained from the first derivative of the LV pressure waveform. Systolic shortening (SS) was defined as the difference in epicardial segment length between end-diastole (L_{ED}) and end-systole (L_{ES}) and was expressed as a percentage of the end-diastolic segment length:

$$SS = (L_{ED} - L_{ES}) \times 100 / L_{ED}$$

All of the cardiac cycles in a 10 second data acquisition period were analyzed, and the beat-to-beat indices were averaged over that period.

Histologic Analysis

Hearts were cut into 5 transverse slices, embedded in OCT freezing medium, and frozen in liquid nitrogen-cooled isopentane. Twelve serial 6 μm thick sections were cut from each slice. Sections were air dried overnight, fixed in 5% paraformaldehyde, and permeabilized in 1% Tween. Sections were stained with primary rabbit-specific monoclonal antibodies to desmin, slow and fast myosin heavy chain (MHC), connexin-43 and α-sarcomeric actin (all from Sigma), and a monoclonal antibody against troponin I (Chemicon), all diluted 1:200 in blocking buffer, for 90 minutes. Secondary antibody staining was performed with a PE-conjugated anti-mouse antibody (Molecular Probes) for 1 hour. Section 11 and 12 from each series were stained with hematoxylin-eosin and Goldner's Trichrome, respectively. Sections were examined under standard light and fluorescence microscopy after background subtraction. Using Image Pro software, engraftment of DAPI-labeled cells was qualified by drawing the engrafted areas and quantifying the areas as a percentage of total infarct area.

Statistical Analysis

All data are reported as a mean±SEM. A difference in response over time was tested using two-way ANOVA with repeated measures. If the overall F test for a group by time interaction was statistically significant, Student's t tests were used to determine statistical significance of pair-wise differences. Differences in proportions were tested for statistical significance using Fischer's Exact Test. Results were considered statistically significant at $P<0.05$.

Results

Cell Culture

Cultures yielded >10^8 myoblasts or bone marrow cells for each animal after 2 weeks. All of the myoblast cultures were >90% desmin positive (92.3±3.2%), indicating the high purity of the injected population (Figure 1A and B). All of the myoblast cultures formed myotubes in vitro. Bone marrow progenitor cells showed >80% CD45 negative cells as analyzed by FACS. Bone marrow cultures showed desmin positivity of 1.1% (±0.4%), (Figure 1C and D).

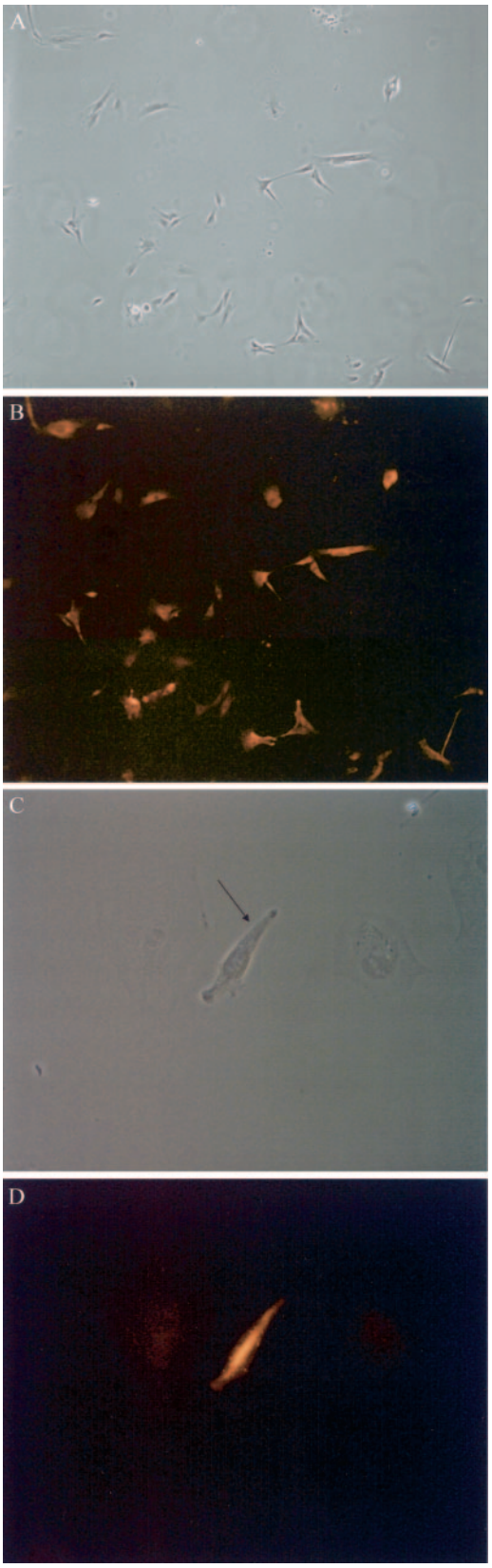

Figure 1. Desmin expression in the injected cell populations. (A), light micrograph and (B) corresponding fluorescence micrograph of desmin-stained rabbit skeletal myoblasts before injection. (C), light micrograph and (D) corresponding fluorescence micrograph of desmin-stained marrow derived cells.

Regional Performance

A stroke work analog, using pressure/segment length measurements, was obtained for each animal at baseline (before injection) and 4 weeks after injection (Table 1). Regional stroke work increased after treatment in 8 of 8 (100%) bone marrow cell-treated animals. Myoblast-treated animals showed improvement in 9 of 12 animals (75%), whereas control animals showed improvement in 4 of 16 animals (25%; $P<0.01$ versus control for SM and BM).

Quantitative analysis of the mean change in stroke work over time showed a significant improvement in the cell-treated groups compared with baseline (Figure 2A). Change in SW was 9.3 ± 3.3 mm*mmHg in the Myoblast group, 7.4 ± 1.6 mm*mmHg in the Bone Marrow group, and -0.9 ± 1.3 mm*mmHg in the Control group ($P=0.0026$ and $P=0.0085$, SM and BM versus Control, respectively). Change in systolic shortening showed a similar treatment effect of both cell types (SM $1.44\pm0.34\%$, BM $1.13\pm1.14\%$, Control $-1.48\pm0.63\%$; $P=0.006$ and $P=0.01$ for SM and BM group versus Control respectively; Figure 2B). There were no significant differences between the SM and BM groups ($P=0.9$ and 0.4 for change in stroke work and systolic shortening, respectively). When the data were reanalyzed using heart rate as an independent covariate, the statistical outcomes were not affected.

Hemodynamics

There were no differences in LV end-diastolic pressure or dP/dt_{max} between groups at baseline or 4 weeks after injection, nor was there a statistical difference in the change of those measures between groups over time (Table 1).

Histology

Sections through the center of the scar from each cell-treated animal were examined using standard light microscopy and fluorescent microscopy to identify engrafted DAPI-labeled cells. No DAPI-positive cells were found in the animals injected with lysed cells (Figure 3C). Transplanted cells from both treatment groups engrafted in all portions of the scar, typically covering ≈70% of the infarct area (Figure 3). Injected myoblasts remained relatively close to the initial injection sites, and were typically surrounded by dense collagen-rich scar tissue. A portion of these areas showed areas of mature myotube formation (Figure 3D and E). However, bone marrow cells were more dispersed throughout the infarct, with some groups of cells at the periphery of the infarct and in close contact with healthy myocardium. Bone marrow-derived progenitors appeared as smaller elongated cells with central nuclei, showing no evidence of myotube formation.

Injected myoblasts showed positive staining for desmin, α-sarcomeric actin, and the slow and fast isoform of MHC (Figure 4), though the slow isoform was more abundant than the fast. Bone marrow-derived progenitors also showed patches of cells that expressed desmin and α-sarcomeric actin throughout the infarct, although not all of the bone marrow cells were positive. In some hearts there was also positive staining for the slow isoform of MHC (Figure 5).

Left Ventricular Function Pre- and Postinjection

	Pre-injection			Post-injection		
	Control (n=16)	Bone marrow (n=8)	Myoblasts (n=12)	Control (n=16)	Bone marrow (n=8)	Myoblasts (n=12)
LV end-diastolic pressure (mm Hg)	8.06±1.39	8.79±2.33	6.43±0.73	6.69±0.66	3.54±1.30	6.45±1.55
Stroke work	−1.3±1.3	−2.3±1.4*	−4.0±1.2*	−2.1±1.1	5.1±2.7*	5.3±2.3*
Systolic shortening (%)	−0.43±0.41	−1.27±0.71*	−0.86±0.20*	−1.92±0.70	−0.15±1.14*	0.59±0.35*
dP/dt_{max} (mm Hg/s)	1997±135	2144±277	1873±131	1641±213	1943±260	1482±121

*$P<0.05$, change between pre- and postinjection vs. control.

None of the animals examined showed positive staining for connexin-43 or cardiac troponin I in the grafted areas. Furthermore, control animals did not show any desmin expression in the scar area, indicating that there was no cardiomyocyte survival 6 weeks after the cryoinjury, which could confound the results.

We used DAPI as a nuclear marker to identify the engrafted cells and performed colocalization with immunohistochemistry. The possibility that the dye had leaked to neighboring cells or had been released on cell death was excluded, since no uptake of dye was seen 7 days after lysed DAPI-labeled cells were injected (Figure 3C). Similar numbers of DAPI-positive cells were seen throughout the infarct in both cell-treated groups.

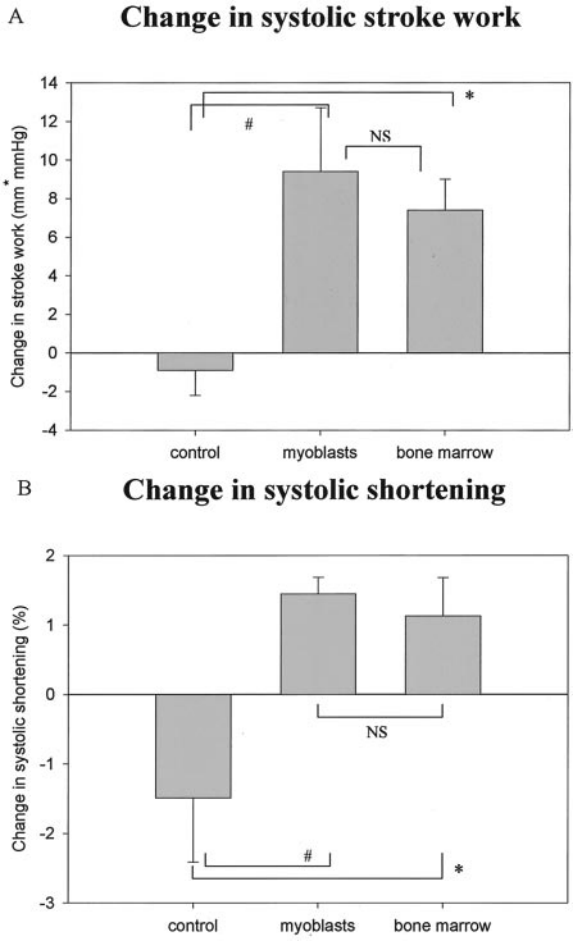

Figure 2. Changes in regional cardiac function after cell transplantation. A, change in stroke work after vehicle, myoblasts, or bone marrow injection. B, change in percentage systolic shortening for control (n=16), myoblasts (n=12), and bone marrow (n=8) groups (#$P=0.0026$ and * $P=0.0085$ versus Control in A; #$P=0.006$ and *$P=0.01$ versus Control in B).

Discussion

The data from this study demonstrate that bone marrow-derived cells and skeletal myoblasts improve regional systolic heart function to a similar degree. This is supported by histological data that showed engraftment of cells throughout the infarct in all of the cell-treated animals and in vivo differentiation of both cell types into cells that expressed contractile proteins.

Study Limitations

It is not clear to what extent the data of this study relate to the clinical setting of human intracardiac cell transplantation. The main difference between this study and the clinical setting is the cryoinfarction model. This model was chosen because the biologic variability associated with an ischemia/reperfusion model makes functional assessment very difficult. However, we have previously injected myoblasts into ligation-induced cardiac injuries and observed similar results to those obtained in this study, suggesting that the model is somewhat comparable.[13]

A second question that is not answered by this study is to what extent both cell types affect cardiac remodeling and global contractile function. It has been shown that both bone marrow-derived cells and myoblasts can prevent LV dilatation and hypertrophy.[10,14] For assessing remodeling and global contractility, echocardiography or MRI should be employed.

Finally, our study was powered to find substantial (>50%) differences in regional contractility between groups, if in fact a difference did exist. Therefore, smaller differences between groups may have gone undetected with the number of animals in the current study.

Cell Types for Cardiac Repair

Transplantation of various types of cells has been shown to augment systolic and diastolic function[15–17] in different animal models and species, but direct comparison of these cells, regarding their effects on function, has thus far not extensively been addressed. Our laboratory has previously examined skeletal myoblasts in comparison with dermal fibroblasts, showing that while both cell types improved compliance, only myoblasts improved systolic function.[15] A

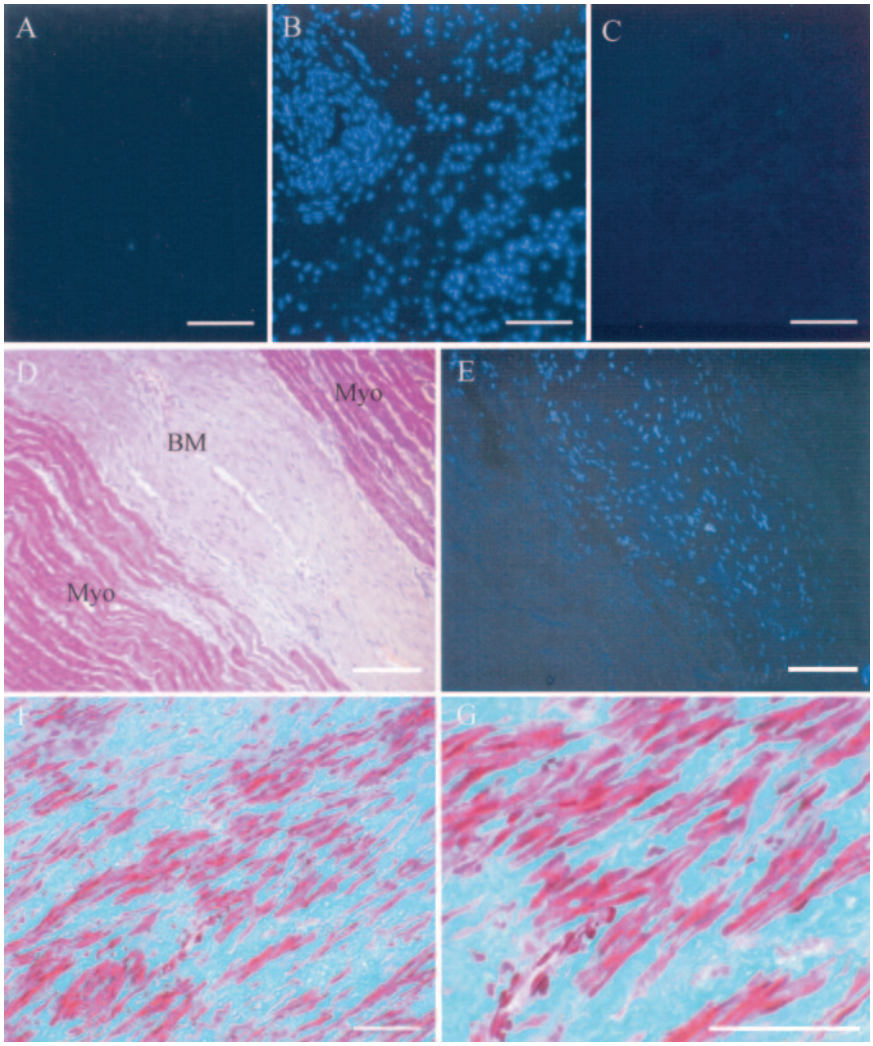

Figure 3. Histologic evaluation of control and cell treated hearts. Fluorescence micrograph of a control heart (A), a bone marrow-engrafted heart (B), and a heart injected with lysed DAPI labeled myoblasts (C; 200X, original magnification). The DAPI nuclear blue stain is easily identified in B. Normal light and corresponding fluorescence micrographs (D and E) of engrafted bone marrow cells (BM) show close contact of cells with the healthy myocardium (Myo). D is hematoxylin-eosin staining and E is DAPI filtered. F (200X) and G (400X) show Trichrome staining of myoblast-engrafted hearts. There is myotube formation with peripheral nuclei surrounded by dense collagen and elastin rich fibrous tissue. Bar represents 100 μm.

study comparing myoblasts and fetal cardiomyocytes in a rat model showed that both cell types improve heart function to the same extent.[16] A recent study compared cardiomyocytes and bone marrow-derived cells.[17] Although these studies indicate that implanting multiple cell types improves function, not every cell type is suitable for clinical use. Fetal or neonatal cardiomyocytes are difficult to obtain, not autologous, and bring ethical dilemmas. Our study examines the only 2 cell types that have gained widespread clinical use.

In our study, we have shown that both autologous skeletal myoblasts and a population of autologous bone marrow-derived progenitor cells improve regional systolic contractility to a similar degree.

Injected Cell Populations

We used desmin staining to assess the number of myogenic cells in our cultures before injection, which indicated a high percentage of myoblasts in our skeletal muscle cultures and a virtual absence of myogenic cells in the bone marrow population before transplant. It remains possible, though, that our muscle-derived cell population contained muscle-derived stem cells with multilineage potential, as described by others.[18,19] Hence, part of the myogenic differentiation seen in vivo could be from these stem-like cells. More extensive characterization of our myoblast cultures is needed to address this issue.

The bone marrow cells showed a high percentage (>80%) of CD45-negative cells by flow cytometry. This indicates that the cultured cells were primarily of mesenchymal origin. It has been shown before that progenitor cells with multilineage capacity copurify with the mesenchymal cell fraction.[20] After injection and engraftment, these cells clearly underwent milieu-dependent differentiation toward a myogenic phenotype. However, the ability of bone marrow-derived cells to repopulate infarcts may not be limited to cells of a myogenic lineage; some investigators have shown that bone marrow mesenchymal cells take part in neovascularization.[21]

Previously, bone marrow-derived cells have also been shown to differentiate into cardiomyocytes both in vivo and in vitro when specific inducers were used, such as 5-azacytidine[9] or when specific subpopulations were selected.[5] Although in this study the engrafted cells started expressing contractile proteins, no differentiation toward a cardiomyocyte phenotype was observed.

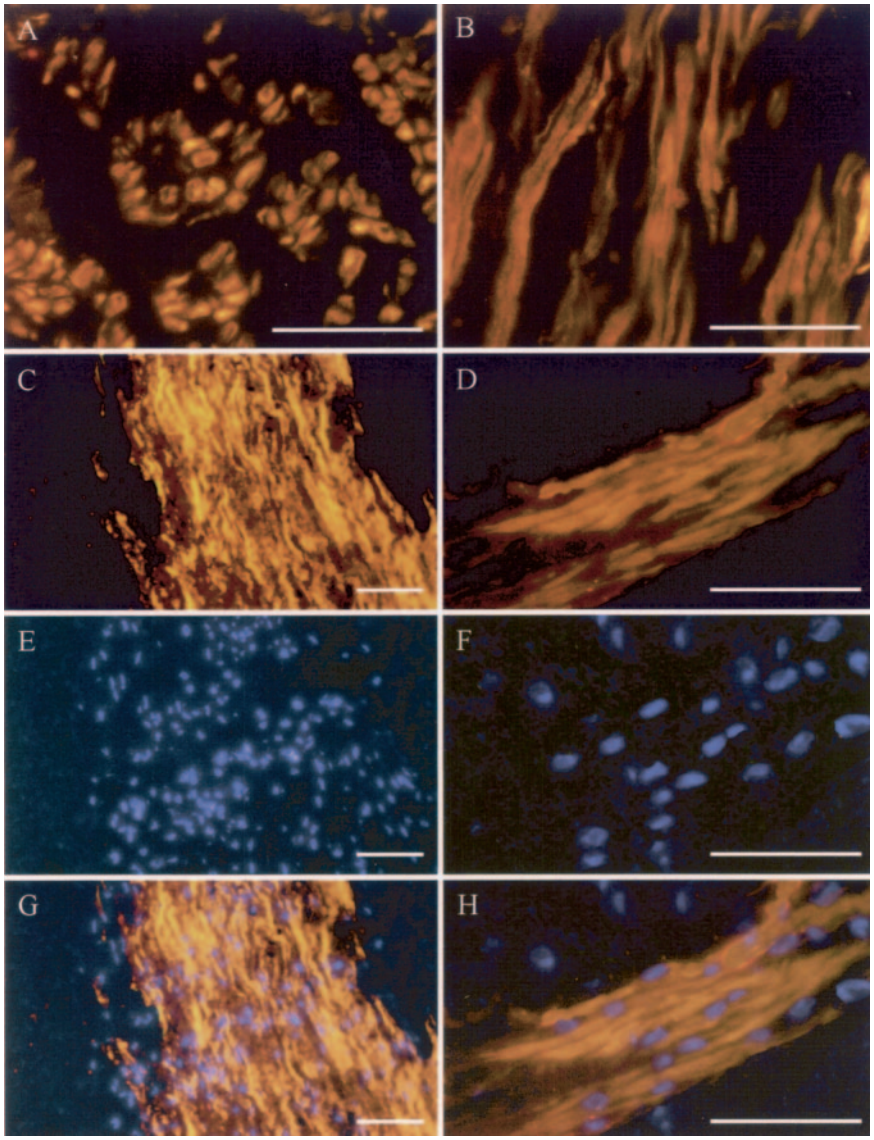

Figure 4. Fluorescence micrographs of sections of scar engrafted with myoblasts. Desmin staining shows that injected myoblasts form patches of unfused single cells (A) or patches of myotubes (B) throughout the scar area (400X). Engrafted myoblasts (identified by their DAPI blue nuclear staining, E and F) additionally show positive immunostaining for the slow (C) and fast (D) isoform of myosin heavy chain (G and H are a merged images of C-E and D-F, respectively; 400X). Bar represents 100 μm.

Global Indices of Cardiac Function

In this study we found an improvement in regional contractile function after cell treatment. However, no improvement in global function (as indicated by dP/dt) was observed after cell treatment. One possible explanation for this is that the infarct area is too small to yield a significant global decrement and, thus, permit global improvement. For example, it has been shown before that global parameters such as ejection fraction only improve in animals when baseline values are markedly decreased, which was not the case here.[23] Furthermore, both dP/dt and ejection fraction can be sensitive to baseline drift of preload and afterload, and, therefore, are not the most representative parameters of global heart function.

Electromechanical Integration

In both cell-treated groups, no expression of the cardiac gap junction protein, connexin-43, was found in the engrafted areas, which indicates that the engrafted cells lack electromechanical integration with the host myocardium. This was additionally confirmed by our observation that most cells were surrounded by scar tissue. However, this does not preclude contractility because of other mechanisms of cell activation. Myotubes may be activated by stretch receptors that respond to the distension of the surrounding myocardium during diastole. This proposed potential mechanism is not novel and has been discussed in the literature previously.[22] Stretch receptor activation may also play a role in contractile activation of bone marrow cells, although additional study is needed to confirm this.

It is commonly believed that, for a piece of myocardium to contribute to the active ejection of blood, it must be organized in a 3-dimensional complex structure that incorporates a well-organized extracellular matrix. However, a possible mechanism for the change in regional contractile function as observed in this study and the global changes observed in other studies is the effect transplanted cells may have on global ventricular remodeling. Changes in small infarct areas during periods of active remodeling may be beneficial to the overall remodeling of the ventricle after infarct.

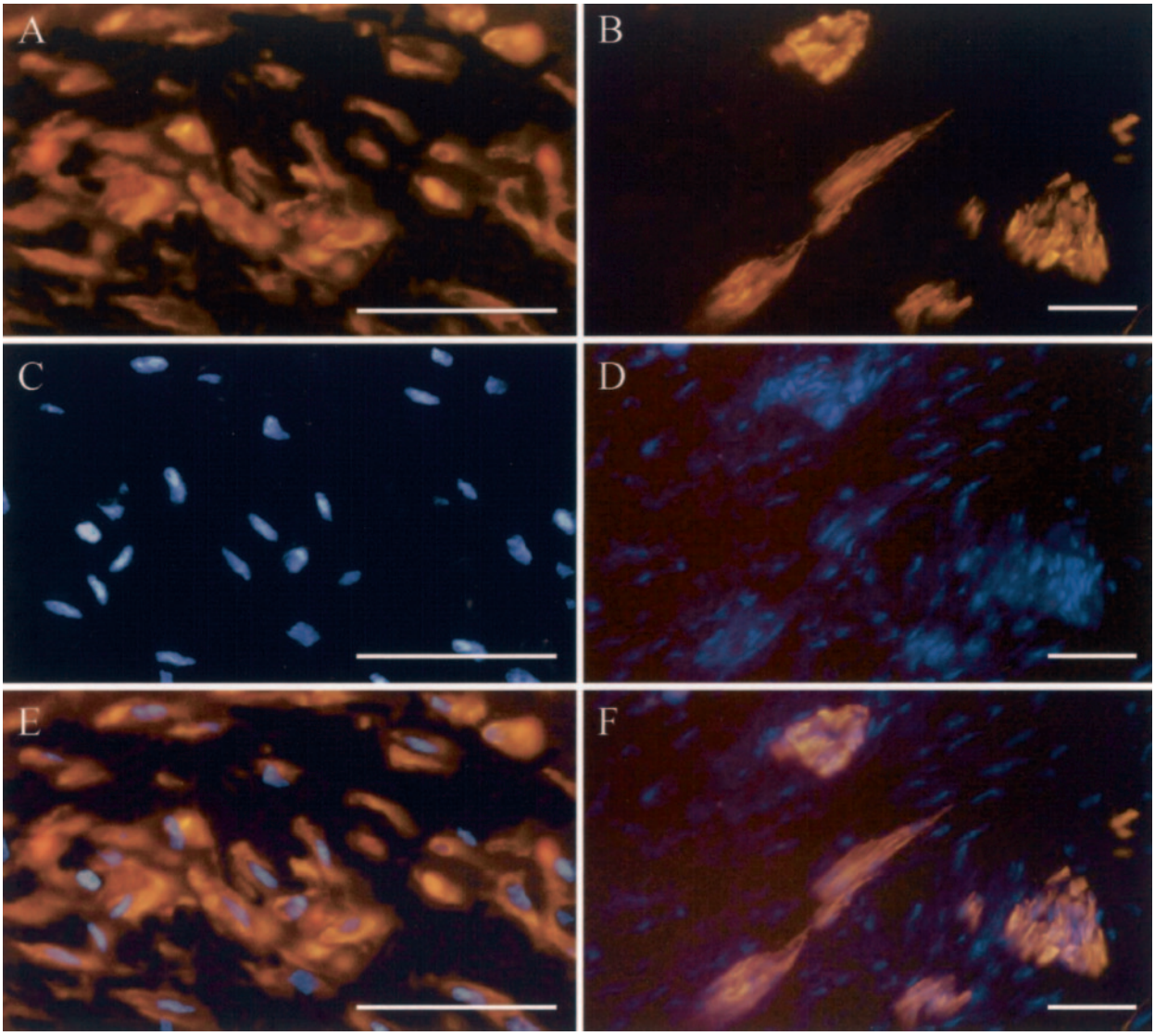

Figure 5. Sections of scar engrafted with bone marrow-derived cells showing patches of positive staining for sarcomeric actin (A) and DAPI (C) throughout the scar area (400X). E presents a merged image. B shows desmin staining, whereas D is DAPI and F is the merged image (200X). Bar represents 100 μm.

Future Studies

These data demonstrate that in the acute setting of myocardial infarction, both skeletal myoblasts and bone marrow cells can improve contraction. Additional studies are needed to examine how different cell types compare when used to repopulate chronically injured myocardium. Similarly, future efforts should be directed toward evaluating combinations of multiple cell types to regenerate vasculature, a pathway for electrical conduction and functioning muscle.

Bone marrow cells represent a very exciting addition to the pool of potential cell types for use in cell transplantation. As we gain more knowledge of their differentiation in vivo and the phenotypes they may be able to generate, it is important to remember the lessons learned from myoblast transplantation. Evaluating the electrical impact of bone marrow cells and their reproducibility as compared with myoblasts will form an important framework for the future as we move cardiomyogenesis from the bench to the bedside.

Acknowledgments

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