Original Article

Assessment of TGF-β3 on production of aggrecan by human articular chondrocytes in pellet culture system

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Abstract

Background: The Autologous Chondrocytes Transplantation (ACT) method is being studied for repair of cartilage diseases. As the chondrocytes dedifferentiated during monolayer culture, three-dimensional cultures are suggested to redifferentiate them. The aim of this study was investigation of the effect of TGF-β3 growth factor on chondrocytes in pellet culture system.

Materials and Methods: The chondrocytes were isolated from three human articular cartilages by enzymatic digestion. The cells of the second passage were transferred to pellet culture system. We determined the chondrogenic medium with TGF-β3 as the experimental group and without it as the control group. After 2 weeks, the aggrecan production was investigated using histological and immunohistochemical (IHC) methods.

Results: The presence of glycosaminoglycans was proved through Toluiden blue staining. Comparison of IHC results using MATLAB software showed that aggrecan in the experimental group was significantly higher than in the control group ($P \leq 0.05$).

Conclusion: The presence of TGF-β3 in the chondrogenic medium could lead to the production of more aggrecan in chondrocytes cultivated in pellet culture system.

Key Words: Aggrecan, chondrocyte, growth factor, pellet system, TGF-β3

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INTRODUCTION

Articular cartilage has few chondrocytes, approximately 1-2% of its volume, and 70-80% of water, 15% of collagens (especially type II), 9% of aggrecan and 3% of other kinds of macromolecules.[1,2] Proteoglycans, especially aggrecan, make the cartilages flexible.[3]

Articular cartilage damage leads to joint dysfunction, and the healed tissue does not have appropriate structural and biomechanical features.[4-6] Various therapies have been used in this regard. Microfracture method was satisfactory in some studies,[7-9] but with some deficiencies.[10] Other therapies, including drugs, debridement of tissue and joint lavage, arthroscopy, surgery and arthroplasty, not only does not heal the tissue but may also cause fibrosis, apoptosis and destruction of the tissue.[11]

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In the Autologous Chondrocyte Transplantation (ACT) method, isolated chondrocytes from articular cartilage were proliferated in a monolayer culture and they gradually dedifferentiated in a way that the expression of type II collagen and protein core of aggrecan genes decreased and the expression of type I collagen gene increased.[12,13] The studies have shown that dedifferentiation improves by increasing the number of passages and using serum in the culture medium.[14-17] Today, three-dimensional culture systems (scaffolds or pellet) are suggested to redifferentiate of dedifferentiate chondrocytes and maintain chondrocytes' phenotype.[18-22] The mechanism of pellet system is similar to the process occurring in the embryonic period, the formation of limb buds and condensation of mesenchymal cells.[23,24]

Growth factors are effective in properties and performance of chondrocytes as lack of growth factors and imbalance between anabolic and catabolic factors is considered as highly influencing factors in osteoarthritis progression.[25] Many studies have been performed on the effect of growth factors like BMP-6, TGF-β3, EGF, IGF-I, PDGF-AB and bFGF under "*in vitro*" conditions on stem cells and chondrocytes with different results.[26-32] The TGF-β family is mainly produced in bones and cartilages. The TGF-β 2 and 3 induce production of type II collagen and proteoglycans in chondrocytes and are effective in chondrogenesis of mesenchymal stem cells.[30] Although the use of TGF-β1 under "*in vivo*" conditions improves cartilaginous damage,[31] it causes side-effects like formation of osteophyte, synovial membrane inflammation and hyperplasia.[32]

Lots of challenges exist about the growth factors during the chondrogenic process due to the inconsistencies among studies. Therefore, in this study, the effect of TGF-β3 growth factor on natural chondrocytes for production of aggrecan was studied in pellet culture system using immunohistochemical (IHC) method.

MATERIALS AND METHODS

Isolation of chondrocytes from articular cartilage

Samples of articular cartilage were removed from three patients' knees. The patients had signed written consent form before the start of experiment. The cartilaginous samples were cut to 1-2-mm pieces. Type II collagenase enzyme solution (Sigma,(Germany) 350 μg/mL) was applied to digest the tissue for 4 h at 37°C. The resultant suspension was centrifuged at 1400 rpm for 10 min and the chondrocytes were cultivated in DMEM/F12, Penicillin/streptomycin 1% and FBS 10% medium.

Pellet culture system

25×10^4 cells of passage 2 were transferred into 15 mm polypropylene conical tubes and were centrifuged with 0.5 mL of chondrogenic medium, including high glucose DMEM (Gibco, USA, Grand island), Penicillin/Streptomycine 1%, ITS 1%, Ascorbate-2-phosphate 50 μg/mL, dexamethason 10^{-8} $_M$, Linoleic acid 5 μg/mL and TGF-β3 10 ng/mL (Sigma) and cultured at 37°C and 5% CO_2.

This medium was also used without TGF-β3 growth factor for the control group. The medium was replaced every 3 days and the samples were studied at the 14th day using histological and IHC methods.

Histological study of the pellets

The samples were fixed in 10% formaldehyde for 24 h, dehydrated in ascending ethanol, clarified with xylol and embedded in paraffin, sectioned into 4-μm thickness and stained by H and E and Toluidine blue. The samples were studied using a light microscope (Nikon, Japan).

Immunohistochemical method

For antigen retrieval, hyaluronidase enzyme (8 mg/mL) was used for 120 min at 37°C and block endogenous peroxidase activity with use of 3% hydrogen peroxide in absolute ethanol for 10 min. The anti-aggrecan primary antibody (Abcam, England) with concentration of 1:50 was added at 4°C for 24 h. The secondary antibody was conjugated with horse radish peroxidase (Abcam) for 60 min, followed by diaminobenzidine (DAB) (DakoCytomation, Denmark), and stained by H and E. The samples were studied using a light microscope (Nikon).

Semi quantitative analysis of the immunohistochemical images

The IHC images were analyzed by MATLAB software. Useless details of the image were eliminated and thickness of the original edges of the image was strengthened. The image holes were filled using suitable morphological operators and the desired area was specified to be studied after eliminating small areas [Figure 1]. The intensity of the brown color of the desired area was quantitatively obtained by extracting the red, blue and green values and combining them.

RESULTS

Monolayer cultivation of chondrocytes

The isolated chondrocytes were gradually attached to the flasks in 24 h. Before adhesion, the cells

were round and, after that, they were fibroblastic like [Figure 2].

Pellet culture results

In pellet culture, passage 2 cells gradually aggregated and separated from the falcon's bottom during the second and third days. After removing, on the 14th day, cellular aggregates were round to oval, with a diameter of about 1 mm [Figure 3].

Histological analyses of samples

Morphological study of the pieces resulting from pellet system was carried out by H and E staining [Figure 4]. The central part of the samples contained more or less round chondrocytes, which were confined in their own lacunas and surrounded by basophilic extracellular matrix. In the peripheral part of the samples, a layer similar to natural perichondrium was formed.

Toluidine blue staining

Toluidine blue is a cationic dye that satins sulfated glycosaminoglycans with negative charge metachromatically. The existence of sulfated glycosaminoglycans in the extracellular matrix of the tissue formed in pellet system was identified by the staining [Figure 5].

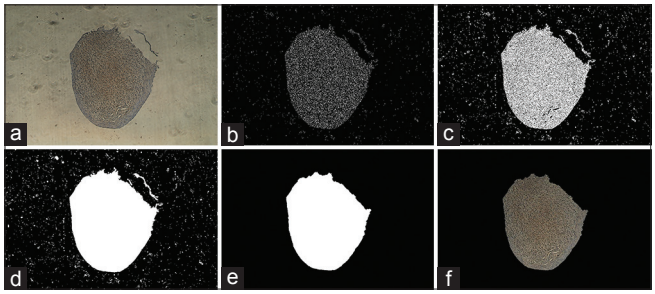

Figure 1: (a-f) Example of operations to analyze pictures by MATLAB software

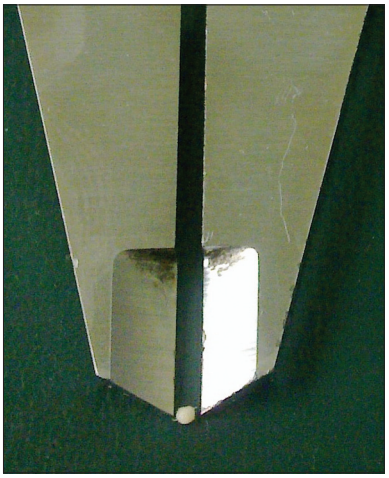

Figure 3: A sample of pellet in day 14 that formed as a 1-mm diameter spherical mass

Immunohistochemical analyses

The brown color resulted from the interaction between DAB and peroxidase conjugated to the secondary antibody, observed in both groups, showing the production of aggrecan in the control and experimental groups [Figure 6]. Then, semiquantitative analysis was performed on the images obtained by immunohistocemical staining of the groups using MATLAB software. Figure 7 shows that color intensity in samples whose medium contained TGF-β3 growth factor was significantly higher than that of the control group.

DISCUSSIONS

In our study, pellet system was used in order to redifferentiate chondrocytes that lost their phenotype during monolayer culture. Today, pellet culture as a three-dimensional culture system is largely used in chondrogenic studies that provide appropriate cell to cell interactions like cellular communications during cartilage formation in the embryonic period.[33]

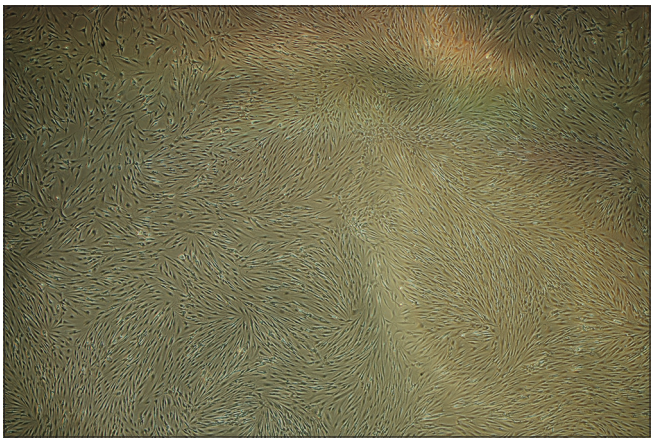

Figure 2: Monolayer culture of articular chondrocytes in passage 2 × 40

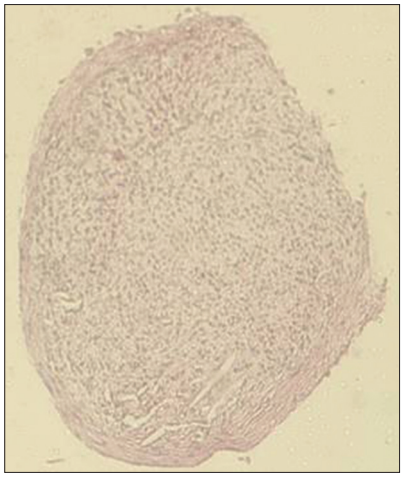

Figure 4: Hematoxylin and eosin staining of pellet

Bernstein *et al.* showed that the expression of type II collagen and SOX9 genes in pellet system was higher than that in alginate scaffold in pig knee joint chondrocytes.[34]

Despite many studies on the effect of growth factors such as HGF, EGF, PDGF-AB, bFGF, IGF-I and TGF-β on chondrogenic process, there is still not enough information for choosing appropriate growth factors. It seems that TGF-β is more suitable for more synthesis of extracellular matrix.[35,36] *In vitro* studies on the effect of TGF-β in chondrocytes have shown quite opposite results. Some researchers found the increase of proliferation in the presence of TGF-β,[37-40] while some others considered this growth factor as an inhibitor to proliferation.[41] This contradiction has also been observed about the effects of this growth factor on the synthesis of extracellular matrix by chondrocytes. As some studies showed that TGF-β increased the synthesis of glycosaminoglycans, both *in vivo* and *in vitro*,[42,43] another study showed that adding this growth factor to chondrocytes inhibited the synthesis of glycosaminoglycans.[44] In Gruber's study, the use of TGF-β increased proteoglycan biglycan and decreased proteoglycan decorin simultaneously.[38] Our results showed that mean aggrecan in the treated group with TGF-β3 was significantly higher than in the control group in the pellet system.

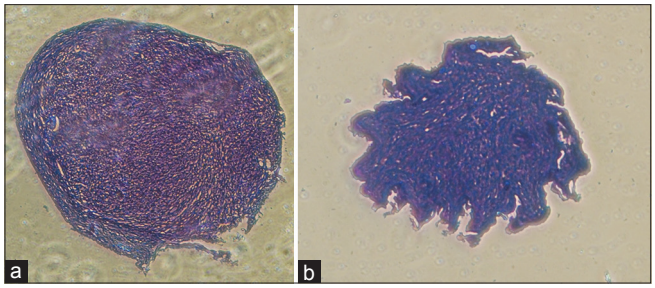

Figure 5: (a) Toluidine blue staining of pellet. Group + TGF-β3 (b) Toluidine blue staining of pellet. Group - TGF-β3

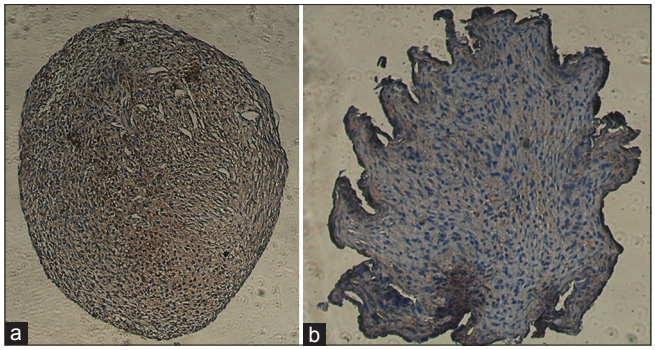

Figure 6: (a) Immunohistochemistry staining of pellet. Group + TGF-β3; (b) Immunohistochemistry staining of pellet. Group - TGF-β3

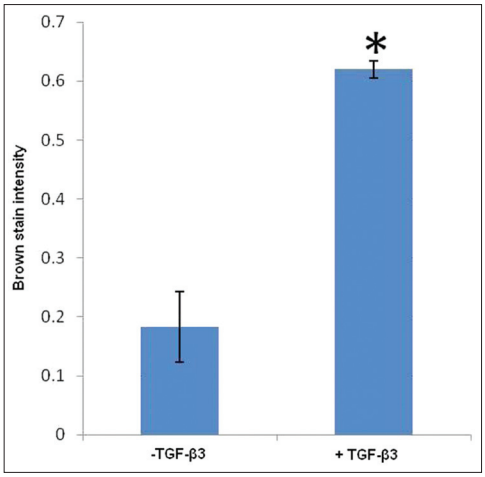

Figure 7: Diagram of analysis of immunohistochemical pictures

An important point that is usually ignored in studies conducted on the effect of TGF-β on chondrocytes and stem cells is that this growth factor exists by different values (from 2.7 ± 0.37 to 20.9 ± 2.2 ng/mL) in FBS used in the culture medium,[45] as the presence or absence and serum concentration can disrupt the results of the experiment. Gunja *et al.* examined concurrent effect of TGF-β and mechanical pressure on cells removed from a rabbit's meniscus tear and tried to minimize FBS concentration in experiments to solve the problem. In the first stage, he reduced the concentration of FBS from 10% to 1% in the medium, which resulted in the reduction of synthesis of collagen and glycosaminoglycan by 50% and the number of cells by 60%. In the second stage, he used TGF-β1 with a concentration of 10 ng/mL in the medium, which resulted in the increase of collagen by 15-times and glycosaminoglycan by eight times.[46] In the present study, the effect of TGF-β in the chondrogenic induction stage in synthesis of aggrecan was examined using pellet system and chondrogenic medium without FBS.

The results of the present study confirm the results of the studies showing a positive effect of TGF-β on the synthesis of extracellular matrix, as the produced proteoglycan in the experimental group was more than that of the control group. However, an important question on the use of growth factors is the mechanism with which these factors increase synthesis of proteoglycans. In normal conditions, synthesis of proteoglycans by chondrocytes is controlled using a negative feedback mechanism, as the chondrocytes are sensitive to proteoglycan concentrations in extracellular matrix and, when these concentrations become adequate, synthesis of proteoglycans reduces. Therefore, without the interference of another factor, the amount of cell proliferation determines the content of proteoglycan of matrix.[47] Because the amount of cell proliferation probably was decreased in pellet system, it could be concluded that the increase

of proteoglycan aggrecan was mainly due to the increase of synthetic activity of chondrocytes than the increase of their proliferation activity. In other words, TGF-β3 increased the cell activity probably through stimulating them, which resulted in higher production of aggrecans in extracellular matrix; therefore, the cell proliferation was less effective in this process.

Regarding the positive effect of TGF-β3 in production of aggrecan by chondrocytes in pellet culture system, it should be considered the other effects of TGF-β3 in this culture condition like production of type I and II collagen and SOX9.

CONCLUSION

The presence of TGF-β3 in the chondrogenic medium leads to higher production of aggrecan by chondrocytes cultivated in pellet system, but we suggest higher evaluation for another effects of this growth factor in pellet system.

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