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Immunological properties of umbilical cord blood-derived mesenchymal stromal cells

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ABSTRACT

Mesenchymal stromal cells (MSCs) are promising candidates for developing cell therapies for intractable diseases. To assess the feasibility of transplantation with human umbilical cord blood (hUCB)-derived MSCs, we analyzed the ability of these cells to function as alloantigen-presenting cells (APC) *in vitro*. hUCB-MSCs were strongly positive for MSC-related antigens and stained positively for human leukocyte antigen (HLA)-AB and negatively for HLA-DR. When treated with interferon (IFN)-γ, the expression of HLA-AB and HLA-DR, but not the co-stimulatory molecules CD80 and CD86, was increased. hUCB-MSCs did not provoke allogeneic PBMC (peripheral blood mononuclear cell) proliferation, even when their HLA-molecule expression was up-regulated by IFN-γ pretreatment. When added to a mixed lymphocyte reaction (MLR), hUCB-MSCs actively suppressed the allogeneic proliferation of the responder lymphocytes. This suppressive effect was mediated by soluble factors. We conclude that hUCB-MSCs can suppress the allogeneic response of lymphocytes and may thus be useful in allogeneic cell therapies.

1. Introduction

MSCs are multipotent cells found in fetal liver, bone marrow (BM), and cord blood [1,2], and have the capacity to differentiate *in vitro* into several mesodermal (bone, cartilage, tendon, muscle, and adipose), endodermal (hepatocyte), and ectodermal (neurons) tissues [3]. Classically, MSCs are defined as being able to adhere to plastic, expressing CD29, CD73, CD44, CD90, CD105, and major histocompatibility complex type [MHC] class I antigens, and not expressing the hematopoietic cell markers CD34, CD45, and MHC class II antigen. MSCs constitutively secrete a large number of cytokines and promote the expansion and differentiation of hematopoietic stem cells (HSCs) [4]. Furthermore, MSCs are not immunogenic, namely, they do not induce allogeneic lymphocytes to proliferate *in vitro* [5]. Indeed, MSCs appear to suppress these allogeneic proliferative responses. For example, in *in vivo* trials, co-infusion of MSCs as a third party delayed donor cell rejection, even when immunosuppressant drugs were not used [6]. These characteristics make MSCs potent candidates for the development of allogeneic cell-based therapeutic strategies. The therapeutic efficacy of MSCs for bone, joint, and neuronal diseases has recently been reported [7,8].

BM is the main source of MSCs and BM-MSCs have already been used in various clinical studies. However, the number of MSCs in the BM and their multi-lineage differentiation capacity decline with age [9]. Therefore, human umbilical cord blood is often used as an alternative source of stem or progenitor cells, namely, HSCs and MSCs [10]. hUCBs also have a practical advantage over BM-derived MSCs in that they are obtained by non-invasive methods that do not harm either the mother or the infant [11]. Furthermore, cord blood stem cells are more immature than adult MSCs and expand readily *in vitro* [12]. Given these features along with their potent differentiation potential [3], hUCB-MSCs are an attractive source for cellular or gene transfer therapy. Moreover, a female patient with chronic spinal cord injury who was given hUCB-MSCs showed functional and morphological improvement [13]. The transfer of hUCB-MSCs was also useful in treating Buerger's disease [14].

The aim of the present study was to investigate the immunological properties of hUCB-MSCs to assess their potential usefulness in allogeneic transplantation. We examined the effect of IFN-γ pretreatment on the expression of MHC molecules on the hUCB-MSC cell surface and assessed whether hUCB-MSCs can provoke an *in vitro* allogeneic reaction. We also asked whether hUCB-MSCs, like other MSCs [15,16], can inhibit allogeneic MLR and mitogen-induced lymphocyte proliferation. In addition, we examined whether these immunological properties of hUCB-MSCs change after they differentiate into various lineages.

2. Materials and methods

2.1. Isolation and culture of hUCB-MSCs

This study was approved by the Institutional Review Board of Medipost Inc., Seoul, Korea. The hUCB-MSCs were separated and

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maintained as described previously [3]. Briefly, hUCB samples were collected from the umbilical vein of deliveries with informed maternal consent. Mononuclear cells were isolated from the hUCBs by centrifugation through a Ficoll-Hypaque gradient (density 1.077 g/cm^3, Sigma, St. Louis, MO, USA). The separated mononuclear cells were washed, suspended in α-minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), and seeded at a concentration of 5×10^6cells/cm^2. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 with a twice weekly change of culture medium. One to 3 weeks later, when the monolayer of fibroblast-like adherent cells colonies had reached 80% confluence, the cells were trypsinized (0.25% trypsin, HyClone), washed, resuspended in culture medium (α-MEM supplemented with 10% FBS) and sub-cultured.

2.2. Neurogenic differentiation

Poly-D-lysine- and laminin (Sigma)-coated plates were used to induce neuronal differentiation. Initially, hUCB-MSCs were pretreated for 2 days in DMEM/10% FBS with 20 ng/ml basic fibroblast growth factor (R&D System, Inc., Minneapolis, MN, USA). Neuronal differentiation was then induced by replacing this medium with neurogenic medium composed of DMEM/F12 medium supplemented with 200 μM butylated hydroxyanisole, 25 μg/ml insulin (Sigma), 25 mM KCl, 2 μM valproic acid (Sigma), 10 μM forskolin (Sigma), and 1 μM hydrocortisone (Sigma). After 72 h of differentiation, neurogenesis was evaluated by measuring the expression of MAP-2 and NeuN by Western blot analysis.

2.3. Chondrogenic differentiation

hUCB-MSCs were induced to differentiate into chondrogenic cells as described previously [3]. After 3 weeks of differentiation in chondrogenic medium [high-glucose DMEM (Gibco BRL) supplemented with 100 nm dexamethasone (Sigma), 50 mg/mL L-ascorbic acid (Sigma), 100 mg/mL sodium pyruvate (Sigma), 40 mg/mL L-proline (Sigma), 10 ng/mL transforming growth factor-β3 (TGF-β3, Sigma), 500 ng/mL bone morphogenic protein 6 (BMP-6, R&D Systems, Minneapolis, MN, USA) and 50 mg/mL ITS$^+$ premix (6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL BSA, and 5.35 mg/mL linoleic acid, Becton Dickinson)], chondrogenesis was evaluated by safranin O staining. To dissect the cells into single cell suspensions, chondrogenic pellets were incubated for 1 h in dissection solution (DMEM medium containing 0.2% collagenase type I) and then pipetted vigorously until single cell suspensions were achieved.

2.4. MLRs

To assess T-cell reactivity against allogeneic cell populations, human responder PBMCs (1×10^5/well) were cocultured with inactivated allogeneic PBMCs (1×10^5/well) or hUCB-MSCs (1×10^2, 1×10^3 or 1×10^4/well) in 96-well tissue culture plates. The PBMCs were purchased from AllCells, LLC (CA, USA). The stimulator PBMCs and hUCB-MSCs were inactivated by treatment with 10 μg/ml mitomycin-C (Sigma) for 1 h at 37 °C. In some experiments, responder PBMCs were cocultured with allogeneic stimulator PBMCs in 96-well tissue culture plates at 1×10^5/well in the presence or absence of 1×10^2, 1×10^3 or 1×10^4/well preplated hUCB-MSCs. Due to the large size of hUCB-MSCs, these cells were not cultured at the same ratio with the PBMC responder cell (1×10^4 hUCB-MSCs in a 96-well plate well are 80% confluent). For experiments using transwell chambers (BD Bioscience), PBMCs from two HLA-type-mismatched individuals were seeded at 1×10^5/well in the presence or absence of 1×10^3 or 1×10^4 hUCB-MSCs. The hUCB-MSCs were separated from the PBMCs by a high density pore membrane (transwell chamber, BD Bioscience) by being added to the upper compartment. T-cell proliferation to alloantigens was determined by adding 20 μl of bromodioxyuridine (BrdU) after 6 days of MLR culture (the final BrdU concentration was 10 μM). The tissue culture plates were then incubated at 37 °C in 5% CO_2 for an additional 18 h. Thereafter, the tissue culture plates were centrifuged at 300*g* for 10 min, the substrate was added as described by the manufacturer's instructions and colorimetric immunoassays were immediately used to measure the BrdU that had been incorporated during DNA synthesis, which estimates cell proliferation (Roche, Boerhringer Mannheim). Thus, the absorbance of the samples was measured in an UV max kinetic microplate reader (Molecular Device) at 370 nm (reference wavelength: 492 nm). The data of three replicates are shown as means and standard deviation (SD).

2.5. Mitogen-induced proliferation assay

PBMCs were seeded in triplicate at a concentration of 1×10^5/50 μl/well in 96-well plates with 5 or 10 μg/ml phytohaemagglutinin (PHA, Sigma). RPMI 1640 medium (50 μl/well) or hUCB-MSCs (1×10^2, 1×10^3 or 1×10^4/well) were added. After 3 days of incubation, 20 μl BrdU was added for 18 h. In the experiments using transwell chambers, 1×10^5 PBMCs per well were added to the lower compartment and then 1×10^3 or 1×10^4 MSCs were added to the upper compartment of the transwell chamber. All studies were performed using HLA-unmatched donor populations of hUCB-MSCs and hPBMCs.

2.6. Phenotypic analysis

hUCB-MSCs were stained with various combinations of saturating amounts of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD45-FITC, CD34-FITC, CD31-FITC, CD14-FITC, HLA-DR-FITC (Becton Dickinson, San Jose, CA, USA), CD29-PE, CD44-PE, CD73-PE, CD90-PE, CD166-PE, HLA-AB-PE, CD80-FITC, CD86-PE, isotype-matched control (Pharmingen, Los Angeles, CA, USA), and CD105-PE (Serotec, NC, USA). At least 10^4 events were analysed by flow cytometry (FACScan, BD Biosciences) with the cellquest software.

2.7. Cytokine ELISA

The IFN-γ, transforming growth factor-beta (TGF-β), interleukin (IL)-2, IL-10, IL-1α, IL-1β, and tumor necrosis factor (TNF)-α levels in the conditioned supernatants obtained from MLR and proliferation assays were measured by enzyme-linked immunoassays (R&D system) according to the manufacturers' instructions.

2.8. Statistics

Statistical analyses of data were performed by the Student's *t*-test to determine statistical significance. Values are given as means ± SD (standard deviation).

3. Results

3.1. Characterization of the hUCB-MSCs

MSCs were successfully isolated from three hUCBs and could be separately cultured until passage 4. As reported previously [3], the hUCB-MSCs had a fibroblast-like morphology (Fig. 1A). Three different hUCB-MSC clones were uniformly positive for MSC-related antigens, namely, CD73 (96.1 ± 2.3%), CD105 (91.3 ± 4.1%), CD90 (96.8 ± 1.5%), CD166 (93.1 ± 3.5%), CD29 (91.1 ± 6.7%), and CD44 (92.1 ± 5.1%) (Fig. 1D). In addition, these cells were positive for HLA-class I (HLA-AB;