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Evaluation of differentiation potential of human bone marrow-derived mesenchymal stromal cells to cartilage and bone cells

İnsan kemik iliği kaynaklı mezenkimal stromal hücrelerin kıkırdak ve kemik hücrelerine farklılaşma potansiyelinin değerlendirilmesi

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Amaç: Bu çalışmada, sağlıklı insan kemik iliğinden ayrılarak çoğaltılan mezenkimal destekdoku (stromal) hücrelerinin (MSH) fiziksel özelliklerine ve yüzey antijenlerine göre tanımlanması, osteoblast ve kondroblast yönünde farklılaşma potansiyelinin incelenmesi amaçlandı.

Çalışma planı: Yaşları dört ay ile 18 yıl arasında değişen 10 kemik iliği transplantasyon vericisinden toplanan kemik iliğinin 1-3 ml'sinden gradiyent yöntemiyle mononükleer hücreler ayrıldı ve içinde %10 fetal dana serumu bulunan tüplerde kültüre edildi. Tabana yapışma gösteren fibroblastik görüntüde olan hücreler çoğaltıldı. Akım sitometri aygıtı ile hücrelerin yüzey antijenlerine göre immünfenotiplendirmesi yapıldı. Tanımlanan mezenkimal stromal hücrelerin osteoblast ve kondroblast uyarıcı medyumları kullanılarak kondroblast ve osteoblasta farklılaşması incelendi. Osteoblastlar Alizarin kırmızısı, kondroblastlar ise Alsiyan mavisi ile boyanarak mikrofotoğrafları çekildi.

Sonuçlar: Canlı-dışı (*in vitro)* çoğaltılan MSH'lerin biçim ve yapışma özellikleri yaşa bağlı farklılık göstermedi. Destekdoku kaynaklı olabilecek hücrelerde karakteristik CD105, CD44, CD166, CD29, CD90 ve CD73 antikorlarına karşı %90-99 arası pozitif sonuç, hematopoietik hücrelere özgü CD45, CD34, CD14 ve HLA-DR antikorlarına karşı negatif (%0-5) sonuç elde edildi. Hücre tipine özel besiyerlerinde çoğaltılan MSH'lerin, ilerleyen altkültürler (pasajlar) de dahil olmak üzere, tüm altkültürlerde (p1-15) osteoblast ve kondroblast hücrelerine farklılaştığı gözlendi.

Çıkarımlar: Mezenkimal destekdoku hücrelerin, kemik iliği aktarımı, kalıtsal hastalıklar ve organ hasarında; ortopedide kullanılması düşünülen biyomalzemelerin biyolojik etkilerinin canlı-dışı incelemesinde; kemik ve kıkırdak dokusundaki hasarlı bölgelerin tamirinde kullanılması mümkün görünmektedir.

Anahtar sözcükler: Kemik iliği; hücre kültürü teknikleri; hücre farklılaşması; mezenkimal kök hücre; osteoblast; rejenerasyon.

Objectives: This study was designed to identify the characteristics and surface antigen properties of mesenchymal stromal cells (MSC) isolated and cultured from bone marrow of healthy subjects and to assess their differentiation potential to osteoblast and chondroblast lineage cells.

Methods: Mononuclear cells were isolated by density-gradient separation from 1-3 ml of bone marrow collected from 10 donors of bone transplantation aged between 4 months and 18 years. These mononuclear cells were cultured in flasks containing %10 fetal calf serum, which resulted in growth of fibroblast-like cells showing adhesion onto the culture flask. Physical properties of the cells were identified and flow cytometric immunophenotyping was performed to specify surface antigen properties. Differentiation of mesenchymal stromal cells in specific media to chondroblasts and osteoblasts was evaluated. Osteoblasts and chondroblasts were stained with Alizarin red and Alcian blue, respectively, and microphotographed.

Results: *In vitro* yield of MSCs showed no age-related differences in terms of morphological and adhesive properties. While cells of stromal origin showed strong positivity (90% to 99%) to characteristic CD105, CD44, CD166, CD29, CD90, and CD73 antibodies, hematopoietic cells remained negative (0% to 5%) to CD45, CD34, CD14, and HLA-DR antibodies. It was observed that MSCs produced in cell-specific media differentiated to osteoblasts and chondroblasts in all passages (p1-15) tested, including late passages.

Conclusion: It seems that the use of MSCs would provide promising treatment strategies in bone marrow transplantation, inherited diseases, and organ repair; in *in vitro* assessment of biological effects of biomaterials in orthopedics; and in repair of bone and cartilage injuries.

Key words: Bone marrow; cell culture techniques; cell differentiation; mesenchymal stem cells; osteoblasts; regeneration.

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Autologous bone grafts used for treatment of bone injury may cause discomfort to the patient. Similarly, transplantation of autologous or allogenic tissues and/or use of synthetic medical products for cartilage injury also necessitates surgical approaches. In addition, the bone and cartilage areas, to be used as a graft, are limited. Recently, the use of autologous or allogeneic stem cells, is suggested as an alternative therapeutic approach for treatment of bone and cartilage defects.[1]

Duo to their self renewal and differentiation capacity, stem cells are promising cells to be used for regenerative purposes. Thus, it is considered that they may have contributions for recovery of organs that lose their functions with loss of tissue. Recent preliminary reports describe safe use and beneficial effects of stem cells in myocardial, renal, bone tissue and cartilage defects in experimental studies.[2-7] Mesenchymal stem cells or recently named as mesenchymal stromal cells (MSC) [8] appear to be the cells that are very suitable for this purpose due to their in vitro expansion, multilineage differentiation capacity and immunosuppressive characteristics and can be isolated easily from the bone marrow.[9] Since the MSCs are very few in all tissues including the bone marrow, they must be expanded in in-vitro culture conditions for experimental or clinical use. Necessary growth factors and other stimulators must also be added to the medium to ensure differentiation of the proliferating cells towards the desired tissue.

In this preliminary study, isolation of MSCs from the bone marrow of healthy donors, in-vitro expansion and differentiation towards osteocytic and chondrocytic lineages were tested.

Patients and methods

Bone marrow from 10 healthy donors aged 4 months through 18 years was studied in the Department of Paediatric Haematology/ Bone Marrow Translantation Unit, Faculty of Medicine, Hacettepe University. Approximately 1-3 ml of harvested bone marrow (300- 800 ml) was used for the following studies after ethical committee approval.

Isolation of MSCs

Mononuclear cells from the marrow were isolated by density gradient separation method. Bone marrow was diluted with PBS (phosphate- buffered saline) at 1:2 and separated in 15 minutes at 2000 rpm after layering on Ficoll with a concentration of 1.077 g/ml. The cells were rinsed twice with PBS, viability was determined with trypan blue stain, cell counts were determined manually and/or by nucleocounter (Nucleocounter YC- 100 System, ChemoMetec A/S, Denmark). Subsequently, the cells were cultured in 5 ml DMEM-LG (Biological Industries, Israel) containing 10% FCS (Biological Industries, Israel), 1% penicillin- streptomycin and 1% L-glutamine at a density of 2×10^6 cells / cm^2 in 25 cm^2 flasks at 37 °C, 5% CO_2 and humid ambient.

The floating cells and the medium were discarded after 24 and 48 hours and the adherent cells were expanded in flasks. Medium changes were done every 3-4 days regularly every day. When the cells were 70-80% confluent after 7-12 days, they were detached from the surface with 0.25% Trypsin-EDTA. Following expansions were made in 75 cm^2 flasks with a density of 2×10^3 cells/ cm^2 and the cells were expanded for several passages.[10]

Immunophenotyping of MSCs (Flow cytometry)

Cells separated from human bone marrow were identified with an antibody panel by flow cytometry. CD105, CD44, CD166, CD29, CD90 and CD73 antibodies specific for stromal cells (Becton Dickinson, USA) as well as CD45, CD34, CD14 (Becton Dickinson) and HLA-DR (Chemicon, USA) antibodies specific to haematopoietic cells were used. Analyses were done on Beckman Coulter Epix Elite ESP (Beckman Coulter, USA) flow cytometry device.[11]

Differentiation of MSCs

Osteoblast differentiation: MSCs at the third passage were trypsinized and cultured in 9.6 cm^2 polypropylene petri dishes at a concentration of 2×10^3 cells/ cm^2. Osteoblast induction medium contained 10% FCS (Euroclone), 100 nM dexametazone (Sigma, USA), 10 mM beta-glicerophosphate (Sigma) and 0.2 mM ascorbic acid (Sigma) in DMEM-LG (Euroclone, UK). Medium changes were made at 3-4 day intervals during 21 days. The differentiated cells at the end of this period were identified by staining with Alizarin red. [12]

Chondroblast differentiation: 2.5×10^5 MSCs were placed into 15 ml polypropylene tubes and centrifuged for 5 minutes at 1500 rpm and a 3-dimentional cell pellet was developed. The cell pellet was incubated for 24

hours in 5% CO_2 and humid incubator at 37 °C. At the end of incubation, the chondrogenic induction medium was added and consisted of 10 nM dexametazone (Sigma), 10 ng/ml TGF-ß 3 (Peprotech, USA), 50 mg/ml ascorbic acid (Sigma) and 50 mg/ml ITS + Premix (Becton Dickinson) and DMEM-HG (Euroclone). Medium changes were made at 3-4 day intervals during 21 days. 5 mm thick frozen sections were obtained from the cell pellet. Chondroblast differentiation in the sections taken was identified by staining with Alcian blue.

Results

MSC cultures were established from all ten bone marrow samples (100%).

Physical characteristics of MSCs: Adherent spindle shaped and fibroblast-like cells were visible at the bottom of the flask after 24 hours of culture (Figure 1). Cells were expanded up to 15 passages without losing their proliferation and differentiation characteristics. No age related differences was noted in term of cell shape, time to confluency or differentiation characteristics.

Immunophenotyping: Culture expanded cells were immunophenotyped by flow cytometry. While 90-99% of cells showed positive staining with CD105, CD166, CD73, CD29, CD90 and CD44 antibodies suggesting stromal origin and adhesive characteristics of MSCs. Hematopoetic markers including CD45, CD34, CD14 and HLA-DR remained negative (<5 %) (Figure 2).

Figure 1. Microphotograph of mesenchymal stromal cells in culture (x40).

Osteoblast and chondroblast differentiation: Third or fourth passage cells were used for differentiation studies. However, passage 1 and P15 cells

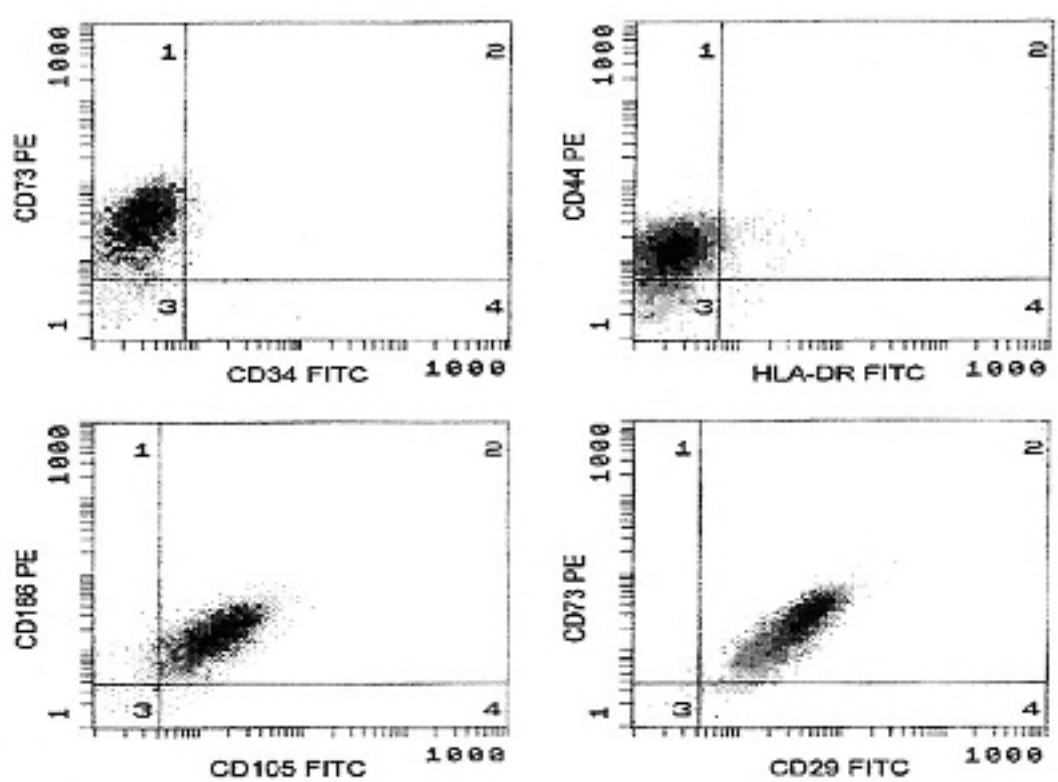

Figure 2. Immunophenotyping of mesenchymal stromal cells

tested in 3 samples showed similar differentiation potential. Cells were checked regularly every day after culturing in differentiation induction medium. Spindle shape osteoblast-like cells lost their spindle-like structure and became rectangular in shape growing on top of each other. Therefore, the cells could not be distinguished clearly on the seventh and tenth days. After twenty one days, black calcium apatite particles were observed with light microscopy. Osteogenic origin of the cells was confirmed by alizarin red staining (Figure 3).

For chondroblast differentiation a3-dimensional pellet was exposed to specific induction medium and frozen section were prepared at day 21. Subsequently, with Alcian blue staining, chondroblast differentiation from MSCs was identified (Figures 4, 5).

No age-related difference was observed for osteoblast and chondroblast differentiation of MSCs separated from bone marrow taken from healthy donors aged 4 months through 18 years.

Discussion

The present study is part of the basic studies conducted within the scope of PEDI-STEM Project for treatment of some genetic diseases in infants. Mesenchymal stromal cell expansion was established in all 10 samples.

Figure 4. Cell pellet rendered for chondroblast differentiation of mesenchymal stromal cells (x 10).

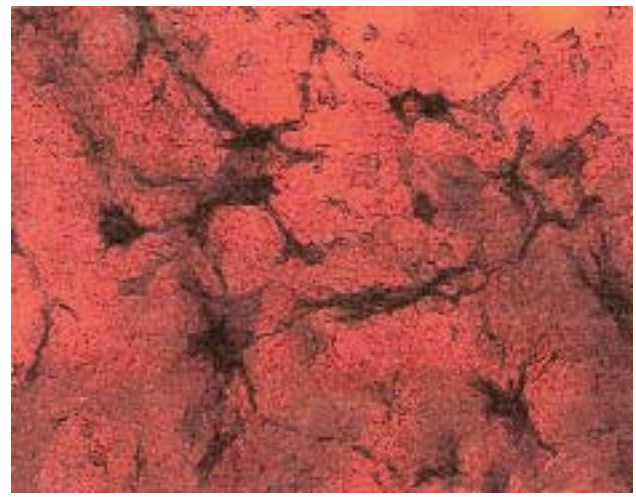

Figure 3. Microphotograph of mesenchymal stromal cells differentiated to osteoblast (Alizarin red x 10).

Previously, we had defined the methods to differentiate osteoblasts from adult mouse and mouse fetus calvarium in an experimental study.[14] Partial cultures were attained from two, and successful cultures were attained from four of ten adult mice. In fetus calvarium cultures, full cultures were attained from six mice, and partially from one mouse, and unsuccessful culture from one of eight mice.[14] In another study, we had separated and defined osteoblast-like cells from rat bone marrow stromal cells and a success rate of 93.8% was attained.[15] In the present study, osteoblasts developed in all of 10 samples stimulated for osteoblast differentiation from bone marrow MSC.

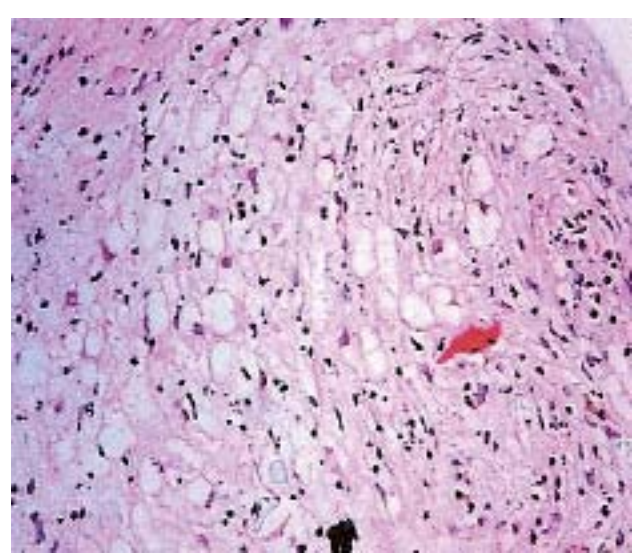

Figure 5. Microphotogrph of mesenchymal stromal cells differentiated to chondroblast (Alcian blue x 10).

1-3 ml bone marrow samples were used to isolate cells. It has been reported that larger volumes of bone marrow aspirate is associated with reduced quantity of progenitor cells.[16] Therefore, small volumes of marrows was used.

It has been suggested that differentiation capacity of MSCs towards connective tissue cells is increased at younger ages.[11, 17] However, no age-related difference was observed in this study.[18] The number of MSCs in the bone marrow of young adult has been reported to range between thousand through hundred thousand and decrease with age.

Recent detailed characterization of stem cells has suggested these cells to be promising vehicles for differentiation towards several tissue cells.[6,11,17,19] Demonstration of differentiation potential of MSCs to the connective tissue cells has made these cells as important cells that can be used for treatment of skeletal disorders.[6,20,21] It has been revealed that the stromal cells in bone marrow are featured to constitute many connective tissue cells including cartilage. [11, 21, 22] Development of chondrocyte nodules have been described on the 16th day and mineralized bone nodules were detected on the 21st day of cultures by dexamethasone.[23]

Bone marrow and lipoaspiration materials are the most frequently used sources of MSCs.[6] In the present study, sufficient number of cells were obtained by in-vitro expansion of MSCs from the bone marrow mononuclear cells derived from healthy marrow donors.[11, 17] Stromal cells constitute 2-3% of the mononuclear cells in the bone marrow and are characterized by adhesive character and differentiation potential.[21] Due to the extremely low numbers of these cells in tissues in vitro expansion is necessary for clinical use.[17] A significant amount of budget and technical infrastructure is needed to create the conditions for clinical grade expansion of cells at internationally accepted standards, the good manufacturing practice standarts. [20]

Long term in vitro expansion of cells may hold several disadvantages including contamination, telomere shortening, clonal evolution, thus carcinogenicity. [24, 25] However, none of these problems have been encountered in the preliminary clinical experimental studies. Long term following of patients is needed to confirm safe clinical use. The use of earlier passage cells is recommended in order to prevent such complication. Development of sarcoma has recently described in animal experiments.[24] Therefore further studies are needed before routine clinical application.

Human bone marrow consists of group of different cells containing MSCs that are the pioneers of the osteoprogenitor cells that have the potential to develop bone tissue besides the haematopoietic and endothelial cells. Mesenchymal stromal cells contain a different cell group that stores bone protein and that responds to TGF-ß, but that does not respond to the hematopoietic growth factors.[26, 27] Two types of MSCs that feature osteogenic progenitor cells have been identified. Undifferentiated, but constituting a larger number of cells and responding to ß-fibroblastic growth factor (ß-FGF), transformative growth factor- ß (TGF-ß) and Vitamin D3, and another group of cells in smaller numbers, constituting very different colonies and non responsive to ß-FGF.[14, 26]

It is possible to divide the bone marrow cells into two categories. STRO-1, CD105, CD73 and CD90 antigen positive MSCs, and the CD34. CD45 and CD14 surface antigen positive hematopoietic cells. [20, 28, 29] Our findings confirmed mesenchymal character of the cells.

In this study, culture expanded and osteo-induced cells constituted calcium deposits that were demonstrated by staining with Alizarin red. Different methods have been used for this purpose.[30] In a previous study, osteoblast-like cells had been stained with pycrotionine stain.[14] Calcium deposits had also been shown by staining with Kossa technique.[31] Other methods to confirm osteogenic lineage include measurement of alkaline phosphate activity, type-1 collagen development, bone gla protein that can be stimulated with Vitamin D3 and osteocalcin.[32]

In order to stimulate the in-vitro cartilage differentiation, a 3D culture medium simulating the in-vivo environment has been used.[16]

The present study has shown that sections taken and prepared from the pellet exposed to chondrogenic media have shown live cartilage tissue. Flat chondrocytes were detected on the surface layers of cartilage cell pellet, and rounder ones were detected in deep zones.

Osteoblasts and chondroblasts have been used for analyzing the biological effects of the new biomaterials produced in orthopaedics.[33] It is aimed at

transferring the cells to the impaired zone in the soluble polymer vessels and using them as innovative to regenerate tissue damage. Mesenchymal stem cells are promising vehicles for bone and cartilage injury repair owing to their characteristics to differentiate to the connective tissue cells.[34]

These findings obtained from the in-vitro experiments suggest that differentiation may be achieved in vivo as well. It is reported that the stimulation necessary for differentiation can be obtained from the soluble factors released in the injury microenvironment. The studies conducted suggest that chemokines, cytokines and other soluble factors released from the defective tissues can play role for mobilization, homing, proliferation and differentiation of stem cells towards the phenotype of damaged cells.[2, 3]

In conclusion, MSCs have been used in preliminary studies in high risk transplantations and/or systemic and progressive disorders in the clinical field. In-vitro experimental studies are being performed in our laboratory as a step towards preparation for later clinical use.

Although great interest has been arisen in orthopedics regarding the use of MSCs for bone and cartilage repair, cautious and conservative approach is recommended for stem cell use for regenerative purposes. Treatment priority for cellular therapies at present is given to life threatening and progressive defects until safety issues are proven.

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