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Research Article

Ultrastructural study of cultured ovine bone marrow-derived mesenchymal stromal cells

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ABSTRACT

Ovine bone marrow-derived mesenchymal stromal cells (oBM-MSCs) represent a good animal model for cell-based therapy and tissue engineering. Despite their use as a new therapeutic tool for several clinical applications, the morphological features of oBM-MSCs are yet unknown. Therefore, in this study the ultrastructural phenotype of these cells was analysed by transmission electron microscopy (TEM). The oBM-MSCs were isolated from the iliac crest and cultured until they reached near-confluence. After trypsinization, they were processed to investigate their ultrastructural features as well as specific surface marker proteins by flow cytometry and immunogold electron microscopy. Flow cytometry displayed that all oBM-MSCs lacked expression of CD31, CD34, CD45, HLA-DR whereas they expressed CD44, CD58, HLAI and a minor subset of the cell population (12%) exhibited CD90. TEM revealed the presence of two morphologically distinct cell types: cuboidal electron-lucent cells and spindle-shaped electron-dense cells, both expressing the CD90 antigen. Most of the electron-lucent cells showed glycogen aggregates, dilated cisternae of RER, moderately developed Golgi complex, and secretory activity. The electron-dense cell type was constituted by two different cell-populations: type A cells with numerous endosomes, dense bodies, rod-shaped mitochondria and filopodia; type B cells with elongated mitochondria, thin pseudopodia and cytoplasmic connectivity with electron-lucent cells. These morphological findings could provide a useful support to identify "in situ" the cellular components involved in the cell-therapy when cultured oBM-MSCs are injected.

1. Introduction

The mesenchymal stromal cells (MSCs), first described by Friedenstein et al. (1970) as population of bone marrow stromal cells that were able to adhere to the plastic culture substrate, are multipotent cells present in bone marrow which can replicate as undifferentiated cells and have differentiation potential giving rise to mesenchymal tissue lineages such as bone, cartilage, fat, tendon, muscle and marrow stroma (Prockop, 1997; Pittenger et al., 1999). These cells can also become neurons, cardiac muscle cells and corneal keratocytes under specific conditions (Makino et al., 1999; Holden and Vogel, 2002; Jiang et al., 2002; Dezawa et al., 2004; Takayama et al., 2009).

In humans, MSCs are defined by several key features: they strongly adhere to a plastic surface in culture; they are clonogenic; they show a surprising proliferation rate (self-renewing); they exhibit a remarkable plasticity, being able to differentiate into many mature cell types of mesodermal and non-mesodermal origin (multipotency) (Bruder et al., 1997; Pittenger et al., 1999; Woodbury et al., 2000; Payushina et al., 2006). In addition to these features, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) considers the MSCs as plastic-adherent cells which express the surface markers CD73, CD90 and CD105 and lack expression of CD14 or CD11b, CD34, CD45, CD79 or CD19 and HLA-DR (Dominici et al., 2006). Besides the markers defined by the ISCT, additional surface proteins have been reported to be expressed by human MSCs including CD29, CD44, CD106 and CD166 (Pittenger et al., 1999; Barry and Murphy, 2004).

In contrast to human MSCs, no uniform characterization criteria are available to date for animal origin MSCs in general, including ovine MSCs. For example, in ovine bone marrow derived MSCs

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(oBM-MSCs) the expression of CD90 has been detected by Rentsch et al. (2010), but not by McCarty et al. (2009) and Rozemuller et al. (2010).

Due to their low immunogenicity and lack of alloreactivity, MSCs are considered optimal candidates for transplantation procedures (Bartholomew et al., 2002; Gotherstrom, 2007; Nauta and Fibbe, 2007; Mobasheri et al., 2009). Recently, numerous experimental studies have demonstrated that oBM-MSCs are good animal model for cell-based therapy and tissue engineering. oBM-MSCs are able to promote bone formation in induced osteonecrosis (Feitosa et al., 2010), tibial defect (Field et al., 2011) and in a ceramic bone substitute (Boos et al., 2011). Additionally, these cells permit the regeneration of injured growth plate cartilage (McCarty et al., 2010) and articular chondrocytes (Marquass et al., 2011). Lastly, oBM-MSCs are effective in regeneration of tendon tissue after induced tendinitis (Crovace et al., 2008; Lacitignola et al., 2014).

In spite of their considerable importance in cell-based therapy, detailed ultrastructural studies of adult MSCs are sparse in mammals (Seed et al., 1986; Raimondo et al., 2006; Pasquinelli et al., 2007; Karaöz et al., 2011; Pieri et al., 2011; Tan et al., 2013) and lack in ovine. The objective of this study has been to examine the ultrastructural features of the oBM-MSCs. Since in the tissues of adult mammals a diffuse network of MSCs exists, the knowledge of their ultrastructural characteristics could represent the basis to identify "in situ" the niches in which such cells reside. Consequently this could provide functional support for maturation of precursors of the primary cellular compartments in adult tissues as has been suggested for humans (Pasquinelli et al., 2007).

2. Materials and methods

2.1. Bone marrow harvesting

This study was part of a larger research that included the harvest of ovine bone marrow. The ethical committee of the University of Bari in accordance with National animal welfare legislation, and in compliance with the guidelines outlined in the NRC Guide for the Care and Use of Laboratory Animals, approved the use of ewes (Bergamasca breed, 2 years of age, female, 45 kg in weight, not inbred) for the study.

Bone Marrow (BM) samples were obtained from the iliac crest of 2 healthy sheep according to Crovace et al. (2008). The procedure was performed under sedation with diazepam[a] (0.05 mg/kg) and local anaesthesia with lidocaine chlorhydrate[b] 2% (20 ml) around the tuber coxae. After aseptic preparation of skin, a 14 gauge (1.0 mm) jamshidi needle was inserted in the tuber coxae to a depth of 3–4 cm. A 20 ml heparinized (2500 I.U. heparin/20 ml BM) syringe was attached to the needle to obtain BM.

2.2. Cell culture

The BM were diluted 1:1 in PBS, then stratified 1:1 on Biocoll Separating solution (FICOLL, gradient 1.077 g/ml, Biochrom, Berlin,Germany) and centrifuged at 2000 rpm × 30 min and seeded in flasks at a concentration of 4–5 × 10^6 cells/cm^2 in complete medium (Coon's) at 37 °C in a humid 5% CO_2/air (carbogen) atmosphere. Non-adherent cells were discarded after 3 days and adherent cells were cultured until they reached near-confluence (10 days) (Fig. 1A). The cultures of BMSCs were trypsin-treated in a routine manner and, after collection in Falcon tubes, the detached cells were rinsed in PBS and centrifuged twice at 1200 rpm for 10 min.

2.3. Electron microscopy

The pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (TBS), pH 7.3, for 2 h at room temperature, and post-fixed or not with 1% OsO_4 in the same buffer for 1 h. Samples were dehydrated in an ethanol series and embedded in Epon 812.

Semithin sections (1 μm thick) were cut and stained with saturated, borax-buffered toluidine blue dye solution and examined under light microscopy to study the general morphology.

Ultrathin sections (50–70 nm) with silver interference were cut, picked up on copper grids and stained with uranyl acetate and lead citrate.

2.4. Immunoelectron microscopy

Ultra-thin sections were mounted on formwar-coated gold grids and incubated overnight with the mouse antibody against human CD 90 (BD Biosciences Pharmingen, San Diego, CA) diluted 1:50 in blocking buffer (TBS 0.1 M, pH 7.4 + BSA1%) at 4 °C. Grids were rinsed with TBS (0.1 M, pH 7.4), and then incubated at a dilution of 1:20 of 10-nm gold-conjugated anti mouse IgG (Sigma) in TBS for 1 h at RT. After several rinses in TBS, the grids were lightly stained with uranyl acetate and lead citrate. Negative controls were performed by substituting the primary antibody with TBS. The grids were observed under a Morgagni 268 electron microscope (FEI, Hillsboro, Oregon, USA).

2.5. Flow cytometric analysis

Flow cytometric analysis was used to characterize the cell-surface antigen expression of oBM-MSCs using antibodies against positively (CD44, CD58, CD90 and HLAI) and negatively (CD31, CD34, CD45 and HLA-DR) markers associated with MSC populations. After trypsinization, cells were resuspended in FACS buffer (pH 7.2 PBS, BSA 0.5%, sodium azide 0.02%) at a concentration of 0.1 × 10^6/100 μl and were labelled for 30 min at room temperature in the dark using 10 μl (1:10 dilution) of fluorochrome-unconjugated mouse primary antibodies anti-ovine CD44 (Serotec®, Milan, Italy) and anti-human CD90 (BD Biosciences Pharmingen, San Diego, CA) or fluorochrome-conjugated monoclonal anti-ovine antibodies anti-CD45FITC,anti- HLA I FITC, anti-CD31PE, anti-CD34PE, anti-CD58PE, and anti-HLA-DR PE (Serotec®, Milan, Italy). Then the cells were washed with FACS buffer to remove non-conjugated antibodies and those incubated with anti CD44 and CD90 were incubated with fluorescein isothiocyanate (FITC)-Goat anti-mouse IgG (H + L) secondary antibody (R&D®, dilution 1:10) for 30 min at room temperature in a dark room. Epics 'XL-MCL' (Beckman Coulter) flow cytometer was used to analyse fluorescent phenotypic marker signals. At least 10,000 events for test sample were acquired. The negative control assay was performed by the reaction of each primary antibody with the respective peptide antigen and in all cases the percent positive cells within the M1 gate were identified. Sample histogram elaboration was performed with EXPO 32 software to assess fluorescent distribution.

3. Results

3.1. Light microscopy

Semithin sections stained with toluidine blue showed that sBM-MSCs contained two distinct kinds of cells: weakly stained large cuboidal cells and strongly stained spindle-shaped cells (Fig. 1B).

3.2. Transmission electron microscopy

TEM observations revealed the presence of two cell types: electron-lucent cells and electron-dense cells. sBM-MSCs exhibited large, irregularly and euchromatic nuclei with one or two nucleoli.