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EXPRESS PUBLICATIONS

Comparison of Human Skeletal Myoblasts and Bone Marrow-Derived CD133$^+$ Progenitors for the Repair of Infarcted Myocardium

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OBJECTIVES The present study was designed as a face-to-face functional comparison of human skeletal myoblasts (SMs) and CD133$^+$ bone marrow-derived hematopoietic progenitors in an animal model of semichronic myocardial infarction.

BACKGROUND Compared with SMs, bone marrow-derived cells have the advantage of plasticity and might more effectively regenerate ischemic cardiac tissue. However, few data exist on the comparative efficacy of these two cell types in semichronic infarcts.

METHODS A myocardial infarction was created by coronary ligation in 32 nude rats. Ten days later, rats received in-scar injections of human SMs, CD133$^+$ progenitors, or culture medium. Left ventricular function was assessed before and one month after transplantation by echocardiography and pressure-volume loops. Immunofluorescence, polymerase chain reaction, and in situ hybridization were used to detect cells grafted in the hearts.

RESULTS One month after transplantation, left ventricular ejection fraction decreased by 8 ± 4% in controls, whereas it increased by 7 ± 3% in CD133$^+$-grafted hearts ($p = 0.0015$ vs. controls) and further by 15 ± 5% in SM-treated hearts ($p = 0.008$ vs. controls). Systolic indices yielded by pressure-volume loops paralleled these data. Engrafted myotubes were identified in all SM-treated hearts by immunofluorescence, whereas in CD133$^+$-grafted hearts, few human cells were only detected by polymerase chain reaction.

CONCLUSIONS In the setting of postinfarction scars, the transplantation of bone marrow-derived CD133$^+$ progenitors improves cardiac function, but this benefit is not superior to that afforded by myogenic cells. (J Am Coll Cardiol 2004;44:458–63) © 2004 by the American College of Cardiology Foundation

During the past decade, a large number of experimental studies have demonstrated that intramyocardial transplantation of skeletal myoblasts (SMs) could effectively repair an ischemically damaged heart (1), thereby setting the stage for early clinical trials (2). More recently, the regeneration of infarcted myocardium also has been reported with the use of bone marrow-derived mononuclear cells (3–6), hematopoietic progenitors (7,8), or mesenchymal stem cells (9). Most of these studies, however, have entailed bone marrow transplantation at the acute stage of myocardial infarction and did not include outcome comparisons with other cell types. Therefore, the present study was designed as a face-to-face functional comparison of SM and hematopoietic progenitors in an animal model of semichronic myocardial infarction.

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METHODS

Experimental model. Ligation of the left coronary artery was performed in seven-week-old immunodeficient rnu/rnu rats (Harlan France SARL, Gannat, France) (10). Ten days later, rats underwent a sternotomy and then were randomly allocated to receive in-scar injections (150 μl) of human SMs (5×10^6, n = 8), CD133$^+$ progenitors (5×10^5, n = 15), or culture medium (n = 9). The viability of the cells after thawing, assessed by fluorescence-activated cell sorting (FACS) using propidium iodide exclusion, was consistently >87%. All experiments were performed in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by

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Abbreviations and Acronyms

FACS = fluorescence-activated cell sorting
HLA = human leukocyte antigen
LVEF = left ventricular ejection fraction
LV = left ventricular
mAb = monoclonal antibody
MyHC = myosin heavy chain
pAb = polyclonal antibody
PCR = polymerase chain reaction
SM = skeletal myoblast

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Isolation and characterization of human SM and CD133$^+$ cells. Human SMs were obtained from a biopsy of the vastus lateralis taken during orthopedic operations and cultured for three weeks (2). The percentage of myogenic cells was assessed by FACS using anti-CD56 antibodies (Becton-Dickinson, Franklin Lakes, New Jersey).

Human CD133$^+$ cells were sorted from blood obtained by leukapheresis after cytokine (granulocyte colony-forming unit) mobilization in patients suffering from hematological disease. CD133$^+$ progenitors were isolated using magnetic beads coated with antibodies against CD133 (Miltenyi Biotec, Paris, France). Purified CD133$^+$ cells were characterized using monoclonal antibodies (mAbs) against human CD34, CD117 (Becton-Dickinson), CXCR4 (BD Biosciences Pharmingen, San Diego, California), CD62E (Santa Cruz Biotechnology Inc., Santa Cruz, California), and CD54 (Caltag Laboratories, Burlingame, California). Isotype-identical antibodies served as controls. Cytometric analyses were performed using a FACS Calibur using Cellquest computer software (Becton-Dickinson). All cells were frozen until implantation.

Colony-forming unit assay. Freshly prepared and cryopreserved CD133$^+$ cells were plated in methylcellulose (Stem Cells Technologies, Meylan, France). All experiments were performed in quadruplicate and at three different cell concentrations (500, 1,000, and 2,000 CD133$^+$ cells per plate). For all the cell concentrations tested, granulocyte/monocyte colony-forming units were obtained on day 14.

Functional assessment. Immediately before and one month after transplantation, we further studied left ventricular (LV) function by using two-dimensional echocardiography (10). Left ventricular ejection fraction (LVEF) was measured by two investigators who were blinded to the experiment.

Pressure-volume loops were only generated one month after transplantation using a LV conductance catheter (Millar Instruments, Houston, Texas) and IOX software (EMKA Technologies, Paris, France). Preload-recruitable stroke work and maximal elastance were measured after temporary occlusion of the inferior vena cava under respiratory arrest (10).

Immunofluorescence. Explanted hearts were cut into three segments and frozen in liquid nitrogen-cooled isopentane. The detection of transplanted cells was primarily based on the presence of human-specific membrane human leukocyte antigen (HLA)-class I (1:20, mAb, Immunotech, Westbrook, Maine) and nuclear lamins-A/C (1:300, mAb, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) antigens. Thereafter, immunolabeling was performed with antibodies directed against neonatal myosin-heavy chain (MyHC) (1:50, rabbit polyclonal antibody [pAb], provided by G. Butler-Browne, CNRS UMR7000, Paris, France), fast-MyHC antibody (1:20, mAb, Novocastra), myosin-binding protein-C (1:200, pAb, provided by L. Carrier, Inserm U582, France), and caveolin-1α (1:50, pAb, Santa-Cruz). To quantify capillary density, nine sections per heart were stained with anticaveolin-1α antibody. Images were processed by a DMRB microscope (Leica, Postfach, Wetzlar, Germany) equipped with epifluorescence optics. Digital images were analyzed using Vision explorer software (Graftek Imaging, Austin, Texas).

Fluorescent in situ hybridization. Heart cryosections (4 μm) were fixed in methanol, followed by a pepsin digestion and formamide treatment. The slides were hybridized (2 h at 37°C) with directly fluorescence-labeled human-specific multicentromeric and rat-specific Y-chromosome deoxyribonucleic acid probes and then counterstained with 4′,6-diamidino-2-phenylindole in antifade solution (Vector Laboratories, Burlingame, California).

Polymerase chain reaction (PCR). Deoxyribonucleic acid was obtained by proteinase K digestion and phenol extraction, and PCR was performed using a human-specific Alu primer (upstream-primer-5′-ACGCCTGTAATCCCAGCACTT-3′; downstream-primer-5′TCGCCCAGGCTGGAGTGCA-3′). Amplification conditions were as follows: 95°C for 3 min followed by 30 cycles consisting of 90°C for 30 s, 58°C for 45 s, 72°C for 60 s, and followed by an extension at 72°C for 10 min. All samples also were amplified to detect the glyceraldehyde-3-phosphate dehydrogenase gene as a control for the presence of amplifiable deoxyribonucleic acid.

Statistics. Data are expressed as mean ± SEM. All analyses were performed with the StatView software (SAS Institute, Cary, North Carolina). Statistical comparison among the three groups was performed using the Kruskall-Wallis test. The Mann-Whitney *U* test with Bonferroni's correction was used for post-hoc pairwise comparisons. A value of $p < 0.0167$ was considered significant.

RESULTS

Characterization of the transplanted human cells. On the day of transplantation, >85% of cultured SMs expressed CD56 and desmin markers. The CD133$^+$ cell population (purity >97%) co-expressed CD34 (91 ± 3%), CD54 (82 ± 6%), and CXCR4 (73 ± 3%), whereas only a few co-

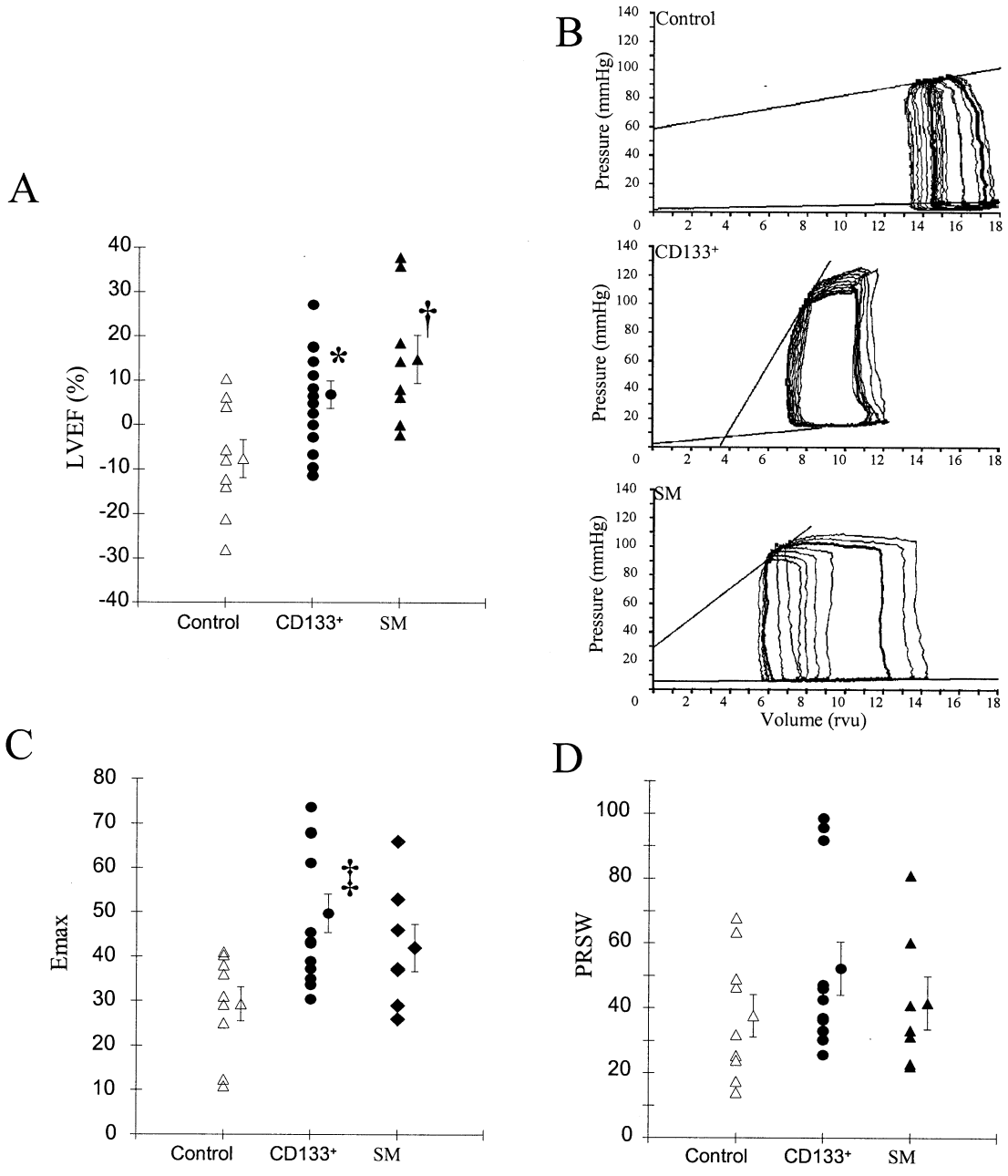

Figure 1. Functional effects of $CD133^+$ and skeletal myoblast (SM) cell transplantation. **(A)** Individual values and mean percentage of change in left ventricular ejection fraction (LVEF) from baseline pretransplantation values in controls (n = 9; **open triangles**), $CD133^+$-treated (n = 15; **closed circles**), and SM-treated (n = 8; **closed triangles**) hearts. **(B)** Representative tracings of pressure-volume occlusion loops. **(C and D)** Individual values and means of **(C)** maximal elastance (Emax) and **(D)** preload-recruitable stroke work (PRSW) in controls (n = 9), $CD133^+$-grafted (n = 12), and SM-grafted (n = 7) hearts. Note the functional improvement in >25% of $CD133^+$-grafted and SM-grafted animals.*p = 0.0015, †p = 0.008, ‡p = 0.01 vs. controls.

expressed CD117 (14 ± 3%) and CD62E (13 ± 3%) surface markers. The functional clonogenic capacities of $CD133^+$ cells were not impaired by cryopreservation because, after 14 days of plating, there was no significant difference in the number of granulocyte/monocyte colony-forming units (18 ± 1.9 and 22 ± 3.2 in cryopreserved and freshly prepared $CD133^+$ cells, respectively).

Functional assessment. Baseline LVEFs were not significantly different between controls (42 ± 3%), SM-grafted (44 ± 2%), and $CD133^+$-grafted hearts (43 ± 2%), thereby suggesting initial ischemic injuries of similar extent. One month later, transplantation of either human SM or $CD133^+$ cells resulted in a superior preservation of function as LVEF decreased from the corresponding baseline values in controls and increased in $CD133^+$-grafted and SM-grafted hearts (Fig. 1A). This effect was primarily the result of a smaller increase in end-systolic volumes from baseline values in SM-treated (0.01 ± 0.01 ml) and $CD133^+$-treated hearts (0.05 ± 0.01 ml) compared with controls (0.08 ± 0.02 ml). In contrast, LV end-diastolic volumes did

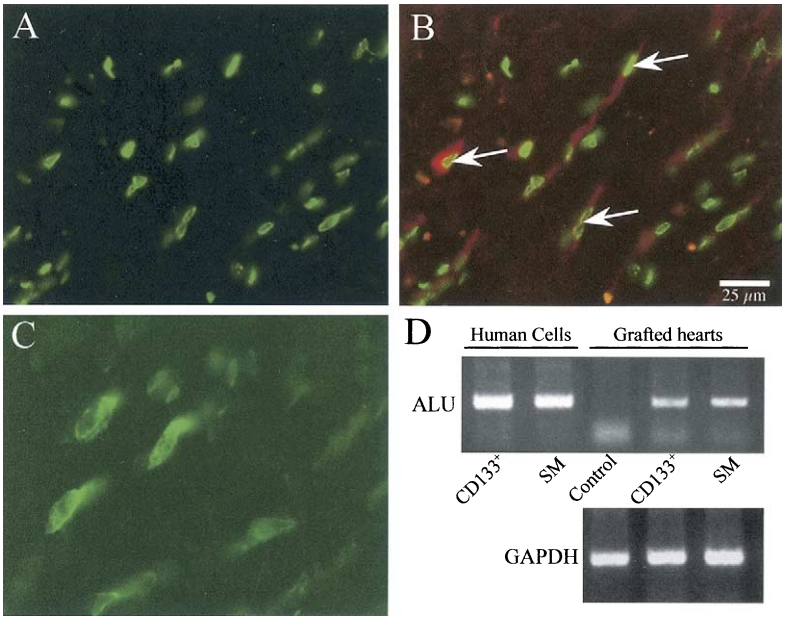

Figure 2. Detection of human cells in grafted rat hearts. One month after skeletal myoblast (SM) grafting, immunofluorescence shows that many cells were positively stained for human lamins-A/C **(A)**; some of them expressed **(B)** neonatal myosin heavy chain (MyHC) **(arrows)** and **(C)** adult-fast MyHC. **(D)** Detection via polymerase chain reaction of human cells in grafted hearts using a human-specific Alu primer.

not differ among the groups. Echocardiographic data were recorded at similar heart rates.

The improvement of systolic function in transplanted rats was further supported by pressure-volume loops, which demonstrated that maximal elastance and preload-recruitable stroke work were shifted leftward in the SM group and to a greater extent in the $CD133^+$-grafted hearts compared with controls (Figs. 1B to 1D).

Phenotypical characterization of transplanted cells. One month after transplantation, the presence of many lamins-A/C (Fig. 2A) and HLA-class I-positive human cells were identified in all SM-grafted hearts. Some of these cells expressed neonatal and adult MyHC (Figs. 2B and 2C), but none co-expressed cardiomyocyte-specific myosin-binding protein-C and endothelial cell-specific caveolin-1α. Conversely, in the $CD133^+$-grafted hearts, human cells could not be detected by either immunofluorescence or by in situ hybridization using human-specific multicentromeric probes and were only identified in 8 of 13 hearts by PCR using human-specific Alu primer (Fig. 2D).

Angiogenesis did not differ among the three groups, with an average capillary density in the border zone of 18.3 ± 1.3 μm^2, 17.8 ± 1.2 μm^2, and $17.8 \pm 0.7/100$ μm^2 in controls, SM-grafted, and $CD133^+$-grafted hearts, respectively.

DISCUSSION

The major finding of this study is that human cryopreserved $CD133^+$ progenitors and SMs both improve the preservation of postinfarction LV function compared with controls that receive culture medium alone. In contrast to SMs which, despite their functional efficacy (1) remain lineage-restricted, bone marrow-derived cells have the purported advantage of plasticity. This finding has led to early clinical trials (3–6) that were reported to be successful. However, our failure to demonstrate the benefits of mononuclear cell transplantation in infarcted sheep myocardium (11) has led us to refocus on specific subpopulations. In this setting, $CD133^+$ were selected because: 1) the immaturity of these cells should make them prone to signal-induced phenotypic changes (12); 2) most $CD133^+$ cells also express the CD34 surface marker, which has been associated with cardioprotective properties (8); and 3) $CD133^+$ cells can be sorted by a clinically approved kit. Indeed, $CD133^+$ cells already have been injected in postinfarction scars during coronary artery surgery but without conclusive evidence for their functional efficacy (13).

Although baseline LVEFs were moderately impaired because the survival of nude rats was only compatible with relatively small infarcts, treatment effects were readily apparent, with the two transplanted groups demonstrating a better and comparable preservation of LV function compared with controls. Although a robust engraftment of SM was consistently demonstrated by the co-expression of human nuclear and skeletal muscle-specific markers, immunofluorescence failed to identify human cells in the $CD133^+$ group. These cells could only be detected by PCR, and none of them expressed muscular or endothelial markers. Technical problems accounting for our negative immunofluoresence data are unlikely because: 1) similar antibodies were consistently used throughout the study and turned out to be successful for detecting engrafted myotubes; 2) even if fusion events had occurred between $CD133^+$ cells and the few residual in-scar cardiomyocytes, thereby leading to loss of staining for HLA-class I antigens,

the nuclear-bound lamins A/C should still have been detectable in these chimeric cells; and 3) additional in situ hybridization using human-specific multicentromeric probes have failed to detect polyploid cells in the $CD133^+$-grafted hearts. The reasons for the $CD133^+$ graft loss over the course of time are unclear and could involve the greater sensitivity of $CD133^+$ cells to ischemia; the low percentage of $CD34^-$ cells, a side population credited for a higher engraftment potential (14); and/or rejection due to B lymphocytes and natural killer cells still present in nude rats that could have affected $CD133^+$ more than myoblasts that lack HLA-class II antigens (15). The smaller number of injected $CD133^+$ cells compared with SM cells was primarily justified by the difference in the proliferation rate of these two cell lineages because $CD133^+$ cells have a greater potential for multiple doublings (16). Our choice of cell dosing was also dictated by clinical considerations. Using a bone marrow aspirate, Stamm et al. (13) injected 2×10^6 $CD133^+$ cells in patients undergoing concomitant bypass surgery, a value only four times higher than the dose that we have used in 1-g rat hearts. Even after cytokine mobilization and apheresis, one is able to obtain at most 200×10^6 $CD133^+$ cells (17), which is still several orders of magnitude lower than the one-billion value that can be achieved with successfully expanded SMs (2). Altogether, the dose used in this study is consistent with that which was reported in previous studies in which murine $Sca\text{-}1^+$ cells (7), which are roughly equivalent to $CD133^+$ cells, or human $CD34^+$ progenitors (8) were found to successfully engraft in infarcted areas and induce myocardial tissue repair. However, in these "positive" studies, cells were injected early after infarction and may thus have found appropriate cytokines and growth factors for enhancing their survival and committing them towards a cardiomyogenic and/or endothelial phenotype. These signals most likely wane when freshly ischemic tissue is replaced by scar tissue. As such, the present observations are consistent with the limited bone-marrow graft survival in a mouse model of doxorubicin-induced cardiomyopathy (18). They also are in line with the recent findings that the almost complete lack of detectable hematopoietic stem cells (c-kit-enriched or $c\text{-}kit^+Thy1.1loLin\text{-}Sca\text{-}1^+$) 30 days after their implantation into myocardial infracted areas did not preclude the echocardiographic demonstration of a limitation of ventricular dilatation and dysfunction in the transplanted mice (19).

The discrepancy between the robust engraftment of SMs and the scarcity of remaining progenitors suggests different mechanisms of action. In the case of SMs, a limitation of remodeling is not supported by the lack of between-group differences in diastolic function. It is therefore more likely that engrafted myoblasts provided added systolic function, either directly through direct transmembrane electrotonic currents fired by neighboring cardiomyocytes or indirectly through paracrine mechanisms (20). Protection of surrounding host cardiomyocytes by such a release of soluble factors or cell-cell interactions is most likely the predominant mechanism of operation after $CD133^+$ cell transplantation to account for the functional improvement persisting beyond the graft loss. Our failure to demonstrate between-group differences in angiogenesis does not exclude a release of other progenitor-derived cytokines or growth factors that could improve function through favorable changes in extracellular matrix remodeling, rescue of reversibly damaged host cardiomyocytes, or recruitment of a resident pool of cardiac stem cells (21). Indeed, a similar discrepancy between the loss of engrafted human umbilical vein endothelial cells over the course of time and a sustained improvement in function recently has been reported in a rat model of myocardial infarction (22) and supports the paradigm of long-lasting cell-mediated paracrine effects that could remain operative even when the original graft is no longer detectable. This hypothesis is indirectly supported by our findings that $CD133^+$ cells can still be detected when hearts are examined at an earlier time point (six days) after transplantation (data not shown).

Conclusions. The $CD133^+$ hematopoietic progenitors effectively preserve postinfarction LV function although, in the setting of fibrous scars, their functional efficacy does not seem to be greater than that of SMs, which may represent a benchmark against which alternate cell types should be tested.

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