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Behavior of multipotent stem cells isolated in mobilized peripheral blood from sheep after culture with human chondrogenic medium

Carlos Landa-Solís[a,1], Anell Olivos-Meza[b,1], Brenda Olivos-Díaz[a], Adriana Hernández-Rangel[a], Víctor Hugo Cárdenas-Soria[b], Maria Cristina Velasquillo-Martínez[a], José Clemente Ibarra-Ponce de León[a,b,*]

[a] Tissue Engineering, Cell Therapy and Regenerative Medicine Unit, Instituto Nacional de Rehabilitación, Mexico City, Mexico
[b] Orthopedic Sports Medicine and Arthroscopy Service, Instituto Nacional de Rehabilitación, Mexico City, Mexico

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ABSTRACT

Today, regenerative medicine requires new sources of multipotent stem cells for their differentiation to chondrocytes using the mediums of differentiation available in the market. This study aimed to determine whether the Mesenchymal Stem Cells (MSCs) isolated from Mobilized Peripheral Blood (MPB) in sheep using the Granulocyte Colony-Stimulating Factor (G-CSF), have the ability of first acquire a fibroblast-like morphology after being forced out of the bone marrow niche by G-CSF and second, if the cells have the capacity to express collagen type-II α I in primary culture using a human commercial media of differentiation. Six Suffolk male sheep with age of 2 years were mobilized using G-CSF. One subcutaneous injection of 10 mcg per kilogram of bodyweight were administered every 24 h during three consecutive days. At day four, a sample of 20 mL of peripheral blood was harvested, afterwards, monocytes cells were separated by ficoll gradient. The mobilized MSCs were expanded in primary culture in DMEM medium supplemented with 10% adult sheep serum for three weeks and characterized by an antibody panel for surface markers: CD105, CD90, CD73, CD34, and CD45, before and after primary culture. Subsequently, an aliquot of cells in the first pass were cultured in a commercial human chondrogenic medium for three weeks. As a result, the percentage of surface markers for MSCs (CD105, CD90, CD73) in expanded cells in primary culture significantly increased, at the same time a decrease in the markers for hematopoietic cells (CD34 and CD45) was observed and the cells morphology was fibroblast-like. After three weeks of differentiation culture, the immunofluorescence analysis evidenced the expression of collagen-type-II. It was concluded that Mesenchymal Stem Cells isolated from mobilized peripheral blood in sheep have the ability to pre-differentiate into chondral like cells and express collagen type-II when are stimulated with a human commercial chondrogenic medium in monolayer culture.

1. Introduction

Articular cartilage has limited capacity to self-repair and minor lesions in young patients can lead to early osteoarthritic joint degeneration. The advent of new techniques of cartilage repair by tissue engineering has increased in the last twenty years with spread options of cell sources. The field provided by autologous chondrocytes have demonstrated the capability to form hyaline like tissue with the presence of some critical limitations, such as poor quantity of donor cartilage, two surgical procedures, and *in-vitro* expansion with the consequence of cell de-differentiation. Recent reports have enhanced the widespread application of Mesenchymal Stem Cells (MSCs) in tissue engineering due to their plasticity that allows expansion of a sufficient number to repair extensive damaged areas. There are different MSCs sources, the most useful has been the Bone Marrow (BM) specialty in the iliac crest. However, this implies an invasive and painful procedure. Issues like this have given rise to investigators turning toward the isolation of MSCs from peripheral blood by means of treatment with the Granulocyte-Colony Stimulating Factor (G-CSF) (Chao et al., 1993; Schmitz et al., 2005), This is based on the negative modulation of Vascular Adhesion Surface Molecule 1 (VCAM-1) and of nestin inhibition in nestin-positive MSCs localized in the vascular compartment of the BM niche (Hopman

* Corresponding author at: Av México-Xochimilco N.289. Col. Arenal de Guadalupe, CP 14389, México City, Mexico.
E-mail addresses: clanda@inr.gob.mx (C. Landa-Solís), aolivos_meza@hotmail.com (A. Olivos-Meza), olivosbrenda@gmail.com (B. Olivos-Díaz), adrih_rangel@hotmail.com (A. Hernández-Rangel), vcardenassoria@gmail.com (V.H. Cárdenas-Soria), cvelasquillo@inr.gob.mx (M.C. Velasquillo-Martínez), cibarra@inr.gob.mx (J.C. Ibarra-Ponce de León).
[1] Equal contribution.

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and DiPersio, 2014; Sousa et al., 2014). This process favors the MSCs release from the niche and their migration through intramedullary space toward the peripheral blood where then the MSCs are obtained with a less invasive technique than Bone Marrow Aspiration (BMA) (Sahin and Buitenhuis, 2012; Salvucci et al., 2012). The cell mobilization of MSCs to peripheral blood presents other advantages: is a less pain process, permit an ambulatory procedure and diminish the surgical risks.

Two stem cell populations have been identified in the Mobilized Peripheral Blood (MPB): Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs) (Fu et al., 2014; Gallardo et al., 2009). The HSCs population has been characterized by means of surface markers such as CD133 +, CD45 +, CD34 +, and CD38– (Azouna et al., 2011; Jin et al., 2008), while characteristic markers described in MSCs are CD105 +, CD90 +, CD73 +, CD34–, and CD45–(Jin et al., 2008; Rammal et al., 2013; Thomasson et al., 2003; Villa-Diaz et al., 2012). For the population of MSCs their plasticity has been demonstrated on being differentiated in adipocytes, chondrocytes, and osteocytes (Calloni et al., 2014; Jin et al., 2008; Tondreau et al., 2005).

This cell plasticity allows the use of MSCs as a viable option for the development of new technology in cartilage repair. However, any *in-vitro* behavior of mobilized mesenchymal stem cells have been described regarding their capacity to pre-differentiate into cartilage linage when they are stimulated in monolayer culture. The use of G-CSF is not risky and pre-clinical models are necessary to evaluate appropriate dosage and secondary effects for MSCs isolation in peripheral blood which is totally different to the parameters used for HSCs mobilization required in bone marrow transplantation. Due to its anatomical similarity with the human knee joint, sheep have been considered as an ideal model for development of novel technology to repair damaged articular cartilage (Pape and Madry, 2013). The objective of this study was to determine whether the mesenchymal stem cells isolated from Mobilized Peripheral Blood (MPB-MSC) in sheep, after the shook induced by G-CSF, could be acquire a fibroblast-like morphology and pre-differentiate into chondral lineage by expression of collagen-type-II α I in primary culture.

2. Methods

Ethic committee approval was obtained and animal used in this study were care in accordance with institution guidelines. An experimental and comparative study was employed to obtain the objective of this research. All experiments were performed in the research and veterinary facilities at the National Institute of Rehabilitation.

2.1. Animal characteristics

Six male Suffolk sheep with ages of two years and weigh between 60 and 70 kg were included in this study. Sheep were placed and cared by Veterinary Medicine Doctors in the animal facility area. Management of the animals was developed establishing the clinical history of each sheep, describing in detail their state of health and body condition; these data was established on their admittance into the animal facility. All animals received human care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication no.85-23, revised 1985)".

2.2. Mesenchymal stem cells mobilization to the peripheral blood

To increase the number of MSCs in the peripheral blood, a mobilization process was performed with the subcutaneous application of a daily injection of G-CSF (Filgrastim; Amgen, Thousand Oaks, CA, USA) at a dose of 10 μg/kg body weight during three consecutive days. At day-four, Mobilized Peripheral Blood (MPB) was harvested from the jugular vein taking a sample of 20 ml of blood in a syringe previously heparinized (100 IU/mL).

2.3. Mobilized peripheral blood harvesting

In the animal care facility, the harvesting of a 20 ml of mobilized peripheral blood was performed with the sheep standing without sedation due to the procedure was minimally invasive. No discomfort in the animals was observed around the procedure. The area of the jugular groove in the neck was shaved and cleaned for antisepsis with a solution of iodine and 70% ethyl alcohol (the working area was washed three times). All procedures were performed in sterile conditions by the Medical Veterinary Staff. The jugular vein was identified, a 16 G needle connected to a 20-ml heparinized syringe was introduced into the vein and a sample of 20 ml of peripheral blood was taken in every animal. The sample was poured and divided immediately into four sterile tubes 12 x 75 mm embedded with heparin. All tubes were gently shaking before send them to the laboratory for processing. After blood harvesting, sheep returned to its yard where they were monitored by the animal facility staff until recovery.

2.4. Isolation of mononuclear cells

The mononuclear cells present in the mobilized peripheral blood were separated by discontinuous density gradient centrifugation. Into a laminar flow hood (Forma Scientific) every sample was poured in 50 mL polypropylene tubes for centrifugation (1500 *g*). After centrifugation, samples were carried out into 1:2 dilution with buffer phosphate solution (BPS, Gibco Invitrogen) and supplemented with 1% antibiotics/antimycotics (Penicillin 10,000 IU & Streptomycin 10,000 μg). Then new 50 mL polypropylene tubes (cat. #CLS430829, Corning) were prepared adding 15 mL of Ficoll Paque (Amersham Biosciences, Piscataway, NJ, USA). Twenty-five milliliters of diluted blood-PBS sample was poured in every Ficoll tube taking care not to break the surface tension obtaining a final volume of 40 mL. This final dilution was centrifuged at 300 *g* during 35 min. The separated fraction of mononuclear cells in the dilution was taken and aliquoted in 1×10^5 cells samples for characterization by flow citometry and pre-differentiation in monolayer culture.

2.5. Expansion of isolated mononuclear cells

In order to establish their immunophenotype three aliquots with 5×10^3 mononuclear cells of every animal were seeded and expanded in monolayer into 25 cm^2 culture flasks supplemented with Dulbecco's Modified Eagle's Medium (DMEM; Gibco-Life Technologies, USA) enriched with 10% adult sheep serum (SBA; BIO-WEST, Inc. cat. #S4190-100) and 1% antibiotic-antimycotic (Gibco-Life Technologies). The cultures were maintained at 37 °C and 5% of CO_2 during three weeks or when they reached a 90% cell confluence (Passage-1). For immunofluorescence analysis, chambers with 16 wells (Lab-Tek cat.178599) were used seeding 1×10^4 mononuclear cells per well in the same culture conditions, those cultures were expanded during three weeks or when they reached a confluence of 80%.

2.6. In-vitro pre-differentiation of mononuclear cells into chondral linage

The previously expanded mononuclear cells until passaged-1 were subcultured into six well plates (9.5 cm^2; Corning cat. 3516) with a density of 1×10^5 cells per well during three weeks at 37 °C temperature and 5% CO_2. Those cells were supplemented with human chondrogenic medium (Advance Stem Tm Chondrogenic Cat. H01-SH3088902), enriched with grown factors (Advancestemt Stem Cell Growth Supplement, cat H01-SH3087801) and 1% antibiotic-antimicotic (Gibco Life Technologies, EU).