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Multilineage potential of adult human mesenchymal stromal cells derived from bone marrow of patients with polycytaemia vera

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Abstract: Human mesenchymal stromal cells were isolated from the bone marrow of patients with polycyteamia vera (the myeloproliferative disorder) with the aim to characterize the properties of the mesenchymal stromal cells originating from the pathologically affected bone marrow. Their *in vitro* growth and potential to differentiate were determined. Isolated mesenchymal stromal cells were able to differentiate into three mesenchymal lineages under appropriate cultivation conditions.

Key words: mesenchymal stromal cells; polycytaemia vera; multilineage differentiation.

Abbreviations: ahMSCs, adult human mesenchymal stromal cells, BM, bone marrow; MSCs; mesenchymal stromal cells; PV, polycytaemia vera.

Introduction

Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic progenitor cells capable to differentiate *in vivo* and *in vitro* into multiple lineages of the mesenchyme, including bone, cartilage, tendon, muscle, adipose tissue and possibly bone marrow stroma. In recent years these cells came to prominence for their potential use in regenerative medicine of connective tissues, tissue engineering, correction of genetic disorders and hematopoietic engraftment enhancement or prevention of graft versus host disease (Le Blanc & Ringdén 2007). Autologous transplantation of adult human MSCs (ahMSCs) derived from bone marrow (BM) is well established technique in traumatology, orthopedics and maxillo-facial surgery practice in our hospital.

BM remains the primary source of MSCs, but they have been found and isolated from a wide variety of tissues like neural (Gage 2000), epidermal (Watt et al. 2006), gastrointestinal (Marshman et al. 2002), skeletal and cardiac muscle (Seale & Rudnicky 2000; Hughes 2002) liver (Forbes 2002), lung and pancreatic tissue (Bonner-Weir & Sharma 2002; Otto 2002), fat tissue (De Ugarte et al.2003), deciduous teeth (Miura 2003), periosteum (Nakahara et al. 1990), trabecular bone (Noth et al. 2002), synovium (DeBari et al. 2001), tendon (Young et a1. 1998), cord blood (Pittenger et al. 1999) and placenta (Parolini et al. 2008). The ahMSCs for therapeutic use are mostly isolated from the bone marrow aspirated from the iliac crest. Even if they represent a small fraction of the mononucleated cells population 0.001–0.01% (Pittenger et al. 1999), it is possible to obtain an adequate quantity of MSCs for further *in vitro* multiplication by standard cell culture techniques. According to the International Society for Cellular Therapy criteria for defining multipotent mesenchymal stromal cells, the MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Dominici et al. 2006). The *in vitro* differentiation of BM-derived ahMSCs into osteoblastic, chondrogenic and adipogenic lineage was described by several working groups (Bruder et al. 1998; Johnstone et al. 1998; Digirolamo et al. 1999; Pittenger et al. 1999; Murgalia et al. 2000; Barry et al. 2001), where the different growth factors and their combinations were used to achieve desired phenotype.

The aim of our study was to evaluate the differentiation potential of MSCs derived from the BM of patients with polycytaemia vera (PV). PV is a non-

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Table 1. Patients characteristics.

Patient (n)	Sex	Age	Diagnosis
1	F	54	PV
2	M	69	PV
3	M	60	PV
4	M	29	PV

leukaemic chronic myeloproliferative disorder with the overlapping erythropoietin independent expansion of erythropoietic components in the BM, which subsequently became hyperplastic (Hoffbrand & Petit 2000). PV usually affects older individuals; 4–7% of patients are younger than 40 years (Perea et al. 2001). Our results show that MSCs derived from PV-afflicted BM maintain their differentiation potential.

Material and methods

BM procurement and culture expansion of stromal cells
BM was obtained under the informed consent from 4 patients with supposed PV from diagnostic BM samples to perform clonogenic assay as diagnostic procedure which confirmed the suspected patients diagnosis (Table 1).

Human MSC isolation
BM was harvested from iliac crest under the local anaesthesia. Approximately 1 mL of aspirate was immediately mixed with 2 mL of Iscove's modified Dulbecco's medium containing 44% of ACD solution, 9% of fetal calf serum, 1% of gentamycin and 2% of penicillin, streptomycin and amphotericin B solution (Gibco BRL, U.K.). Mononucleated cells were subsequently isolated by gradient centrifugation (Ficoll-Paque, Amersham Biosciences AB, Sweden) at $400\times g$, 25 min, 4 °C. Isolated cells were washed in Dulbecco's modified Earle's medium and resuspended in minimum essential medium (both from Biochrom AG, Berlin, Germany) supplemented with 20% fetal calf serum and 1% of antibiotic/antimycotic solution at a density 1×10^5/mL and seeded into 25-cm^2 flask (Sarstedt AG & Co., Nümbrecht, Germany). Cells were cultured at 37 °C in atmosphere of 5% CO_2. After 24 hours of cultivation, non-adherent cells were washed out. Fresh medium was replaced every third day of cultivation. At 70% monolayer confluence, the MSCs were enzymatically detached using 0.25% trypsin-EDTA (Gibco BRL, U.K.). Single cell clones were obtained by the limiting dilution and cultivation at the 6 well tissue culture plates (Sarstedt AG & Co., Nümbrecht, Germany). After enzymatic dettachment the harvested clones of MSCs were seeded into 8 well Nunc Lab-TekTM II-Chamber SlideTM System (NUNC A/S, Roskilde, Denmark) at cell density 20 000 cells/well.

In vitro differentiation of PV BM derived MSCs
Twenty-four hours after seeding the monolayers were washed with Dulbecco's modified Earle's medium and treated according to Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Inc., Minneapolis, MN, USA) which contains specially formulated media supplements to effectively differentiate MSCs into osteogenic, adipogenic and chondrogenic lineages. For the adipogenic and osteogenic differentiation, minimum essential medium was supplemented with fetal calf serum (10%), antibiotic/antimycotic solution (1%) and growth factors (hydrocortisone, isobutylmetylxanthine, indomethacin and dexamethasone, ascorbate phosphate, β-glycerol phosphate, respectively); chondrogenic differentiation medium Dulbecco's modified Earle's medium/F-12 medium (Gibco BRL, U.K.) contained 1% insulin-transferin-selenium, 1% of antibiotic/antimycotic solution and growth factors (dexamethasone, ascorbate phosphate, proline, pyruvate and transforming growth factor-β3). The *in vitro* differentiation was completed at the 21st day.

Phenotype confirmation of MSCs and differentiated cells
Non-differentiated BM-derived MSCs were evaluated morphologically. The expression of CD105 (endoglin) and the absence of the surface HLA-DR expression were detected by immunofluorescent staining. For CD105 immunofloresccent staining cells were fixed with 3.7% formaldehyde for 30 min and non-specific sites were blocked overnight with 5% normal goat serum and permeabilized with 0.05% tween20. Mouse monoclonal antibody against human endoglin (DakoCytomation, USA) was used for antigen detection. HLA-DR immunocytochemistry was performed after the fixation of cells with 4% paraformaldehyde and blocked with 1% BSA in PBS overnight. Primary purified mouse anti-human HLA-DR (DakoCytomation, USA) antibody was used for searching of HLA-DR existence.

The osteoblastic differentiation was confirmed by Von-Kossa staining and by immunofluorescent staining of osteocalcin and osteonectin. For Von Kossa staining, the cells were fixed with 10% formol for 30 min at room temperature, washed with deionized water and stained with 5% silver nitrate for 30 min, subsequently washed with deionized water and developed with 5% sodium carbonate dissolved in 25% formol. The fotochemic reaction was stopped with sodium thiosulphate (all from Merck, Germany). For immunofluorescent staining of osteocalcin and osteonectin, cells were fixed with 0.5% glutaraldehyde for 15 min and blocked overnight with 1% BSA in PBS. Mouse monoclonal antibodies against human osteocalcin and osteonectin (R&D Systems, Inc., Minneapolis, MN, USA) were used.

Adipocytic cell phenotype was proved by oil-red staining with 0.5% Oil Red (Sigma) dissolved in 60% isopropyl alcohol; cell monolayer was incubated with the dye for 60 min at room temperature.

Differentiation of MSCs to chondrocytes were demonstrated by the detection of aggrecan produced by differentiated chondrocytes. Cells were fixed with 4% paraformaldehyde in PBS, blocked for 45 min at room temperature with blocking buffer containing 10% normal donkey serum, 1% BSA and 0.3% triton-X. Cells were incubated overnight with primary goat-anti human aggrecan antibody and after washing, the secondary fluorescein isothiocyanate conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc., USA) was used.

In all immunofluorescent staining procedures performed the primary antibodies bound to antigens were visualized by secondary fluorescein isothiocyanate conjugated goat anti-mouse antibody (BD Biosciences, USA).

Results

Morphological evaluation of non-differentiated ahMSCs
Non-differentiated PV BM-derived ahMSCs were evaluated morphologically (Fig. 1). The typical spindle-shaped morphology of PV BM-derived ahMSCS was

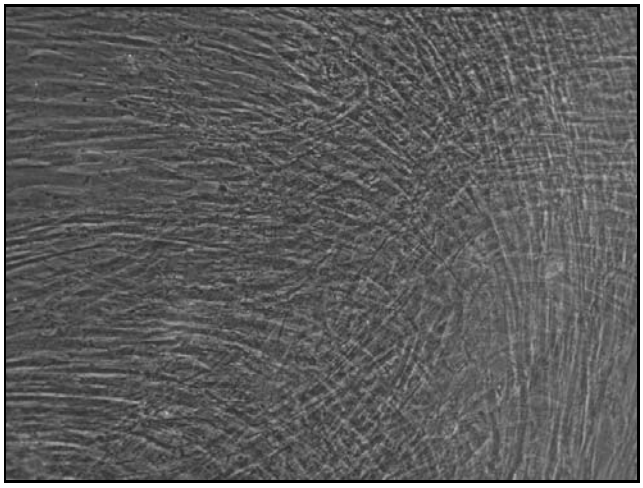

Fig. 1. Confluent monolayer of non-differentiated MSCs. Typical fibroblast-like cellular shape. Magnification 200×, phase-contrast native picture photograph, 21st day of culture.

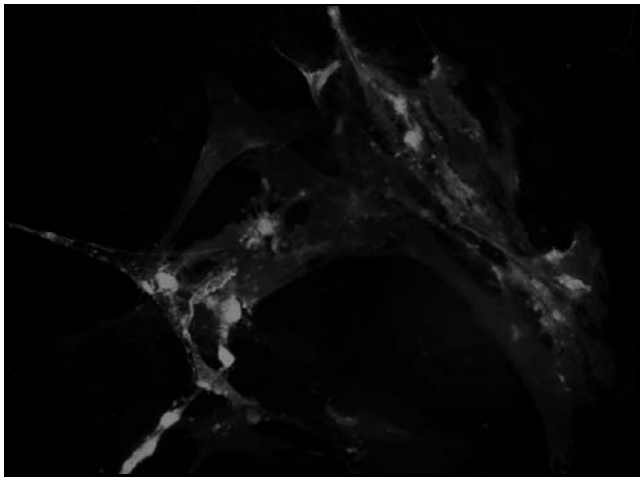

Fig. 2. Non-differentiated MSCs. Positive immunocytochemistry for CD105. Magnification 400×, 21st day of culture.

Fig. 3. Non-differentiated MSCs. Negative immunocytochemistry for HLA-DR. Magnification 200×, 21st day of culture.

maintained, as is characteristic for MSCs of healthy BM donors.

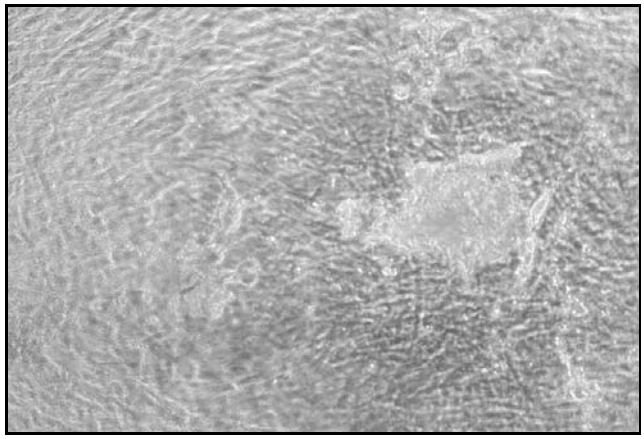

Fig. 4. Monolayer of differentiated osteocytes with extracellular calcium-matrix deposits over the monolayer. Magnification 100×, phase-contrast native picture photograph, 21st day of culture.

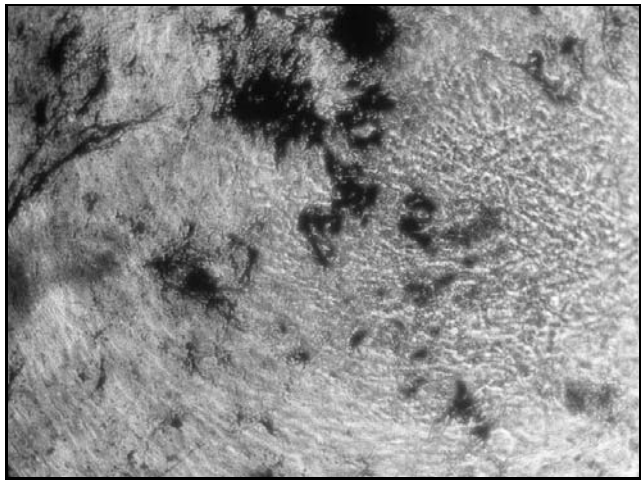

Fig. 5. Differentiated osteocytes and positive Von Kossa staining of extracellular calcium-matrix deposits. Magnification 200×, phase-contrast photograph, 21st day of culture.

Immunofluorescent staining for CD105 and HLA-DR antigens

MSCs cultured without any differentiating factors added to cultivation medium (minimum essential medium supplemented with 20% fetal calf serum and 1% of antibiotic/antimycotic solution) were immunofluorescently stained for endoglin (Fig. 2) and compared to the negative control. Cells stained for HLA-DR antigen were negative (Fig. 3) as defined in the International Society for Cellular Therapy criteria for MSCs.

Osteogenic differentiation

MSCs cultured in the presence of osteogenic medium acquired osteoblastic morphology and started to produce an extracellular matrix containing calcium (Fig. 4). Extracellular deposits of calcium and its salts were stained according to Von Kossa staining method (Fig. 5), the indirect method for identifying the process of mineralization in cell culture, marking the location of calcium in extracellular deposits. Osteblastic phenotype of differentiated cells was also confirmed by im-

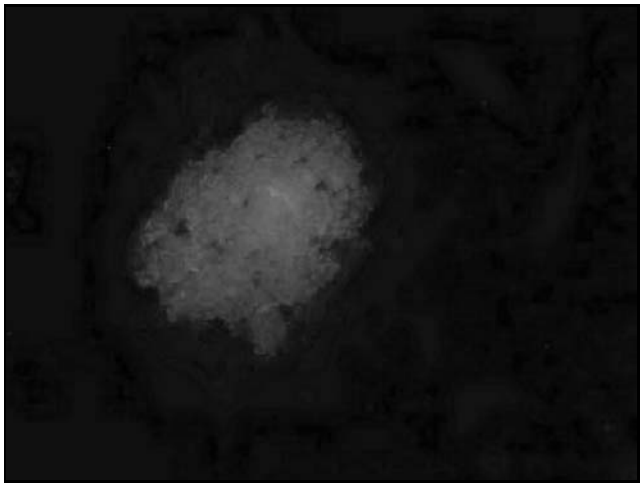

Fig. 6. Differentiated osteocytes, positive immunocytochemistry for osteocalcin in extracellular calcium-matrix deposits. Magnification 200×, 21st day of culture.

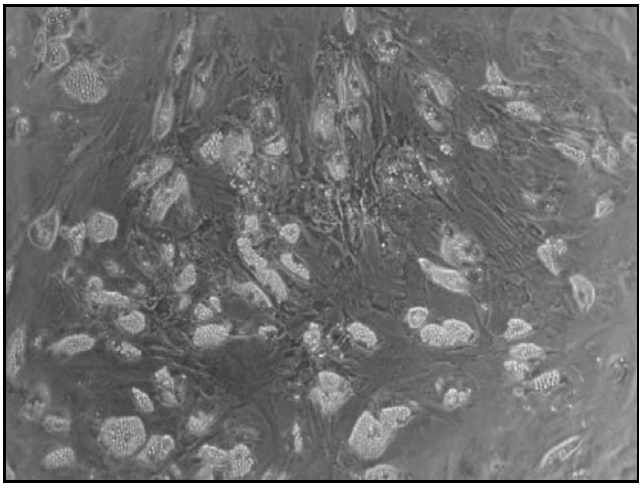

Fig. 8. Monolayer of differentiated adipocytes with fat-filled vacuoles inside adipocytes. Magnification 100×, phase-contrast native picture photograph, 21st day of culture.

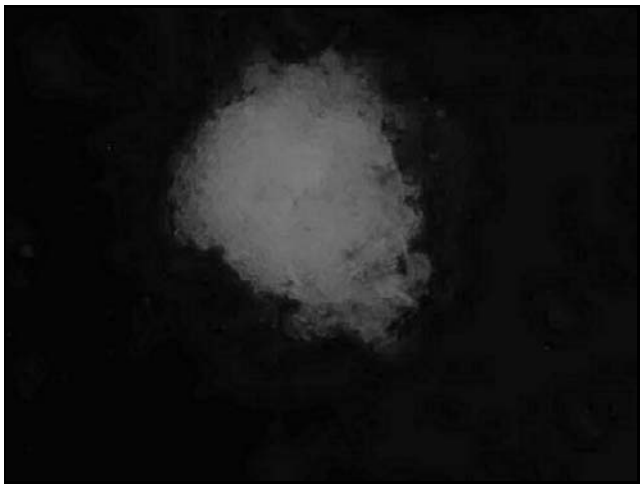

Fig. 7. Differentiated osteocytes, positive immunocytochemistry for osteonectin extracellular calcium-matrix deposits. Magnification 200×, 21st day of culture.

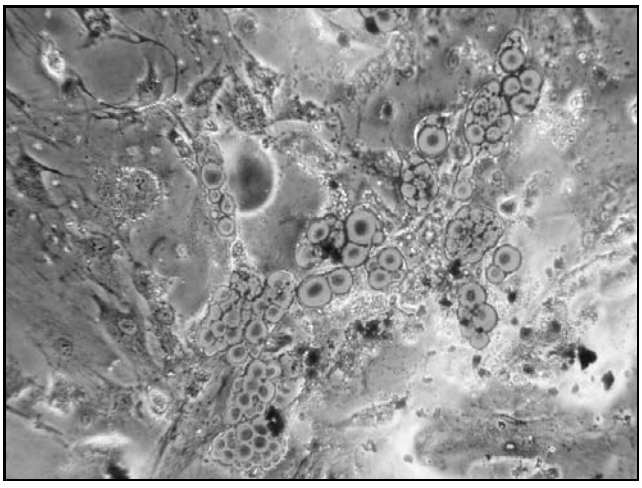

Fig. 9. Differentiated adipocytes with red stained lipid vacuoles (positive oil-red staining). Magnification 400×, phase-contrast photograph, 21st day of culture.

munofluorescent staining by using monoclonal antibodies against osteocalcin (Fig. 6) and osteonectin (Fig. 7), proteins secreted by osteoblasts, considered as biomarkers of the bone formation process.

Adipogenic differentiation
Differentiated adipocytes began to produce lipid-filled vacuoles in their cytoplasm within 2–5 days, which is the typical feature of the adipocytes morphology (Fig. 8). Lipids and fatty acids in vacuoles of adipocytes were coloured red with oil-red dye (Fig. 9).

Chondrogenic differentiation
Morphological evaluation of isolated MSCs under the influence of chondrogenic differentiation factors in monolayer culture revealed very rapid loss of their fibroblastic morphology within 24 hours (Fig. 10). The presence of aggrecan in differentiated chondrocytes culture was apparent after its immunocytochemical staining (Fig. 11). Aggrecan is an extracellular matrix proteoglycan specifically produced by chondrocytes.

Discussion

In the presented work we used a small portion of the BM aspirates taken from the patients for colony forming unit evaluation. MSCs isolated from these samples were *in vitro* expanded and evaluated for their ability to differentiate into cell types of three mesenchymal lineages. In PV, genetic alterations are thought to influence a hematopoietic stem cell in terms of its behavior. Progenitor cells produce more peripheral blood cells and increased number of erythroid cells, often with the rise of granulocytes and megakaryocytes (Fernandez-Luna et al. 1998; Hoffbrand & Petit 2000).

The aim of our study was to characterize the differential potential of the ahMSCs derived from the BM containing the pathologically changed components.

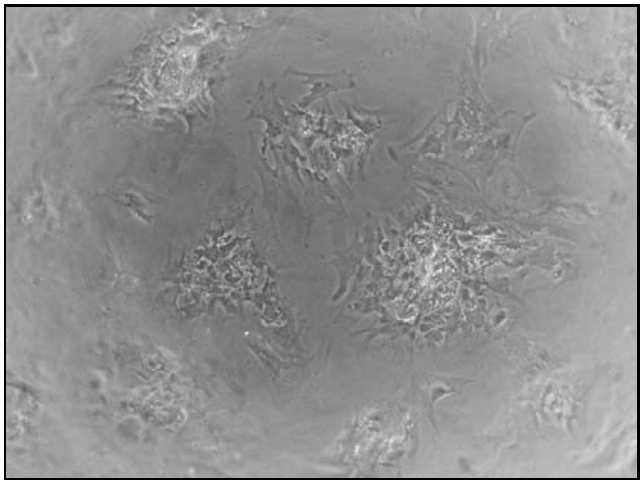

Fig. 10. Differentiated chondrocytes. Magnification 100×, phase-contrast native picture photograph, 21st day of culture.

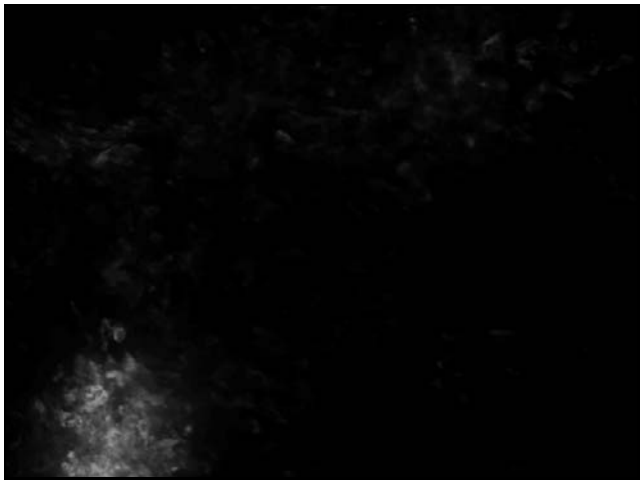

Fig. 11. Differentiated chondrocytes, positive immunocytochemistry for aggrecan. Magnification 200×, 21st day of culture.

The MSCs phenotype of isolated cells derived from the PV afflicted BMs was identified by the capacity of cells to adhere to plastic surface, by their typical spindle-shaped morphology, by the CD105, a cell-surface glycoprotein presentation; by the negative staining results to surface HLA DR and by the ability of cells to differentiate into three mesenchymal lineages. Three-lineage mesenchymal differentiation is the biological property that most uniquely identifies MSCs (Dominici et al. 2006). Even if the PV BM contains an excessive number of cells of the hematopoietic line origin, our results show the occurrence of the satisfactory quantity of MSCs and excellent growth *in vitro*. MSCs were able to differentiate into adipocytes, chondrocytes and osteoblasts without any difficulties. We suggest, if need required, MSCs of PV patients could be expanded *in vitro* and used for the ahMSCs biology investigation, as the BM procurement is a painful procedure; it is not easy to obtain a BM from healthy donors.

Several works have reported that hematopoietic progenitors could also be expanded *ex vivo* by co-culture with BM stromal cells for application to the BM transplantation (Kohler et al. 1999; Tsuji et al. 1999; Yamaguchi 2001). Human MSCs enhanced the engraftment of the cord-blood derived HSC in NOD SCID mice and in a sheep fetus (Bruder et al. 1998; Hoffbrand & Petit 2000; Barry et al. 2001), which was particularly important when the dose of hematopoietic stem cells was low (Barry et al. 2001). Koc et al. (2000) after the first autologous MSCs transplantation at the time of autologous peripheral blood progenitor-cell transplantation in patients with breast cancer observed rapid hematopoietic recovery after the myeloablative therapy, that indicates a positive impact of MSCs on hematopoiesis. The safety and the efficiency of the *in vitro* expanded MSCs infusion were proven in 46 recipients of the allogenic hematopoietic stem cells (Frassoni et al. 2002).

MSCs are known to sustain hematopoietic cells in their function, providing a stromal microenvironment for maturing blood cells precursors and serving as a source of a broad range of cell-derived signals driving the commitment, differentiation and maturation of hematopoietic cells (Tavassoli & Takahashi 1982; Strobel et al. 1996; Koller et al. 1997). MSCs provide an important role in the creation of the hematopoietic stem cell niche, and in some cases were able to support hematopoesis *in vitro* (Noth et al. 2002). MSCs give rise to cells constituting the hematopoietic microenvironment stromal cells, endothelial cells, vascular smooth muscle cells, adipocytes, and osteoblasts. Stromal cells act through a number of mediators, cytokines, adhesion molecules, peptides, hormones, and other molecules like Wnts and eicosanoids. Cytokines and chemokines include the colony stimulating factors, interleukin-6, leukemia inhibitory factor, interleukin-1, interleukin-7, interleukin-8, stem cell factor, flt3 ligand, hepatocyte growth factor, thrombopoietin, insulin-like growth factor-1, transforming growth factor-β, γ-interferon-inducible protein-10, monocyte chemoattractant protein-1, and stromal-derived factor-1. Extracellular matrix collagens, laminins, and fibronectins appear to have a role in hematopoiesis through binding to a number of cell adhesion molecules, which indicates a highly controlled, multifactorial, and redundant regulation of hematopoiesis by MSCs. Stromal cells constitute the cell population that assists the hematopoietic stem cell and its progeny, to set of cells modulating quiescence, self-renewal and commitment of hematopoietic stem cells and the proliferation, maturation, and apoptosis of more mature hematopoietic cells (Charbord 2004).

The development of methods for simultaneous application of the BM-derived and *ex vivo* expanded MSCs to the patients with hematopoietic cells disorders during an autologous hematopoietic stem cell transplantation is considered to be a very promising idea for improvement of the stem cells engraftment, hematopoietic reconstitution and prevention of the BM transplantation failure. It will need a further investigation. In the

study of Lazarus et al. (2000) autologous MSCs derived from hematological malignancies BM were infused intravenously in patients without toxicity and decreased the incidence of acute and chronic GVHD in patients who received hematopoietic stem cells and MSCs co-transplantation. Similarly, study of MSCs derived from chronic myeloid leukemia t(9;22) patients provides the evidence that in a stem cell transplantation setting of chronic myeloid leukemia patients, autologous MSCs could be a source of stem cell support in future cell therapy applications. Isolated MSCs from the BM of patients with chronic myeloid leukemia did not demonstrate any presence of the Philadelphia chromosome, retained their ability to differentiate into osteoblasts and could support expansion of cord blood stem cells (Jootar et al. 2006). MSCs from patients with chronic myeloid leukemia expressed the typical MSCs phenotype and differentiated into osteogenic and adipogenic lineages (Carrara et al. 2007). The supposition of the risk that MSCs after the *in vitro* cultivation prepared for application to the patient would be contaminated by hematopoietic cells is low, since cultures of human MSCs become free of hematopoietic precursors after one or two passages (Sekiya et al. 2002) and according to our experiences they are washed out after the first cultivation medium exchange after the seeding.

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