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Mesenchymal progenitor cells derived from traumatized human muscle

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Abstract

Mesenchymal stem cells (MSCs) derived from adult tissues are an important candidate cell type for cell-based tissue engineering and regenerative medicine. Currently, clinical applications for MSCs require additional surgical procedures to harvest the autologous MSCs (i.e., from bone marrow) or commercial allogeneic alternatives. We have recently identified a population of mesenchymal progenitor cells (MPCs) in traumatized muscle tissue that has been surgically debrided from traumatic orthopaedic extremity wounds. The purpose of this study was to evaluate whether MPCs derived from traumatized muscle may provide a clinical alternative to bone-marrow MSCs by comparing their morphology, proliferation capacity, cell surface epitope profile and differentiation capacity. After digesting the muscle tissue with collagenase, the MPCs cells were enriched by a direct plating technique. The morphology and proliferation rate of the muscle-derived MPCs was similar to bone-marrow derived MSCs. Both populations expressed cell surface markers characteristic for MSCs (CD 73, CD 90 and CD105), and did not express markers typically absent on MSCs (CD14, CD34 and CD45). After 21 days in specific differentiation media, the histological staining and gene expression of the MPCs and MSCs was characteristic for differentiation into osteoblasts, chondrocytes and adipocytes but not into myoblasts. Our findings demonstrate that traumatized muscle-derived MPCs exhibit similar phenotype and resemble MSCs derived from the bone marrow. MPCs harvested from traumatized muscle tissue may be considered for applications in tissue engineering and regenerative medicine following orthopaedic trauma requiring circumferential debridement.

Keywords

Mesenchymal Stem Cells; Multipotent Progenitor Cells; Muscle Stem Cells; Heterotopic Ossification; Regenerative Medicine; Orthopaedic Trauma

1. Introduction

Mesenchymal stem cells (MSCs) have generated significant interest in the fields of regenerative medicine and tissue engineering (Chen et al., 2006; Kolf et al., 2007; Kuo and Tuan, 2003). These adult tissue derived stem cells are capable of differentiation into a

variety of cellular lineages, particularly osteoblasts, adipocytes and chondrocytes (Prockop, 1998; Pittenger et al., 1999). There are also established protocols to differentiate MSCs into myoblasts (De Bari et al., 2001), cardiomyocytes (Shim et al., 2004; Xu et al., 2004), hepatocytes (Lysy et al., 2008; Lee K-D et al., 2004) and neurons (Jori et al., 2005). Therefore, MSCs have great potential to regenerate damaged tissue and organs, such as the liver (Chamberlain J et al., 2007), heart (Wollert et al., 2004; Tomita et al., 1999), muscle (Dezawa et al., 2005) and peripheral nerves (Hu et al., 2007), and MSCs are an important component to many clinical strategies of engineering tissues such as bone (Arinzeh et al., 2003; Bruder et al., 1998) and cartilage (Caterson et al., 2001). There is also substantial evidence that systemically administered MSCs enhance local wound healing responses by producing trophic mediators at the sites of tissue regeneration (Caplan, 2007). Finally, MSCs derived from adult tissues are not burdened by the ethical concerns associated with embryonic stem cells. However, to take advantage of the regenerative benefits of MSCs, it must be possible to obtain these cells when they are needed clinically. As a result, there is a need to explore novel approaches that improve the availability of autologous MSCs.

A number of tissues that have been reported to yield MSCs when properly harvested. Most commonly, MSCs are derived from bone-marrow aspirates or from the marrow space of long bones (Tuli et al., 2003a; Kuo and Tuan, 2003). There are varying definitions of the MSC cell type, and there are no definitive cell markers for these cells. However, there are three generally agreed upon guidelines to categorize progenitor cells as MSCs (Beyer Nardi and da Silva Meirelles, 2006; Kolf et al., 2007). First, they should form colonies when initially plated on tissue culture plastic. Second, they must be capable of extended *in vitro* expansion while maintaining the potential to differentiate into osteoblasts and adipocytes. Third, they must not exhibit CD14, CD34 and CD45, which are characteristic epitope markers of hematopoietic stem cells (HSCs), and they should be positive for the markers CD73, CD90 and CD105 (Chamberlain G et al., 2007). In addition to bone marrow, cell populations have been derived from other connective tissues, such as trabecular bone (Song et al., 2005; Tuli et al., 2003b; Noth et al., 2002), periosteum (Nakahara et al., 1991; Choi et al., 2007), adipose (Gimble and Guilak, 2003; Ryden et al., 2003), synovial fluid (Jones et al., 2004; Vandenabeele et al., 2003), periodontal ligament (Jo et al., 2007; Gay et al., 2007) and umbilical cord (Baksh et al., 2007; Lee OK et al., 2004), which meet these requirements to be considered MSCs. However, the clinical significance of these tissues as a source of MSCs is limited by the invasive procedures that are required to collect the cells.

Orthopaedic traumatology is one field in particular that could benefit from a defined, readily available source of MSCs (Pountos and Giannoudis, 2005). Current treatment strategies for orthopaedic extremity trauma can be augmented by MSCs (Quarto et al., 2001; Vacanti et al., 2001), which may be obtained by performing a procedure to harvest autologous cells or from commercial allogenic alternatives. Many orthopaedic traumas routinely require circumferential debridement of the contaminated and devitalized tissue (Attinger et al., 2000), and this procedure thus generates a substantial amount of waste tissue that may be a potential source of MSCs. Although murine muscle tissue contains cells similar to murine bone marrow-derived MSCs (Qu-Petersen et al., 2002), there is limited evidence that human muscle contains a similar MSC population. Our laboratory has recently reported that the muscle debridements obtained from patients with extremity trauma also contains mesenchymal progenitor cells (MPCs) (Nesti et al., 2007). Therefore, the overall goal of this study was to assess whether MPCs derived from traumatized muscle may provide a clinical alternative to MSCs derived from standard tissues. Our specific aims were to compare (1) cell morphology, (2) proliferation capacity, (3) cell surface epitope profile, and (4) differentiation capacity of the traumatized muscle-derived MPCs to bone-marrow derived MSCs.

2. Methods

2.1 Harvesting traumatized muscle derived MPCs

Traumatically injured muscle was collected with Institutional Review Board (IRB) approval from the Walter Reed Army Medical Center (WRAMC). Patients were considered for inclusion in this study if they were exposed to orthopaedic trauma and sustained extensive soft tissue extremity wounds ($n=20$; age: 24.4 ± 5.3 years; sex: 100% male). Informed consent was obtained from each patient prior to tissue collection. These patients arrived at WRAMC approximately 3–7 days after injury and underwent serial surgical debridement and irrigation procedures every 2–3 days until their wounds were clinically acceptable for definitive orthopaedic treatment. Tissue was collected at each debridement and irrigation until definitive closure of the wounds was attained.

The tissue was dissected to excise approximately 500 μL of non-necrotic muscle that was not contaminated by granulation, adipose or fibrous tissues. The excised muscle was transferred into a Petri dish containing Dulbecco's Minimum Essential Medium (DMEM) and minced until the slurry could easily passed through the tip of a 25 mL serological pipette (Falcon, BD Biosciences, San Jose, CA). The minced muscle tissue was then transferred to a 50 mL conical tube containing DMEM and 0.5 mg/mL Collagenase Type 2 (Worthington Biochemical Corp., Lakewood, NJ), and incubated at 37°C for 2 hours with gentle agitation. At the end of the digestion, the suspension was vortexed briefly and passed through a 5 mL serological pipette to mechanically break down any tissue remnants. The digestate was strained through a 40 μm sieve into a new 50 mL conical tube and centrifuged for 5 minutes at 200g. After aspirating the supernate, the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 5 units/mL of penicillin, streptomycin and fungizone (PSF). The cell suspension was plated in a T175 tissue culture flask (Falcon) and incubated for 2 hours at 37°C , and then the cells were washed extensively with Hank's Buffered Saline Solution (HBSS) to remove any cells that did not adhere to the tissue culture plastic. The adherent cells were cultured in DMEM supplemented with 10% FBS and 3 units/mL of PSF. On each of the first three days, the cells were washed with HBSS and the medium was replaced. The cells were trypsinized and subcultured into new flasks after tightly packed colony forming units (CFUs) were observed and maintained in Growth Medium (GM: DMEM supplemented with 10% FBS and 1 unit/mL of PSF) from that point forward. Subsequent subcultures were performed when the cells were approximately 85% confluent.

2.2 Harvesting bone-marrow derived MSCs

Bone-marrow derived MSCs were harvested from femoral heads obtained from patients undergoing elective total hip replacement ($n=4$, age: 63.8 ± 8.5 , sex: 50% male) according to an IRB approved protocol and with patient consent. Using a published protocol (Caterson et al., 2002), whole bone segments were placed in a large Petri dish, and any loose marrow was generally scraped out. The remaining marrow space was washed by inserting 28G needle and perfusing with DMEM, and the resulting slurry was transferred to a 50 mL conical tube and vortexed briefly. The tissue slurry was passed through a 40 μm cell strainer into a new 50 mL conical tube and centrifuged for 5 minutes at 200g. After aspirating the supernate, the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum and 1 unit/mL of PSF, and the cell suspension was plated in T175 tissue culture flasks. The medium in the flask was changed once a week, and the cells were subcultured once tightly packed CFUs are observed. Subsequent subcultures were performed when the cells were approximately 85% confluent.

2.3 MTT Assay

For proliferation assays, cells were plated in 24-well plates at an initial density of 1,000 cells/cm². 1, 3 and 7 days after seeding, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to the medium in 4 replicate wells to a final concentration of 1 mg/mL, and the cells were incubated at 37°C for two hours. The medium was aspirated, and the cells were rinsed with HBSS and then allowed to air-dry at 4°C. The incorporated MTT reduction product was eluted with DMSO and quantified spectrophotometrically (A₅₅₀) using a Synergy HT Plate reader (Bio-Tek, Winooski, VT). The concentrations of MTT reduction product were normalized to that on day 1.

2.4 Flow Cytometry

Approximately 250,000 cells were plated in a 150 cm² cell culture flask for immunophenotyping analysis by fluorescence activated cell sorting (FACS). When the cultures were approximately 80% confluent, the cells were rinsed once with HBSS, released with 0.25% trypsin, pelleted by centrifugation, and resuspended in 1.2 mL of FACS buffer (0.1% bovine serum albumin and 0.01% sodium azide in HBSS). 100 µL of the cell suspension (approximately 100,000 cells) was aliquoted into FACS tubes, and 2 µL of phytoerythrin (PE) conjugated antibodies (approximately 0.4 µg) were added to each tube. All antibodies were mouse IgG1, κ isotype and reactive against human antigens (isotype control: clone MOPC-2I; CD14: clone M5A2; CD34: clone 563; CD45: clone HI30; CD73: clone AD2; CD90: clone 5E10; BD Biosciences; CD105: clone 6N6; Serotec, Oxford, UK). The cells were incubated in the dark at 4°C for 40 minutes, washed once in FACS buffer and resuspended in 100 µL of fresh FACS buffer. The fluorescent intensity profiles of the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) by acquiring 10,000 cells for each surface marker. Positive staining was defined by a >10-fold shift in the median fluorescence intensity histogram relative to the isotope control.

2.5 Differentiation Assays

At the third passage, the MPCs and MSCs were plated onto tissue culture plastic and cultured for up to 28 days in either (1) osteogenic induction medium (OM) containing GM supplemented with 10 mM β-glycerolphosphate (Sigma, Saint Louis, MO), 50 mg/mL ascorbic acid (Sigma), 10 nM 1,25-dihydroxyvitamin D₃ (Biomol, International, Plymouth Meeting, PA) and 0.01 mM dexamethasone (dex, Sigma) or (2) adipogenic induction medium (AM) containing GM supplemented with 0.5 mM IBMX (Arcos Organics, Geel, Belgium), 1 mM dex and 1 mg/ml insulin (Sigma). Chondrogenic differentiation was performed in pellet cultures containing 2.5×10⁵ cells per pellet and by culturing the cells in chondrogenic medium (CM) containing DMEM supplemented with 1% ITS (BD Biosciences, San Jose, CA), 10 ng/ml transforming growth factor-β₃ (TGF-β₃, R&D Systems, Minneapolis, MN), and 0.1 mM dex for 21 days. Myogenic differentiation was performed in myogenic medium consisting of α-MEM (Gibco), 5% horse serum (Hyclone, Logan UT) and SkGM differentiation supplements (Lonza, Basel, Switzerland). Primary human myoblasts obtained from Lonza were similarly cultured and analyzed as a positive control for myogenesis.

2.6 Histology and Immunohistochemistry

Cells in monolayer were fixed with buffered 4% paraformaldehyde (FD Neurotechnologies, Ellicott City, MD). To assay for osteogenesis, the cells were stained using a kit for alkaline phosphatase activity (Sigma) and alizarin red (Rowley Biochemical Institute, Danvers, MA) to detect mineralized matrix. To assay for adipogenesis, the cells were stained with oil red O (Sigma) for evidence of lipid droplets. Chondrogenic pellets were fixed with paraformaldehyde, dehydrated, paraffin-embedded and sectioned at 5 µm thickness.

Histological sections were stained with alcian blue (Rowley) for presence of sulfated glycosaminoglycans.

Aggrecan and collagen type II in the chondrogenic pellets were detected by immunohistochemistry. The sections were pre-incubated with hydrogen peroxide to quench endogenous enzyme activity, and then pre-digested with 300 U/mL of hyaluronidase (Sigma) or 20 mg/mL of Proteinase K (Roshe Diagnostics, Indianapolis, IN) for 30 min at 37°C for the detection of collagen type II or aggrecan, respectively. The sections were incubated overnight at room temperature in Tris-buffered saline containing 0.1% bovine serum albumin and antibodies raised against either collagen type II or aggrecan (Developmental Studies Hybridoma Bank, Iowa City, IA). Immunostaining was detected histochemically using the streptavidin-peroxidase Histostain SP Kit for DAB (Zymed Laboratories, San Francisco, CA), and imaged with a color CCD camera and an inverted microscope.

2.7 Reverse Transcription-Polymerase Chain Reaction

The differentiated cells were lysed in TRIzol (Invitrogen, Carlsbad, CA), homogenized using QIAshredder Columns (Qiagen, Hilden, Germany) and the RNA was extracted in the TRIzol according to the manufacturer's protocol. The RNA preparations were cleaned up using RNeasy Mini columns (Qiagen), and RNA concentrations were estimated on the basis of A₂₆₀. The RNA samples were reverse transcribed with the SuperScript III System for qRT-PCR (Invitrogen) using Oligo-dT and random hexamers. The expression levels of osteogenic, adipogenic, chondrogenic genes were assayed by conventional PCR using Platinum Taq (Invitrogen) following the manufacturer's protocol (See Table 1 for gene primers, annealing temperatures, PCR cycles and product sizes). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a control for RNA loading of samples. The PCR products were visualized electrophoretically using the DNA 1000 Labchip Series II on a Bioanalyzer (Agilent, Santa Clara, CA). Gene expression analysis was performed on cells from three patients for each cell type, and the results shown are characteristic of all three populations.

2.8 Statistical Methods

Quantifiable data comparing the traumatized muscle-derived MPCs and bone marrow-derived MSCs were analyzed using Student's t-tests. Statistical significance was assigned to $p < 0.05$.

3. Results

3.1 Morphology and proliferation capacity of MPCs derived from traumatized muscle

Twenty traumatized muscle samples were obtained from 16 patients (four patients had injuries on two extremities and a specimen was obtained from each injury). After processing the traumatized muscle tissue, an average of 148 million cells was harvested per gram of tissue. Approximately 2% of the cells were adherent within two hours, and the non-adherent cells were washed away. On the day after plating, three of the cultures were found contaminated and discarded. MPCs from the remaining 17 samples formed nearly confluent colonies within 2–3 weeks. The morphology of the isolated MPCs was long, spindle shaped, resembling that of the isolated MSCs (Figure 1A–D), and they maintained this morphology as they were expanded *in vitro*. Based on the MTT assay (Figure 1E), the traumatized muscle-derived MPCs proliferated at approximately the same rate as the MSCs.

3.2 Surface epitope profile of traumatized muscle- derived MPCs

There were also similarities in the cell surface epitope profile between traumatized muscle-derived MPCs and bone marrow-derived MSCs (Figure 2). The MPCs were positive for surface markers that are characteristic of MSCs (CD44, CD49e, CD73, CD90 and CD105), and negative for the markers characteristic of HSCs (CD14, CD31, CD34 and CD45). For each antibody, there was no significant difference in the normalized fluorescence intensity between MPCs and bone marrow-derived MSCs (t-test, n=3).

3.3 Differentiation of MPCs into multiple cell lineages

MPCs derived from traumatized muscle demonstrated evidence of differentiation into osteoblasts and adipocytes. In osteogenic medium, MPCs exhibited increased ALP activity compared to MPCs cultured in growth medium (Figures 3A and B) and produced a mineralized matrix that could be observed by staining with alizarin red (Figure 3D and E). Up-regulated expression of core binding factor $\alpha 1$ (CBFA1/RUNX2), a master regulator of osteogenesis, as well as the osteoblastic genes, ALP and osteocalcin, was also observed (Figure 3G). Compared to cells cultured in growth media, MPCs cultured in adipogenic medium exhibited characteristic intracellular lipid droplets that could be stained using oil red O (Figure 4A and B). These cells also up-regulated the expression of PPARG2, a master regulator of adipogenesis, as well as lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4), which are adipocyte-specific genes (Figure 4D). Differentiation of MPCs occurred in a manner similar to that observed for bone-marrow derived MSCs, although the latter showed baseline expression osteoblast specific genes under growth conditions (Figure 3G).

MPC pellets cultured under chondrogenic conditions morphologically resembled MSC pellets cultured under identical conditions (Figure 5A and D). Both pellets were of similar size, with a shiny exterior surface characteristic of cartilage nodules. Alcian blue staining indicated the presence of a sulfated proteoglycans-rich extracellular matrix in both types of pellets (Figure 5B, C, E and F), although chondrocytes differentiated from MPCs appeared to be somewhat more elongated than those derived from MSCs, which were more spherical. In pellets made from both cell types, less intense staining was seen in regions near the center, where nutrient diffusion could have been limiting because of the size of the pellet (Cooper et al., 2007). Immunohistochemistry showed that both cell types produced collagen type II (Figure 6G and I) and aggrecan (Figure 5H and J), although the muscle-derived MPCs did not appear to generate as much collagen type II as the bone marrow derived MSCs. In response to chondrogenic induction, both cell types up-regulated the expression of SOX9, a master regulator of chondrogenesis, and the chondrocyte specific genes collagen type II (COL2A1), aggrecan (AGC), COMP and collagen type X (COL10A1). The expression of these chondrocyte-associated genes was greater for both cell types in the mature pellet at day 21 compared to day 7 (Figure 5K).

Under myogenic culture conditions, neither MPCs derived from traumatized muscle nor bone marrow-derived MSCs appeared to undergo significant differentiation into myoblasts (Figure 6). The MPCs in the myogenic medium did not form the multinucleated myotubes that could be observed in the primary myoblasts under similar conditions (Figure 6B and C). Furthermore, the myoblast specific genes, MYOD and myosin heavy chain (myosin HC), were only slightly up-regulated in the MPCs compared to the high levels observed in primary myoblasts, and were not expressed in the bone-marrow derived MSCs (Figure 6D).

4. Discussion

The main objective of this study was to assess whether MPCs derived from traumatized human muscle may offer a clinical alternative to MSCs derived from standard tissues, such as bone marrow or adipose. The traumatized muscle-derived MPCs were harvested from debrided tissues (i.e., the surgical waste material) following traumatic orthopaedic injury, and they were enriched by allowing the cells to adhere to tissue culture plastic for two hours and then rinsing away the contaminating cell types. The readily adherent cells exhibited similar morphology and proliferation characteristics as bone marrow derived MSCs. They were negative for the HSC markers, CD14, CD34 and CD45, and positive for CD73, CD90, CD105, thus presenting a surface epitope profile that is characteristic of MSCs (Chamberlain G et al., 2007). Furthermore, there were no significant differences in the measured fluorescence intensities of the positive surface markers, which indicate that they were likely to be present on the cell surface at similar concentrations. Finally, the MPCs readily exhibited evidence of differentiation into osteoblasts, adipocytes and chondrocytes under the appropriate induction conditions, but they did not undergo myogenesis under culture conditions that were sufficient for terminal differentiation of myoblasts. These results suggest that traumatized muscle-derived MPCs are also functionally similar to the bone marrow-derived MSCs.

That muscle-derived MPCs and bone marrow MSCs are similar is supported by a number of observations made in this study. First, the morphology, surface epitope profile and multipotentiality of the traumatized muscle-derived MPCs were evaluated against a well-characterized and extensively studied population of MSCs (Prockop, 1998; Pittenger et al., 1999; Bruder et al., 1998; Tuli et al., 2003a; Kuo and Tuan, 2003; Caterson et al., 2002; Kolf et al., 2007). Second, the surface epitope profile was determined using flow cytometry, so quantitative data could be collected on the relative fluorescent intensity of cells stained with antibodies for each marker. In addition to determining whether the cells were positive or negative for characteristic surface markers, flow cytometry permits the comparison of the surface concentration of these markers that were present on the MPCs and MSC. Third, the differentiation capacity of the cells was assayed on the basis of multiple phenotypic characteristics, including histology and the expression of at least two lineage-specific genes. As a result, lineage-specific differentiation could be verified for the comparison of the differentiation capacity between MPCs and MSCs. Finally, the myogenic differentiation potential of these cell types was also assayed, and the negative results strongly suggest that the traumatized muscle-derived MPCs were unlikely to be muscle stem cells, such as satellite cells, or derived from de-differentiated primary myoblasts that exhibit MSC characteristics.

Despite the strength of these observations, there are a few caveats that must be applied to our findings. First, all of the differentiation assays in this study were performed after the third passage, or approximately 10–12 population doublings. Although the traumatized muscle-derived MPCs were capable of differentiating into osteoblasts, adipocytes and chondrocytes after being expanded *in vitro*, we have not necessarily demonstrated “robust *in vitro* expansion,” which usually implies that differentiation can still occur after 15 or more population doublings (Bruder et al., 1998; Colter et al., 2000). Although not reported here, we have differentiated the MPCs into osteoblasts and adipocytes at later passages, and as late as passage 8, but we have not yet performed a rigorous study to verify their differentiation potential at these higher passages. Second, the surface markers in this study that defined the surface epitope profile of the MPCs met the minimum requirement for characterizing an MSC phenotype. However, a broader set of markers could more conclusively determine whether these MPCs are indeed identical to bone marrow-derived MSCs, and whether there are differences between the two cell types. Finally, in this study,

we only investigated the *in vitro* behavior of the traumatized muscle-derived MPCs. From these results, it is impossible to predict whether the cells will maintain their MSC properties and differentiation abilities after they are implanted in an *in vivo* environment, and this question must be addressed in future investigations.

The effect of trauma on the MPCs derived from the traumatized muscle is another variable that was not addressed in this study. These MPCs were harvested from muscle detriments within days of a traumatic orthopaedic injury that involved a substantial amount of soft tissue damage, and they were exposed to inflammation and wound healing factors. It is unknown what effects these trauma-induced mediators may have had on the progenitor cell population within the injured muscle tissue. One area of current investigation in our laboratory is to identify whether untraumatized muscle also contains a population of MPCs, and whether the untraumatized muscle-derived MPCs have the same similarities to bone-marrow derived MSCs. Other investigators have identified populations of muscle-derived multipotent stem cells in murine muscle (Qu-Petersen et al., 2002), as well as multipotent stem cells in human muscle that can be isolated with immuno-selective techniques (Zheng et al., 2007; Lecourt et al., 2007). There are currently no reports of plastic-adherent multipotent cells derived from healthy human muscle tissue. The identification of a multipotent progenitor cell population in traumatized muscle tissue reported here raises the possibility that these MPCs could have been recruited to the site of injury and may be involved in the process of tissue repair. Therefore, the biological properties of the traumatized muscle-derived MPCs may reflect *in vitro* modeling of the natural healing response as a stem cell population that has been recruited from its niche to participate in tissue regeneration. This model may permit a systematic analysis of the functional roles of specific biochemical factors in the wound healing environment during the reparative process.

One of the most striking differences between the traumatized muscle-derived MPCs and bone-marrow derived MSCs is that the latter express the osteogenic genes CBFA1 and osteocalcin under growth medium conditions. This result is consistent with previous observations that suggest a subset of cells predisposed towards osteogenic differentiation is present in the population of bone marrow-derived MSCs (Muraglia et al., 2000; Hicok et al., 1998; Minguell et al., 2001). Although the MPCs are capable of differentiating into osteoblasts, osteoblastic gene expression is not seen unless the cultures are induced with osteogenic medium. This difference between the two cell types may be clinically advantageous for the traumatized muscle-derived MPCs, since it implies that they remain in a more plastic, undifferentiated state while being expanded *in vitro*, and that they do not demonstrate an inclination towards differentiation along a particular cell lineage.

The identification of a cell type in traumatized muscle capable of osteogenic differentiation is relevant to the study of heterotopic ossification (HO). HO is characterized by pathological bone formation in the soft tissues (Kaplan et al., 2004), and it occurs at higher frequencies in patients that have sustained severe orthopedic trauma (Potter et al., 2007; Pape et al., 2004). Although the mechanism of traumatic HO is not precisely known, it is generally accepted that progenitor cells within the muscle become dysregulated by conflicting wound healing responses and initiate osteogenesis (Kaplan et al., 2004). The results of this study suggest that the traumatized muscle-derived MPCs may be the putative osteoprogenitors responsible for HO and could be used in future investigations to better understand the mechanisms that lead to ectopic bone formation.

In conclusion, this study has demonstrated that MPCs derived from traumatized muscle have many important similarities to bone-marrow derived MSCs. Based on the results of this study, the MPCs could provide a clinically significant source of MSCs that are readily available using surgical waste as a tissue source and do not require any additional surgical

procedures. Furthermore, if the MPCs are actively involved in the reparative processes at the time of harvest, they may provide a clinical advantage over other populations of quiescent stem cells. In future studies, we will investigate whether these cells can maintain their multipotentiality *in vivo*, and whether they can be used in tissue engineering and regenerative medicine applications.

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Figure 1.

Morphology of MPCs derived from traumatized muscle (A and C) and MSCs derived from bone marrow (B and D). The MPCs resembled the long, spindle shaped MSCs when they are isolated (A and B) and when they were near confluent (C and D). Scale bar = 25 μ m. (E) The proliferation of muscle-derived MPCs and bone marrow-derived MSCs measured using the MTT reduction assay. The amount of MTT reduction product was not significantly different between the MPCs and MSCs on day 3 or 7, suggesting that the proliferation rates of the two cell types were the same. Data from cells derived from three different patients (mean $\pm$ SD).

Figure 2.

Flow cytometric analysis of surface epitope profiles of traumatized muscle-derived MPCs and bone marrow-derived MSCs. (A) The traumatized muscle-derived MPCs were positive for CD73, CD90 and CD105 and negative for CD14, CD34 and CD45. The black lines represent the fluorescent intensity of cells stained with the indicated antibodies and the gray lines represent the negative control cells, which were stained with a non-immunoreactive isotype control antibody. (B) There were no significant differences in the normalized fluorescence intensity of MPCs or bone marrow derived MSCs that were stained with each antibody. The fluorescence intensities were normalized to the negative control cells. Data from cells derived from three different patients (mean $\pm$ SD).

Figure 3.

Osteogenic differentiation of MPCs derived from traumatized muscle. MPCs cultured in osteogenic medium exhibited increased ALP activity (B) compared to MPCs cultured in growth medium (A) and produced a mineralized matrix stained with alizarin red (E). MPCs cultured in growth medium exhibited little alizarin red staining (D). Positive controls: bone marrow-derived MSCs cultured in osteogenic medium - ALP activity (C) and alizarin red staining (F). Scale bar = 5 mm. (G) MPCs cultured in osteogenic medium (Medium: O) up-regulated the expression of osteoblast-specific genes (CBFA1/RUNX2, ALP: alkaline phosphatase, and osteocalcin) compared to cells cultured in growth medium (Medium: G). This gene expression profile was similar to that of bone marrow-derived MSCs, although the expression of the osteoblast specific genes was also seen under growth conditions in the MSCs.

Figure 4. Adipogenic differentiation of MPCs derived from traumatized muscle. MPCs derived from traumatized muscle and cultured in adipogenic medium formed intracellular lipid droplets that were stained using oil red O (B). MPCs cultured in growth medium did not produce any oil droplets (A). (C) Positive control: bone marrow-derived MSCs cultured in adipogenic medium with oil red O staining. Scale bar = 50 μ m. (D) MPCs cultured in adipogenic medium (Medium: A) up-regulated the expression of adipocyte-specific genes (PPARG2, LPL - lipoprotein lipase, and FABP4 - fatty acid binding protein 4) compared to cells cultured in growth medium (Medium: G). This gene expression profile was characteristic of bone marrow-derived MSCs.

Figure 5.

MPCs derived from traumatized muscle and cultured under chondrogenic pellet conditions (A, B, C, G and H) compared to bone marrow-derived cells cultured under identical conditions (D, E, F, I and J). The gross morphology of the chondrogenic pellets was similar for both cell types (A and D; scale bar = 500 μ m). Alcin blue stained sections of the pellets were imaged at 4x (B and E; scale bar = 250 μ m) and 10x (C and F; scale bar = 200 μ m) showing the presence of a sulfated proteoglycans-rich extracellular matrix. Pellets of both cell types stained positively for collagen type II (G and I) and aggrecan (H and J; scale bar = 250 μ m). (K) The expression of chondrogenic genes (SOX9, COL2A1, aggrecan - AGC, COMP and COL10A1) at day 7 and 21 in traumatized muscle-derived MPCs cultured under

chondrogenic conditions was characteristic of bone marrow-derived MSCs undergoing chondrogenic differentiation.

Figure 6.

Myogenic differentiation of MPCs derived from traumatized muscle. MPCs derived from traumatized muscle and cultured in myogenic medium (B) became less spindle shaped and more tightly packed compared to MPCs cultured in growth medium (A), but they did not fuse to form multinucleated myotubes, as is characteristic of primary myoblasts cultured under identical conditions (C). Scale bar = 50 μm. (D) Low levels of myoblast specific genes were detected in traumatized muscle-derived MPCs (MYOD and myosin heavy chain) when cultured in either growth medium (Medium: G) or myogenic medium (Medium: M). These genes were not detected in bone marrow-derived MSCs but were expressed at high levels in primary myoblasts cultured in myogenic medium (1° MB).

Table 1

Primer sequences, annealing temperatures, cycle numbers and product sizes for the primers used in the RT-PCR analysis.

Gene	Primer Sequence (5' → 3')	T _A [°C]	Cycles	Product Size [bp]
GAPDH	CAAGGCTGAGAACGGGAAGCAGGGGGCAGAGATGATGACC	58	26	194
COL1A2	GGCTCCTGCTCCTCTTAGCGCATGGTACCTGAGGCCGTTT	58	26	132
CBFA1/RUNX2	ACTGGGCCCTTTTTCAGAGCGGAAGCATTCTGGAA	53	26	317
ALP	TCAGAAGCTCAACACCAACGGTCAGGGACCTGGGCATT	55	26	199
Osteocalcin	GCCTTTGTGTCCAAGCGGACCCACATCCATAG	52	28	315
PPARG2	TGAATGTGAAGCCCATTGAACTGCAGTAGCTGCACGTGTT	53	26	161
LPL	GAGATTTCTCTGTATGGCACCTGCAAATGAGACACTTTCTC	52	26	276
FABP4	TGGGCCAGGAATTTGACGAAGTTCAACGTCCCTTGGCTTATGCT	59	24	215
SOX9	ACATCTCCCCAACGCCATCTCGCTTCAGGTCAGCCTTGC	58	34	173
COL2A1	GGAAACTTTGCTGCCCAGATGTCACCAGGTTACCAGGATTGC	58	34	167
AGC	TGCGGGTCAACAGTGCCTATCCACGATGCCTTTCACCACGAC	58	36	182
COMP	TGTCCCCAGAAGAGCAACCCATTGTCTGTCGTCGTCGTCGC	57	22	195
COL10A1	CCAGGAAAACCAGGTCTCGCAGCCTCTCCATTGTGTCCG	56	26	149