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Expansion of Human Marrow Derived Mesenchymal Stem Cells and their Transdifferentiation Potential

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Abstract

Background: The ability of mesenchymal stem cells (MSCs) to differentiate into other cell types makes these cells an attractive therapeutic tool for cell transplantation. In order to provide a source of human MSCs for autologus cell-based therapy, we have expanded MSCs from the bone marrow and analyzed the biological identities and transdifferentiation potential.

Methods: The bone marrow of healthy donors was aspirated from the iliac crest. The adjacent cells expanded rapidly and maintained with periodic passages until a relatively homogeneous population was established. The identification of these cells was carried out by differentiation potential into the osteocytes and adipocytes. Transdifferentiation of human MSCs into hepatocyte-like cells was undertaken in response to a specific culture condition.

Results: The differentiation of MSCs into osteoblast is determined by deposition of a mineralized extracellular matrix. Adipocytes are identified by their morphology and staining. Hepatic cells were demonstrated in vitro functions characteristic of liver cells.

Conclusion: We have defined conditions under which human MSCs can be isolated and expanded from human bone marrow. These cells can be amplified about 10^8-fold in 6 weeks, and are capable of transdifferentiation into the cells of another developmental lineage.

Keywords: Mesenchymal stem cells; Human; Differentiation; Bone marrow

Introduction

Mesenchymal stem cells (MSCs) have generated a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types that constitute the tissue in which they exist.[1-3] They were first described by Fridenstein *et al.* in 1976,[4] as the clonal, plastic adherent cells, being a source of the osteoblastic, adipogenic and chondrogenic cell lines. The interest in MSCs rapidly grows with expanding knowledge about their exceptional characteristics and usefulness in the clinic.[5-8] The primary source of MSCs in adult individuals is the bone marrow, where they are immersed in the stroma.[9] They are present at a low frequency in the bone marrow, and recent studies suggest that in humans there is one MSC per 34,000 nucleated cells.[10] Apart from the bone marrow, MSCs are also located in other tissues of the human body.[11]

Human MSCs may participate in cell therapy protocols through two mechanisms. First, MSCs may contribute physically to injured sites when administrated locally or systemically. Second, MSCs may have a supportive role through means of factors. Examples of these applications are in treating children with hematopoietic recovery,[11] osteogenesis imperfect,[12] and bone tissue regeneration strategies.[13] Furthermore, the MSCs may be directly obtained from

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individual patients, thereby eliminating the complications associated with immune rejection of allogenic tissue. Despite diverse and growing information concerning MSCs and their use in cell-based strategies, the mechanisms that govern MSC self-renewal and multilineage differentiation are not well understood and remain an active area of investigation. Therefore, research efforts focused on biological and physiological characteristics of this highly useful stem cell type are crucial. Moreover, the identification and maintenance of MSCs in the undifferentiated phenotype depend on efficient methods of isolation as well as optimal conditions for subsequent culture in vitro, such as the tissue culture substrate, specific culture media,[14] starting and passaging cell-plating density,[15] and supplementation with proliferative, differentiation and transdifferentiation factors.[2,16] Therefore, establishing an optimal cell culture system is of critical importance.

The present study describes a simple method for isolation and rapid expansion of MSCs from human bone marrow, provided for autologous source of stem cell transplantation. Further analysis of MSCs confirmed the differentiation potential of these cells into mesodermal lineage as well as successful transdifferentiation into other specific cell lineages.

Materials and Methods

Human MSCs were obtained from 5 ml iliac crest aspirates of normal donors who underwent bone marrow collection for a related patient (age range of 19-49 years). A written informed consent was obtained-from each patient. Each 5 ml of the aspirate was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM)-low glucose (1,000 mg/l glucose) (Invitrogen, Merelbeke, Belgium) and layered over about 5 ml of ficoll (Lymphoprep; Oslo, Norway). The isolation method was performed according to a previously reported method[17] by some modifications which will be mentioned completely. After centrifugation at 2000 rpm for 30 min, the mononuclear cell layer was removed from the interface. The cells were suspended in DMEM and centrifuged at 1200 rpm for 15 min and then resuspended in basal DMEM medium containing 10% fetal calf serum (Invitrogen, Merelbeke, Belgium), 100 ug/ml penicillin (Invitrogen, Merelbeke, Belgium), 100 ug/ml streptomycin (Invitrogen, Merelbeke, Belgium), and 2 mM glutamine (Invitrogen, Merelbeke, Belgium). The cells were seeded at a density of 800.000/cm^2 in 25 cm^2 T-flasks and maintained at 37°C with an atmosphere of 5% CO2. After 4 days, the non-adherent cells were removed and the media were changed every 3 days. In order to expand the MSCs cells, the adhered monolayer was detached with trypsin- EDTA (Invitrogen, Merelbeke, Belgium) for 5 min at 37°C, after 15 days for the first passage and every 7 days for successive passages. During in vitro passaging, the cells were seeded at a density of 8.000/cm^2 and expanded for several passages until they no longer reached confluence.

At each passage, the cells were counted and analyzed for viability by trypan blue staining analysis. The functional potential of differentiation into osteocyte and adipocyte and transdifferentiation into hepatocyte-like cells was achieved in response to specific culture conditions. Each experiment described here was replicated for 3 times.

For osteogenic differentiation, the 4th- passage cells were treated with osteogenic medium for three weeks with medium changes twice weekly.[18] Osteogenic medium consisted of DMEM supplemented with 10^{-8} M/L dexamethasone (Sigma-Aldrich, St. Louis, USA), 10 mmol/L glycerol phosphate (Sigma-Aldrich, St. Louis, USA), 3.7 gr/L sodium bicarbonate (Sigma-Aldrich, St. Louis, USA), and 0.05 gr/L ascorbic acid (Sigma-Aldrich, St. Louis, USA). Osteogenesis was assessed by alizarin red staining.

To induce adipogenic differentiation, the 4th-passage cells were treated with adipogenic medium for 3 weeks. Medium changes were performed twice weekly.[19] Adipogenic medium consisted of DMEM supplemented with 1 mol/L hydrocortisone (Sigma-Aldrich, St. Louis, USA), 0.05 gr/L ascorbic acid, 0.05 gr/L indomethacin (Sigma-Aldrich, St. Louis, USA), and 10^{-6} M/L dexamethasone. Adipogenesis was assessed by oil red O staining.

For hepatogenic differentiation, the 4th- to 5th-passage cells, at 10^5/cm^2, were seeded in basal medium to reach a confluency about 50% prior to induction. After 24 hours, the media were removed and cells were induced by treating MSCs with basal DMEM medium supplemented with 20 ng/mL hepatocyte growth factor (HGF) (Peprotech, Paris, France), 20 ng/mL insulin growth factor (IGF) (Peprotech, Paris, France), and nicotinamide 0.61 g/L, for 7 days. It was followed by treatment with basal DMEM medium supplemented with 10 ng/mL oncostatin M (Peprotech, Paris, France), 10^{-3} mol/L dexamethasone (Sigma-Aldrich, St. Louis, USA), 20 ng/mL HGF, and 20 ng/mL IGF thereafter. Medium changes were performed twice weekly and hepatogenesis was assessed by

immunofluorescence analysis for albumin and α-feto protein production, and by in vitro assays for functions that are characteristic of liver cells.

For staining of intracellular proteins, the cells were fixed overnight with 4% formaldehyde at 4°C, and permeabilized with 0.4% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 10 minutes. After blocking with bovine serum albumin, the plates were incubated with mouse primary antibodies against human albumin and α-feto protein (Sigma-Aldrich, St. Louis, USA) for 1 hour, followed by fluorescein- or phycoerythrin-coupled goat anti-mouse immunoglobulin G secondary antibody for 1 hour. Between the incubations, the samples were washed with PBS - 0.05% Tween.

Results

Adherent cells were observed in all the samples after 3 days' culture and in the following 15 days an adherent monolayer was achieved (Figure 1A and Figure 1C, respectively). The cells were of two types: a type of cells with large and flat morphology, and a type of smaller spindle-shaped cells. The rapid expansion of the MSCs in the culture was found to depend on the presence of single cell-derived colonies composed of a few fibroblast-like cells (Figure 1B). The bone marrow cells rapidly generated a confluent layer of cells possessing an elongated, fibroblastic shape (Figure 1D). The cells increased in size and showed a polygonal morphology with evident filaments in the cytoplasm especially when the early passage cells were compared with late passage ones. The MSCs isolated from healthy donors were expanded for up to 10 passages.

At each passage, the cells were counted and analyzed for viability by trypan blue staining analysis showing viability between 98% and 100% in the samples.

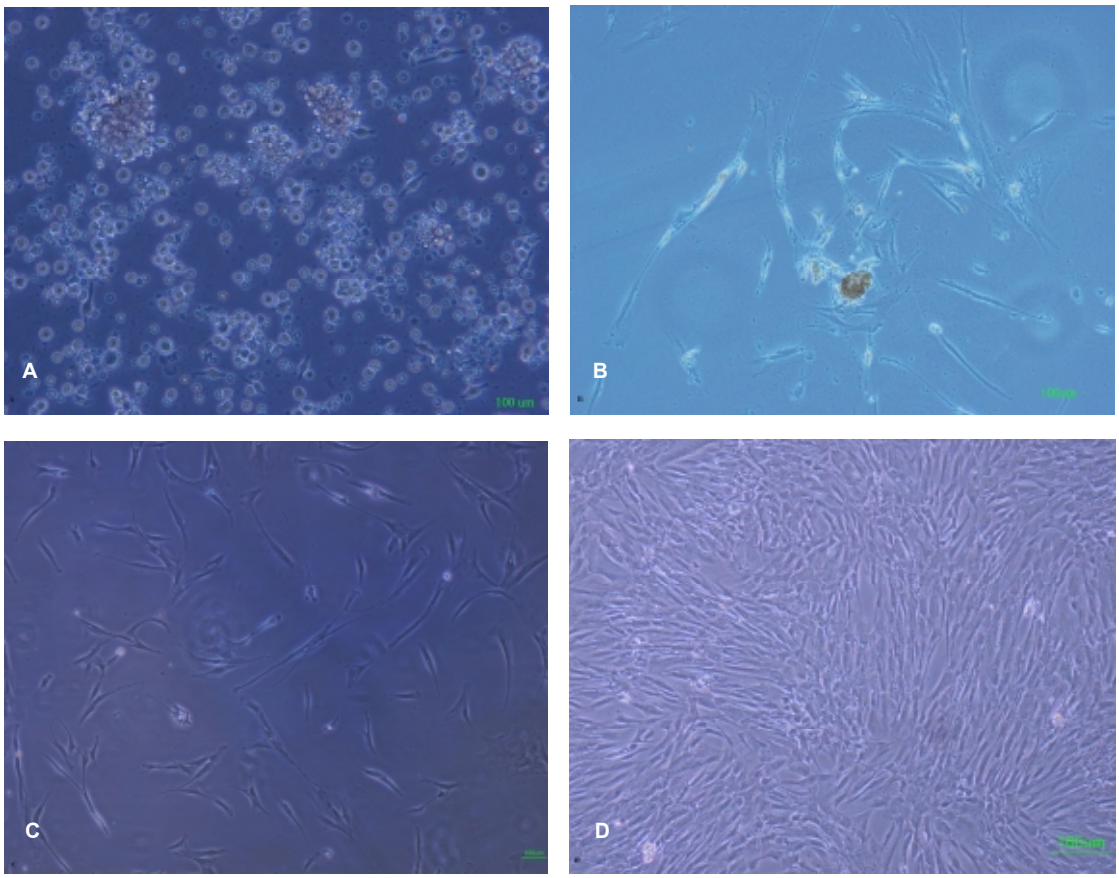

Fig. 1: Human bone marrow cells culture. Morphological features of in vitro expanded adherent spindle-shaped cells which appeared on day 3 **(A)**. The presence of single cell-derived colonies composed of a few fibroblast-like cells on day 6 **(B)**. Bone marrow derived cells 15 days after isolation **(C)**. Purified populations of spindle-shaped cells appeared on the first passage **(D)**. Original magnification, ×10

The differentiation of MSCs to osteoblasts in vitro involved incubating a confluent monolayer of MSCs with the osteogenic media for 2–3 weeks. The MSCs formed aggregates or nodules, and calcium accumulation could be seen over time. These bone nodules were stained positively by alizarin red technique (Figure 2A).

To promote adipogenic differentiation, MSC cultures were incubated with adipogenic media. An accumulation of lipid rich vacuoles within the cells was seen after 3 weeks. Eventually, the lipid vacuoles combined and filled the cells. Accumulation of lipid in these vacuoles was assayed histologically by oil red O staining (Figure 2B).

Transdifferentiaion of human MSCs to hepatocyte-like cells was achieved by incubation of MSC with hepatogenic media consisting of defined cytokines and growth factors in a 2-step protocol. Differentiation was induced by treating MSCs with step 1 differentiation medium consisting of IGF as a growth factor, and HGF as a potent mitogen for hepatocytes. It was followed by treatment with step-2 maturation medium consisting of oncostatin M, a member of the interleukin 6 cytokine families thereafter. The oncostatin M together with HGF increased the cell size and enhanced maturation of hepatocyte-like cells. To confirm albumin and α-feto protein secretion in human MSC-derived hepatocytes, we examined transdifferentiated human MSCs by immunocytochemistry. On day 12 after differentiation, the cells were fixed and immunostained with anti-albumin and anti α-feto protein antibodies. Human MSCs cultured in the media containing HGF and oncostatin M showed positive immunostaining to albumin (Figure 3A) and α-feto protein antibodies (Figure 3C).

Discussion

The ability of MSCs to modulate immune responses implies the potential role of MSCs in cellular therapy by facilitating engraftment in organ transplantation,[20,21] and in gene therapy by delivering genes into the tissues of interest.[22] The bone marrow-derived adherent cells were found to contain different cell types including fibroblasts, hematopoietic progenitor cells, macrophages, endothelial cells and adipocytes.[23]

On the basis of mesenchymal progenitor's capacity to adhere to plastic, we isolated MSCs from BM of healthy donors using a ficoll gradient and expanded them in DMEM and 10% FBS without adding any growth factors. Using the method described in this study, the rapid expansion of the MSCs in culture was found to depend on the presence of single cell derived colonies composed of a few fibroblast-like cells. The cells contained two populations: a major population of large and flat cells referred to as type I cells, and a minor population of smaller spindle-shaped cells referred to as type II cells. After replating the cultures at low density, the population of type I rapidly expanded, and the small spindle-shaped cells declined in number. The stationary cultures of MSCs contained a major population of large and flat cells which increased in size and number during the

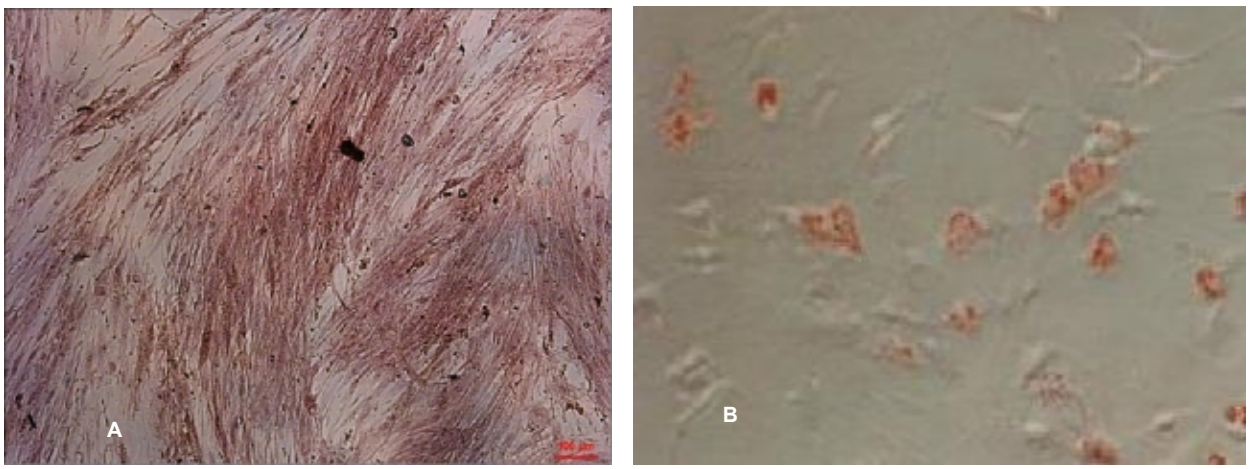

Fig. 2: In vitro differentiation of the human bone marrow derived MSCs cells. Osteogenic and adipogenic differentiation of human MSCs were examined after 4th passage. Arrowheads indicate the nodule like structures stained with Alizarin red **(A)**, and adipose droplet stained with Oil red **(B)**. Original magnification, ×40

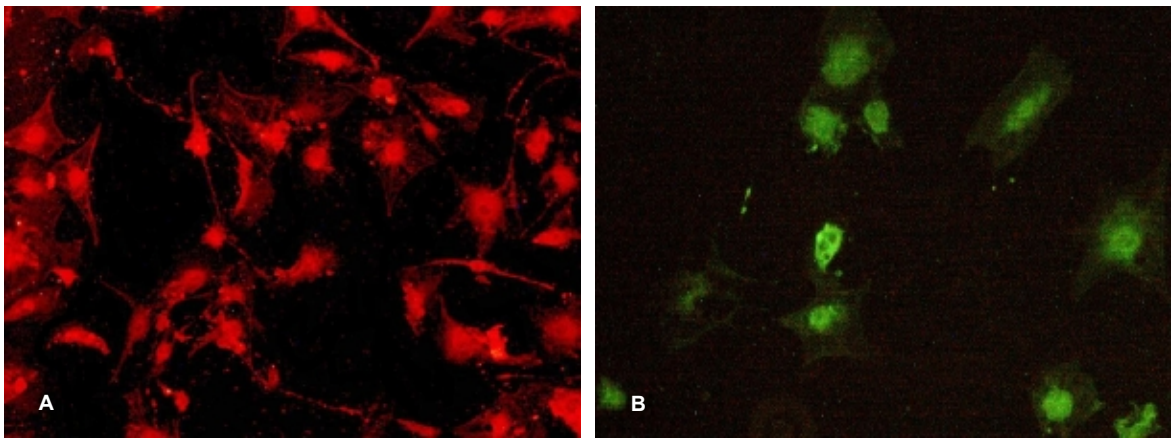

Fig. 3: In vitro transdifferentiation of the human bone marrow derived MSCs cells.
Characterization at the protein level of MSC differentiation into hepatocyte-like cells upon sequential exposure to liver-specific factors on day 12. Immunocytochemistry was performed for albumin (A), and α-feto protein (B). Original magnification, ×40

log phase growth and became the predominant cell as the cultures approach senescence. It was also noted that bone marrow MSCs generated an adherent layer initially formed by individual cells or colonies composed of a few fibroblast-like cells, which rapidly reached confluence. We observed an exponential growth of these cells with 98% of viability at each passage and noted that these multipotent cells had substantial proliferation and expansion in culture and did not differentiate spontaneously during culture expansion, but did differentiate when they grew in lineage-specific culture conditions.

A way to identify the supposed MSC populations is by their capacity to be induced to differentiate into the bone, fat, and cartilage in vitro.[1,10] We have also introduced a system of culturing MSCs that supports and maintains their optimal differentiation potential during the long term culture expansion. The human bone marrow-derived MSCs differentiated into adipocytes and osteoblasts when they grew in specific culture conditions. Osteoblastic differentiation was demonstrated by the accumulation of a bone-like mineralized matrix. Adipocytic differentiation was shown by the presence of cytoplasmic lipid accumulation. These conditions, however, are unlikely to reflect the physiological signals MSCs receive and induce in vivo. There have been some recent reports investigating the role of bone morphogenic proteins (BMPs) on osteogenesis.[24]

This piece of evidence together with fibroblastic morphology, clonogenic capacity of the cells, negative and positive surface markers, and differentiation functions led to the conclusion that these cells (fibroblast-like cells) were human MSCs. In this study, we have developed an in vitro differentiation strategy to assess if human MSCs that have differentiated into a given mesenchymal cell lineage can transdifferentiate into other cell types in response to inductive extracellular condition.

The phenotypic change of one differentiated cell type into another is referred to as transdifferentiation.[2,25] It has been demonstrated as a physiological property of amphibian species, such as during limb regeneration and the conversion of pigmented epithelia into lens and neural retinal cells.[26] Transdifferentiation may also occur in mammalian systems. It has been shown that in vertebrates the adult stem cells maintain the multidifferentiation potentials even after being exposed to certain inductive factors. Although several lines of evidence have suggested the existence of transdifferentiation potential of the adult stem cells in mammalian systems, other findings have been used to argue against and invalidate this notion, namely cell fusion,[27,28] and cell heterogeneity.[29]

In this study, we have developed an in vitro differentiation strategy to assess if differentiated MSCs were able to maintain their multidifferentiation potential. Since freshly isolated human MSCs were the sole cell source in this in vitro culture system, it is reasonable to conclude that the transdifferentiation phenomenon we observed was the very property of differentiated human MSCs but not the

result of cell fusion.

Our results showed that fully differentiated cells from human MSCs were capable of transdifferentiation into cells of another lineage. This study opens new perspectives which are not only applicable to the study of in vitro cell differentiation but it also offers the possibility to isolate and culture human nonembryonic multipotent stem cells as an unlimited cell source for autologous cell-based therapy. It might open a road to trigger cell fate and ‘‘trans’’-differentiate uncommitted cells from different tissues towards endodermal lineages.

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