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The Effect of Conditioned Medium of Adipose-Derived Stem Cells on the Expression Level of Cartilage Specific Genes by Chondrocytes

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Abstract

Objectives: Osteoarthritis (OA) is the most common multifactorial disease that deteriorates articular cartilage in the elderly. Recently, mesenchymal stem cells have been introduced as candidates for OA treatment but some researchers showed that implantation of stem cells into the lesion site of patients can lead to some side effects. Accordingly, many scientists switch their field of research to the application of stem cells supernatant. The purpose of this study is to evaluate whether supernatant of adipose-derived stem cells (ASCs) can result in the expression of cartilage-specific genes in chondrocytes.

Materials and Methods: Mesenchymal stem cells were isolated from adipose tissue and their supernatant was collected and added to chondrocytes. After 3 days, chondrocytes were prepared for expression of collagen type II, sox9, COMP and aggrecan genes using real-time RT-PCR.

Results: Data analysis showed that in the presence of ASCs conditioned medium (CM), the gene expression level of collagen II, sox9, and aggrecan did not change but that of the COMP significantly increased after 3 days.

Conclusions: The obtained results may be related to the unknown factors secreted by stem cells and more investigations are necessary for understanding the exact mechanisms underlying these findings.

Keywords: Stem cells, Adipose tissue, Osteoarthritis, Supernatant

Introduction

Articular cartilage is a semi-solid and flexible load-bearing connective tissue that acts as a shock-absorber at the articulating surfaces of synovial joints (1). This translucent load-bearing tissue is composed of a delicate and precisely arranged extracellular matrix (ECM) which is maintained by the only cell type known as chondrocyte (2). Due to scarce number of chondrocytes and lack of vessels, nerve, and lymph, cartilage tissue has a poor ability to self-repair and regenerate (3), so repeated trauma and damage typically result in joint disorders (4). Osteoarthritis (OA) which is the most prevalent type of musculoskeletal diseases is featured by the degradation of cartilage ECM, synovial inflammation, and involvement of subchondral bone (1). At the molecular level, inflammatory mediators such as interleukin 1 beta (IL1β) and tumor necrosis factor-alpha (TNFα) have been implicated to stimulate matrix-degrading enzymes such as matrix metalloproteinases (MMPs), aggrecanase and nitric oxide and further suppress the synthesis of normal ECM component (4).

Traditional techniques to heal cartilage damage include microdrilling, lavage , microfracture and so on (2). During the past few years, autologous chondrocyte transplantation (ACT) has evolved with some promising results for cartilage regeneration. However, some investigations have pointed to the little difference between ACT and microfracture technique in which blood elements such as mesenchymal stem cells (MSCs) can penetrate from marrow cavity into the defect site (4).

Early cartilage tissue engineering attempts have relied on the application of primary isolated chondrocytes, but the difficulty in sample harvesting, expanding and dedifferentiation of these cells in culture condition shifted the research focus to the application of stem cells (4). MSCs can be extracted from different adult organs such as bone marrow, adipose tissue, and muscular tissue and have the potential to differentiate into a wide range of cells including adipocytes, chondrocyte, muscle and bone (1). Among different sources, adipose tissue seems to be an ideal candidate for MSCs isolation (5). Its abundance in the body, easy accessibility and the greater number of stem cells which can be isolated introduced this tissue as a viable alternative source for cell therapy strategies (2,5).

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Some investigations revealed that adipose-derived stem cells (ASCs) have greater potential for cartilage repair compared to cultivated chondrocytes (6) and can maintain their ability to synthesize collagen type II even after the fifteenth passage (7). It has been reported in different studies that stem cells exert their enhancing effects, at least in part, through synthesis and secretion of cytokines and growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2) and hepatocyte growth factor (HGF) in a paracrine manner (8). Particularly, the immunomodulatory, proliferative and anti-apoptotic effects of these secreted factors have been shown previously (9,10). Despite the numerous advantages of stem cell application in regenerative medicine, transplantation of these cells in human can be hazardous due to the risk of inducing cancer (11). Therefore, due to the presence of trophic factors in stem cells conditioned medium, it can overcome the limitations of stem cell application and can be used as an alternative for stimulation of chondrocyte ECM synthesis and further cartilage damage healing.

Regarding the potential role of secreted growth factors in conditioned medium of ASCs, the aim of our study is to understand whether it can promote expression of some major cartilage genes including sox-9 (the major cartilage transcription factor), cartilage oligomeric protein (COMP), aggrecan and collagen type II.

Materials and Methods

Cartilage Harvesting and Chondrocyte Isolation

The consent was obtained from patients undergoing joint replacement surgery. The articular cartilage samples were transferred to the cell culture lab in phosphate buffer saline (PBS). The cartilage samples were cut into l × l mm^2 pieces and then were washed 3 times with PBS containing 1% penicillin/streptomycin (p/s). Chondrocyte isolation was performed by cartilage digestion in Pronase 1% for 60 minutes in shaking water bath followed by incubation for 4-6 hours in collagenase 0.2%. After enzyme neutralization, digested pieces were centrifuged at 1500 rpm for 5 minutes. The cells were counted, seeded at a density of 5 × 10^5 in T75 culture flasks and incubated at 37°C, 5% CO2. The first medium exchange was done after 24 hours and was followed three times per week. The cells were trypsinized after reaching about 70% confluency.

Isolation of Mesenchymal Stem Cells from Adipose Tissue

Adipose tissue samples were collected following written informed consent from patients undergoing laparotomy surgery without confirmation of any malignancy. The obtained adipose samples were transferred to the lab and were washed 3 times with PBS. Then, they were cut into small pieces using a sterile scalpel. Adipose tissue pieces were weighed and then were digested with collagenase type II (0.2%) in shaking water bath for 60-90 minutes. The enzyme activity was neutralized by adding Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% FBS and then centrifugation was performed at 1500 rpm for 5 minutes. The obtained MSCs were counted with trypan blue dye exclusion test on the hemocytometer slide and then plated at a density of 5×10^5 in T75 culture flask in humid Co_2 incubator (37°C, 95% humidity, 5% CO_2). The medium exchange was performed 3 times per week. When the culture reached 70% confluency, the first cell passage was done. ASCs at the fourth and seventh passages were used for this study.

Experimental Design

To evaluate whether ASCs-conditioned medium (CM) can influence the chondrocyte gene expression, the cells at the third passage were divided into control and CM-treated groups. Cultivated chondrocytes (density of 1 $\times10^6$) received DMEM as control or were treated with ASCs-CM for 72 hours. Afterwards, the samples were prepared for the expression of cartilage-specific genes such as sox-9, aggrecan, collagen II and COMP by real-time RT-PCR.

Collection of Conditioned Medium from ASCs

For applying the ASCs conditioned medium, the cells at the fourth and seventh passages were used. For this purpose, after reaching about 70% confluency, the supernatant was removed using PBS, the attached cells were washed and then fresh free DMEM was added to them. After 48 hours, the conditioned medium was aspirated and centrifuged at 1500 rpm for 5 minutes and subsequently centrifuged at 3000 rpm for 3 minutes to remove any debris and further kept at -80°C until use.

TGF-β1 and IGF-1 Measurement Using Enzyme-Linked Immunosorbent Assay (ELISA) Method

Using human transforming growth factor-ß1 (TGF-β1) ELISA kit (BOSTER, Cat No. EKOSB, CA) and human insulin like growth factor (IGF-BP-1) ELISA kit (BOSTER, Cat No. EKO382, CA), the concentration of these two growth factors in ASCs conditioned medium was measured. Based on the manufacture's protocol, the incubation of the samples and standards was performed at 37°C for 90 minutes. Then, biotinylated antibodies were added (37°C, 60 minutes) and the plates were washed 3 times with 0.01M Tris-buffered saline (TBS). After the addition of avidin-biotin-peroxidase, TMB color developing agent was added while the samples were incubated in dark place. Finally, the absorbance was measured at 450 nm using a microplate reader (Tecan).

Gene Expression Using Real-Time RT-PCR

The gene expression profile of sox-9, aggrecan, collagen II and COMP in all groups was evaluated by real-time RT-PCR method. For this purpose, the total RNA content was extracted using RNX-PLUS reagent kit (Yekta Tajhiz Azma, Cat No. YT9065). According to the manufacture's

protocol, chloroform was added to samples that have been placed on ice and then centrifuged at 12 000 rpm for 15 minutes. In the next step, the samples were incubated in Isopropanol and further centrifuged at 12 000 rpm for 5 minutes. The supernatant was removed and 75% ethanol was added. After another centrifugation (7500 rpm, 8 minutes), the obtained pellet was dissolved in DEPC treated water. Approximately 500 ng/μL of total RNA was used for cDNA synthesis using a reverse transcription kit (Takara, RR037I, Japan). The RT-PCR reactions were performed using Rotor-Gene 6000 (Corbett, 010755, Australia) with SYBR Green PCR Master Mix (Takara, RR820L, Japan) under the condition of 2 seconds at 98°C and 5 seconds at the annealing temperature of 60°C. Table 1 shows the primer sequences used in this study. The gene expression levels of samples were analyzed by the ΔΔCT method using β-actin as internal control. All experiments were done in triplicate.

Statistical Analysis

The data are reported as means ± standard deviation (SD). One-way ANOVA and Bonferroni post-test were used to understand the statistical difference between different groups. A *P* value <0.05 was set as significant.

Results

The Elevation of the Secretion of TGF-β and IGF-1 by Increasing the Number of Cell Passages

To evaluate whether increasing the number of cell passages can influence the amount of secreted factors by ASCs, we measured the TGF-β and IGF-1 levels in cells at the fourth and seventh passages. As it can be observed in Figure 1A and B, there is a significant difference in the amount of both TGF-β and IGF-1 secretion at the seventh passage of ASCs compared to the fourth passage cells (*P*=0.01 and *P*=0.001, respectively).

The Effect of ASCs-Conditioned Medium on Chondrocyte Gene Expression

After the treatment of chondrocytes with adipose derived stem cells conditioned medium (ASC-CM) for 3 days, the expression of cartilage-specific genes including collagen type II, sox-9, aggrecan and COMP was evaluated using real-time RT-PCR. As it can be understood from Figure 2, the expression of collagen type II, sox-9 and aggrecan genes in ASCs-CM treated chondrocytes decreased compared to the control chondrocytes, but that of COMP increased significantly in treated chondrocyte compared to the control group (*P*<0.0001).

Discussion

In this in vitro study, we found that treatment of chondrocytes with ASC-CM resulted in up-regulation of COMP gene expression but not other genes. Furthermore, our study showed the presence of TGF-β and IGF-1 in the conditioned medium of ASCs.

Table 1. Primer Sequences Used in Real Time RT-PCR and Related Annealing Temperature

Gene	Primer	Annealing Temperature
sox9-F	AGAGAGGACCAACCAGAATTC	57°C for 30 s
sox9-R	TGGGTAATGCGCTTGGATAG	57°C for 30 s
Coll2-F	GGCAATAGCAGGTTCACGTACA	59°C for 30 s
Coll2-R	CGATAACAGTCTTGCCCCACTT	59°C for 30 s
Comp-F	TGCAATGACACCATCCCAG	56°C for 30 s
Comp-R	ACACACACTTTATTTTGTCCTCTC	56°C for 30 s
ACAN-F	CAACTACCCGGCCATCC	56°C for 30 s
ACAN- R	GATGGCTCTGTAATGGAACAC	56°C for 30 s
B actin-F	TCCTCCCTGGAGAAGAGCTA	58°C for 45 s
B actin-R	TCAGGAGGAGCAATGATCTTG	58°C for 45 s

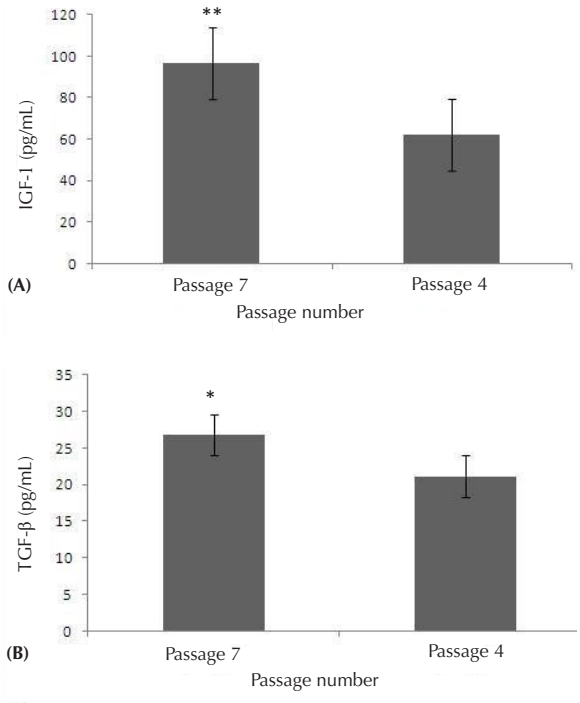

Figure 1. Measurement of IGF-1 (A) and TGF-β (B) Secretion by ASCs in Different Cell Passages. *P<0.01, **P<0.001.

OA which is the most common form of musculoskeletal system disorders occurs as a result of cartilage ECM degradation (12). In normal cartilage tissue, chondrocytes are responsible for maintaining the synthesis of ECM components (3). Cartilage cells are fed through diffusion process, therefore, this tissue has inherently poor capacity for regeneration (13). In recent decades, chondrocyte implantation techniques have introduced for repairing the damaged cartilage (14). The major problem with this method is obtaining enough chondrocytes because these cells have poor proliferative capacity and lose their phenotype after some passages in monolayer culture (15). Therefore, finding a suitable and safe promoting factor for maintaining chondrocyte phenotype and the potency of these cells for ECM synthesis is of importance.

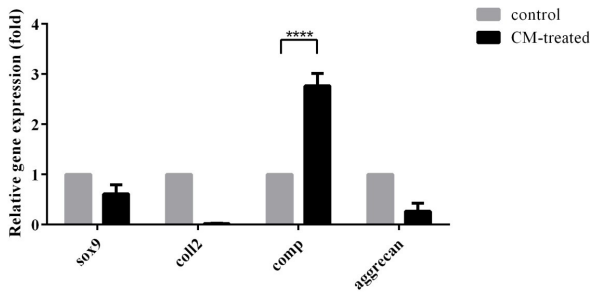

Figure 2. The Analysis of the Expression of Cartilage-Specific Genes in the Chondrocytes Cultured in Monolayer and Mass Culture System. **** $P<0.00001$.

In this study, we treated chondrocytes with ASC-CM to examine whether it can enhance the expression of cartilage-specific genes such as collagen type II, COMP, aggrecan and sox-9. We applied ASC-CM instead of ASCs alone due to some disadvantages of these cells as tumorigenesis (16). Various studies reveal that stem cells secrete different growth factors as secretome and microvesicles in their supernatant, therefore, the application of CM alone without stem cells can be advantageous for the repair of damaged tissues (17). To evaluate whether ASCs can secrete anabolic growth factors into the conditioned medium, we measured the amount of TGF-β and IGF-1 in the supernatant of cells in different passages. This evaluation revealed that by increasing the cell passage number, the amount of these growth factors increases. Therefore, the promoting effects of ASC-CM on chondrocytes can be interpreted in part because of the presence of growth factors. ASC-CM certainly contains many factors other than those we have measured. In this study, we found that treatment of chondrocytes with ASC-CM resulted in reduced expression of collagen II, Aggrecan and sox-9 genes, however, the expression of the COMP was increased. In accordance with our result, it has been previously reported that ASCs can secrete angiogenic factors such as VEGF-A, which is able to induce chondrocyte apoptosis and reduce proteoglycan synthesis (18). Furthermore, it has been observed that cartilage explants exposed to MSC-CM showed down–regulation in collagen type II and aggrecan gene expression (8).

Our study showed that in the presence of ASC-CM, the expression of COMP gene was up-regulated significantly compared to the control chondrocyte. COMP is an important non-collagenous protein in cartilage tissue that interacts with collagen fibers and proteoglycans and its mutation results in skeletal malformations (19). Except for COMP, the expression of other cartilage-specific genes such as collagen II, Aggrecan and sox-9 was down-regulated in chondrocytes treated with ASC-CM. The data obtained in this study can be attributed to the unknown factors secreted into the supernatant by ASCs.

Conclusions

These data suggest that despite the potential effects of ASCs and their secreted factors, ASCs have a limited ability to promote the chondrocyte gene expression at least in part because of the unmeasured factors in it, therefore, finding these factors and reducing their amounts can reverse its inhibitory effects on cartilage regeneration.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

The institutional review board and Medical Ethics Committee of the Tabriz University of Medical Sciences approved the study protocol (ethical number: 5/104/512).

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