Iranian Journal of Basic Medical Sciences
Vol. 10, No. 3, Autumn 2007, 146-153
Received: Feb 27, 2007; Accepted: Oct 4, 2007

Mesenchymal Stem Cell Purification from the Articular Cartilage Cell Culture

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Abstract

Objective
Articular cartilage as an avascular skeletal tissue possesses limited capacity to heal. On the other hand, it is believed that the regeneration capacity of each tissue is largely related to its stem cell contents. Little is known about the presence of mesenchymal stem cells in articular cartilage tissue. This subject is investigated in the present study.

Materials and Methods
Articular cartilage was obtained from rats of Wistar strain and subjected to enzymatic digestion using 1:1 ratio of 0.2% collagenase I and 0.1% pronase. The released cells were then collected and cultivated in 25 cm^2 culture flask, which was resulted in the appearance of two morphologically distinct cell populations. Fibroblastic population were purified and expanded through several subcultures, using their loose adherence on culture surfaces and examined in terms of differentiation potential.

Results
The primary culture mainly contained polygonal cells with few fibroblastic cells among them. Upon the first subculture, the fibroblastic cells appeared as aggregates of few cells among the polygonal cartilage cells. It was found that the fibroblastic cell population could easily detach from the culture surface following to a brief exposure to tripsin solution. Using this property, these cells purified and expanded by several subcultures. According to the results, the isolated cells were able to easily differentiate into chondrocytic, osteocytic and adipocytic cell lineages.

Conclusion
It seems that articular cartilage contains a distinct fibroblastic cell population with mesenchymal stem cell nature. These cells can be purified and expanded by cell culture methods, thanks to their loose adherence on the culture dish surfaces.

Keywords: Articular cartilage, Bone and fat differentiation, Cartilage, Mesenchymal stem cells

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Introduction

Mesenchymal stem cells (MSCs), also known as marrow stromal cells (1) or mesenchymal progenitor cells (2), are recognized by two abilities, first their potential to undergo extensive self-renewal proliferation and second their capacity to differentiate into three distinct lineages as osteocytic, chondrocytic and adipocytic cells (3). These cells were first isolated and described by Fridenstein (4) who provided definitive evidences that bone marrow contained non-hematopoietic cells in addition to well-recognized hematopoietic precursors. The most striking feature of these cells was reported to be their clonogenic property; the clone that they produce shows close resemblance to bone and cartilage tissue.

Several reports have indicated that MSCs possess more differentiation potential than it was originally thought. MSCs differentiation into hepatocyte, neuron, liver epithelial cells, and renal epithelial cells has previously been reported (5-8). This property is referred to as MSCs plasticity.

To date, the isolation of MSCs has been reported from bone marrow of human and several adult vertebrate specious. These cells that are able to undergo extensive self-renewal ability provide an appropriate cell source for cell-based treatment of bone and cartilage defects in animal models (9-14). According to some reports MSCs are also appropriate to be used in tissue engineering strategies especially those that are intended for curing of skeletal defects (15,16).

Although MSCs are first described to be isolated from the bone marrow samples but there are reports of isolation and characterization of the cells from variety of other tissues. According to previous investigations, trabecular bone, synovial membrane, bone periosteom, adipose tissue, dental pulp and muscular tissue may also contain MSCs (17-22).

Since articular cartilage is an avascular tissue unable to fully regenerate its damages, the isolation and characterization of MSCs using this tissue has been gained some attentions. In one attempt it has been reported that MSCs have been isolated and characterized from articular cartilage (23), but it has not been clear whether the isolated cells have been dedifferentiated chondrocyte or alternatively a distinct cell population existed in articular cartilage. In the present study in an attempt to establish an articular cartilage cell culture, few fibroblastic cells observed that were appeared among the cartilage polygonal cells. The initial examination revealed that whereas cartilage cells had established very firm attachment on the culture surfaces, fibroblastic cells had adhered very loosely on it, so that they could be lifted easily by a brief tripsinization. Using this property, fibroblastic cell population was isolated and purified. These cells were then expanded through several successive subcultures and evaluated in terms of *in vitro* differentiation into three skeletal lineages as osteocytic, chondrocytic and adipocytic cells.

Materials and Methods

Chondrocyte culture establishment

Rats of Wistar strain (8-10 weeks age and average weight of 200-250 g) were sacrificed by CO_2; the skin around the knee joint was incised and the soft tissues were cleaned off. The knee joint was then removed, placed in DMEM (Dulbecco's Modified Eagls Medium, Gibco, USA) containing 15% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 100 U/ml streptomycin and transferred to inside of laminar hood. Under sterile

conditions, the knee joint was opened and the articular cartilage from the tibia's upper and femur's lower surfaces were carefully dissected out, washed with PBS (phosphate buffer solution, Gibco, USA) and placed within the enzyme solution prepared from 1:1 ratio of 0.2% collagenase I (Sigma, USA) and 0.1% pronase (Sigma, USA). Enzymatic digestion was carried out overnight at 37 ºC and followed by collection of the released cells which were cultivated in 25 cm^2 culture flask in DMEM containing 10% FBS and 100 U/ml streptomycic/ penicillin antibiotics. All cultures were incubated at 37 ºC with an atmosphere of 5% CO_2 till confluency reached. During this period the culture medium was changed two times a week. Confluenced culture were lifted by exposing to tripsin/EDTA and subcultured at 1:2 ratios. The initial examination of the passaged-1 culture indicated that while chondrocytes appeared firmly to be attached to culture surfaces the cells with fibroblastic morphology had loose adherence. Using this property the fibroblastic cells were purified and expanded.

Cell purification

Chondrocyte passaged-1 culture was exposed to tripsin/EDTA solution and examined by invert phase contrast microscope to observe the lifting of the fibroblastic cells. Upon observation of the detached cells, approximatly 2 ml FBS was added in culture to neutralize tripsin effects. The lifted cells were then collected, washed by two times centrifugation, cultivated in new 25 cm^2 culture flasks and expanded through several successive subcultures.

Chondrogenesis

To induce cartilage differentiation, a micro mass culture system was used. For this purpose, 2.5×10^5 passaged-3 cells were pelleted under 1200 g for 5 minutes and cultured in a chondrogenic medium (1, 9) containing DMEM supplemented by 10 ng/ml transforming growth factor-ß3(Sigma, USA), 10 ng/ml bone morphogenetic protein-6 (Sigma,USA), 50 mg/ml insulin transferin selenium+ premix (Sigma, USA) and 1.25 mg bovine serum albumin (Sigma, USA) and 1% fetal bovine serum (Gibco, UK). The chondrogenic culture was maintained at 37 ºC, 5% CO_2 for 21 days with a medium replacement of three times a week. At the end of this period the cultures were evaluated for cartilage differentiation by histochemistry and RT-PCR analysis.

To examine cartilage differentiation, two specific staining for proteoglycan detection were utilized. For this purpose, the pellets were subjected to the following: fixing in 10% formalin; dehydrating in an ascending ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5 µ by microtome. Some sections were stained in toluidine blue for 30 seconds at room temperature and the others with alcin blue for 20 minutes at 37 ºC.

Osteogenesis

Confluenced passaged-3 cells cultured in 6-well plates used to induce bone differentiation. The proliferation medium of the cultures was replaced by osteogenic medium (1, 9) that consisted of DMEM supplemented with 50 mg/ml ascorbic2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM glycerole phosphate (Sigma, USA). The cultures were incubated at 37 °C and 5% CO_2 for 21 days with medium replacement of three times a week. Occurrence of differentiation was examined by histochemistry and RT-PCR analysis.

Alizarin red staining was used to detect whether the mineralized matrix was formed in the cultures. For staining, the cultures were first fixed by methanol for

10 minutes and then subjected to alizarine red (Sigma, USA) solution for 2 minutes.

Adipogenesis

Confluenced passaged -3 cells were used to evaluate the adipogenic ability of the isolated cells. The proliferation medium of the cells was replaced by adipogenic DMEM medium (1, 9) containing 100 mM dexamethazone (Sigma, USA) and 50 mg/ml indomethacin (Sigma, USA). The cultures were then incubated for 21 days at 37 ºC, 5% CO_2. The medium was changed 3 times a week. Occurrence of adipogenic differentiation was evaluated by histochemistry (Oil red staining) as well as RT-PCR analysis. The culture was fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red (Sigma, USA) solution in 99% isopropanol for 15 minutes. At the end, the stain solution was removed and the cultures were washed with 70% ethanol before they were observed by light microscopy.

RNA extraction and RT-PCR analysis of gene expression

Total RNA was collected from cells that had been induced to differentiate into osteoblastic, chondrocytic or adipocytic lineages as detailed above, using RNX-Plus™ solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove contaminating genomic DNA. Standard reverse-transcription reaction was performed with 5 µg total RNA using Oligo (dT) $_{18}$ as a primer and RevertAid ™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR was as follows: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were utilized to detect differentiations.

Table 1. Primers used in RT-PCR.

Genes	Primer sequences(5′-3′)	Annealing temp (°C)	Length of the bp
Collagen X	F: 5′-ACAAAGAGCGGACAGAGACC-3′ R: 5′-AGAAGGACGAGTGGACATAC -3′	61	183
Collagen II	F: 5′-GCCTCGCGGTGAGCCATGATC -3′ R: 5′-CTCCATCTCTGCCACGGGCT-3′	59	240
Aggrecan	F: 5′-TAGAGAAGAAGAGGGGTTAGG-3′ R 5′-AGCAGTAGGAGCCAGGGTTAT-3′	58	180
Osteopontin	F : 5′-GATTATAGTGACACAGAC-3 R: 5′-AGCAGGAATACTAACTGC -3′	55.9	331
Osteocalcin	F : 5′-GTCCCACACAGCAACTCG -3′ R : 5′-CCAAAGCTGAAGCTGCCG-3′	61.7	381
ALP	F: 5′-GGACCCTGCCTTACCAACTCATTTGT-3′ R: 5′-CGCACGCGATGCAACACCACTCAGG -3′	59	401
PPAR-alpha	F : 5′-CCCTGCCTTCCCTGTGAACTGAC -3′ R: 5′-GGGACTCATCTGTACTGGTGGGG-3′	62	387
PPAR- gamma2	F: 5′-GGTGAAACTCTGGGAGATCCC -3′ R: 5′-CTCCATCTCTGCCACGGGCT-3′	62	392
C/EBP-alpha	F: 5′-ACGTGGAGACGCAGCAGAA-3′ R: 5′- AGGCGGTCATTGTCACTGG -3′	61	211
GAPDH	F: 5′-TCGGTGTGAACGGATTTG-3′ R: 5′-ACTCCACGACATACTCAGCAC-3′	58	276

Amplification conditions were as follows: initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of denaturation at 94˚C for 45 minutes; annealing at 65 (insulin), 57 (GLUT1), 55 (GLUT2), 56 (glucagon), 65 (Oct4) and 60˚C (β-actin) for 45 minutes; extension at 72˚C for 30 minutes; and a final polymerization at 72˚C for 10 minutes. Each PCR was performed in triplicate and under linear conditions. The products were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

Results

Cell culture

In primary culture, one day after culture initiation, chondrocytes observed to be attached on culture surfaces. On the following days the chondrocytes started to proliferate and the culture was reached into confluency on the day 10 after initiation. At this time it was mainly consisted of polygonal cells along with few individual fibroblastic cells (Figure 1A). Upon first subculture the fibroblastic cells were appeared as aggregates of several cells surrounded by polygonal chondrocytes (Figure 1B).

Purification

According to the observation, 1 minute after tripsinization, fibroblastic cells were lifted and became floating. The polygonal chondrocytic cells resisted tripsinization, so they were detached after 5 minutes of being exposed to tripsin/EDTA. Using this property, fibroblastic cells were purified and expanded by three successive subcultures (Figure 1C and D). The cells maintained their fibroblastic morphology during the cultivation period.

Chondrogenesis

As it was noticed, the cell pellet subjected to chondrogenic medium grew larger as the culture time progressed, so that at the end of differentiation period, it seemed to be as twice of its initial size (Figure 2A).

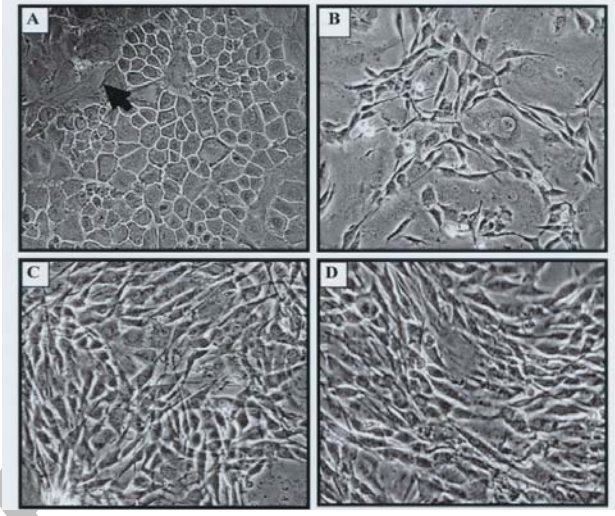

Figure 1. Chondrocyte culture prepared from rat's articular cartilage. A) In primary culture, the cells appeared mainly to be polygonal in shape with a few fibroblastic cells among them (arrowhead). Upon subculture, fibroblastic cells proliferated and observed as aggregates of a few cell surrounded by chondrocytes (B). These cells were purified thanks to their loose attachment on the surfaces (C). They were expanded through several successive subcultures (D).

Upon toluidine blue staining, the matrix secreted among the cells was stained purple (Figure 2B) indicating that the glycosaminoglycan-rich material was produced. Similarly, with alcian blue staining, this matrix stained bluish green (Figure 2C). RT-PCR analysis revealed that the mRNA of collagen II, X and aggrecan macromolecule were largely produced in differentiated cells (Figure 2D). These data strongly imply the occurrence of cartilage differentiation in the isolated cells.

Osteogenesis

Based on the daily checking, 5 days after culture initiation, nodule-like aggregation was appeared in some area of osteogenic cultures (Figure 3A).

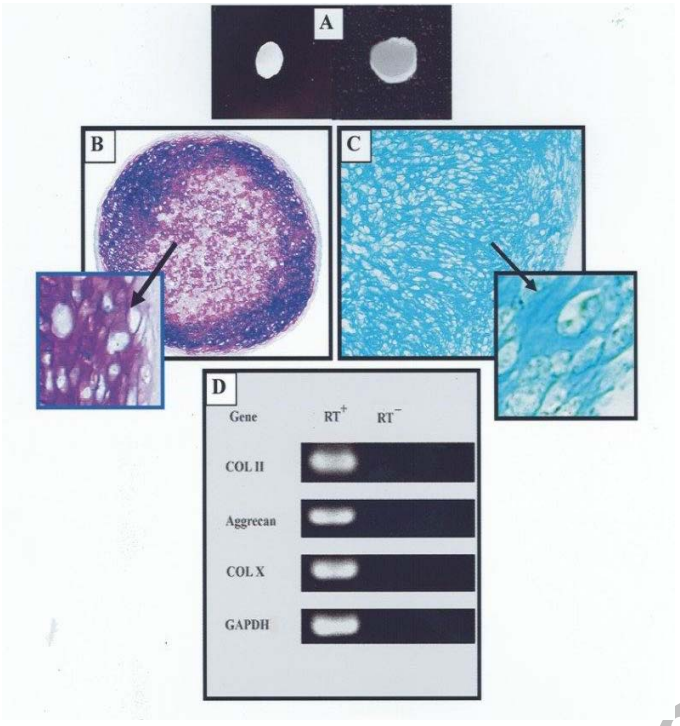

Figure 2. Chondrogenesis. A) At the end of cultivation periods, pellets seemed to be grown larger. They stained purple by toluidine blue (B) and bluish green by alcian blue(C). RT-PCR analysis indicated that cartilage specific genes were largely expressed (D).

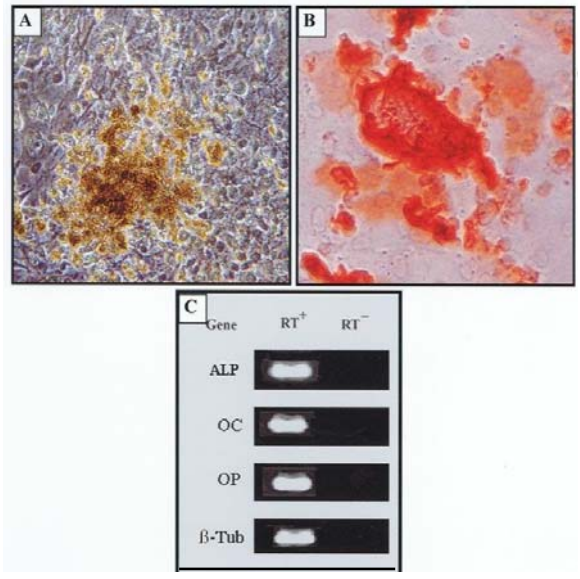

Figure 3. Osteogenesis evaluation. A) Few days after culture initiation, nodule-like area was appeared. These were stained red by alizarin red staining (B). RT-PCR analysis indicated that bone specific genes has been largely expressed (C).

The amount of these nodules increased as the time progressed. Upon alizarin red staining, these nodules strongly stained red (Figure 3B) indicating that they were heavily mineralized during the induction period. RT-PCR analyses indicated that bone specific proteins including osteocalcin, osteopontin and alkaline phosphatase were largely expressed in the cells (Figure 3C). All of these data implied that bone differentiation occurred in the culture of the purified cells.

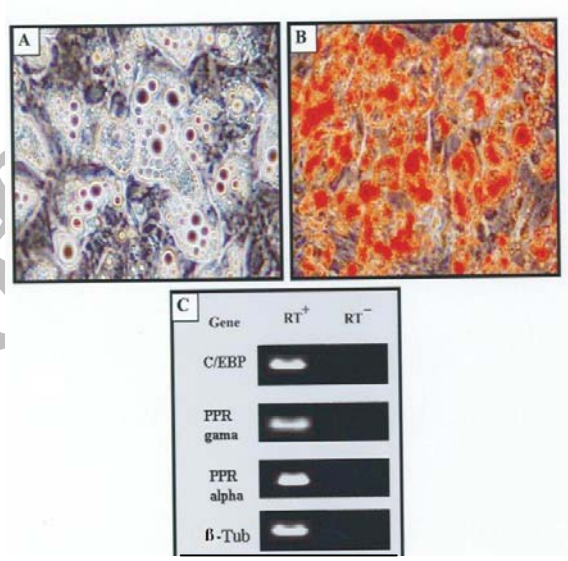

Figure 4. Adipogenesis analysis: A) a few days after culture initiation, lipid droplet was appeared in some cell. These droplet stained red with oil red (B). RT-PCR evaluation of the cells revealed that the mRNA of adipocyte specific genes has been largely produced.

Adipogenic cultures

3-4 days after culture initiation, some lipid droplets appeared inside of the cells subjected to adipogenic medium. Lipid droplet-containing cells were increased during the induction period (Figure 4A). The lipid droplets stained red upon oil red staining (Figure 4B) and the adipocyte marker genes including PPAR-alpha, PPAR-gamma2 and C/EBP-alpha appeared to be expressed in lipid-containing cells by RT-PCR analysis (Figure 4C).

Discussion

In the present study, fibroblastic cells appeared among the chondrocytes in culture prepared from rat's articular cartilage were purified using their loose adherence on the culture surfaces and expanded by several passages. These cells appeared to have tripotent differentiation; hence the mesenchymal stem cell nature. So far, in one attempt to isolate MSCs from articular cartilage, it remained unclear whether the isolated cells were a distinct cell population or they are chondrocytes undergone dedifferentiation. The observations were mostly in favor of distinct cell population rather than dedifferentiated chondrocytes.

In this work, it was observed that the fibroblastic cells existed in a very rare number as an individual cells even in early days of primary culture, and appeared as aggregations of few cells in passaged culture. According to these observations, it seemed to be logical to think that the purified fibroblastic cells were as distinct cell population rather dedifferentiated chondrocytes. In this regard, these observations differ with that of Fickert et al (23) who observed that in primary culture, the acquisition of fibroblastic shape became increasingly apparent as the cells were cultured on plastic. This description "acquisition of fibroblastic shape" imply that the fibroblastic cells were produced following chondrocytes dedifferentiation.

The other reason for this claim that the isolated cells seemed to be distinct cell population rather than dedifferentiated chondrocytes is considering the fact that dedifferentiation process occurs gradually in a long term during several successive subcultures. Indeed, in some previous research works, to induce this process, investigators utilized specific factors such as TGF-β, FGF and PDGF (24, 25) in chondrocytes culture. In this project, fibroblastic cells were observed even in early days of primary culture.

In spite of the considerable attempts that have been made to define the antigenic profile of human MSCs, no definitive single marker has so far been introduced. In this regard, several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 have been proposed to be as markers of human MSCs (26-28). However, the identification of MSCs, especially those from animal source, among the other cells and hence their isolation would be a difficult task owing to the lack of distinct specific marker (29). In almost all studies, the isolation of the cells is mostly performed on the base of their plastic adherence properties. After isolation, it is required to indicate their mesenchymal-stem cell nature. In the lack of specific marker it was proposed that the golden standard to identify the MSCs is to differentiate them into two or more cell lineages (9). In the present study, evaluation of the purified cells indicated their ability to produce differentiated progenies of osteoblastic, chondrocytic and adipocytic lineages, therefore; their mesenchymal stem cell nature.

In conclusion, it seems that articular cartilage contains a distinct population of fibroblastic cells in a very rare amount. These cells can be purified and expanded with standard culture techniques thanks to their loose adherence on the plastic culture dish.

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