Original Article

A comparative study of aggrecan synthesis between natural articular chondrocytes and differentiated chondrocytes from adipose derived stem cells in 3D culture

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Abstract

Introduction: The main obstacle for tissue engineering is to find the most appropriate cell which is able to produce extracellular matrix (ECM) similar or better than natural chondrocytes *in vitro*. This study compared aggrecan synthesis's potential between differentiated chondrocytes (DCs) from adipose-derived stem cells (ADSCs) and natural articular chondrocytes (NCs) in 3D culture *in vitro*.

Materials and Methods: Human ADSCs were isolated from sub-cutaneous adipose tissue and then the surface markers including CD 14, 45 CD105, CD90, CD44 were analyzed by flow cytometry. Also human articular chondrocytes were yielded of non-weight bearing area of Knee cartilage. Both types of the cells were encapsulated in alginate scaffolds and cultured in chondrogenic medium with and without TGFβ3 for 3 weeks. Then the extent of aggercan (AGC) production was evaluated by ELISA on days 14 and 21.

Results: Our findings indicated that differentiated chondrocytes (DCs) with and without TGFβ3 synthesized more AGC than natural chondrocytes (NCs) on day 14. But DCs without TGFβ3 had higher production than other groups on day 21. Application of TGFβ3 resulted in an increase of amount of AGC in DCs on day 14 but a decrease on day 21 than same group.

Conclusion: Since, aggrecan is an important chondrogenic marker, it was concluded that ADSCs can be possible reliable alternative cell source for cartilage tissue engineering in future.

Key words: Adipose-derived stem cell, articular chondrocyte, alginate, scaffold, TGFβ3

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INTRODUCTION

Articular cartilage is an avascular and aneural tissue so it cannot self-regenerate once damaged by sports and accidental injuries or articular diseases.[1,2] Different surgical strategies such as: Articular debridement, subchondral drilling, periosteal, or perichondrial resurfacing have relieved pain and improved joint function but the repaired tissues are fibrocartilage rather than hyaline

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cartilage.[3-5] So the investigators have been employed a new strategy, tissue engineering, for repairing of articular cartilage damages during the past two decades. The basis of tissue engineering involves the use of cells, scaffolds, and signaling factors.[6] Since 1994, Autologous Chondrocyte transplantation (ACT) procedure has been used for articular cartilage repair with successful clinical results.[7,8] But, several disadvantages have been reported such as donor-site morbidity, low number of available cells, and dedifferentiation of chondrocytes *in vitro* culture.[9] Due to limitations, stem cells seem to be a promising alternative source for repairing cartilage defects. Mesenchymal stem cells (MSC) are obtained easily from different tissues such as bone marrow,[10] adipose tissue,[11] synovial membrane,[12] trabecular bone[13] muscle,[14] and other tissues. Their proliferation capacity and high multilineage differentiation potential make them attractive alternative for chondrocytes in cartilage regeneration.[5,15] These cells could be easily differentiated into chondrocytes *in vitro*.[16] Bone marrow is the most frequently used source of mesenchymal stem cells applied for cartilage repair but it is not the optimal source, because its sampling procedure is dangerous and painful.[17]

In recent years, human adipose-derived stem cells (ADSCs) are considered as a suitable alternative source for cartilage tissue engineering.[18-20] Adipose tissue can be easily obtained without invasive manner and a large number of stem cells can be harvested from low proportion of fat which is 100 times more, compared to bone marrow.[21] Several researchers have studied and compared chondrogenic potential between ADSCs and BM-MSCs conflicting but they rendered different results. Some of them concluded that differentiation potential of ADSCs is good or similar to BM-MSCs and others reported BM-MSCs were better.[22] It is expected, when MSCs are employed for articular cartilage repair, they could be differentiated into chondrocytes which comparable to native articular chondrocytes.[17,23] Nowadays, the main obstacle for cartilage tissue engineering is to use the most appropriate cell which is able to produce extracellular matrix (ECM) similar or better than natural chondrocytes. However, this challenge has been extended up to now and needs to be studied more.[5,24] Selecting a suitable scaffold is the other important factor in tissue engineering. Alginate---a natural polysaccharide found in brown seaweed---in the presence of calcium or other 2-valent cations transforms into hydrogel. It is a nontoxic and injectable scaffold that stimulates the expression of chondrogenic phenotype.[25-27] Proteoglycans constitute the important part of extracellular matrix of articular cartilage which can resist against to mechanical loading. Aggrecan is a large proteoglycan (80-90%) consist of a core protein with glycosaminoglycan side chains.[1,28]

In this study, we decided to compare the amount of AGC produced by differentiated chondrocytes (DCs) from ADSCs and natural articular chondrocytes (NCs) in alginate, with and without TGFβ3, on days 14 and 21.

MATERIALS AND METHODS

Isolation and proliferation of adipose derived stem cells

Human ADSCs were extracted from subcutaneous abdominal adipose tissue harvested from four patients (30-50 years). Consent was obtained from the patients previously. Adipose tissue was mechanically minced and washed with PBS (Sigma) and then it was digested with 0.075% type I collagenase (Sigma) solution at 37°C for 30 min. After inactivation of the collagenase with DMEM- LG (Sigma) and 10% fetal bovine serum (FBS) (Invitrogen), the cell solution was centrifuged at 1500 rpm for 10 min. The supernatant was removed and the resultant pellet was resuspended in culture medium contained DMEM-LG supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco) and then cultured at 37°C, 5% CO_2 conditions. Medium was replaced every 4 days. When the cells reached 80% confluence, they were passaged with 0.05% trypsin/0.53 mM EDTA (Sigma) solution. For preparation of alginate beads, the cells at passage 3 to 5 were detached by trypsin/EDTA, centrifuged, and counted.

Flow cytometry

The cell markers were quantified by flow cytometry. ADSCs should characteristically show positive for CD105 (a receptor for TGFβ), CD90 (marker of thymic antigen), CD 44 (hyaluronic acid receptor) and negative for CD 14, CD 45(hematopoietic markers). Cells were released with trypsin-EDTA, rinsed, and suspended in PBS. Cell suspension were split into aliquots (100 µl); an unstained group, 5 µl mouse antibody $IgG_{1,2}$ (negative control), 5 µl mouse anti-human monocolonal CD105(Abcam)(unconjugated) and mouse anti-human monocolonal CD 44(DAKO Cytomation) conjugated with phycoerythrin (PE), 5 µl mouse anti-human monocolonal CD 14,45(IQ Product) and mouse anti-human monocolonal CD 90(IQ Product) conjugated with fluoroisothiocyanate (FITC). Then the samples were incubated for 30 min in the dark at 4°C. The cells were washed with PBS and centrifuged at 1500 rpm for 10 min. The supernatant was removed, the labeled cell pellet resuspended in 200 µl PBS, and

subjected to FACS analysis (BD FACS Caliber).

Chondrocyte isolation

In this study, articular cartilage was obtained from knee joints of 5 patients, 20 to 30 years old, by arthroscopy. The cartilage specimens were removed from non-weight bearing areas and transported to laboratory in PBS.Consent was obtained from the patients previously. Then the cartilage slices were minced into 1-2 mm pieces and digested by type II collagenase solution (350 u/ml) (Sigma) for 4 h at 37°C. After digestion, the solution was centrifuged and cells cultured in DMEM/F12 (Gibco) 10% FBS, 1% penicillin-streptomycin. Medium was replaced every 4 days. When the cells reached 80% confluence, they were passaged with trypsin/ EDTA solution. For preparation of alginate beads, the cells at passage 2 to 4 were detached by trypsin/EDTA, centrifuged, and counted.

Encapsulation of ADSC and chondrocytes in Alginate

The isolated ADSCs (P3 to P5) and monolayer chondrocytes (P2 to P4) were separately resuspended in 1.5% alginate (Sigma)[29] at 5 million cells/ml. The alginate/cell suspension was expressed through a 23-gauge needle into a 102 mM $CaCl_2$ solution (Merck). The alginate beads after 15 min were washed twice in 0.9% saline solution and once in DMEM. The new alginate beads cultured in 12-well plate and 2 ml chondrogenic culture media was added to each well and incubated at 37˚C, 5% CO_2 for 14 and 21 days.

Chondrogenic culture media contained: DMEM-HG (High Glucose)(Gibco), penicillin and streptomycin 1% (Gibco), dexamethasone 10/7 M(Sigma), ascorbat-2-phosphate 50 µg/ml(Sigma), bovine serum albumin 1% (Sigma), linoleic acid 5 µg/ml (Sigma), insulin-transferrin-selenium (ITS) 1% (Sigma), with and without adding transforming growth factor-β_3 (TGFβ_3) 10 ng/ml (Sigma). Medium was replaced every 4 days and l ml of supernatant medium on days 14 and 21 was frozen at –20°C for enzyme-linked immunosorbent assay (ELISA).

MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay

The viabilities of NCs and DCs in alginate beads were assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on days 14 and 21. At first, the medium of each well was removed, rinsed with PBS, and replaced with 800 µl serum free medium and 80 µl MTT solution (5 mg/ml in PBS). Then it incubated at 37˚C, 5% CO_2 for 4 h, so that purple formazan crystals formed in the alginate beads. Then the medium was discarded and added 800 µl DMSO (Sigma) to each well, and incubated in dark for 2 h. DMSO dissolved the formazan crystals and created a purple color. Then 100 µl of the solution transferred to 96-well plate and absorbance of each well was read at 570 nm with ELISA reader (Hiperion MPR4). The MTT assay was also applied to the scaffolds without cells as controls and the data was subtracted from measured values. The assays were performed in triplicate.

Enzyme-linked immunosorbent assay

Aggrecan was quantified in supernatant culture media on days 14 and 21, according to protocol of Human aggrecan Direct ELSA kit (Invitrogen). Briefly, supernatant media as antigens were added to ELISA plate and AGC molecules bound to coated antibodies. Then secondary enzyme conjugated antibodies were added which linked to antigens and formed sandwich. Finally, enzyme`s substrate was added and the absorbance of the mixture was measured at a wavelength of 450 nm by spectrophotometer.

Statistical tests

The Kolmogorov-Smirnov test was used for assessing normal distribution of variables and ANOVA (one-way-analysis of variance) with the LSD *post hoc* test was used for the comparison of MTT and concentration of ELISA results in different groups.

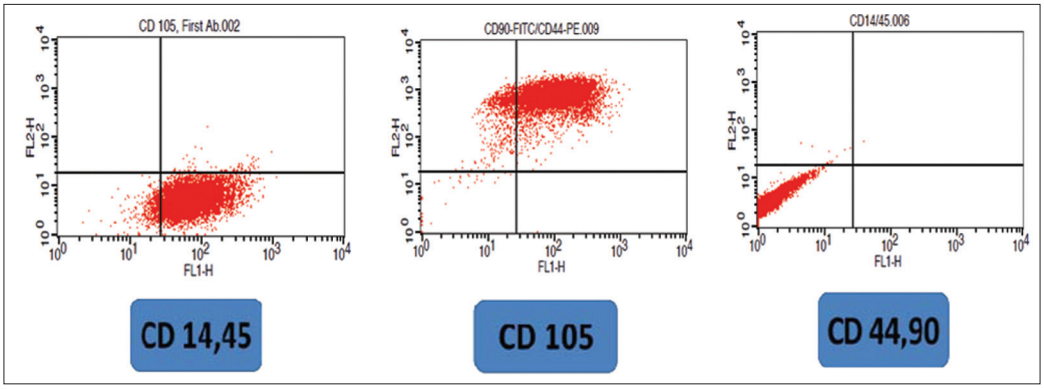

Figure 1: Flow cytometry dot plots of CD 105, CD 90, CD 44, CD 14, 45 in human adipose-derived stem cells

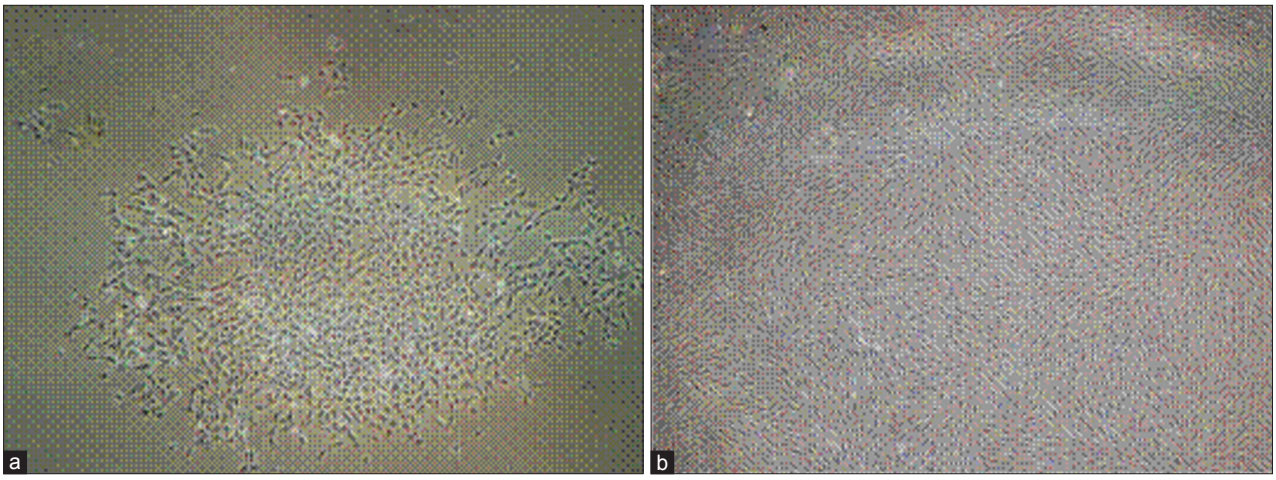

Figure 2: (a) Inverted microscope images of monolayer chondrocytes (a) at passage 0 after 7 days. Note the cells make colonies. (b) at passage one showing fibroblast like morphology (×60)

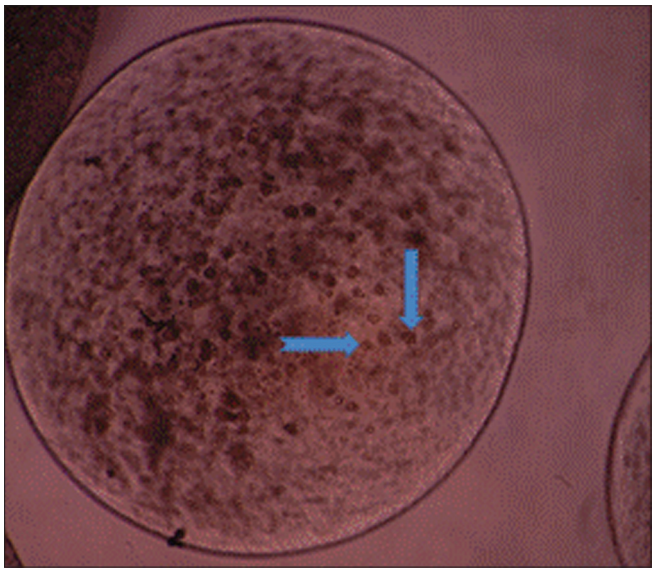

Figure 3: Inverted microscope images of chondrocytes seeded in alginate beads. Note the spherical shape of the cells (Arrows) within the bead (×60)

RESULTS

Flow cytometry

Flow cytometric analysis of undifferentiated human adipose-derived stem cells (ADSCs) was performed. The results showed they were negative for CD 14, 45(0.14%) but expressed CD 44, CD90 (89.69%), and CD105 (94.64%) at high level [Figure 1].

Chondrocyte culture

Some chondrocytes in primary culture adhered to plastic flasks after 7 days and made cell colonies. They lost their round phenotype gradually and transformed to fibroblast-like morphology at passage one [Figure 2]. But, when monolayer chondrocytes at passage 2 to 4 seeded in alginate, they gained their spherical shape [Figure 3].

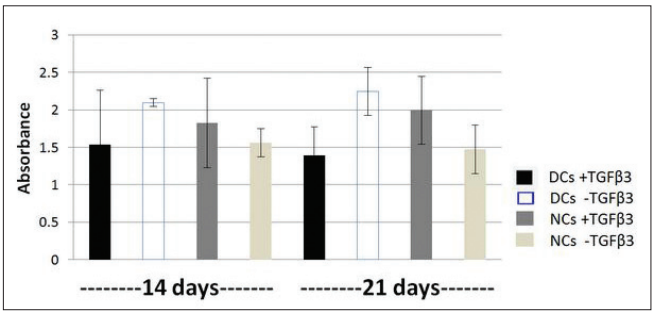

Figure 4: Comparison of MTT assay results between eight groups. They have not significant differences ($P > 0.05$)

MTT

MTT assay was applied for eight groups of alginate beads, NCs and DCs in two different chondrogenic media (with and without TGFβ3) on days 14 and 21. Followed by treatment with MTT solution, the dark blue formazan crystals were seen in cells seeded in alginate beads, which indicated their metabolic activity. However, the addition of TGFβ3 improved viability in NCs and declined in DCs at two time points, but the comparison of all results showed [Figure 4] that they have not significant differences ($P > 0.05$).

ELISA

The results showed that the content of AGC in tissues produced by DCs with and without TGFβ3 are more than NCs with same conditions on day 14 and there was a significant difference ($P < 0.05$) [Figure 5]. The addition of TGFβ3 to chondrogenic media results in enhancing the AGC production in DCs than the same group without TGFβ3, and they have significant differences ($P < 0.05$). However, its amount, to some extent was superior in tissues generated by

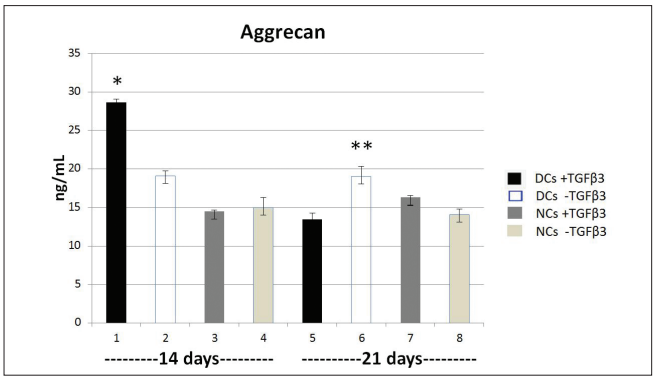

Figure 5: The concentration of aggrecan in eight groups on days 14 and 21. *P < 0.001,** P < 0.05

NCs without TGFβ3 and they have not significant differences (P>0.05). The results showed that AGC synthesis in four groups on day 21 significantly decreased than DCs with TGFβ3 on day 14 (P <0 .05). However, its proportion in DCs without TGFβ3 on day 14 is significantly higher than all groups on day 21 (P <0.05) with exception of DCs without TGFβ3, which their difference was not significant (P >0.05). TGFβ3 led to decreasing of AGC production in DCs significantly on day 21 than the other group without it (P <0 .05). Our findings indicated that in the presence of TGFβ3, NCs could produce more AGC than other same group, but there was no significant difference (P <0 .05)

DISCUSSION

Chondrocytes synthesize the extracellular matrix components, collagens, and proteoglycans (PGs).[30] Aggrecan, the main part of PGs, consists of a core protein and glycosaminoglycan chains are attached to it covalently.[29] A great number of AGC molecules are attached to a central hyaluronic acid by non-covalent link proteins.[31] Side chains of AGC have negative charges and lead to a high osmotic environment for resistance against mechanical loading.[30]

The TGFβ family including five types (TGFβ1-5) and TGFβ1, 2, 3 are able to stimulate chondrocytes to synthesis type II collagen and proteoglycans and they also are used for inducing chondrogenic differentiation of different MSCs.[32] According to previous studies, the day 14 is the most important time point in chondrogenesis *in vitro*, because the highest proportion of extracellular matrix is produced at that time. They showed the concentration of extracellular matrix components can negatively reduce the metabolic activity of chondrocytes[33,34] which is inconsistent with our findings.

The characterization of ADSCs was analyzed by flow cytometry. Our results revealed the positive expression of CD90, CD44, CD105 and negative of CD14, 45. These results are inconsistent with marker list of International Society for Cellular Therapy.[35,36] MTT results showed the metabolic activity in eight groups of cells in alginate beads but there was no significant difference (P >0.05). Some authors reported that the viability of encapsulated chondrocytes in alginate increased as the time passed.[37-39] However, other experiments found lower viability of chondrocytes in alginate beads over the time.[40-42] The review of literature denoted that TGFβ led to improved proliferation of chondrocytes[43] but some investigators reported its inhibitory function.[44] However, in our study, TGFβ3 resulted in increasing of viability in NCs and decreasing in DCs on days 14 and 21 than the same groups without TGFβ3 but these differences were not statistically significant (P >0 .05).

ELISA results indicated DCs with and without TGF β3 produced more AGC than NCs on day 14 (P <0.05) but on day 21 only DCs without TGF β3 has significantly higher production than other three groups on day 21 (P <0 .05). Interestingly, the proportion of AGC between DCs without TGFβ3 on day14 and 21 was stable continuously. Our results of two DCs groups on day 14 and DCs group without TGF β3 on day21 are in agreement with the recent findings of Tigli *et al.* They demonstrated that embryonic stem cell-derived MSCs, embryonic stem cells, BM-MSC and ADSCs had superior ability in expression of chondrogenic markers such as, AGC, collagen type II and sox-9 than articular chondrocytes in silk and chitosan scaffolds.[45] Our results about the DCs group with TGFβ3 on day 21 is in agreement with previous findings.[24,46,47] They indicated ADSCs in hyaluronic acid, BM- MSCs in alginate, and agarose have lower capacity to produce cartilage matrix than articular chondrocytes and in comparison BM-MSCs in hyaluronic acid showed equal matrix to articular chondrocytes. Mahmoudifar *et al.* reported that AGC gene expression of ADSCs seeded in polyglycolic acid (PGA) scaffold was less than fetal chondrocytes.[48]

Also TGFβ3 led to promotion of the AGC production in DCs on day 14 and reduction on day 21 compared to the same group without TGFβ3. This growth factor did not show significant AGC production variations on NCs groups on two time points which may be due to the maturation of these cells. It is suggested that different isoforms of TGF β are able to regulate chondrogenesis. For instance, $TGF\beta_2$ lead to synthesis of proteoglycan in chondrocytes seeded in alginate beads.[49,50] Also authors reported that TGFβ2 and TGFβ3 resulted in improved synthesis of AGC in chondrocytes differentiated from BM-MSCs in pellet

and micromass cultures.[51,52] A recent report indicated that fetal chondrocytes without TGF-β1 expressed more AGC than the same group with TGF-β1 in polyglycolic acid (PGA) scaffolds (48). Also, other members of TGF-β such as TGF-β1, BMP-2, and BMP-9 resulted to increased expression of AGC gens and type II collagen in MSCs encapsulated in alginate beads.[53,54] According to our observations, NCs have constantly generated equal AGC on days 14 and 21, so they maintained their phenotype after 3 weeks, as reported previously.[55]

The findings of this study indicated that DCs from adipose-derived MSCs have higher potential to synthesis aggrecan than natural articular chondrocytes *in vitro* and may be a reliable alternative cell source for cartilage tissue engineering in future.

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