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Induction of Chondrogenic Differentiation of Human Adipose-Derived Stem Cells with TGF-β3 in Pellet Culture System

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Abstract

Objective

Adult stem cells which are derived from different tissues, with their unique abilities to self-renew and differentiate into various phenotypes have the potential for cell therapy and tissue engineering. Human adipose tissue is an appropriate source of mesenchymal stem cells with wide differentiation potential for tissue engineering research. In this study isolated stem cells from human subcutaneous adipose tissue were investigated for chondrogenic potential of adipose-derived stem cells (ADSCs) in pellet culture system treated with transforming growth factor- β3 (TGF-β3).

Materials and Methods

Human ADSCs were isolated from subcutaneous adipose tissue and digested with collagenase type I. Immunocytochemical method for cell surface antigens was done in order to characterize the cells. The isolated cells were treated with chondrogenic medium, supplemented with TGF-β3 in pellet culture system and harvested after 21 days. Histological staining was used to evaluate the presence of proteoglycan, with alcian blue. Immunohistochemical method performed for the assessment of cartilage–specific type II collagen and aggrecan. Also, in order to confirm our results, we managed RT-PCR technique.

Results

Chondrogenesis of ADSCs in pellet culture, induced by TGF-β3 growth factor. Histological and immunohistochemical methods showed deposition of typical cartilage extracellular matrix components in pellets. RT-PCR analysis of cartilage matrix genes, such as type II collagen and aggrecan, also, confirmed the induction of the chondrocytic phenotype in high-density culture upon stimulation with TGF-β3.

Conclusion

TGF-β3 promoted chondrogenesis of ADSC in pellet culture system. We suggest that human subcutaneous adipose stem cells could be excellent candidates for the cartilage tissue engineering.

Keywords: Adipose, Chondrogenesis, Stem cell, Tissue engineering, TGF- β3

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Introduction

Cartilage tissue damage is a rather worldwide problem of many people (1). Osteoarthritis as a result of lesions in the articular cartilage is the most common cause of disability, after trauma or degenerative joint diseases.

Cartilage tissue is an avascular, aneural tissue with limited capacity for self- repair (2). Current treatment methods for cartilage tissue injuries include chondral shaving, subchondral drilling, tissue debridement, microfracture of the subchondral bone, transplantation of autologous and allogeneic osteochondral grafts. The most common clinical treatment for an articular cartilage lesion is to create bleeding microfracture within the subchondral bone in an attempt to stimulate cartilage regeneration (3). While some of these approaches show promise, many lead to formation of fibrous tissue, apoptosis, and further cartilage degeneration (4-6). In addition the repaired tissue in these circumstances lacks the biomechanical properties of normal articular cartilage, leading to inconsistent long-term outcomes (7).

Recently many efforts have been undertaken to repair articular cartilage lesions by transplantation of autologous chondrocytes. A major problem of human chondrocytes in culture is the phenomenon of dedifferentiation. In addition, autologous chondrocyte transplantation has been associated with significant donor-site morbidity and the initiation of osteoarthritic changes in the joint (8). Thus, an alternative cell source is necessary for the repair of cartilage.

Mesenchymal stem cells (MSCs) provide a source for fascinating models of differentiation (9, 10), regenerative medicine (11) and cell therapy (12). There are increasing reports that MSCs can be isolated from various adult mesenchymal tissues such as bone marrow (13), periosteum (14), synovium (15), skeletal muscle (16), adipose tissue (17), dermis (18), blood (19) and trabecular bone (20).

Mesenchymal stem cells with chondrogenic potential are present in many tissues of the body. Those of the adipose tissue are particular interest because, could be harvest easily in large quantities with minimal possibility of morbidity and discomfort, clinically (21). In addition, the uncomplicated enzyme-based isolation procedures make adipose tissue an attractive source for researchers and clinicians of nearly all medicinal subspecialization (22).

The induction of chondrogenesis of ADSCs appears to require a rounded cell shape through pellet culture or a 3-Dimentional (3-D) hydrogel (23). Pellet culture system that was originally described as a method for preventing the phenotypic modulation of chondrocytes *in vitro* (24) allows cell- cell interactions, analogous to those that occur in precartilage condensation during embryonic development (25).

However, this cell configuration is not sufficient for the induction of chondrogenesis. The chondrogenic differentiation of the ADSCs required the certain bioactive factors. Many studies have shown that various growth factors such as bone morphogenetic protein-2 and -3 (26, 27) as well as transforming growth factor-$ß_1$ and $ß_2$ (28, 29) can induce mesenchymal stem cells to differentiate into chondrocytes under certain culture conditions. The transforming growth factor-$ß_3$ is the essential growth factor for promoting chondrogenesis *in vivo* (30). This growth factor has significantly increased Sox-9 expression; however, TGF- $ß_3$ cannot alone induces human bone marrow-derived stem cells into completed chondrocyte (31). The effect of TGF- β3 on chondrogenesis of ADSCs in pellet culture system has not yet been recorded. Therefore, in this study we decided to isolate and culture the subcutaneous adipose derived stem cells and evaluate chondrogenic differentiation potential of ADSCs in pellet culture system, using TGF- β3.

Materials and Methods

Isolation of ADSCs

Human subcutaneous adipose tissue was isolated from healthy donors (n=10; age: 24-50) undergoing cesarean surgery.

Adipose tissue was minced into small pieces (1-2 mm^3), washed with phosphate-buffered saline (PBS) and digested with 1.5 mg of collagenase type I per mg of fat tissue for 60 min at 37 °C, with continuous shaking. The

floating adipocytes separated from the stromal cells fraction, by multiple centrifugations and washing steps. Red blood cells were removed, using lysis buffer. Stromal cells counted by hemocytometer and plated with 3000 cells/cm^2 in tissue culture flasks containing DMEM, 10% FBS, 1% Penicillin-streptomycin and incubated at 37 ˚C, 5% CO_2 conditions. After 24 hrs with changing of medium, nonadherent cells discarded.

Culture media replaced every 2-3 days and the cultures allowed to reach 80% confluence, before trypsinization and replating.

Investigation of cell marker by immunocytochemical method

Although, the isolation of ADSCs was based on the adherence to plastic surface, but in order to characterize these cells further, immunocytochemical study used for detecting of cell surface antigens.

Cell markers of hematopoietic lineage cells CD34, CD45, and mesenchymal cell markers CD44, CD105, receptors of hyaluronan, and TGF-β3 were investigated respectively.

Cells of the passage 4 plated on cover slips and grown until reaching the confluence. They were washed again with PBS two times and fixed with 4% Para formaldehyde for 20 min. Then they washed with PBS, and incubated in acetone and goat serum for 30 min at room temperature. Primary antibodies for CD34, CD44, CD45 and CD105 markers (DAKO Cytomation, Denmark) were applied for 60 min at 37 ˚C and then washed with PBS. The HRP- labeled secondary antibody applied for 2 hrs at 37 ˚C, followed by washing with PBS. Diaminobanzidin was used fop staining. Counterstaining of cells was done using hematoxylin (Merck, Germany).

Chondrogenic differentiation

ADSCs from the passage 4, harvested with trypsin-EDTA and 2×10^5 cells/ml centrifuged at 1400 rpm for 8 min in a 15 ml polypropylene conical tube. Then cells were resuspended in chondrogenic medium (CM), consisting of DMEM-high Glucose (Gibco), 1% insulin-transferring-selenium (Sigma), 1.25 mg/ml bovine serum albumin (Sigma), 5 mg/ml linoleic acid (Sigma), 50 mg/ml ascorbate-2-phosphate (Sigma), 1% penicillin-streptomycin (Gibco), and 10 ng/ml transforming growth factor-β3 (Sigma). After repeating centrifugation at 1400 rpm for 8 min, pellets were cultivated at 37 ˚C, 5% CO_2 for 21 days. The medium changed every 3 days, and the same medium without TGF- β3 used as the control.

Histological and immunohistochemical analysis

After 21 days of culturing, pellets were harvested, fixed in 10 % formalin for 2 hrs, dehydrated with ethanol, cleared with xylen and finally embedded in paraffin. Then, they sectioned at 5 μm thickness for hematoxylin and eosin (H-E) staining. Sulfated glycosaminoglycans (GAG) were assessed by alcian blue staining.

Immunohistochemical method for evaluation of type II collagen and aggrecan was performed. Briefly, the tissue sections were deparaffinized, treated with trypsin for 30 min to facilitate antibody access. Then, 3% hydrogen peroxide in ethanol for 30 min was added to suppress the endogenous peroxidase activity. Monoclonal antibodies against type II collagen (Serotec) and aggrecan (Serotec) were applied and allowed to react for overnight at 4 ˚C. Sections incubated with the secondary antibody, rabbit anti-mouse immunoglobulin (DAKO Cytomation, Denmark) for 60 min and the existence of collagen type II plus aggrecan in the extracellular matrix was revealed.

RT-PCR analysis

Total cellular RNA isolated by RNX-plus solution (CinnaGen Inc Iran). First strand complementary DNA (cDNA) synthesized from 1 μg total RNA using oligo(dt) as a primer and Revert Aid TM First-strand cDNA synthesis kit (Fermentase), according to the manufacturer's instructions. Subsequent PCR performed as follows: 2.5 μl cDNA, 1X PCR buffer, 200 μM dNTPs, 1 μM of each primer pair (Table 1), and 1 unit / 25 ml reaction Taq DNA Polymerase (Fermentas). Amplification conditions were: initial denaturation at 95 ˚C for 5 min, followed by 30 cycles of denaturation at 95 ˚C for 30 sec, annealing at 56 ˚C for 45 sec and extension at 72 ˚C for 45 sec. The products analyzed on 1% agarose gel and visualized by ethidium bromide staining.

Table 1. Sequences of different primers were used for RT-PCR.

Name of genes	Sequences of primers	Product size
Aggrecan	F- AGGAGGGCTGGAACAAGTACC R- GGAGGTGGTAATTCCAGGGAACA	350 bp
Collagen II	F- CAGGTCAAGATGGTC R- TTCAGCACCTGTCTCACCA	370 bp
GAPDH	F- GGGCTGCTTTTAACTCTGGT R- GCAGGTTTTTCTAGACGG	680 bp

Results

Cell culture

After 2 days of culturing and at the first changing of medium, spindle bipolar to polygonal fibroblast -like cells, attached to the flask were observed (Figure 1). After 10 to 13 days, the cell culture reached to confluence. Cells in the passage 4 showed uniform monolayer (Figure 2) and needed approximately 1 week to reach 80% confluence. Expanded mesenchymal stem cells displayed a phenotypic single population, which was uniformly negative for hematopoietic antigens such as the early hematopoietic stem cell marker (CD34) (Figure 3), the leukocyte common antigen (CD45) (Figure 4). These MSCs were positive for typical MSC – related antigens, expressing a receptor for TGF-ß3 (endoglin, CD105) (Figure 5), and the hyaluronan receptor (CD44) (Figure 6).

Chondrogenic differentiation

Adipose–derived mesenchymal stem cells in the passage 4 were trypsinized to detach from the surface of flask, cultured for 21 days in high–density system and treated with TGF-ß3. The resulted products, spherical in shape, appered with more than 1 mm in diameter and 1×10^{-3} g in weight (Figure 7). The chondrogenic potential of ADSCs characterized histologically by H-E and alcian blue staining.

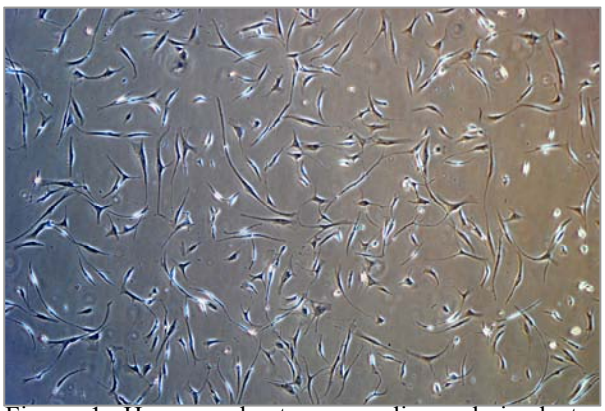

Figure 1. Human subcutaneous adipose-derived stem cells in primary culture after 2 days (×40).

The histological sections showed a chondrocyte-like cell morphology which located in lacunae (Figure 8). Furthermore, sections of pellets showed the accumulation of extracellular matrix. For showing the existence of proteoglycans in extracellular matrix, metachromatic staining with alcian blue used and the results, indicated matrix of cartilage in sections (Figure 9). To confirm the results, immunohistochemical (IHC) analysis for type II collagen and aggrecan carried out using monoclonal-specific antibody. Positive immunostaining was observed in sections and the existence of type II collagen and aggrecan revealed (Figures 10, 11).

RT-PCR

Total RNA was extracted from pellets on the day 21. Both type II collagen and aggrecan mRNA expression were detected in pellets culture after 21 days (Figure 12). In pellets that were not treated by TGF-β3, type II collagen and aggrecan mRNA were not depicted. In addition there was no expression of chondrocyte- specific genes in ADSCs from the monolayer culture (Figure 12).

Overall the results showed that, chondrogenesis of subcutaneous adipose stem cells was induced with TGF- β3 in pellet culture system.

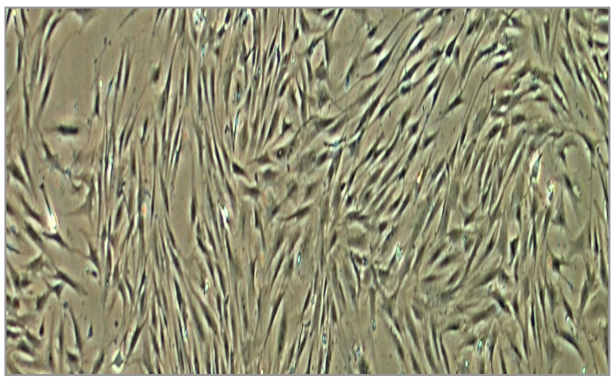

Figure 2. Adipose-derived stem cells have reached 80% confluence in the passage 4. Cells show uniform shape in monolayer culture (×40).

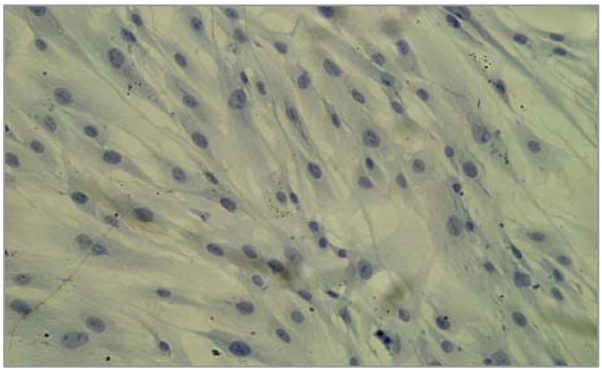

Figure 3. Immunocytochemical method shown that ADSCs were not expressed CD34, a hematopoietic marker, thus cells stained with hematoxylin (×400).

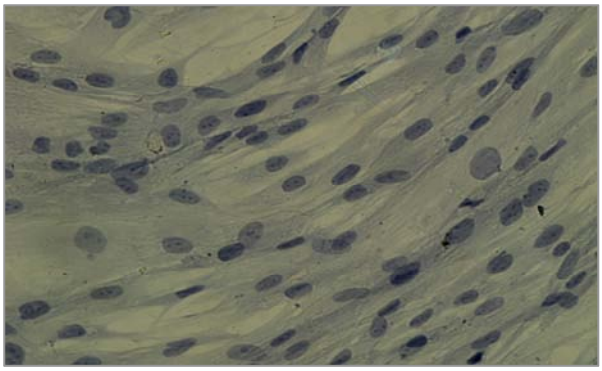

Figure 4. Immunocytochemical method shown that ADSCs were not expressed CD45, a leukocyte common antigene, thus cells stained with hematoxylin (×400).

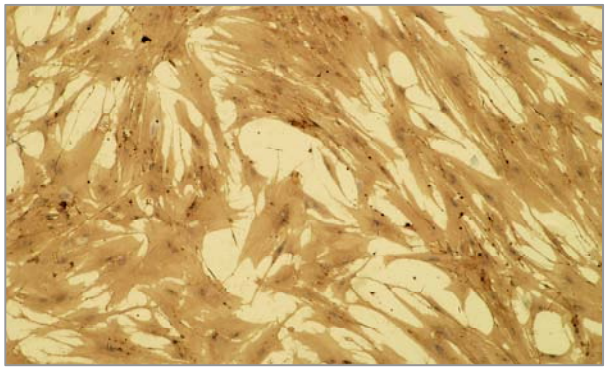

Figure 5. Immunocytochemical method shown ADSCs express CD105 marker, transforming growth factor-ß3 receptor (×400).

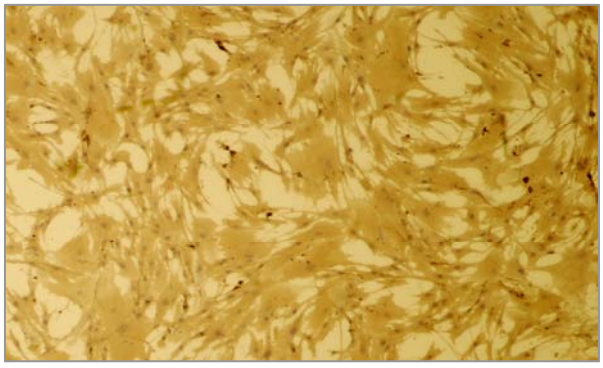

Figure 6. Immunocytochemical method shown ADSCs express CD44 marker, hyaluronan receptor (×400).

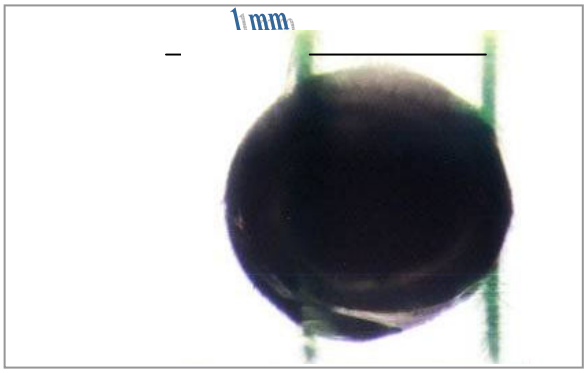

Figure 7. Gross appearance of construct after 21days treatment with TGF-ß3 in pellet culture system (×40).

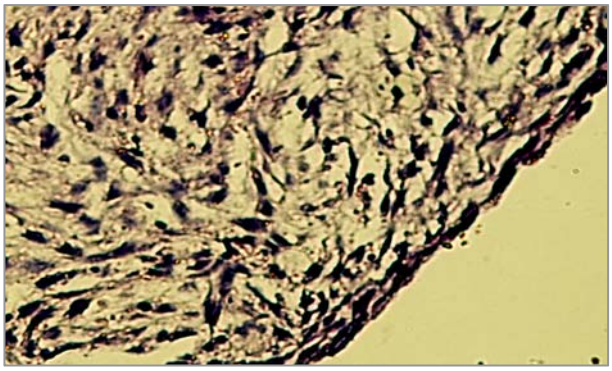

Figure 8. The section of the resulted construct. Chondrocytes are located in lacuna (H-E) (×400).

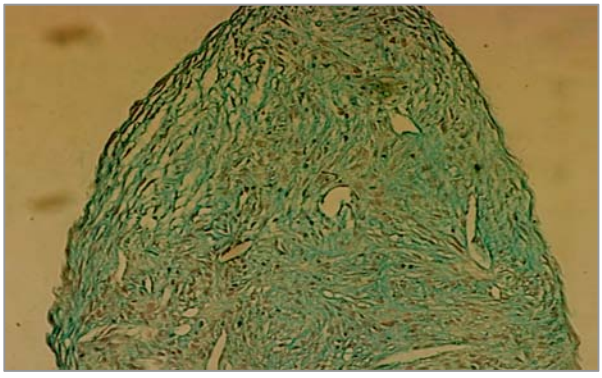

Figure 9. Alcian blue staining of resulted tissue. Existence of methachromatic extracellular matrix in tissue was revealed (×200).

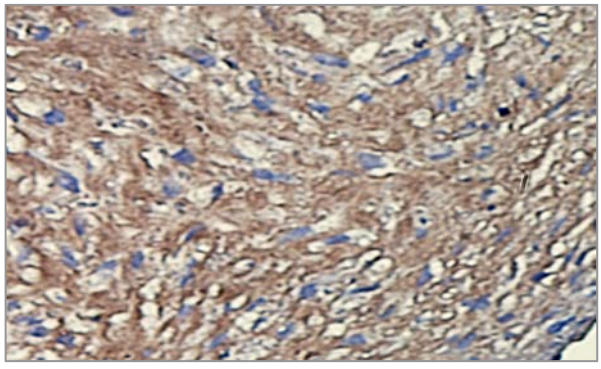

Figure 10. Immunohistochemical method revealed existence of aggrecan in extracellular matrix after chondrogenic induction (×400).

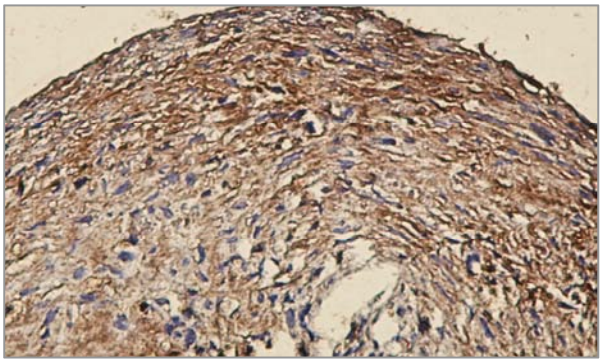

Figure 11. Immunohistochemical method revealed existence of type II collagen in extracellular matrix after chondrogenic induction (×300).

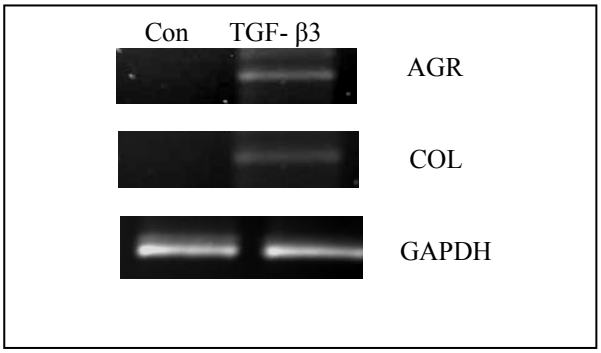

Figure 12. RT-PCR results showed expression of cartilage specific molecules, aggrecan (AGR) and type II collagen (COL) in TGF-ß3 treated group. GAPDH used as housekeeping gene.

Discussion

At the present study, we confirmed the chondrogenic capacity of ADSCs in the presence of TGF- β3. ADSCs were isolated from the human adipose tissue that obtained from cesarean surgery. Previous studies characterized ADSCs and revealed that ADSCs have multi – lineage potential, but, in those studies fat tissue isolated in the liposuction surgery (17, 31, 32).

Characterization of MSCs has been performed using the expression of cell – specific proteins and CD markers (27, 33, 34). We showed that like MSCs, ADSC cells express CD44, CD105 markers but, do not express the CD34 and CD45 antigens. Our results demonstrated that surgery procedure had no effect on the surface markers.

Many researches have shown that the essential conditions for chondrogenesis from human MSCs are a 3-D culture or high density cell culture, and the growth factors (17, 27, 31, 32).

Pellet or micromass culture system was originally described as a method for preventing the phenotypic modulation of chondrocytes (24). Since the introduction of micromass culture system by Johnston *et al* (27), scientists have utilized this system for different purposes. Some researchers have used this culture system to evaluate the chondrogenic potential of the cells isolated by different methods (35, 36). Others used it to study the role of macromolecules such as TGF-β family in promoting chondrogenic differentiation (37, 38).

Our investigation showed that the pellet having been cultured in chondrogenic medium supplemented with TGF-β3 for 21 days, differentiated into tissue composed of chondrocyte-like cells and a plenty of matrix accumulated among them.

Since the cartilage–specific molecules were produced in pellets as evidenced by RT-PCR and immunohistochemical methods, we concluded that the tissue could be cartilage.

Zuk *et al* isolated processed lipoaspirate cells (PLA) from human adipose tissue and induced them into adipogenic, osteogenic, chondrogenic and neurogenic lineage cells. Also, they used TGF- β1 for chondrogenic differentiation in micromass culture, during 2 weeks (17), while in the present study we used TGF-β3 growth factor, in order to do chondrogenic induction for the first time in pellet culture, during 3 weeks.

Some researchers that investigated chondrogenic potential of human adipose-derived stem cells, used growth factor of TGF-β1 in pellet culture system or various scaffolds (23, 39, 40).

Estes and colleagues studied chondrogensis of ADSCs influenced with TGF-β3 and other growth factors on alginate scaffold, during 1 week (32), however, we did not used any scaffold in our study.

Jin *et al* in 2007 demonstrated that human ADSC could be induced into chondrocyte-like phenotype in pellet culture system using human TGF- ß2 treatment *in vitro* (31). They used TGF-ß2 while we used TGF- ß3 growth factor. In addition Jin et al. showed the

expression of the chondrocyte marker genes at 7 and 14 days of the induction but, in our study the expression of cartilage specific-genes was revealed after 21 days of induction.

To our knowledge, this is the first report that TGF-β3 can induce differentiation of ADSCs into the chondrocyte in pellet culture system.

Conclusion

In summary, our study demonstrated the chondrogenesis of human subcutaneous adipose-derived stem cells, induced by TGF-β3 in the pellet culture, for the first time in Iran. Therefore, ADSCs, induced with appropriate growth factors, may serve as a valuable source of easily accessible mesenchymal stem cells, for producing stem cell derived cartilage.

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References

1. Anonymous. Prevalence and impact of arthritis by race and ethnicity-US, 1989-1991. Morb Mortal Wkly Rep 1996; 10:373-378.
2. Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffold and mesenchymal stem cells. Biomaterial 2005; 26:7082-7094.
3. Gillogly SD, Voight M, Blackburn T. Treatment of articular cartilage defects of the knee with autologous chondrocyte implantation. J Orthop Sports Phys Ther 1998; 28:241-251.
4. Furukawa T, Eyre DR, Koide S, Glimcher MJ. Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. J Bone Joint Surg Am 1980; 62:79-89.
5. Mitchell N. Healing of articular cartilage in intra-articular fractures in rabbits. Clin Orthop Relat Res 2004; 62:628-634.
6. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW. The reactions of articular cartilage to experimental wounding: role of apoptosis. Arthrit Rheum 2000; 431:215-225.
7. Minas T, Nehrer S. Current concepts in the treatment of articular cartilage defects. Orthop 1997; 20:525-538.
8. Lee JD, Hwang O, Kim SW, Han S. Primary cultured chondrocytes of different origins respond differently to b-FGF and TGF-ß. J Life Sci 1997; 61:293-299.
9. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. *In vitro* cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci USA 2002; 99:4397-4402.
10. Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). J Bone Miner Res 2004; 19:256-264.
11. Wakitani S, Imoto K, Saito M, Yamamoto T, Kawabata HA. Case report: reconstruction of a damaged knee following treatment of giant cell tumor of the proximal tibia with cryosurgery and cementation. Osteoarthritis Cartilage 2002; 10:402-407.
12. Horwitz EM, Hanlon AL, Pinover WH, Hanks GE. There a role for short-term hormone use in the treatment of nonmetastatic prostate cancer? Radiat Oncol Investig 1999; 7:249-259.
13. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997; 4, 276:71-74.
14. Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA, *et al*. Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis *in vitro*. Osteoarthritis Cartilage 2003; 11:55-64.
15. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 2001; 44:1928-1942.
16. Cao B, Zheng B, Jankowski RJ, Kimura S, Ikezawa M, Deasy B, *et al*. Cummins J, Epperly M, Qu-Petersen Z, Huard J. Muscle stem cells differentiate into hematopoietic lineages but retain myogenic potential. Nat Cell Biol 2003; 5:640-646.
17. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. A Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13:4279-4295.
18. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, *et al*. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 2001; 264:51-62.
19. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. Mesenchymal precursor cells in the blood of normal individuals. Arthrit Res 2000; 2:477-488.

20. Nöth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS. Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 2002; 20:1060-1069.
21. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am 1994; 76:579-592.
22. Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M, Helder MN, Klein-Nulend J, Schouten TE, Ritt MJ, van Milligen FJ. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy 2006; 8:166-177.
23. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 2004; 25:3211-3222.
24. Holtzer H, Abbott J, Lash J, Holtzer S. The loss of phenotypic traits by differentiated cells *in vitro* dedifferentiation of cartilage cells. Proc Natl Acad Sci USA 1960; 46:1533-1542.
25. Fell HB. Confocal images of marrow stromal (Westen-Bainton) cells. J Morphol Physiol 1925; 40:417-459.
26. Majumdar MK, Wang E, Morris EA. BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. J Cell Physiol 2001; 189:275-284.
27. Johnston B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. *In vitro* chondrogenesis of bone marrow derived mesenchymal progenitor cells. Exp Cell Res 1998; 238: 265 -272.
28. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow.Tissue Eng 1998; 4:415-428.
29. Minguell JJ, Conget P, Erices A. Biology and clinical utilization of mesenchymal progenitor cells. Braz J Med Biol Res 2000; 33:881-887.
30. Indrawattana N,Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, *et al*. A growth factor combination for chondrogenic induction from human mesenchymal stem cell. Biochem Biophys Res Commun 2004; 320:914-919.
31. Jin XB, Sun YS, Zhang K, Wang J, Ju XD, Lou SQ. Neocartilage formation from predifferentiated human adipose derived stem cells in vivo. Acta Pharmacol Sin 2007; 28:663-671.
32. Estes BT, Wu AW, Guilak F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. Arthritis Rheum 2006;54:1222-1232.
33. Bruder SP, Horowitz MC, Mosca JD, Haynesworth SE. Monoclonal antibodies reactive with human osteogenic cell surface antigens. Bone. 1997; 21:225-235.
34. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999 2; 284:143-147.
35. Sun S, Guo Z, Xiao X, Liu B, Liu X, Tang PH. Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. Stem Cells 2003; 21:527-535.
36. Eslaminejad MB, Nikmahzar A, Taghiyar L, Nadri S, Massumi M. Murine mesenchymal stem cells isolated by low density primary culture system. Dev Growth Differ 2006; 48:361-370.
37. Sekiya I, Colter DC, Prockop DJ. BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun 2001; 284:411-418.
38. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ. Comparison of effect of BMP-2, -4, and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 2005; 320:269-276.
39. Lee RH, Kim BC, Choi IS, Kim H, Choi HS, Suh KT. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose. Tissue Cell Physiol Biochem 2004; 14:311-324.
40. Erickson GR, Gimble JM, Franklin DM, Rice HE,Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. Biochem Biophys Res Commun 2002; 290:763-769.