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Characterisation and intracellular labelling of mesenchymal stromal cells derived from synovial fluid of horses and sheep

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ABSTRACT

Multipotent mesenchymal stromal cells (MSCs) derived from synovial fluid (SF) are considered to be a promising cell type for therapeutic applications in joint disease. However, despite their potential relevance for clinical and experimental studies, there is insufficient knowledge about SF-derived MSCs isolated from horses and sheep. In this study, cells were recovered from healthy SF and bone marrow (BM) of sheep, and from healthy and osteoarthritic SF of horses. Ovine SF-MSCs were used to assess the efficiency of intracellular labelling with quantum dots (QDs). Colony forming units, generation times, trilineage differentiation potential and expression of CD73, CD90 and CD105 at mRNA level were assessed. QD labelling was efficient, with >98% positive cells directly after labelling at 10 nmol/L and >95% positive cells directly after labelling at 2 nmol/L. The label decreased over 7 days of culture, with more persistence at the higher labelling concentration. No significant differences in proliferation were observed. All MSCs had trilineage differentiation potential, but adipogenesis was more distinct in equine samples and chondrogenesis was most pronounced in ovine SF-MSCs. CD73, CD90 and CD105 were expressed in equine and ovine MSCs.

Introduction

Multipotent mesenchymal stromal cells (MSCs) isolated from synovial fluid (SF) are promising as an autologous therapy for osteoarthritic conditions or cartilage defects (de Sousa et al., 2014). Multipotent MSCs, also referred to as mesenchymal progenitor cells or mesenchymal stem cells, are found in many tissues and are currently defined by their plastic adherence, trilineage differentiation potential and expression of surface markers, including CD73, CD90 and CD105 (Dominici et al., 2006).

Due to their mesenchymal lineage differentiation potential, MSCs are considered to be a suitable cell type for tissue engineering in orthopaedics. Their use for tissue grafts could replace other cell types, such as chondrocytes, avoiding local damage to donor sites (Mardones et al., 2015). Furthermore, MSCs can modulate immune responses (Yagi et al., 2010) and angiogenesis (Conze et al., 2014). MSCs from different species or different tissue sources appear to share the same basic characteristics and to have a similar morphology, yet also have clear differences (Burk et al., 2013; Hillmann et al., 2016). Specific subtypes of MSCs may be advantageous for certain clinical applications, based on practical reasons and potentially higher potency.

SF can be harvested minimally invasively by arthrocentesis and, therefore, is a practical source of MSCs, particularly for autologous cell therapy. SF from human beings contains cells with a phenotype that is similar to bone marrow (BM)-derived MSCs (BM-MSCs) (Jones et al., 2004). MSCs are found at higher concentrations in joints with osteoarthritis and in joints with meniscal injury than in healthy or rheumatoid joints and it is hypothesised that SF-MSCs originate from damaged joint tissues (Jones et al., 2004, 2008; Sekiya et al., 2012; Matsukura et al., 2014; Garcia et al., 2016). The chondrogenic potential of SF-MSCs appears to be higher than BM-MSCs, whilst their adipogenic capacity is lower than BM-MSCs (Jones et al., 2008; Murata et al., 2014). SF-MSCs also have immunosuppressive and immunoregulatory properties (Hagmann et al., 2013; Garcia et al., 2016).

Most in vitro studies have used human SF as a source of MSCs, reflecting the minimally invasive approach to harvesting SF (Jones et al., 2004, 2008; Sekiya et al., 2012; Matsukura et al., 2014). Studies in rabbits have suggested that SF-MSCs can be used for the repair of meniscal injury or osteochondral defects (Horie et al., 2012; Shimomura et al., 2014). Bovine and porcine SF-MSCs have been characterised by Jones et al. (2008) and Tang et al. (2015),

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respectively. SF-MSCs from horses and sheep have not been well characterised, even though equine joint disease is important clinically and sheep are widely used in experimental models of joint disease. To our knowledge, only one study has characterised equine SF-MSCs (Murata et al., 2014). Therefore, the first aim of this study was to gain more knowledge of the proliferation and differentiation potentials of SF-MSCs from horses and sheep, which may provide a basis for future in vivo studies in these species.

Labelling of MSCs is used in several research applications, such as co-culture studies investigating cellular interactions and studies on the in vivo distribution of injected cells. The second aim of the study was to determine if equine and ovine SF-MSCs can be labelled with intracellular quantum dots (QDs) (Bilan et al., 2016). We hypothesised that SF-MSCs can be labelled with QD without a negative impact on their cell properties.

Materials and methods

Ovine and equine mesenchymal stromal cell recovery

SF was harvested from the tibiotarsal joints of six healthy sheep under sedation and local anaesthesia; the sheep had a mean ± standard deviation (SD) age of 2 ± 0 years and a mean ± SD body weight of 45 ± 5 kg. SF was collected under general anaesthesia from paired radiocarpal or tibiotarsal joints from three horses (mean ± SD age 4 ± 2 years; mean ± SD body mass 520 ± 26 kg) with osteoarthritis in one of their radiocarpal or tibiotarsal joints based on clinical and radiographic findings. Arthrocentesis was performed using 18 or 20 Ga injection needles after aseptic preparation of the skin and SF was aspirated into sterile syringes. Paired BM samples were obtained from two of the sheep by inserting a 14 Ga Jamshidi needle into the tuber coxae and aspirating the BM into heparinised (2500 IU/20 mL BM) syringes. Sample collection procedures were approved by the ethical committee of the University of Bari (approval number 264/2013B; date of approval 4 November 2013).

SF and BM samples were immediately taken to the laboratory to isolate the plastic-adherent cell fraction. The SF was diluted 1:5 in phosphate buffered saline (PBS), centrifuged at 750 *g* for 15 min and the cell pellet was collected, rinsed with PBS and cells were seeded at a density of 4–5 × 10^5/cm^2. The BM was diluted 1:1 in PBS, stratified 1:1 on Biocoll separating solution (1.077 g/mL; Biochrom) and centrifuged at 350 *g* for 30 min. The separated cells were rinsed twice with PBS and seeded at a density of 4–5 × 10^6/cm^2. The medium was replaced twice weekly and cell cultures were passaged at confluency by trypsinisation.

Cells from all samples were frozen and stored in liquid nitrogen until the start of the experiments. Freezing was performed in culture medium containing 10% dimethyl sulphoxide (Sigma Aldrich) using a freezing container (Mr Frosty, Nalgene) for controlled cooling (approximately 1 °C/min). Cryovials were thawed in a 37 °C water bath; cells were immediately washed when thawed and prior to seeding on culture flasks. Passage 3–5 MSCs cultured in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose supplemented with 10% foetal bovine serum, 1% penicillin–streptomycin and 0.1% gentamicin; Gibco/Thermo Fisher Scientific) were used for all experiments. Standard culture conditions included incubation at 37 °C and 5% CO_2 in a humidified atmosphere and a medium change twice each week.

Quantum dot labelling of ovine mesenchymal stromal cells

Samples of MSCs from three sheep underwent QD labelling (Qtracker 655 Cell Labelling Kit, Thermo Fisher Scientific) at 2, 5 and 10 nmol/L, whilst paired control cells remained unlabelled. MSCs from these samples were analysed by flow cytometry directly after labelling, as well as after 7 days of culture. Cells were stained with Fixable E-Fluor 780 (eBioscience) to be able to exclude dead cells in the analysis. Stained and unstained labelled and control cells were subjected to flow cytometry using a FACS Canto II (BD Biosciences) and analysed with FlowJo v10.

MSCs obtained from the three remaining sheep again underwent QD labelling at a 10 nmol/L concentration. These labelled MSCs, as well as paired unlabelled control MSCs, were then subjected to the MSC characterisation assays detailed below. Cells were also cultured in chamber slides (Nunc, Thermo Fisher Scientific), fixed in 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI; Carl Roth) for examination using an inverted confocal laser scanning microscope (TCS SP5, Leica Microsystems). To avoid cross-talk in excitation at 405 nmol/L (DAPI) and 488 nmol/L (QD), a sequential scanning mode was executed. Images were acquired with photomultipliers and micrographs were processed and analysed using the Leica Application Suite Advanced Fluorescence 2.6.0 software (Leica Microsystems) and Adobe Photoshop CS3 (Adobe Systems).

Colony forming units and generation time

For the colony forming unit (CFU) assay, MSCs were seeded at low density (20 cells/cm^2) and incubated under standard conditions for 7 days. Colonies consisting of at least 50 fibroblast-like cells were counted.

For calculation of the generation time (GT), population doubling (PD) was assessed. MSCs were seeded at a density of 3000 cells/cm^2, incubated under standard conditions for 7 days, trypsinised and counted. Experiments were performed in duplicate with MSCs from each ovine or equine sample. The calculation was based on the following formulae:

$$PD = \frac{\ln \frac{\text{Cell count harvest}}{\text{Cell count seeding}}}{\ln 2}$$

$$GT = \frac{\text{Days in culture}}{PD}$$

Trilineage differentiation

Differentiation was induced using the StemPro assay kits (Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kits; Gibco/Thermo Fisher Scientific) according to the manufacturer's instructions except that: (1) seeding densities for adipogenesis and osteogenesis were reduced, using only half of the recommended cell numbers (i.e. 5000 cells/cm^2 for adipogenesis and 1500 cells/cm^2 for osteogenesis); and (2) for adipogenesis, in addition to using the complete StemPro medium alone for ovine MSC, equine and ovine MSCs were incubated in complete StemPro medium supplemented with 5% rabbit serum (Sigma Aldrich).

After an incubation period of 7 days for adipogenesis or 21 days for osteogenesis and chondrogenesis, cells or chondrogenic micromasses were fixed in 4% paraformaldehyde. Monolayer cells were stained with oil red O for detection of intracellular lipids or by the von Kossa method for detection of extracellular mineralisation. Paraffin sections prepared from the micromasses were stained with Alcian blue, Masson's trichrome and safranin O. Chondrogenesis was evaluated using a score system including safranin O staining, matrix accumulation and cell morphology as separate parameters, with a maximum of nine total achievable score points (Grogan et al., 2006). Experiments were performed in duplicate with MSCs from each ovine or equine sample.

Real-time PCR

Gene expression of the MSC-related surface markers CD73, CD90 and CD105 was assessed by real-time PCR as described previously (Burk et al., 2014). Total RNA of MSCs was isolated and DNase-treated RNA was reverse transcribed using the RNeasy Mini Kit (Qiagen) and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Relative quantification of cDNA was performed with a 7500 Real Time PCR System (Applied Biosystems) and SYBR Green as double-strand DNA-specific dye (iQ SYBR Green Supermix; Bio-Rad). B-Actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were used as housekeeping genes and relative quantification of gene expression was performed as described previously (Pfaffl, 2001). Primers amplifying the respective genes are listed in Table 1.

Statistical analysis

Using SPSS Statistics 22 (IBM), Kruskall–Wallis one-way analyses of variance (ANOVAs) were performed to compare the different sample groups. Differences between paired samples were analysed using Wilcoxon tests. The level of significance was set at $P < 0.05$.

Results

Quantum dot labelling

Quantum dot labelling of ovine MSCs was efficient in all samples and at all concentrations. Directly after the labelling procedure, the mean percentages of labelled cells were >98.8% in all samples labelled with 5 or 10 nmol/L QD and >95.4% in all samples labelled with 2 nmol/L QD. The mean fluorescence intensity on flow cytometry directly after labelling was more than two-fold higher in MSCs labelled with 10 nmol/L QD than in those labelled with 2 nmol/L QD (Fig. 1).

After 7 days of culture, the percentage of labelled cells decreased and was more variable at all labelling concentrations. The mean fluorescence intensity decreased after 7 days of culture at all labelling concentrations. The most persistent QD labelling was observed in MSCs labelled with 10 nmol/L QD; the percentage of