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Expanded cryopreserved mesenchymal stromal cells as an optimal source for graft-versus-host disease treatment

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ABSTRACT

Mesenchymal stromal cells (MSC) are fibroblast-like cells present in different types of tissues. Their immunomodulatory potential represents a promising method for post-transplant immunotherapy in the treatment of GVHD (graft-versus-host disease) with suboptimal response to standard immunosuppression. In this study we tested influence of 1–8 month-long cryopreservation on ability of MSC to suppress activation of non-specifically stimulated lymphocytes.

We did not observe any changes in proliferation capacity of MSC after thawing. Lymphocytes metabolic activity was inhibited by 30% and number of dividing cells was three times smaller in the presence of MSC. Two activation markers were studied (CD25 and CD69) to confirm preservation of functional cell integrity. Expression of CD25 antigen on $CD3^+CD4^+$ and $CD3^+CD4^-$ cells was decreased in all co-cultivated samples. Level of CD69 expression on $CD3^+CD4^+$ cells was lower in samples with added MSC (10–15% on day +2) but without reaching statistical significance. The lower expression (approximately 5%) was observed also on CD4-cells.

The study confirms the preservation of immunomodulatory properties of cryopreserved and re-expanded MSC. Aliquots with cryopreserved cells can represent an optimal source for a quick preparation of MSC cell product with the possibility to apply the same cells repeatedly.

1. Introduction

Mesenchymal stromal cells (MSC) are non-hematopoietic stem cells with a multi-lineage potential first identified by Friedenstein et al., in 1976 [1]. These fibroblast-like cells are present in different types of tissues like bone marrow, adipose tissue [2], cord blood [3], dental pulp [4] and others. MSC can be distinguished from hematopoietic cells by being positive for cell surface markers like CD73, CD90, and CD105, whereas being negative for CD45, CD34, CD14, and HLA-DR [5]. They are capable to differentiate into fat, bone, cartilage [6] or even neural cells [7]. A further important function is the modulation of the immune response [8,9]. The interaction of MSC and the immune system is complex and includes a number of mechanisms. Their influence on immune cells is a combination of direct cell-to-cell interactions and soluble factors production. An example of a cell-to-cell modulation of immune cells is the inhibition of lymphocyte proliferation by engagement of the inhibitory molecule programmed death 1 (PD-1) to its ligands PD-L1 and PD-L2 leading to activation of programmed cell death [10]. MSC-dendritic cell interaction increases the production of anti-inflammatory IL-10 which is associated with a higher presence of T_{REG} lymphocytes [11]. MSC also express indoleamine 2,3-dioxygenase (IDO) – catalyst of tryptophan conversion to kynurenine – which was identified as a major immunosuppressive effector of the pathway that inhibits T-cell responses to auto- and alloantigens [12]. They can also inhibit two of the most important proinflammatory cytokines – TNF-α and IFN-γ [13] and thereby suppress the immune system. The above mentioned and/or other mechanisms can change the inflammatory environment to immune tolerant. It looks suitable the use of the immunosuppressive activity of MSC to the treatment of autoimmune diseases [14,15] as well as of graft-versus-host-disease (GVHD), an important complication after allogeneic hematopoietic cells transplantation [16]. GVHD is

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caused by an alloreactive interaction between the donor's immune system and the recipient's tissues. Severe forms of GVHD are a major source of morbidity and transplant-related mortality. The disease and its long-term treatment with corticosteroids reduce quality of life post-transplant and diminish the outcome of transplantation therapy. Especially GVHD poorly responding to a standard immunosuppressive treatment indicates a major problem in allogeneic transplantation. The application of mesenchymal cells and utilization of their immunomodulatory potential represent a promising method for post-transplant immunotherapy in the treatment of GVHD with suboptimal response to immunosuppressive therapy. The use of immunomodulatory potential of MSC is subject of several clinical trials.

The aim of this study is to prove the immunomodulatory potential of cryopreserved and re-expanded MSC, and to emphasize the advantages of this approach to MSC preparation for their application in clinical trials for steroid-refractory GVHD treatment.

2. Methods

2.1. Mesenchymal stromal cell cultivation and cryopreservation

MSC were isolated from bone marrow of healthy donors using the gradient centrifugation technique (LSM 1077 medium, PAA, USA) followed by the separation of MSC by adherence to plastic (175 cm^2 culture flask; Corning, USA). Non-adherent cells were washed out with PBS (PAA) after 48 h. The cells were growing for 2–3 weeks in complete culture medium (alpha-MEM without Nucleosides with L-Glutamine (PAA, USA), 10% pHPL (pooled human platelet lysate, local source) with addition of 560 IU heparin (Biochrome, Germany) until 80–90% confluence. The medium was changed every 2nd day. Then the cells were released with TrypLE-Select solution (Life Technologies – Gibco, CA, USA) and re-seeded at concentration 1×10^6 on 175 cm^2 flasks. Cell numbers were determinate using hematology analyser (Coulter AcT diff2, Beckman Coulter, USA). The subculturing was repeated two times and then at 3rd passage the cells were cryopreserved. The cells were frozen at concentration 1×10^6 cells in 1 ml cryomedium using programmable controlled rate freezer. The cryomedium was prepared by a combination of 60% alpha-MEM medium, 30% pHPL and dimethylsulphoxide at 10% (CryoSure-DMSO, WAK-Chemie, Germany), as the most widely used cryoprotectant [4,17]. The freezing rate was set at 1 °C/min to −80 min like in other protocols for freezing of mammalian cells [18–20]. Cryotubes with frozen cells were immediately transferred into liquid nitrogen for long term storage.

Five different MSC (from different donors) were thawed after 1,3,6,7 and 8 months. The cells from one aliquot containing 1×10^6 cells were seeded into two 175-cm^2 culture flasks. Fresh medium was replaced after 24 h and then every 2nd day. MSC were re-cultivated until reaching 80–90% confluence, usually in 5–8 days. The samples tested of sterility and for presence of *Mycoplasma* were collected before the cryopreservation and during the last medium exchange in defrost culture.

2.2. Immunophenotype and viability determination

The cells were stained for typical immunophenotype at each passage. A mix of antibodies 1) CD45-Horizon V500 (BD Bioscience, USA), CD19-Pacific Blue, CD105-FITC (Exbio, CZ), CD73-PE, CD90-APC (both Biolegend, USA), CD34-PECy7 (Immunotech, USA) and 2) CD45-Horizon V500 (BD Bioscience, USA), HLA-DR-Pacific Blue, CD13-FITC, 7-AAD, CD14-APC (all Immunotech) was added into 100 μl of cell suspension. The washing with PBS 5 min/1500 RPM followed after 15 min of incubation in the dark (room temperature). The samples were analyzed on FACSCanto II flow cytometer using FACSDiva Software (both BD Bioscience).

2.3. Preparation of PBMC samples

PBMC (peripheral blood mononuclear cells) of healthy donors were isolated by gradient centrifugation (Histopaque – 1077, Sigma, USA) and diluted with cultivation medium RPMI 1640 (Lonza, Switzerland) to final concentration 1×10^6 cells/ml. Eighteen different samples were used in this study.

2.4. Metabolic activity

PBMC suspension (100 μl with 10^3 cells) was stimulated by addition of 5 μl phytohemagglutinin (PHA, Sigma–Aldrich, USA) at the concentration of 1 μg/ml. The control well contained stimulated PBMC only and the experiment well was prepared by mixing PBMC with 10 μl of MSC suspension at the concentration of 10^4/ml. 50 μl of MTT (soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-difenyl tetrazolium bromide, Sigma, USA) and 10 μl of RPMI 1640 were supplemented. The cells were cultivated at 37 °C in 5% CO_2 for 24 h. Then solution in wells was outpoured by gently flip and 400 μl the solvent (1 DMSO: 1 absolute alcohol) was added for 3 h (37 °C, gently shaking). The absorbance was measured thereafter on MRX II (Dynex, CZ). Unstimulated PBMCs with MSC addition were used as a control.

2.5. Cell proliferation

BD Cycletest™ Plus DNA Reagent Kit (BD Bioscience) was used to determinate dividing cells. Peripheral blood (100 μl) was stimulated with 500 μl PHA (concentration 2.5 μg/ml, Sigma–Aldrich). MSC were added at the concentration of 10^5/ml. RPMI 1640 (Lonza) was supplied to the final volume of 2 ml. The cells were incubated at 37 °C in 5% CO_2 for 72 h. The staining procedure was done according to the manufacturer's recommendations. The samples were measured using flow cytometer Cytomics FC 500 (Beckman Coulter, USA). The data were analyzed by Multicycle AV software (Phoenix Flow System, USA). The proportion of dividing cells was defined as the sum of cells in S and G2/M phases of cell cycle.

2.6. Analysis of activation markers

MSC were mixed with nonspecific stimulated or unstimulated (control group) PBMC (10 μl of phytohemagglutinin at the concentration of 1 μg/ml; PHA, Sigma–Aldrich, USA) in a ratio 1:2 and co-cultivated in 2 ml of RPMI 1640 (Lonza) with 10% pHPL for 5 days. The flow cytometry analysis of CD69, CD25 and HLA-DR on $CD3^+$ cells was performed on day +1 up to +4. 200 μl of mixed cell suspension was incubated with CD45-Horizon V500 (BD Bioscience), CD4-FITC, CD25-APC, CD69-PE (all Exbio), CD3-PerCP and HLA-DR-PB (both Immunotech) for 10 min/RT. The stained suspension was washed with 2 ml of PBS and centrifuged for 5 min/1500 RPM. The samples were immediately measured on BD FACSCanto II using FACSDiva Software (both BD Bioscience). The proportion of activation marker expression on $CD3^+CD4^+$ and $CD3^+CD4^-$ cells was analyzed using FlowJo software (Tree Star, USA).

2.7. Statistical analysis

A non-parametric Wilcoxon test was used to investigate statistical significance in differences of proliferation, in metabolic activity, and in the level of activation markers between groups with and without the presence of MSC. The kinetics of activation