MULTILINEAGE POTENTIAL OF EQUINE BLOOD-DERIVED FIBROBLAST-LIKE CELLS

+* Osteoarticular Research Group, Institute of Pathology, University of Bern, Bern, Switzerland.
dobrila.nesic@pathology.unibe.ch

ABSTRACT INTRODUCTION:
Stem cells represent attractive candidates for cell based therapy and tissue engineering for cartilaginous and bone tissues. Autologous bone marrow derived mesenchymal stem cells (BM MSC) are currently employed for tendon repair in horses. Peripheral blood would be an important alternative source of MSC given the easier access and less invasiveness. In a preliminary study in our laboratory, fibroblast-like cells were isolated from equine peripheral blood (ePB-FLC). Under the standard differentiation methods established for human BM MSC, ePB-FLC underwent osteogenesis and stained positive for alkaline phosphatase while the adipogenic response was very weak and no chondrogenic differentiation was detected. This study focused on the optimisation of the differentiation conditions to provide better evaluation of the multilineage differentiation potential of the circulating ePB-FLC.

METHODS:
Peripheral blood was collected from a mix between venous and arterial blood of slaughtered horses (after stunning) and of venous blood from living horses. Bone marrow was collected from the sternum of slaughtered (after stunning and bleeding) and living horses with the approval of local ethical committee. ePB-FLC were isolated by density gradient centrifugation and adhesion to plastic. Cell colonies were detected 10 days after seeding. Cells were expanded in DMEM-F12 medium supplemented with 20% FCS for three passages prior to differentiation assays. Human and equine BM MSC were used as positive controls and human foreskin fibroblasts and human umbilical vein endothelial cells (HUVEC) cells as negative controls. The adipogenic and osteogenic differentiation assays were performed in monolayers at cell confluence and chondrogenesis in pellet cultures. Adipogenic differentiation was induced with standard method for hBM MSC with 4 cycles of induction (DMEM:F12, 10% FBS, 1% penicillin/streptomycin supplemented with 1µM dexamethasone, 100µM indomethacin, 500µM 3-IBMX, and 10µg/ml insulin) followed by one week in maintenance (DMEM:F12, 10% FBS, 1% penicillin/streptomycin supplemented with 10µg/ml insulin). Alternatively, cells were induced for 3 days with 5% rabbit serum (RS). Lipid droplets were stained with 5% Oil red O and cells counterstained with Mayer hematoxylin. Osteogenic differentiation was induced with basic media (DMEM:F12, 10% FBS, 1% penicillin/streptomycin) supplemented with 0.1 mM L-ascorbate-2-phosphate, 10nM β-glycerophosphate and 10nM or 100nM dexamethasone). Alkaline phosphatase staining was performed after 1-2 weeks. Calcium-deposition was assessed with 2% Alizarin red S staining after three weeks. Chondrogenesis was induced in pellet cultures with DMEM (high glucose) supplemented with ITS+, 0.1mM ascorbate-2-phosphate, 10⁻³ M dexamethasone and TGF-β3. Different concentrations of TGF-β3 (0, 1, 5, 10ng/ml) were tested. Cartilage specific matrix synthesis was analyzed after three weeks with real-time RT-PCR and histology. TaqMan real-time PCR was performed and monitored using the ABI Prism 7700 Sequence Detection System. Analyzed genes included Collagens type I and II, Aggrecan, and Versican. Comparative Ct method was used for analysis and 18S RNA served as internal control for cDNA input. Proteoglycan synthesis was evaluated via Alcian blue and Safranin O staining, and collagen deposition via Masson Trichrome staining.

RESULTS SECTION:
Adipogenic differentiation using the standard method for hBM MSC resulted in minimal lipid droplets production in ePB-FLC. A substantial increase in number and size of lipid droplets was observed with 5% RS after only three days of induction (Figure 1) indicating the capacity of eBP-FLC to undergo adipogenesis. Very few lipid droplets were observed in human fibroblasts and HUVEC. Osteogenesis was detected via alkaline phosphatase staining in ePB-FLC and eBM MSC 1-2 week after induction. Weak spontaneous osteoblast formation was also detected in untreated control cultures but not in induced human fibroblasts or HUVEC. Matrix mineralisation assessed by calcium deposition was detected with Alizarin red S after 3 weeks of induction (Figure 2). An increase in dexamethasone to 100nM resulted in faster osteogenic differentiation and matrix mineralisation. Cell seeding density, proliferation, confluence and individual variations were observed to influence the time needed for osteoblast formation and calcium deposition.

Chondrogenic differentiation resulted in an increase in the cartilage specific gene expression given as ratios of Collagen II/ I and Aggrecan/Versican in ePB-FLCs and eBM MSC in the presence of TGF-β3 thereby indicating their chondrogenic potential (Figure 3). The increase in TGF-β3 concentration correlated with the increase of gene expression ratios but was less pronounced in ePB-FLC. Incubation of ePB-FLC with TGFβ1 did not result in cartilage specific matrix production. In histology however, proteoglycan deposition was only detected in eBM MSC, consistent with much higher Aggrecan/Versican gene expression ratios observed.

DISCUSSION:
This study demonstrates that ePB-FLCs have the potential to differentiate into the three mesenchymal lineages: adipogenic, osteogenic and chondrogenic, providing that differentiation conditions are optimized. Adipogenic differentiation requires 5% RS, osteogenic differentiation is more efficient in the presence of 100nM dexamethasone, and chondrogenesis is induced with TGF-β3 and not TGF-β1. These conditions differ from conditions established for human BM MSC and in part of equine BM MSC suggesting that circulating ePB-FLC may not be the same cell type as MSCs present in bone marrow. Alternatively, stem cells leaving bone marrow to enter the blood system undergo changes due to the different environment. Nevertheless, the possibility to isolate multipotent cells from peripheral blood has very important clinical implications for cell therapy and tissue engineering for treatment of cartilaginous and bone tissues.