Myogenic fusion of human bone marrow stromal cells, but not hematopoietic cells

Daqing Shi, Hans Reinecke, Charles E. Murry, and Beverly Torok-Storb

Following marrow transplantation in both patients and animals, cells containing donor nuclei have been detected in a variety of nonhematopoietic tissue. Whether this phenomenon represents transdifferentiation of marrow-derived cells, infiltration of blood cells, or cell fusion is still controversial. In muscle, where cell fusion occurs during normal myogenesis, fusion of marrow-derived cells with resident myotubes is a likely explanation. We tested 8 subpopulations of human bone marrow for their ability to fuse with mouse C2C12 myoblasts. Relatively high fusion efficiency was observed with marrow stromal cells whereas hematopoietic cells, including populations enriched for stem cells, progenitor cells, and monocytes were refractory to fusion. Mouse myotubes containing human nuclei also contained transcripts for human muscle–specific genes. Injection in vivo of human stromal cells expressing green fluorescent protein (GFP) into the regenerating tibialis anterior muscle of nonobese diabetic–severe combined immunodeficient (NOD/SCID) β2m−/− mice resulted in regenerating muscle fibers expressing GFP. These data suggest that marrow-derived cells contribute to myogenesis through fusion and that stromal cells are better fusion partners than hematopoietic cells. (Blood. 2004;104:290-294)

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Introduction

Recent reports suggest that bone marrow–derived cells can be detected in regenerated muscle tissue of mice.1-4 In addition, bone marrow transplantation in the dystrophin mutant (mdx) mouse, a model for Duchenne muscular dystrophy (DMD), resulted in the detection of donor cell–derived dystrophin in affected muscle fibers.5,6 The presence of donor cells in muscle fibers was also observed in a patient with DMD who received an allogeneic marrow transplantation at one year of age.7 However, in both the mdx mouse and the patient with DMD, the contribution of donor cells to affected muscle was very low and did not impact muscle function.7,8 Whether this low frequency event represents transdifferentiation of marrow stem cells into muscle or rare fusion events remains controversial.9-11

Resolving this controversy is critical for understanding the real potential and limitations of marrow transplantation. In addition, understanding how marrow cells contribute, even as rare events, to myogenesis is an important step toward developing strategies to increase the frequency of these events. We hypothesized that, regardless of the mechanism, bone marrow–derived cells could be therapeutically valuable if a higher frequency of donor cell contribution to myogenesis could be achieved. This concept is supported by studies in which wild-type mesangioblast cells were shown to rescue the α-sarcoglycan null dystrophic mice through myogenic fusion.12 To identify human marrow cells with myogenic fusion potential we investigated the ability of human cells to contribute to myogenesis using first a xenogeneic in vitro model combining mouse C2C12 myoblasts with various subsets of human marrow cells, and second using an in vivo regeneration model.

C2C12 cells represent an early stage of muscle differentiation. Depending on the culture conditions, they can either proliferate as myoblasts or differentiate into myotubes.13 Previous studies have shown that coculturing C2C12 cells with dermal fibroblasts resulted in cell fusion and the subsequent correction of the dysgenic membrane in regenerating muscle.14-16 The myogenic potential of neural stem cells was also demonstrated by coculture with C2C12 cells.17,18 In this report we cocultured human hematopoietic and nonhematopoietic bone marrow cells with C2C12 and found that stromal cells have a significantly greater potential to contribute to myobute formation than enriched populations of hematopoietic progenitor cells, and that this contribution is due to cell fusion. Human stromal cells also contributed to regenerating muscle in vivo in the nonobese diabetic–severe combined immunodeficient (NOD/SCID) β2m−/− mouse.

Materials and methods

Human cell preparations

Human CD34+ cells from cadaveric marrow were provided by the Cellular Therapy Laboratory, Fred Hutchinson Cancer Research Center (FHCRC). Flow sorting was used to prepare CD34+ and CD34+/CD38− populations, both more than 95% pure. A more differentiated CD33+ population was expanded from the CD34+ cells by culturing for 6 days in RPMI with 10 ng/mL interleukin 3 (IL-3), IL-6, granulocyte colony-stimulating factor...
(G-CSF), and stem cell factor (SCF). More-mature CD14+ cells were sorted from peripheral blood mononuclear cells obtained from healthy donors. Long-term marrow cultures (LTCs) and mesenchymal stem cells (MSCs) were prepared from normal donor marrow as described previously.19,20 Both the LTC and MSC populations were depleted of CD45+ cells prior to coculturing with C2C12 myoblasts. All samples of human blood and bone marrow were obtained from healthy donors after informed consent as required by the FHCRC institutional review board.

**Fusion assay**

Stromal cells (2500/cm²) or hematopoietic cells (125 000/cm²) from human marrow were obtained from healthy donors after informed consent as required by the FHCRC institutional review board. Marrow preparations were cocultured with C2C12 myoblasts (2500/cm²) for 4 days in Dulbecco modified Eagle medium (DMEM) 10% fetal bovine serum (FBS), then cultured for 2 additional days in DMEM with 10 μg/mL insulin and transferrin to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse antihuman nuclei immunoglobulin G1 (Ig) monoclonal antibody (Chemicon, Temecula, CA) and the antiseromicermyosin heavy chain IgG2b monoclonal antibody MF-20 (a gift from Dr Haushka, University of Washington). Total cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). The slides were visualized with appropriate Alexa-fluor-conjugated goat antimouse IgG1 and IgG2b secondary antibodies (Molecular Probes, Eugene, OR). Total cell nuclei were visualized by antibodies specific to human nuclei (Invitrogen, Carlsbad, CA) and human specific MYH-2 primers (forward, 5'-ctgctgaaggagagggagct-3'; reverse, 5'-tgtgattcgcgctacctt-3') and actin primers (forward, 5'-agagctacgagctgcctgacggcc-3'; reverse, 5'-agatgagctcttgctagctgctc-3'). The PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

**RT-PCR**

Total RNA was isolated from cell cultures using RNaseasy Mini kit (Qiagen, Valencia, CA) and reverse transcriptase–polymerase chain reaction (RT-PCR) were performed using superscript one-step RT-PCR system (Invitrogen, Carlsbad, CA) and human specific MYH-2 primers (forward, 5'-ctgctgaaggagagggagct-3'; reverse, 5'-tgtgattcgcgctacctt-3') and actin primers (forward, 5'-agagctacgagctgcctgacggcc-3'; reverse, 5'-agatgagctcttgctagctgctc-3'). The PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Lentiviral transduction**

Vesicular stomatitis virus glycoprotein (VSV-G)–pseudotyped HIV-derived lentiviral vectors expressing the enhanced green fluorescent protein (GFP) were provided by Dr. Hans-Peter Kem (FHCRC). Vector stocks were prepared and titered as described.21 The MSC cells were cultured overnight, with the GFP-expressing vector at a multiplicity of infection of 10. GFP-positive MSCs were sorted by FACS Vantage SE Cell Sorters (Becton Dickinson, San Jose, CA) and injected into the tibialis anterior muscle of mice.

**Cardiotoxin injury and cell injection**

NOD/SCID β2m-/- mice, 8-10 weeks of age, were anesthetized with Avertin (0.018 mL/g-0.022 mL/g body weight), followed by muscle injury to the tibialis anterior (TA) by injection of 40 μL cardiotoxin (20 μM in phosphate-buffered saline [PBS]). After 24 hours, 3 × 10^5 human MSCs were injected into the injured TA muscle. Mice were euthanized 7 days after injection by pentobarbital overdose, and the TA muscles were harvested and processed for frozen sections.

**Immunohistochemistry**

The TA muscles were frozen in OCT embedding medium and sectioned as 6-μm longitudinal sections. The sections were fixed in 4% paraformaldehyde and incubated with antihuman nuclei antibody (1:250) overnight at 4°C followed by Alexa-fluor 488 goat antimouse IgG1 secondary antibody (Molecular Probes). The myofibers were revealed by rhodamine-phalloidin staining (Molecular Probes). The images were captured from frozen sections by Nikon Eclipse E800 microscopy and followed by hematoxylin and eosin (H&E) staining of the same sections. To reconstruct a 3-dimensional image of GFP-positive regenerating TA muscle fibers, a stack of 44 one-μm sections of a frozen TA muscle were acquired as green and red 2 channel images by a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) and imported into MetaMorph software (Molecular Devices, Sunnyvale, CA) as a series of .tif files. A 3-dimensional reconstruction was created by Velocity software (Improvement, Lexington, MA) and used to generate a Quicktime movie (Apple, Cupertino, CA) showing a 180-degree horizontal rotation of the stack. This short video is available as supplemental data (Video S1; see the Supplemental Video link at the top of the online article at the Blood website).

**Results**

**Detection of human marrow cell contribution to myotubes in an in vitro myogenesis model**

In an effort to better understand the contribution of bone marrow–derived cells to muscle repair, we established a xenogeneic in vitro cell culture model in which different populations of human marrow cells were tested for their ability to fuse with mouse myotubes. In this model, human marrow–derived cells were cocultured with C2C12 for 4 days under proliferative conditions then switched to differentiation conditions for an additional 2 days. Figure 1 provides an example of how human nuclei in the myotubes were detected. Multinucleated myotubes were revealed by the presence of sarcomeric myosin heavy chain (Figure 1A). Human nuclei are unequivocally identified by staining with an antihuman nuclei
monoclonal antibody (Figure 1B), while the numerous mouse nuclei present in this field as shown by DAPI staining (Figure 1D) are negative. The contribution of human marrow cells to the myotubes was determined by the presence of human nuclei in the myotubes, which were easily distinguished from those outside the myotubes by spatial correlation of human nuclei staining with nuclear spaces revealed by MF20 staining (Figure 1A,C).

The results in Figure 1 showed that coculture of human LTC cells with the C2C12 mouse myoblasts resulted in the contribution of human nuclei to mouse myotubes. The results were further confirmed by confocal microscopy. To further identify the cells in LTCs responsible for myogenic fusion, we isolated stromal cells by sorting out CD45+ hematopoietic cells. The stromal cells showed the same fusion frequency with C2C12 as adipocytes, chondrocytes, osteocytes, and stromal cells under mouse C2C12 myoblasts.

Recently, MSCs from marrow were reported to give rise to adipocytes, chondrocytes, osteocytes, and stromal cells under different culture conditions. Given their pleiotropic potential, we tested the ability of MSCs to fuse with C2C12. Although the stromal cells generated in LTC media and MSC media are morphologically different and MSCs proliferated more than LTC cells, a similar number of fusion events were observed (Table 1). To determine whether fusion was a common feature of adherent stromal cells we tested 2 immortalized stromal cell lines derived from human LTCs. One line, designated HS-5, has small fibroblast-like cells that secrete many cytokines. The other, HS-27A, has large cells that support cobblestone area formation. Both HS-5 and HS-27A failed to fuse with myotubes (Figure 1E-F), suggesting that only specific subpopulations of marrow stromal cells have the potential to fuse.

Given both LTC and MSC populations are reported to have stem cell potential, we considered whether the C2C12 coculture conditions actually induced myogenic differentiation of these cells rather than cell fusion. We hypothesized that fusion would require cell-cell contact whereas "transdifferentiation" would not. To address this, the MSCs and C2C12 were initially cultured separately, then added together when myogenic fusion was induced. After 2 days, no human nuclei-containing myotubes were observed. In a separate set of experiments we tested whether C2C12 can induce a myogenic program in MSCs through secreted molecules. In this case MSCs and C2C12 were cultured in transwells to avoid direct cell-cell contact but allow an exchange of culture media throughout the 6 days of culture. No myosin heavy chain positive-staining cells or myotubes were observed in the MSC culture, while normal myogenesis occurred in the chamber containing the C2C12 (data not shown). Therefore, stromal contribution to myotubes requires contact with C2C12 myoblasts.

**Lack of myogenic fusion with hematopoietic cells**

We next assessed the ability of various hematopoietic cells to fuse with C2C12. We studied 4 populations of hematopoietic cells: the CD34+/CD38− population that is enriched for stem cells, the CD34+ population containing hematopoietic progenitor cells, CD33+/CD34− relatively mature myeloid progenitor cells, and CD14+ monocytes. In these experiments 250 000 cells of each type were cocultured with C2C12, and only one myogenic fusion event was detected. The studies are summarized in Table 1. The negative results raised the concern that the C2C12 culture conditions may adversely affect hematopoietic cells and thereby prevent their fusion. To address this concern we repeated the experiments using LTC media to support hematopoietic cells during the first 4 days of culture. Under these conditions myotube formation still occurred and more human cells were retained; however, there were no detectable fusion events (data not shown). Therefore, we conclude that hematopoietic cells are refractory to myogenic fusion.

**Fusion induces human myosin heavy chain expression**

The presence of human nuclei in mouse myotubes raised the question as to whether any human muscle-specific genes were transcribed. In the in vitro myogenesis model, we observed that most "hybrid myotubes" were positive for multiple human nuclei. However, the staining intensity of these nuclei was not consistent, but rather appeared as a decreasing gradient from one brightly stained nucleus. This observation suggested that the human nuclear proteins from one nucleus were transported to neighboring mouse nuclei. Transportation of proteins among nuclei in myotubes has been described previously. We hypothesized that mouse muscle-specific transcription factors may translocate in a similar fashion to human nuclei and turn on transcription of human muscle-specific genes. To measure the human muscle gene expression, total RNA was isolated from C2C12, LTCs, MSCs, and cocultures of LTCs and MSCs with C2C12. RT-PCR was performed using human-specific primers. Among several myosin heavy chain genes tested, we detected elevated myosin heavy chain IIa gene expression in the cocultures, indicating that muscle-specific genes in stromal cells can be turned on through myogenic fusion (Figure 2).

**Human stromal cells deliver GFP to the regenerating TA muscle in vivo**

Our cell culture experiments indicated that bone marrow stromal cells are capable of fusing with myotubes. To test whether the MSCs can fuse with skeletal muscle fibers undergoing repair after injury in vivo, cardiotoxin was injected into the tibialis anterior of NOD/SCID β2m−/− mice. Human MSCs were injected 24 hours later into the cardiotoxin-injured muscles. TA muscles were examined after 7 days for the presence of human nuclei in skeletal muscle fibers by immunofluorescent staining with anti-human nuclei antibody in combination with rhodamine-phalloidin staining for muscle fibers. Data indicate that 0.44% of injected human cells was incorporated into myotubes. One example of a human nucleus inside a myotube is shown in Figure 3A-C. In comparison to uniform staining of the intact human nuclei shown in Figure 1, the staining of the nucleus in Figure 3B has a stippled pattern due to the fact that this image represents a section through the nucleus.

<table>
<thead>
<tr>
<th>Human cell populations</th>
<th>Events/no. of cells tested</th>
<th>Frequency</th>
</tr>
</thead>
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<tr>
<td>Hematopoietic compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38−</td>
<td>0/45 000</td>
<td>0</td>
</tr>
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<td>CD34+</td>
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<td>.000004</td>
</tr>
<tr>
<td>CD33−</td>
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<tr>
<td>CD14+</td>
<td>0/250 000</td>
<td>0</td>
</tr>
<tr>
<td>Stromal compartment</td>
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<td></td>
</tr>
<tr>
<td>LTCs</td>
<td>145/5 000</td>
<td>.029</td>
</tr>
<tr>
<td>MSCs</td>
<td>190/5 000</td>
<td>.038</td>
</tr>
<tr>
<td>HS-5</td>
<td>0/5 000</td>
<td>0</td>
</tr>
<tr>
<td>HS-27a</td>
<td>0/5 000</td>
<td>0</td>
</tr>
<tr>
<td>Control cell</td>
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<td></td>
</tr>
<tr>
<td>HFF</td>
<td>4/5 000</td>
<td>.0008</td>
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Each myotube containing one human nucleus or more was counted as one event.
To verify the fusion of human MSCs with mouse muscle, GFP-expressing MSCs were prepared by lentiviral infection. The GFP-positive MSCs were then injected into the regenerating TA muscle. After a week, the TA muscles were examined for the presence of green fibers. No GFP signals were observed in PBS-injected control TA muscle. However, GFP-positive fibers were present in GFP-MSC injected muscle (Figure 3D). H&E staining of the same section suggested that the GFP signals originated from regenerating TA myofibers but were absent in undamaged muscles (Figure 3D–E, arrows). The presence of GFP-positive myofibers was confirmed by confocal microscopy (Figure 3F and Supplemental Video S1). In these experiments more than 100 6-μm longitudinal sections were cut per TA muscle, and one section per every 20 sections was analyzed for GFP-expressing fibers. The average number of myotubes per section was 4,396 and had on average one GFP-positive fiber. These data indicate that 0.023% of total TA muscle fibers were GFP-positive.

**Discussion**

The detection of bone marrow–derived cells in muscle as well as many other tissues has generated considerable enthusiasm for rethinking the differentiative potential of adult stem cells and their possible role for various tissue replacement therapies. Although there are multiple reports of marrow-derived cells in nonhematopoietic tissue, the significance of the findings remains unclear due to the low frequency of these events, as does an understanding of the mechanism responsible for the observation.

In bone marrow transplant recipients, donor blood cells will be present in every tissue in the body. Therefore, when searching for donor cell contribution to recipient tissue, care must be taken to control for blood-derived lymphocytes and macrophages. This is a particular issue for in vivo experiments in which tissue damage is used to trigger regeneration, resulting in recruitment of blood, ultimately marrow-derived cells, to the damaged area. Donor cell contribution to regeneration can, at least theoretically, take several forms: macrophages can scavenge dead cells and secrete factors that favor endogenous regeneration, lymphocytes may also produce beneficial factors, totipotent stem cells may differentiate into the needed tissue, or other undefined populations may fuse with endogenous regenerating cells. Distinguishing among these possibilities is critical for understanding the real potential of adult stem cells and shaping future research efforts accordingly.

In the current study, we first used differentiating mouse C2C12 myoblasts as an in vitro model of myogenesis to test the capability of different human bone marrow cells to fuse with myotubes. We found that marrow stromal cells cultured either as LTCs or MSCs, but not hematopoietic cells, fuse with C2C12 myoblasts. The fusion required cell-cell contact, and resulted in the expression of human myosin heavy chain.

We tested 4 populations of human cells in the hematopoietic lineage: CD34+CD38−, an enriched stem cell population, CD34+ enriched for stem/progenitor cells, CD33+ committed myeloid progenitor cells, and CD14+ monocytes. With the exception of one rare event, these cells were not able to fuse with C2C12 cells and contribute to myotubes. The one exception was in the CD34+ cell population which could, at least theoretically, be attributed to stromal precursors known to express CD34.26 The lack of hematopoietic fusion with C2C12 was not due to the culture conditions: experiments were also conducted using culture media that supported the survival of hematopoietic cells, yet no fusion occurred between hematopoietic cells and myotubes. Our failure to demonstrate a contribution of hematopoietic cells to myogenesis in vitro is consistent with recent publications that showed only rare hematopoietic cell contribution to myogenesis in vivo, in both the cardiotoxin injury and mdx mouse models.27,28 These reports question the efficacy of hematopoietic stem cell transplantation for the treatment of muscle diseases.

The nonhematopoietic cells in the marrow, or stromal cells, are a heterogenous population. They provide the microenvironmental (ME) niche for stem cells and their progeny. The ME can be approximated in vitro in primary LTCs established from marrow aspirates. More-recent studies have described culture conditions that allow the outgrowth of stromal cells reported to have stem cell potential. These are termed MSCs. In our C2C12 fusion study, cells from both LTC and MSC cultures were able to fuse with C2C12. In addition, an increased level of human myosin heavy chain expression in the C2C12 cocultures indicated that both LTCs and MSCs were able to initiate a myogenic program. Importantly, immortalized human stromal cell lines HS-5 and HS-27a failed to fuse with C2C12, suggesting that fusion was not necessarily promiscuous among adherent, fibroblastic cells. Although fusion of fibroblasts with myogenic cells in vitro has been reported previously,15-16 and may suggest little in the way of specificity for fusion partners, our results suggest otherwise. We tested 9 populations of human cells and found significant differences among them in terms of fusion.
frequencies (Table 1). Considering the heterogeneity of LTC and MSC cultures, further studies are needed to identify the subpopulation of cells in these cultures capable of fusion.

Taken together, these data indicate that marrow-derived cells in LTC and MSC cultures can fuse with relatively high efficiency to myoblasts in vitro. Given the ease with which LTCs or MSCs can be expanded in vitro and transduced, they appear to be an attractive source of cells for fusion in vivo. To test this, we injected GFP-labeled MSCs into damaged mouse TA muscles and showed regenerating muscle fibers expressing GFP, indicating a contribution of human cells to regenerating muscle. The frequency of fusion events was low, comparable with that reported for the detection of donor cells after stem cell transplantation. This suggests therefore that the detection of “donor”-derived cells following transplantation can be as easily attributed to fusion as to transdifferentiation. Whether such fusion events could eventually prove therapeutic is unclear. Much work is needed to establish the in vivo relevance of this observation; paramount among this work is the development of ways to deliver therapeutic cell preparations to target tissue and to increase the frequency of the fusion events. Understanding the molecular mechanisms involved in stroma/C2C12 fusion in vitro could provide insights needed to increase the fusion efficiency in vivo.

Acknowledgments

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References


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