Veterinary Immunology and Immunopathology 200 (2018) 1–6

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Differentiation of equine bone marrow derived mesenchymal stem cells increases the expression of immunogenic genes

Laura Barrachina[a,b], Ana Rosa Remacha[a], Antonio Romero[a,b], Pilar Zaragoza[a], Francisco José Vázquez[a,b], Clementina Rodellar[a,*]

[a] Laboratorio de Genética Bioquímica LAGENBIO (Universidad de Zaragoza), Instituto Agroalimentario de Aragón– IA2, (Universidad de Zaragoza-CITA), Instituto de Investigación Sanitaria de Aragón (IIS), C/Miguel Servet, 177, 50013 Zaragoza, Spain
[b] Servicio de Cirugía y Medicina Equina, Hospital Veterinario, Universidad de Zaragoza, C/Miguel Servet, 177. 50013 Zaragoza, Spain

ARTICLE INFO

Keywords:
Horse
Mesenchymal stem cells
Allogeneic
Immunogenicity
Differentiation

ABSTRACT

Mesenchymal stem cells (MSCs) are a promising treatment for equine musculoskeletal injuries because of their ability to regulate the inflammation and to differentiate into other cell types. Since interest in allogeneic therapy is rising, concerns about MSC immunogenicity need to be addressed. Differentiated MSCs from several species increase their expression of immunogenic molecules and induce alloresponses, but equine MSC immunogenic profile after differentiation has not been reported. Therefore, the aim of this study was to assess the gene expression of immunogenic markers in tri-lineage differentiated equine bone marrow derived MSCs (eBM-MSCs). For this purpose, eBM-MSCs (n = 4) were differentiated into osteoblasts, adipocytes and chondrocytes. Differentiation was confirmed by specific staining and gene expression of lineage-related markers. Subsequently, gene expression of *MHC-I*, *MHC-II*, *CD40* and *CD80* was analyzed in undifferentiated (control) and tri-lineage differentiated eBM-MSCs. Osteogenesis and adipogenesis, but not chondrogenesis, significantly upregulated *MHC-I*; *MHC-II* expression significantly increased in the three lineages, while *CD40* and *CD80* expression did not change. Despite this, *MHC-I* and *MHC-II* upregulation after differentiation might lead to increased immunogenicity and risk of allorecognition, either eBM-MSCs differentiate in vivo after administration or they are differentiated prior to administration, with potential negative consequences for effectiveness and safety of allogeneic therapy.

1. Introduction

Mesenchymal stem cells (MSCs) are raising great interest for the treatment of equine musculoskeletal injuries since these have a huge impact in this species (Thorpe et al., 2010), and because of the suitability of the horse as animal model (Colbath et al., 2017). Mesenchymal stem cells exert their therapeutic effects through different mechanisms, including anti-inflammatory and immunomodulatory properties, and their ability to differentiate into cells such as chondrocytes, osteoblasts or tenocytes (da Silva Meirelles et al., 2009). The regulatory properties of MSCs are currently focusing most of the interest, but differentiation should also be taken into account as it can occur in vivo after administration even if at low rates (Murphy et al., 2003; Mokbel et al., 2011). Furthermore, some therapeutic strategies are based on the implantation of differentiated MSCs, such as chondrocytes for treating joint pathologies (Broeckx et al., 2014a; Ham et al., 2015). The implantation of chondrocytes derived from MSCs may overcome the limitations of other techniques using autologous chondrocytes or chondroprogenitor cells, which are technically demanding and may involve donor site morbidity related to tissue harvesting (Jayasuriya and Chen, 2015; Cokelaere et al., 2016). Moreover, bioreactors are being developed to produce tissue-engineered constructs derived from MSCs for the repair of tissues such as cartilage, bone or tendon (Xie et al., 2013; Youngstrom et al., 2016).

The optimal moment for MSC administration has yet to be determined, but several studies suggested enhanced effects with early administration (Koch et al., 2009; Mokbel et al., 2011), which might be limited by the use of autologous MSCs. Autologous therapy may also be limited in elderly patients or patients with genetic disorders. Hence, allogeneic therapy has raised great interest (Zhang et al., 2015). However, MSCs are not truly immune-privileged as they may induce both cellular and humoral immune responses, thus their immune allorecognition would lead to negative effects both in terms of effectiveness, because of the elimination of the cells, and safety, because of

* Corresponding author.
E-mail address: rodellar@unizar.es (C. Rodellar).

https://doi.org/10.1016/j.vetimm.2018.04.004
Received 13 September 2017; Received in revised form 14 March 2018; Accepted 9 April 2018

potential adverse effects for the patient (Berglund et al., 2017b).

In spite of the aforementioned, clinical implications of allogeneic equine MSCs administration are not entirely clear. While single and repeat intra-articular administration of allogeneic MSCs have been reported as clinically safe in both healthy (Carrade et al., 2011; Pigott et al., 2013a,b; Ardanaz et al., 2016) and pathologic equine joints (Broeckx et al., 2014a,b), other authors reported altered synovial parameters compared to autologous MSCs after second administration (Joswig et al., 2017).

These contradictory results might be due to different levels of major histocompatibility complex (MHC) expression and MHC compatibility between donor and recipient, since positive and heterogeneous MHC-II expression level (Schnabel et al., 2014) and in vivo generation of antibodies against MHC-mismatched equine MSCs (Berglund and Schnabel, 2016) have been reported. MSC differentiation might affect their MHC expression and, consequently, their immunogenicity. Hence, differentiation may imply negative consequences for allogeneic MSC survival, potentially hampering their effectiveness and compromising patient safety (Lohan et al., 2014).

Upregulation or induction of MHC-I and MHC-II and costimulatory molecules such as CD40 or CD80 has been shown after MSC differentiation into several lineages in human and small laboratory animals (Le Blanc et al., 2003; Liu et al., 2006a,b; Huang et al., 2010; Ryan et al., 2014; Yang et al., 2017). However, to the best of our knowledge, immunogenic profile of differentiated MSCs has not been assessed in the horse. Since allogeneic MSCs may differentiate in vivo albeit at low rates (Mokbel et al., 2011) or they can be differentiated to create tissue constructs prior to implantation (Youngstrom et al., 2016), questions about immunogenicity of equine differentiated MSCs need to be addressed.

The aim of this study was to assess the gene expression of immunogenic markers after tri-lineage differentiation of equine bone marrow derived MSCs (eBM-MSCs) in order to provide preliminary results about the potential consequences of eBM-MSC differentiation for their allogeneic administration.

2. Material and methods

2.1. Isolation of equine BM-MSCs

Twenty ml of BM from sternum were collected in heparinized syringes using a 4″11G Jamshidi needle from four healthy horses (Shetland ponies geldings, 4–7 years, 138–162 kg) under approval of the Ethic Committee for Animal Experiments from the University of Zaragoza (Project License 31/11). Gradient density separation technique (Lymphoprep, Atom) was used to isolate mononuclear cells, which were expanded in basal culture medium consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1%Glutamine, 1%Streptomycin/Penicillin and 10%Fetal Bovine Serum (FBS) (Sigma-Aldrich) until passage three and then characterized as eBM-MSCs by their phenotype (Ranera et al., 2011) and by their tri-lineage differentiation potential as it will be explained in the next section.

2.2. Tri-lineage differentiation of equine BM-MSCs

Equine BM-MSCs (n = 4) were exposed to induction media (differentiation) or basal medium (undifferentiated control) in triplicates according to each differentiation assay. Differentiation potential was assessed by specific staining and gene expression of markers for each lineage. Methodology used was previously described by Ranera et al. (2011).

For inducing osteogenic differentiation, 20,000 cells/cm^2 were seeded in 24-well plates. Osteogenic medium consisted of basal culture medium described above supplemented with 10nmol/l dexamethasone, 10 mmol/l β-glycerophosphate and 100 µmol/l ascorbate-2-phosphate (Sigma-Aldrich). After 7 days, cells were fixed with 70%ethanol for 1 h at room temperature (RT), stained with 2%Alizarin Red stain (pH 4.6) (Sigma-Aldrich) for 10′ RT and washed with PBS (Gibco).

Equine BM-MSCs were seeded at 5000 cells/cm^2 in 12-well plates for the adipogenic differentiation with induction medium consisting of 1 µmol/l dexamethasone, 500 µmol/l 3-isobutyl-1-methylxanthine, 200 µmol/l indomethacin and 15% rabbit serum (Sigma-Aldrich) supplemented basal medium. After 15 days, cells were fixed with 10% formalin (Sigma-Aldrich) for 15′ RT, stained with 0,3%Oil Red O stain (Sigma-Aldrich) (dissolved in 60:40; isopropanol:distilled water) for 30′ at 37 °C and washed with distilled water.

To achieve chondrogenic differentiation, approximately 300,000 eBM-MSCs were transferred to conic bottom 15 ml tube, 400 µl of differentiation medium were added and then centrifuged at 1750 rpm 5′ to pellet the cells. Chondrogenic medium consisted of 10%FBS, 10 ng/ml TGFβ-3 (R&D Systems), ITS + premix (Beckton Dickinson), 40 µg/ml proline, 50 µg/ml ascorbate-2-phosphate and 0.1 µmol/l dexamethasone supplemented high glucose DMEM (Sigma-Aldrich). After 21 days, pellets were fixed in 10%formalin, embedded in paraffin and cut into 5 µm sections. The sections were hydrated with increasing gradients of alcohols, stained with Mayer's haematoxylin and 3%Alcian Blue dyes, rinsed with distilled water, dehydrated with decreasing amounts of alcohol and mounted.

Differentiation was also assessed by analyzing the gene expression of the osteogenic markers *Alkaline phosphatase (ALP)* and *Runt-related transcription factor 2 (RUNX2)*, the adipogenic markers *Lipoprotein lipase (LPL)* and *Peroxisome proliferator-activated receptor γ (PPARγ)*, and the chondrogenic markers *Collagen type II alpha I (COL2A1)* and *Aggrecan (ACAN)*. Methodology used for gene expression analysis will be further explained in the next section.

2.3. Real time quantitative polymerase chain reaction (RT-qPCR)

Expression of genes coding for the lineage-associated markers aforementioned and immunogenic markers *MHC-I*, *MHC-II*, *CD40* and *CD80* was analyzed by RT-qPCR. Isolation of mRNA and complementary DNA (cDNA) retrotranscription from all samples were performed with the kit Cells-to-cDNA II (Ambion) according to manufacturer's instructions.

RT-qPCR reactions were performed and monitored with a StepOne RT-PCR System device (Applied Biosystems), using Fast SYBR Green Master Mix (Applied Biosystems) and 2 µl of cDNA as template. Amplification was performed in triplicate as follows: 20'' at 95 °C, followed by 40 cycles consisting of 3''/95 °C and 30''/60 °C. A dissociation curve protocol was run after every reaction. Gene expression levels were obtained using the comparative Ct method. Normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, *GAPDH* and *B2M* (Ranera et al., 2011). Primers were designed with the Primer Express 2.0 software based on known equine sequences and cDNA obtained from equine peripheral blood mononuclear cells (PBMCs) was used as positive control to validate the primers (Remacha et al., 2015). Information about primers is shown in Table 1.

2.4. Statistical analyses

Statistical analyses were performed using the SPSS 15.0 (SPSS Inc.). Normality of each data group was tested with the Shapiro-Wilk test. Differences in the expression level of each gene were assessed between undifferentiated (control) and differentiated cells within each lineage by the non-parametric paired Wilcoxon test. Significance level was set at $P < 0.05$ for all analyses.

3. Results and discussion

Equine MSCs were successfully isolated from BM from all animals and displayed similar phenotype (data not shown) and differentiation