Chondrogenesis of Mesenchymal Stem Cells Derived from Human Umbilical Cord Blood

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Abstract : In the current study, the mesenchymal stem cells (MSCs) isolated and propagated from the human umbilical cord blood (UCB) were tested for their capabilities of differentiation into chondrocytes *in vitro*. The mesenchymal progenitor cells (MPCs) collected from UCB were cultured in a low glucose DMEM medium with 10% FBS, L-glutamine and antibiotics. The human MSC colonies were positively stained by PAS reaction. When the immunophenotypes of surface antigens on the MSCs were analyzed by fluorescence-activated cell sorter (FACS) analysis, these cells expressed positively MSC-related antigens of CD 29, CD44, CD 90 and CD105, whereas they did not express antigens of CD14, CD31, CD34, CD45, CD133 and HLA-DR. Following induction these MSCs into chondrocytes in the chondrogenic differentiation medium for 3 weeks or more, the cells were stained positively with safranin O. We clearly confirmed that human MSCs were successfully differentiated into chondrocytes by RT-PCR and immunofluorescent stain of type-II collagen protein. These data also indicate that the isolation, proliferation and differentiation of the hUCB-derived MSCs *in vitro* can be used for elucidating the mechanisms involved in chondrogenesis. Moreover this differentiation technique can be applied to developing cell-based tissue regeneration or repair damaged tissues.

Key words : mesenchymal stem cells, human umbilical cord blood, chondrogenesis, FACs analysis, RT-PCR, Safranin-O staining, immunocytochemical staining.

Introduction

The repair of articular cartilage damage by trauma or disease is limited. Both full-thickness and partial-thickness cartilage defects is not covered, because injured cartilage occurs degradation of the full functional articular surface. The avascularity and low cellularity of articular cartilage is related to ineffective repair. In particular, cells with chondrogenic potential may fail to be recruited to the site of injury in sufficient numbers. For these reasons, there is considerable interest in developing cell therapies to treat damaged cartilage.

The mesenchymal stem cells (MSCs) can be isolated from a variety of tissues, including bone marrow, adipose tissue, skeletal muscle, liver, fetal tissues, amniotic fluid, placenta, peripheral blood and umbilical cord blood (1,4,5,9,14). The MSCs from these tissues have capacity to expand and differentiate into tissues of three germ layers including bone, cartilage, muscle, ligament, tendon, fat, dermis, hepatocyte-like cells, and neuron-like cells in *vivo* and *vitro* (2,3,6,13,19,22). These cells have various characteristics such as multipotential,

easy isolation and culture and high expansion potential. The MSCc are considered a good candidate cell for cartilage tissue therapy (16). Especially, the MSCs from umbilical cord blood have several benefits of non-invasive easy collection, capability of differentiation into three germ layers, and low immune reaction after transplantation (16,12,21).

The proposes of this study were to assess the potential of MSCs in future clinical applications. We investigated the differentiation capacity of the human MSCs derived from human umbilical cord blood and evaluated these cells in the morphological, biochemical and immunohistochemical characteristics towards chondrogenic lineages.

Materials and Methods

Isolation and culture of MSCs from human umbilical cord blood

Human umbilical cord bloods (UCBs) were obtained during Cesarean section after receiving informed consent from patients. This study was approved by the Institutional Review Board at Seoul National University Bundang Hospital. The blood was collected into 250 ml standard blood collection bags (Green Cross, Yongin, Korea) that contained citrate-phosphate

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dextrose anticoagulant.

The buffy coat cells were obtained by centrifugation (400 g for 20 min) and the low-density mononuclear cells (MNC) were isolated using Ficoll-Paque Plus (< 1.077 g/ml, Amersham Biosciences, Sweden). The cells were then resuspended in Dulbeco's Modified Eagle low glucose medium (Gibco-BRL, Gaithersburg, MD, USA) that was supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) and antibiotics. The total numbers of nucleated and viable cells were determined with a haemocytometer and by using trypan blue stain.

The cells were cultured in the low glucose Dulbeco's Modified Eagle Media (DMEM) with 10% FBS, 2 mmol/l L-glutamine (Gibco-BRL) and 0.3% penicillin-streptomycin (Gibco-BRL) under 5% CO_2 at 37°C. The cells were initially plated into the 10 cm² culture dishes or 175 cm² tissue culture flasks (Nunc, Rochester, NY, USA) at a density of 1.5×10^6 mononuclear cells/cm². The cells were transferred to new flasks to remove the platelets after 1 day of culture. Half of the medium was changed weekly thereafter. The cells were passaged by trypsinization (0.05% trypsin/EDTA solution; Gibco BRL) upon reaching 70% to 90% confluence (5,000-6,000 cells/cm²), and they were replated at a density of 0.5~ 1.5×10^6 mononuclear cells/cm².

Periodic acid-Schiff (PAS) treatment for glycogen

For the identification of glycogen in the UCB-derived MSCs, the cultured cells were rinsed 3 times with PBS and dried in the air. The cells were fixed with formaline-ethanol fixative solution for 1 minute. The cells were oxidized in 10 g/L periodic acid for 5 min and rinsed three times in dH_2O. Then, the cells were treated with Schiff's reagent for 15 min, rinsed in dH_2O for 10 min, and stained with hematoxylin for 90 seconds. The dried specimens were observed under an inverted microscope.

Fluorescence-activated cell sorting (FACS) analysis

To analyze the expression of surface markers in hUCB-MSCs, FACSAria was used (Becton & Dickinson, Franklin Lakes, NJ, USA). Briefly, hUCB-MSCs were fixed with 70% ethanol for 10 min at 4°C and stained for 30 min on ice with anti-human antibodies that recognize various surface molecules of CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD133, and HLA-DR. The antibodies were conjugated to the fluorescein isothiocyanate (FITC) (Abcam, Cambridge, UK) or the phycoerythrin (PE) (Becton & Dickinson).

Induction of chondrogenic differentiation

To induce chondrogenic differentiation, fourth- to seventh-passage MSCs were transferred into the 60 mm dishes or 15-mL polypropylene tubes. The cells in the tubes were centrifuged at 400 g for 5 minutes, to form a pelleted micromass at the bottom of the tube and then treated with a chondrogenic medium (Cambrex Bio Science Inc. Walkersville, MD, USA).

The chondrogenic medium consists of differentiation basal medium supplemented with dexamethasone, ascorbate, ITS + supplement, sodium pyruvate, proline, penicillin/streptomycin, L-glutamine and TGF-beta3. Medium changes were carried out twice weekly and chondrogenesis was assessed at intervals of a week.

Histological staining (Safranin-O staining)

For the Safranin-O staining, the specimen was fixed in the formaline-ethanol fixative solution for 5 minute. Safranin-O (0.1%) was applied for 1 min. and washed with distilled water. Also, for the evaluation of chondrogenic differentiation, the cells were collected and fixed in 4% paraformaldehyde and embedded in paraffin. Embedded blocks were sectioned into 5 µm slices. After deparaffin, the specimens were stained with 0.1% aqueous safranin-O for 1 minute. The stained specimens were washed in distilled water and dehydrated. The specimens were coversliped for observation under light microscopy.

Immunocytochemical staining

The dried specimens were fixed in the formaline-ethanol fixative solution for 1 minute and then rinsed with dH_2O. Following three washes with distilled water, 1% normal goat serum was applied for 1 hour at room temperature. Then polyclonal antibody to human-collagen type II (Millipore, Billerica, MA, USA), diluted at 1 : 20 in goat serum, was reacted overnight at 4°C. After washing in PBS, FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 2 hrs.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction from the specimens and RT-PCR was performed before and after chondrogenic differentiation of MSCs. Total RNA was isolated from the pellet using TRIzol reagent (Invitrogen, Taipei, Taiwan). RNA concentrations were determined by absorbance at 260 nm with a spectrophotometer. The isolated RNA samples were converted to first-strand complementary DNA(cDNA) using reverse transcriptase (Super-Script TMII, Life Technology, Gaithersburg, MD) and oligo (dT) primers and amplified by PCR using ELITE Taq Polymerase (Bioprince, Beijing, China) and gene-specific primer sets.

The primer sequences were as follows; Type II collagen (forward: 5'-ACGGCGAGAAGGGAAGTTG-3', reverse: 5'-GGGGGTCCAGGGTTGCCATTG-3'), and β-actin (forward: 5'-GCGGGAAATCGTGCGTGACATT-3', reverse: 5'-GATGGA GTTGAAGGTAGTTTCGTG-3'). Amplification was performed using 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 40 sec. PCR products were stained with ethidium bromide on a 1% agarose gel and visualized by UV light using a Bioprofile image analysis system (Viber Lourmat, Marne la Vallee, France).

Results

Growth and morphology of MSCs

After the initial three days of primary culture, the mesenchymal progenitor cells adhered to a plastic surface and presented a small population of single cells with spindle shape. On days 7-10 after initial plating, the cells looked like long spindle-shaped fibroblastic cells, began to form colonies and became confluent (Fig 1A). After re-plating the fibroblast-like cells appeared polygonal or spindly with a long process. Following staining with PAS, the cells were observed under a microscope. The umbilical cord MSCs displayed blue smooth nuclear and red cytoplasm (Fig 1B).
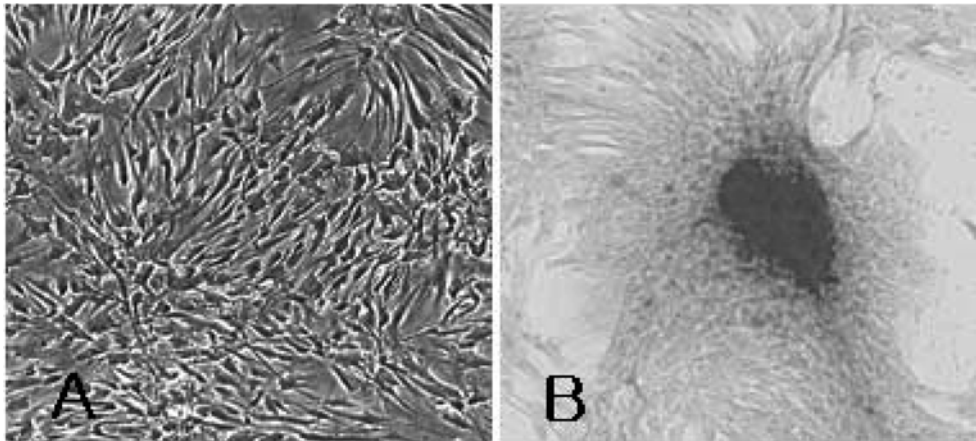

Fig 1. Phase contrast photograph (A) and PAS staining (B) of MSCs derived from human umbilical cord blood. The UCB-derived MSC colonies showed dominant growth of bipolar fibroblast-like cells (A) and positive reaction of PAS staining (B). A: × 50, B: × 100.
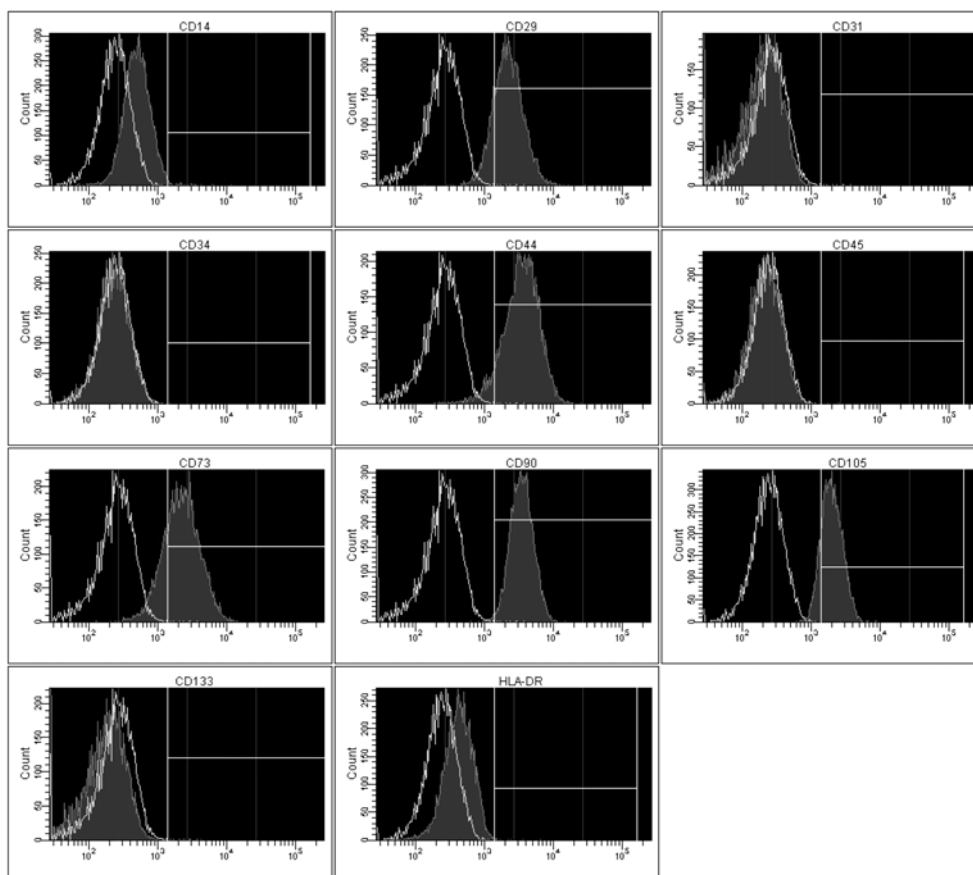

Fig 2. Immunophenotype of human mesenchymal stem cells derived from umbilical cord blood by FACS analysis. These cells positively expressed antigens CD29, CD44, CD90 and CD105, but the cells did not express antigens CD14, CD31, CD34, CD45, CD133 and HLA-DR.
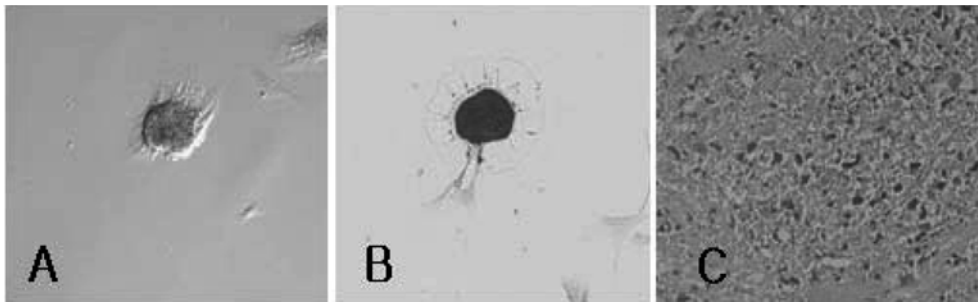
Fig 3. Phase contrast photograph (A) and Safranin O staining (B) of chondrogenic differentiation from mesenchymal stem cells. C showed Safranin O staining of colony paraffin block section in chondrogenic differentiation from mesenchymal stem cells (A and B: 50×, C: 100×). Red cell clusters indicate positive cells of Safranin O staining.

Immnuophenotypic characterization of surface antigens of UCB-derived MSCs

Immunophenotype of surface antigens on human MSCs derived from umbilical cord blood were analyzed by FACS analysis. These cells expressed positively with the antigens of CD29, CD44, CD73, CD90 and CD105 (Fig 2). However, they showed negative expression of surface markers of CD14, CD31, CD34, CD45, CD133 and HLA-DR (Fig 2).

Differentiation of MSCs into chondrocytes

With chondrogenic supplementation, the differentiation was apparent after one week of incubation. By the end of the second week, part of the MSCs became positive to the Safranin-o staining (Fig 3B). Similarly, the micropellet analysis showed positive stain (Fig 3C) and non-treated control culture did not show spontaneous chondrocyte formation even after 3-4 weeks of cultivation.

Expression of specific genes for chondrocytes

This study performed to evaluate chondrogenic differentiation from MSCs using immunostaining and RT-PCR methods. We analyzed the expression of type II collagen as an important chondrogenic marker gene. The positive reaction of type II collagen was detected in the chondrogen-induced MSCs (Fig 4A). In RT-PCR reaction, the type II collagen mRNA in MSCs was expressed at low level. However, following chondrogenic differentiation, the type II collagen mRNA was expressed at higher level compare to that of MSCs (Fig 4B). This result revealed that the UCB-derived MSCs underwent chondrogenic differentiation.

Discussion

Mesenchymal stem cells (MSC) are pluripotent progenitors that can be isolated from umbilical cord blood (5) as well as fetal tissues (7) and placenta (8) in new borne babies, also from adult tissues of bone marrow (16,18), fat (15), liver (7), lung (7), spleen (7), *et al*. MSCs are capable to renew themselves without differentiation in long-term culture (9,12). These cells also have the capabilities to differentiate into tissues of three germ layers including bone, cartilage, muscle,
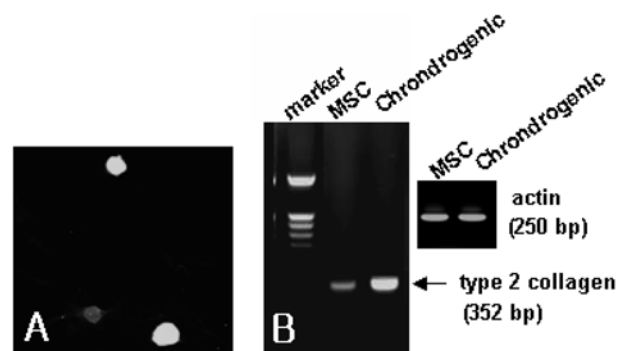

Fig 4. Immunocytochemical staining (A) and RT-PCR analysis (B) of type II collagen in chondrogenic differentiation from mesenchymal stem cells. A: Green-labeled clusters indicate positive cells of type II collagen. B: The arrow indicates PCR-product of type II collagen.

ligament, tendon, fat, dermis, hepatocyte-like cells and neuron-like cells (1,2,4,5,6,9,13,22). But the MSCs derived from umbilical cord blood have some beneficial merits compare to those from other tissues. The feasible collection of specimens is non-invasive compare to collection from bone marrow. Also, it was suggested that the UCB-derived MSCs had the characteristics of their low immunogenicity and suppression of alloreactive T cell responses (17,20). Thus the UCB-derived MSCs could serve as a potential alternative source of MSCs for allogenic application in future. Moreover, the chondrogenesis from UCB-derived MSCs provides a valuable method for researching differentiation of cartilage and bone formation and clinical application for repair of joint damage.

In this experiment the morphology of the hUCB-derived mesenchymal progenitor cells showed spindle-shaped fibroblastoid appearance and plastic-adherence character. The MSCs showed a high proliferative capacity, which were passed over 12 passages. When the immunophenotypes of these MSCs were examined by FACS analysis. The cultured MSCs expressed positively the MSC-specific surface markers of CD29 (B1-integrin), CD44 (hyaluronidase receptor), CD90 (THY-1) and CD105 (endoglin). However, the MSCs had negative expressions of surface markers of CD14, CD31 (PECAM-1), CD34

(hematopoietic), CD45 (leukocyte antigen), CD 133 and HLA-DR (histocompatibility).

Moreover, we confirmed whether these cells have the MSC specific characters and under appropriate culture conditions they can differentiate into chondrocytes during *in vitro* development. Our results showed that culture of human MSCs in chondrogenic differentiation medium led to the expression of type II collagen, one of the chondrocyte specific markers. Furthermore, the chondrocytes were stained positively with Safranin-O dye and immunofluorescence stain of type II collagen protein.

In conclusion, the mesenchymal progenitor cells isolated from hUCB were proliferated and developed to MSCs *in vitro*. Further the MSCs were successfully differentiated into chondrocytes under chemical induction and these cells expressed the morphological, biochemical and immunohistochemical characteristics towards chondrogenic lineages. Our data also indicate that the isolation, proliferation and differentiation of the hUCB-derived MSCs *in vitro* can be used for elucidating the mechanisms involved in chondrogenesis. Moreover this differentiation technique can be applied to developing cell-based tissue regeneration.

Acknowledgments

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사람 제대혈 유래 간엽줄기세포로부터 연골세포 분화

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요　약 : 본 연구에서는 사람 제대혈로부터 간엽줄기세포를 분리하고, 이들을 체외에서 다량 증식시키며, 나아가 이들 간엽줄기세포를 특정한 세포로 분화시키고자 하였다. 사람의 제대혈로부터 단핵세포를 Ficoll-density gradient 법으로 분리하고, 이를 10% 우태아혈청, L-glutamnie, 및 항생제가 첨가된 DMEM 배양액과 Keratinocyte 배양액으로 37℃ 5% CO_2 배양조건에서 계대배양으로 증식시키고 현미경으로 줄기세포의 발달과 형태학적 성상을 확인하였으며, PAS 염색 및 PACS 분석으로 간엽줄기세포임을 확인하였다. 이들은 체외에서 연골세포로 분화를 유도하였고, 이들 분화된 줄기세포는 면역조직화학적 검사법으로 연골세포 특이 물질에 대한 Safranin O 염색법 및 Type II collagen 염색법을 실시하여 이들의 발현을 확인하였으며 RT-PCR을 실시하여 특이 mRNA 발현을 확인함으로서 연골세포로 분화된 것임을 확인하였다.

주요어 : 간엽줄기세포, 사람 제대혈, 연골세포, 유속세포분석, RT-PCR, 면역조직세포검사.