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Culture and characterisation of equine peripheral blood mesenchymal stromal cells

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ABSTRACT

Although the use of mesenchymal stromal cells (MSCs) for the treatment of orthopaedic injuries in horses has been reported, no official guidelines exist that classify a particular cell as an equine MSC. Given the limited characterisation of peripheral blood (PB)-derived equine MSCs in particular, this study aimed to provide more detailed information in relation to this cell type. Mesenchymal stromal cells were isolated from equine PB samples and colony forming unit (CFU) assays as well as population doubling times (PDTs) (from P_0 to P_{10}) were performed.

Two types of colonies, 'fingerprint' and dispersed, could be observed based on macroscopic and microscopic features. Moreover, after an initial lag phase (as indicated by a negative PDT at P_0 to P_1) the MSCs divided rapidly as indicated by a positive PDT at all further passages. Immunophenotyping was carried out with trypsin- as well as with accutase-detached MSC to evaluate potential trypsin-sensitive epitope destruction on particular antigens. Isolated MSC were positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79α, MHC II and a monocyte/macrophage marker, irrespective of the cell detaching agent used. Trilineage differentiation of the MSCs towards osteoblasts, chondroblasts and adipocytes was confirmed using a range of histochemical stains.

Introduction

Stem cells are defined as cells displaying a capacity for 'self-renewal' either with or without differentiation, depending on the symmetry of the division (Horvitz and Herskowitz, 1992). More specifically, mesenchymal stromal cells (MSCs) are adult stem cells derived from mesoderm. In 2006, the International Society for Cellular Therapy (ISCT) carefully determined the qualities human cells must possess in order to be defined as MSCs (Dominici et al., 2006) as follows: plastic-adherent, positive for the markers CD73, CD90 and CD105, negative for the markers CD14 (or CD11b), CD34, CD45, CD79a (or CD19) and MHC II, and exhibiting the ability to differentiate into cells of mesodermal origin such as osteoblasts, chondroblasts and adipocytes. The use of other human MSC markers such as CD29 and CD44 was also reported (Pittenger et al., 1999; Majumdar et al., 2003).

No such guidelines exist for equine MSCs, although these would be of great benefit to researchers in this field (De Schauwer et al., 2011b). Sources of equine MSCs include bone marrow (BM), adipose tissue (AT), umbilical cord, amniotic fluid, umbilical cord blood (UCB), peripheral blood (PB), gingiva and periodontal ligament (Koerner et al., 2006; Koch et al., 2007; Ahern et al., 2011; Carrade et al., 2011; Mensing et al., 2011; Park et al., 2011). For MSCs isolated from equine BM, AT and UCB, the use of several markers and successful trilineage differentiation have been described (Hoynowski et al., 2007; Guest et al., 2008; Koch et al., 2009; Radcliffe et al., 2010).

To date, the only characterisation of equine PB-derived MSCs has been immunophenotyping using two of the proposed positive markers CD44 and CD90, and two of the proposed negative markers, CD34 and CD45 (Martinello et al., 2010). For these negative markers, no information was provided on the positive controls used to confirm cross-reactivity with equine cells, and the potential influence of the detachment product on epitope expression was not evaluated. This latter feature is potentially important, since Hackett et al. (2011) describe the destructive effect of trypsin on the CD14 epitope of equine BM-derived cells, indicating that care is needed when evaluating negative stem cell markers on trypsin-detached cells. Aside from immunophenotyping, the results of different studies on the differentiation of equine PB MSC into cartilage are contradictory (Koerner et al., 2006; Giovannini et al., 2008), highlighting the need for their greater characterisation.

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Current MSC therapies in horses mainly use BM-derived MSCs for the treatment of tendinopathies (Smith et al., 2003; Crovace et al., 2007; Smith, 2008) and BM- or AT-derived MSCs for the treatment of osteoarthritis (Frisbie et al., 2009). The most obvious disadvantages of BM and AT are the difficulty and invasiveness of the harvesting procedure. An excellent alternative source of cells is blood, such as UCB collected at birth, or PB from adult horses. Despite the safety and high success rate of collecting UCB for use as a source of MSCs (Bartholomew et al., 2009), a potential disadvantage is the fact that autologous UCB is not always available at the time of injury, highlighting the potential use of PB as an alternative source.

Given that such blood samples can be readily taken in a sterile manner at the time of injury, they may provide a readily accessible source of autologous MSCs for regenerative therapies. In fact the first clinical applications of heterogenous populations of PB-derived stem cells have been recently described for the treatment of equine ophthalmological conditions (Marfe et al., 2011; Spaas et al., 2011). In order to standardise the promising results of such therapy, it is essential that well-characterised and homogenous stem cell populations are used.

The objective of the current study was to further characterise equine PB-derived MSCs by determining their growth efficiency and proliferation rate, immunophenotyping them using a wide-range of complementary markers, and performing trilineage differentiation experiments.

Materials and methods

Isolation of putative peripheral blood-derived mesenchymal stromal cells

Ten millilitres of blood from the jugular vein of four adult Warmblood horses were collected into EDTA tubes and transported at 4 °C to the laboratory within 4 h of sampling. The blood was centrifuged at 1000 *g* for 20 min at room temperature (RT) and the buffy coat collected and diluted 1:1 with phosphate buffered saline (PBS). Subsequently, the cell suspension was gently layered on a Percoll gradient (density 1.080 g/mL; GE Healthcare) and centrifuged at 600 *g* for 15 min at RT, as previously described (De Schauwer et al., 2011a). The interphase was collected, washed three times with PBS by centrifuging at 200 *g* for 10 min, and the cells planted at 16×10^4 cells/cm^2 in a T_{75} flask in culture medium consisting of low glucose (LG) Dulbecco's modified Eagle medium (DMEM) (Invitrogen), supplemented with 30% fetal calf serum (FCS) (GIBCO), 10^{-11} M low dexamethasone, 50 μg/mL gentamicin, 10 μL/mL antibiotic–antimycotic solution, 250 ng/mL fungizone (all Sigma), and 2 mM ultraglutamine (Invitrogen). The medium was refreshed twice weekly and the putative MSCs were maintained at 37 °C and 5% CO_2. At 70% confluency, cells were trypsinised with 0.25% trypsin–EDTA (P_0) and were further cultured for 10 subsequent passages (P_1 to P_{10}) in expansion medium, which was identical to the culture medium but without dexamethasone.

Colony forming unit assay

Ten, 50 and 100 MSCs were plated/94 mm plate and fixed 8 days later at −20 °C for 10 min using 90% ethanol. Crystal violet staining was used to visualise the colony forming units (CFUs) macroscopically and the total number of CFUs/plate were counted. These experiments were carried out in triplicate for all samples.

Determination of population doubling time

Cell doubling time (CDT) was calculated from P_0 to P_{10} (Hoynowski et al., 2007), using the following formula: $CDT = \ln(N_f/N_i)/\ln 2$, with N_f the final, and N_i the initial, number of cells. For the population doubling time (PDT), the cell culture time (in days) was divided by the CDT (Hoynowski et al., 2007).

Immunophenotyping using flow cytometry

In order to characterise undifferentiated equine MSCs immunophenotypically, the expression of several MSC markers was evaluated simultaneously by flow cytometry. Cells were detached using either trypsin (Invitrogen) or accutase (Innovative Cell Technologies). Per series, 2×10^5 cells were labelled using the following panel of primary antibodies: CD29-Alexa^{488} (Biolegend, clone TS2/16), CD44-APC (BD, clone IM7), CD45-Alexa^{488} (Serotec, clone F10-89-4), CD79α-Alexa^{647} (Serotec, clone HM57), CD90 (VMRD, clone DH24A), CD105-PE (Abcam, clone SN6), MHC II (Serotec, clone CVS20) and a monocyte/macrophage marker-Alexa^{488} (Serotec, clone MAC387). For the detection of the CD79α and monocyte/macrophage marker, fixation and permeabilisation pretreatment was carried out with commercially available reagents (Invitrogen). In general, cells were incubated for 15 min on ice in the dark with the primary antibodies and then washed twice in LG DMEM with 1% BSA.

Incubation for 15 min on ice in the dark with the secondary Alexa^{647}- and PE-linked antibodies (Invitrogen) was performed to label the CD90- and MHC II-positive cells, respectively. In addition, viability assessment was performed on the non-fixed cells with the nucleic acid stain 7-amino-actinomycin D (7-AAD, Sigma). A minimum of 10,000 cells were acquired using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 488 nm solid state and a 633 nm HeNe laser, and these data were subsequently analysed with FACS Diva software. To assess cross-reactivity of the differentiated blood cell markers, for which stem cells should be negative, positive control equine peripheral blood mononuclear (PBMC) and endothelial cells were used. In addition, cells were incubated with or without (autofluorescence) isotype-specific murine IgG_1 and IgM and rat IgG_{2b} in parallel to establish the background signal.

Trilineage cell differentiation

To identify osteogenic differentiation, 3×10^3 cells/cm^2 were planted in a four-well plate and incubated in expansion medium until the cells were 70% confluent. At that point, osteogenic differentiation medium was added and refreshed twice weekly. This medium consisted of LG DMEM (Invitrogen), supplemented with 10% FCS (GIBCO), 0.2 mM L-ascorbic acid-2-phosphate (Fluka), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL gentamycin and 10 μL/mL antibiotic–antimycotic solution (all Sigma) (Koch et al., 2007; De Schauwer et al., 2011a). Three weeks later, osteogenic differentiation was evaluated using alkaline phosphatase (Millipore detection kit) and alizarin red S staining to evaluate calcium phosphate deposition. To assess chondrogenic differentiation, 2.5×10^5 cells/5 mL in a three-dimensional culture system, were centrifuged at 150 *g* for 5 min at RT and resuspended in 0.5 mL chondrogenic-inducing medium which was refreshed twice weekly. This medium was based on the basal differentiation medium (Lonza), supplemented with 10 ng/mL transforming growth factor (TGF)-β_3 (Sigma).

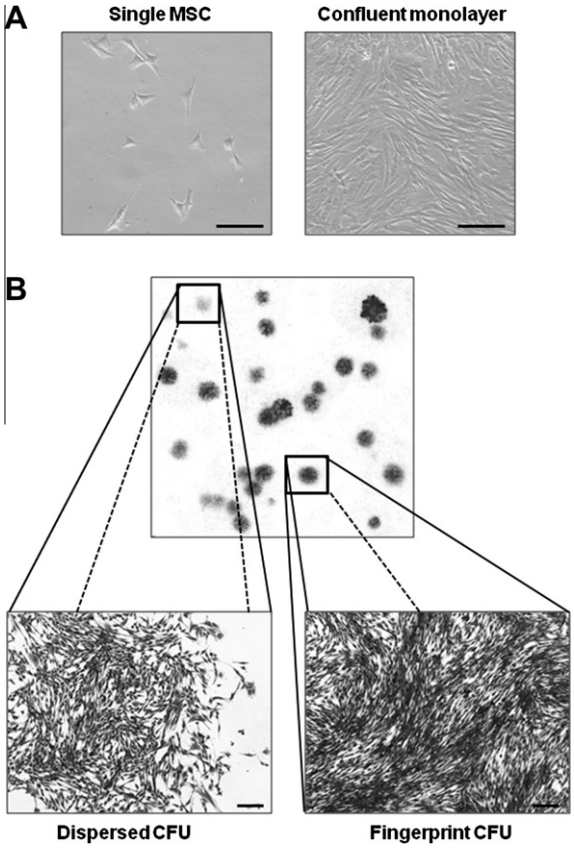

Fig. 1. Adherent putative equine mesenchymal stromal cells (MSCs): (A) representative images of a putative single MSC and of a MSC monolayer; (B) representative macroscopic and microscopic images of dispersed colony forming units (CFUs) and 'fingerprint' CFUs (crystal violet stain). Scale bars, 50 μm.