COMPARATIVE STUDY OF THE DIFFERENTIATION POTENTIAL OF RAT BONE MARROW MESENCHYMAL STEM CELLS AND RAT MUSCLE-DERIVED STEM CELLS

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Abstract - We present a comparative study of the plasticity of rat bone marrow mesenchymal stem cells (MSCs) and rat muscle-derived stem cells (MDSCs). The study was performed on two cell populations that were isolated by aspiration from the femur bone marrow and gastrocnemius muscle biopsy of 6-week-old albino rats. Both cell populations were exposed to identical stimulation conditions. The cells were capable of undergoing osteogenic, chondrogenic, adipogenic and epithelial differentiation, as shown by histochemistry and immunostaining techniques. The MDSC population showed behavior and characteristics similar to the bone marrow MSC population; however, the osteogenic and adipogenic potential was more reduced compared to MSCs. Our results indicate a positive expression of E cadherin and Cytokeratin 10 after 28 days under epithelial stimulation, suggesting a potential use for gastrocnemius muscle MDSCs as a promising source for regenerative therapies, including re-epithelialization and skin regeneration.

Key words: Multi-lineage potential, epithelial, regenerative therapies

INTRODUCTION

The mature functional skeletal muscle cell, the multinucleated myofiber, is surrounded by satellite cells, which lie outside the sarcolemma, within the basal lamina. Satellite cells, first described by Maurer (1961) based on their location and morphology, are referred to by many as muscle stem cells (Hill, 2003). The satellite cells are myogenic precursors that are capable of regenerating skeletal muscle and demonstrate self-renewal properties, but are still considered to be committed to the myogenic lineage (Yablonka-Reuveni, 2011). Muscle-derived stem cells (MDSCs) may represent a predecessor of satellite cells (Fano et al., 2004) and are not considered to be restricted to myogenic lineage (Wu et al., 2010). MDSCs are believed to be a unique population of cells capable of in vitro hematopoietic activity, unlike satellite cells that are unable to undergo hematopoiesis (Asakura et al., 2002). MDSC origin is still unclear, but it is thought that these cells reside within the muscle and exhibit stem cell characteristics that allow them to differentiate into myogenic, osteogenic, chondrogenic cell lineages and even into tissues of other organs, such as the hematopoietic system (Fano et al., 2004, Montarras et al., 2005). Since the discovery that satellite cells from a normal donor could restore dystrophin expression within the dystrophic host muscle (Deasy et al., 2004), skeletal muscle biology and development have been intensively studied (Jankowski et al., 2002). Studies aiming to detail the MDSCs' po-
tential use in cellular therapies have been undertaken for skeletal and cardiac tissue repair (Montarras et al., 2005), bone and articular cartilage (Bueno et al., 2009), liver and hematopoietic regeneration (Bellayr et al., 2010).

In this paper we focused on comparing the multilineage potential of rat MDSCs and bone marrow MSCs. Differentiation of both cell populations was induced towards osteocytes, adipocytes, chondrocytes and epithelial lineage followed by histochemical, immunohistochemical and PCR expression analyses. MDSCs showed a similar differentiation potential to bone marrow MSCs, including towards epithelial lineage. The multilineage potential of MDSCs indicates these myogenic precursors as suitable candidates for regenerative therapies, including for skin re-epithelialization.

MATERIALS AND METHODS

Animals

MSCs were isolated by aspiration from femur bone marrow and MDSCs were isolated by gastrocnemius muscle biopsy of 6-week-old albino rats under anesthesia. The anesthetic procedure used an induction with 5 mg/kg body weight Ketamine, while maintenance was done by Sevoflurane and tracheal intubation (peripheral catheter 14G). Animal housing and manipulation were done according to international animal welfare legislation.

Cell cultures

Cells were isolated by their capacity to adhere to the plastic surface of culture flasks and were grown in a 1:1 mixture of Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F-12 Nutrient Mixture (Sigma-Aldrich Company, Ayrshire, UK), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% penicillin/streptomycin (Pen/Strep, 10,000 IU/ml; PromoCell, Heidelberg, Germany). The medium was restored twice a week and subcultures were done before cells reached confluence. Cells were analyzed for MSC and MDSC potential to differentiate to osteocytes, adipocytes and chondrocytes, i.e. three classic lineages known to be specific for MSC (Quiroz et al., 2008). After immunohistochemical evidence of MSC and MDSC differentiation towards all three lineages, the cell populations were induced to differentiate towards epithelial lineage in order to evaluate their potential utility for re-epithelialization and skin regeneration. The induced differentiation conditions were identical for MSCs and MDSCs.

Differentiation towards osteogenic lineage

For MSC/MDSC induction towards the osteoblastic lineage, the cells were cultured at a density of 15,000 cells/cm² on Lab-Tek Chamber Slides (Nalgene Nunc International, New York, USA). The differentiation medium, OsteoDiff Medium (Miltenyi Biotec, Bergisch Gladbach, Germany), was supplemented with 1% Pen/Strep solution (PromoCell, Heidelberg, Germany). Osteoblast morphological characteristics were evaluated in culture after 10 days.

Potential to form a mineralized matrix (von Kossa staining)

The mineralization of the bone matrix was analyzed by histochemical von Kossa staining (Murshed et al., 2005). The technique allows detection of calcium phosphate deposits. The cultures were rinsed with cold (4°C) PBS (phosphate buffer saline) and fixed with 4% formaldehyde (4°C, 10 min). After washing with cold distilled water, the fixed cultures were covered with 5% silver nitrate solution and kept for 30 min in a dark room. Next, the monolayer cultures were rinsed and covered with distilled water and exposed to ultraviolet light for 1 h. The cultures were treated for 2 min with a 5% sodium thiosulfate solution, while for the nuclear counterstaining a hematoxylin solution (Hematoxylin, Mayer’s Lillie’s Modification, Dako, Carpenteria, USA) was used. The calcium phosphate deposits were stained black by the von Kossa technique. Microscopy analysis was performed on a Nikon Eclipse E800 microscope.
Amelogenin extracellular matrix localization

Immunohistochemical analysis of amelogenin presence was performed using an amelogenin antibody (Santa Cruz Biotechnology, INC, Santa Cruz, USA). The cultures were rinsed with cold (4°C) PBS and fixed with cold methanol (-20°C, 10 min). The primary antibody dilution was 1:50. The staining protocol was preformed according to the manufacturer’s instructions. Staining protocol included secondary biotinylated anti-rabbit antibody, the addition of streptavidin peroxidase, followed by the addition of substrate (DAB) and hematoxylin counterstaining of the nuclei (LSAB2 System-HRP, Dako, Carpenteria, USA).

Differentiation towards chondrogenic lineage – Alcian blue staining

After 24 days cultivation in Chondro Diff (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) media, the cultures on the Nunc Permanox chamber slides were rinsed with cold (4°C) PBS and fixed with 4% formaldehyde (4°C, 10 min). The cells were treated with hematoxylin solution for 3 min. After washing with tap water, the fixed cultures were covered with a mixture of 1% Alcian Blue solution in 3% acetic acid for 20 min, followed by washing with tap water. Finally, the cultures were treated with 50% ethanol for 10 s and mounted.

Differentiation towards adipogenic lineage – Oil Red staining

Oil Red O staining is an assay performed to stain induced adipogenic cultures and it can reveal mature adipocytes. After 21 days of adipogenic stimulation using Adipo Diff (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) ready-to-use media, the MDSCs and MSCs from the monolayer cultures were rinsed with PBS and fixed in 10% formalin for 30 min at room temperature. After washing with sterile water, the fixed cultures were treated with 60% isopropanol for 2 min and then incubated with freshly prepared Oil Red O working solution for 5-10 min to stain lipid vacuoles. After washing with tap water, the chamber-slides were mounted.

E-cadherin is a calcium-dependent adhesion molecule, required for epithelial histogenesis, tissue stability and differentiation functions (Gopalakrishnan et al., 2003). E-cadherin acts as key regulator of epithelial phenotype (Puhr et al., 2012). Cytokeratin 10 (CK 10) is considered a specific marker of terminal differentiation of the keratinocytes, as it is frequently found in stratified keratinized epithelia (Jacques et al., 2009). After 28 days of cultivation in DMEM/F12 (1:1) media supplemented with FGF (Fibroblast Growth Factor), EGF (Epidermal Growth Factor) and KGF (Keratinocyte Growth Factor), the cells were rinsed with cold (4°C) PBS and fixed with cold methanol (-20°C, 10 min). The staining was performed using E cadherin and cytokeratin 10 antibodies (Santa Cruz Biotechnology, INC, Santa Cruz, USA) according to the manufacturer’s instructions. The primary antibody dilution was 1:50.

RNA extraction and PCR analysis

After 28 days cultivation of MDSCs and bone marrow MSCs under epithelial differentiation specific conditions, total RNA extraction was performed. For RNA extraction a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, USA) was used and RNA concentration was measured on a Nanodrop ND-1000 (Wilmington, DE, USA) spectrophotometer. The PCR analysis was set up to confirm the presence of E-cadherin, an epithelial positive marker. For E-cadherin detection, a primer pair with the following sequence: 5’CGTCCCCGTCCAGCCAATCC3’/3’TGGTGCCACACAGGAACGACTC5’ was used which amplifies a 281bp product. Primer design was performed using NCBI Blast primer design tool.

RESULTS

Differentiation towards osteogenic lineage

Under osteogenic induction by OsteoDiff Medium, the MSCs showed strong matrix mineralization ca-
capacity both by von Kossa staining and amelogenin expression compared with the MDSCs (Fig. 1). Based on these observations, after 10 days of osteogenic stimulation it was obvious that the mineralization
Fig. 3 Analysis of adipogenic-lineage marker of bone marrow MSCs (a) and MDSCs (b) at 21 days under induced adipogenic stimulation using Adipo Diff Medium indicated the presence of red oil droplets within the cell cytoplasm, positive markers of adipogenic cell differentiation. The adipogenic process was more intense in bone marrow MSCs compared to MDSCs; magnification 200x.

Fig. 4 Changes in the morphological aspect of rat bone marrow MSCs and MDSCs. Bright field images were taken on day 1 (a, b); magnification 100x, and day 20 (c, d); magnification 200x, under epithelial stimulation, and indicate the acquisition of epithelial phenotype by both cell populations.
process was faster in differentiating MSCs; however, it also occurred, although at more reduced rate, in the MDSCs.

**Differentiation towards chondrogenic lineage**

After 20 days of growth in the chondrogenic medium, the monolayer cultures of MSCs and MDSCs started to form chondrocyte colonies. The colonies continued to expand during extended cultivation, forming a densely organized mass of cells. The tendency to form chondrocyte colonies was similar in the cultures differentiated from bone marrow MSCs and those differentiated from MDSCs. The histochemical results of Alcian Blue staining performed at 24 days of chondrogenic stimulation showed that the differentiated chondrocyte-like cells produced chondrocyte-specific extracellular matrix in similar quantities in cultures derived from both MSCs and MDSCs (Fig. 2).
Differentiation towards adipogenic lineage

Eleven days after adipogenic induction of MDSCs, tiny oil droplets were observed. The number of oil droplets increased during cell cultivation. Adipogenic differentiation was evaluated after 21 days based on the production and accumulation of neutral lipids in differentiated cells. Adipocytes were rounded and filled with lipid droplets, which could fuse to form vacuoles that can be stained by Oil Red O, a lipophilic red dye (Fig. 3). The results obtained indicated a higher adipogenic differentiation potential within the MSCs compared to the MDSCs.

Differentiation towards epithelial-like lineage

Starting at day 20, under FGF, EGF and KGF stimulation cells started to change their morphological aspect from fibroblastic to the polygonal aspect specific to epithelial lineage (Fig. 4). The epithelial markers were assessed after a 28-day cultivation under specific media conditions. For epithelial differentiation, E-cadherin and Cytokeratin 10 (CK 10) presence was assessed using E-cadherin and Cytokeratin 10 polyclonal antibody labeling (Fig. 5). Both cell populations showed positive for E cadherin and Cytokeratin 10 epithelial markers. The PCR analysis of MSCs and MDSCs indicated E cadherin expression.

DISCUSSION

A number of studies have confirmed the differentiation ability of MSCs, at least towards osteogenic, chondrogenic and adipogenic lineages (Calvi et al., 2003, Kasrinaki et al., 2008). At the same time, different reports have mentioned the MSCs’ capacity to induce tissue repair in physiological and pathological conditions, in different nonhematopoietic-origin tissue, including different epithelial types (Wu et al., 2010). Regarding the MDSCs’ multilineage differentiation ability, reports have shown that MDSCs can spontaneously differentiate into myotubes when cultivated in basic medium. Depending on the additional type of inductive factors, they have the capacity to differentiate into cell types with the characteristics of ectodermal or mesodermal origin, including neural, hematopoietic, osteogenic, adipogenic, chondrogenic and endothelial cell types (Montarras et al., 2005, Bueno et al., 2009, Bellayr et al., 2010). However, there have been no reports on the MDSCs’ ability to differentiate towards epithelial-like cells (Wu et al., 2010).

After 10 days under the described experimental conditions, the osteogenic potential of rat MSC and MDSC populations analyzed by histochemical and immunostaining techniques indicated a more substantial matrix mineralization for MSCs compared to MDSCs. Bueno et al. (2009) examined MDSCs’ myogenic, adipogenic, chondrogenic and osteogenic potential. Their results confirm the in vitro plasticity of MDSCs towards the mesodermal tissues tested. The MDSCs’ plasticity was considered consistent with the observed lineage-specific differentiation of bone marrow and other stem cells, but no related data were presented.

Amelogenin is a major protein of enamel matrix comprising more than 90% of the organic fraction produced by ameloblasts, and it seems to be essential for the organization of the crystal pattern and regulation of enamel thickness (Gibson et al., 2001, Xu et al., 2006). Studies have indicated that besides having a role in mineralization and enamel matrix formation, amelogenin is involved in bone development (Haze et al., 2007). Our study confirms Haze et al. (2007) conclusions that suggest the involvement of amelogenin in osteogenesis, though the previous studies involved only bone marrow progenitor cells.

Cartilage extracellular matrix (ECM) contains large amounts of proteoglycans consisting of a protein core to which highly sulfated sugar chains, glycosaminoglycans (GAGs), are attached (Settembre et al., 2008). After 24 days under experimental conditions, sulfated proteoglycan deposit indicators of functional chondrocytes were evidenced by Alcian Blue staining. The study made by Bueno et al. (2009) that involved Toluidine blue staining for 21 days of MDSC chondrogenic-induced differentiation indicated a similar chondrogenic potential compared to that of the MDSCs observed in our experiments.
Adipocyte differentiation involves a pluripotent stem cell precursor that has the potential to differentiate along mesodermal lineages. Under appropriate environmental conditions, preadipocytes undergo clonal and subsequent terminal differentiation (Tanasie et al., 2011). In experimental conditions, both MSCs and MDSCs accumulated tiny oil droplets starting from the eleventh day. Adipogenic differentiation was evaluated after a 21-day cultivation based on the production and accumulation of neutral lipids in differentiated cells, by Oil Red O staining. The results obtained indicated a higher adipogenic differentiation potential within the MSCs compared to the MDSCs. Rebelatto, et al. (2008) indicated a similar result when comparing bone marrow MSCs and umbilical cord-derived adult stem cells (UCB-derived MSCs). The same study suggests that the adipogenic process was present in the UCB-derived MSCs at more initial stages compared to bone marrow MSCs and would require a prolonged time interval in order to acquire the same level of differentiation. We believe a similar situation is required for MDSC osteogenic and adipogenic differentiation. Different studies examining MDSC osteogenic or adipogenic differentiation performed sample evaluation at later time intervals for osteogenic (2-4 weeks) and adipogenic (24 days) lineages and did not mention a reduced differentiation potential for MDSCs; however, their studies did not include bone marrow MSCs (Kim et al., 2008, Bueno et al., 2009).

Human MSCs isolated from human bone marrow can differentiate into epithelial-like cells and might represent valuable candidates for tissue engineering and epithelial tissue cell therapy (Rebelatto et al., 2008). In vivo studies revealed MSCs' homing and differentiation in retina pigment epithelial cells, the epidermis, ductal epithelial cells and renal tubules epithelial cells (Kim et al., 2008, Wang et al., 2005). Under FGF, EGF and KGF stimulation, both rat MSC and MDSC populations expressed positive E-cadherin and Cytokeratin 10 cells. E-cadherin is a calcium-sensitive cell adhesion molecule in human epithelial tissues and type I cytoskeletal 10 (cytokeratin-10), along with actin microfilaments and microtubules, compose the cytoskeleton of epithelial cells, being responsible for the structural maintenance of the cells and tissue (Peinado et al., 2004). The presence of both markers and an epithelial-like phenotype acquired by MDSCs and MSCs suggests their capacity to differentiate towards an epithelial-like lineage.

In this study, we used qualitative assays to evaluate the differentiation potential of MSCs isolated from rat bone marrow and gastrocnemius muscle after exposure to specific culture conditions. Under experimental conditions, both MSC and MDSC populations expressed positive markers for all four specific lineages towards which they were induced; however, the MDSCs presented a more restricted or at least delayed osteogenic and adipogenic capacity. The chondrogenic and epithelial-like phenotype was achieved by both cell populations in similar proportions. This study points to a potential use of MDSCs for regenerative therapies in other less studied areas such as skin regeneration.

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