Chondrogenic differentiation of Human Bone Marrow Mesenchymal stem cells Induced by Synovial Fluid in Vitro.

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Abstract: Mesenchymal stem cells (MSCs) have the potential to differentiate into distinct mesenchymal tissues including cartilage, which suggest these cells as an attractive cell source for cartilage tissue engineering approaches. Our objective was to study the effects of synovial fluid on chondrogenic differentiation of human MSCs in monolayer and micromass cultures. The cells in passage 2 were induced into chondrogenic differentiation with different concentration of synovial fluid (0,100,150 and 200 μl/ml. Differentiation along the chondrogenic lineage was documented by Sox9 and type II collagen expression for 21 days. The expression of the identified genes was confirmed by RT-PCR. RT-PCR showed that synovial fluid could promote expression of Sox9 and collagen II mRNA in an dose-dependant manner, especially at the concentration of 150 and 200 μl/ml. In summary, synovial fluid induce chondrogenesis of human mesenchymalstem cells, which encourage tissue engineering applications of MSC in chondral defects, as the natural environment in the joint is favorable for chodrogenic differentiation.
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Key words: Human mesenchymal stem cells; synovial fluid; Chondrogenic differentiation; RT-PCR.

Introduction

Cartilage is frequently damaged but only shows a limited capacity for repairing. Considering cartilage damage and increasing the number of patients with degenerative disease of the skeletal system such as OsteoArtheritis (OA), demand for effective treatment strategies of cartilage defects (1).Treatment of a cartilage defect always begins with conservative treatments. These include medications, physical therapy, possibly injections, and other options. If these treatments do not work, some other options would be useful such as: Micro fractur, Cartilage Transfer and Cartilage Implantation. Cartilage implantation, also called autologous chondrocyte implantation (ACI), is another procedure used to grow cartilage cells. In this case, autologous chondrocytes are isolated, expanded ex-vivo and re-implanted into the defect site, either as a cell suspension combined with a periosteal flap or seeded onto a biocompatible carrier material (2). Because of supply limitations of autologous chondrocytes for transplantation procedures, much attention was drawn to human mesenchymal stem cells (hMSC). hMSC have the potential to differentiate into distinct mesenchymal tissues including cartilage, which suggest these cells as an attractive cell source for cartilage tissue engineering approaches.

For inducing chondrogenesis of hMSC, it can be helpful to use some materials that have seen in the joint like Synovial (SF).Synovial fluid were shown to have the potential to induce chondrogenic differentiation in chicken limb bud bioassays (3,4). SF nourishes articular cartilage, lubricates articular joint surfaces, functions as a shock absorber and contains growth factors like transforming growth factor-β1(TGF-β1) (5), bone morphogenetic proteins(BMP) and Hyaluronic acid(HA) (6). We hypothesized that human synovial fluid has the potential to differentiate hMSCs along the chondrogenic lineage by increase expression of chondrogenesis genes. One of the most important of these genes is Sox9 (7). Sox9 is a member of the family of Sox (Sry-type HMG box) genes and has been shown to be expressed predominantly in mesenchymal condensations and cartilage(8).Sox9 regulates expression of the gene encoding type II procollagen, the major matrix protein characteristic of chondrocytes (9,10).These observations indicate that Sox9 plays a key role in chondrogenesis (11). Therefore, the purpose of the study reported here was to determine the chondrogenic effects of SF on high-density micromass and monolayer cultures of hMSCs by study of Sox9 and Col2a1 expressions.

Materials and methods

Cell cultures:

hMSCs at passage0 were purchased from cell Bank, Royan institute. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM,Gibco) with 10% fetal bovine serum (FBS;Gibco) with 100u/ml penicillin and 100µg/ml streptomycin solution,at 37ºc with 5% CO_2 and 96%humidity and cultured for 21 days.

Cell differentiation studies: The chondrogenic differentiation of hMSCs was induced by adding synovial fluid, that was provided by Dr. Shiari,from arthritis rheumatoid patients's knees. For cell differentiation we were used two methods:

1-monolayer culture: MSCs (passage2) were plated with a density of $6×10^5$cells/dish in 6-well plates. To induce chondrogenic differentiation, the cells were treated with Synovial fluid. In the control group, the cells were cultured in control medium alone.

2- micromass culture: for the micromass culture, approximately 200,000 hMSCs (passage2) were placed in four 15-ml polypropylene tube(Falcon), and centrifuged to pellet.

In both methods, these cells were divided into 4 groups. In group A (negative control group) medium containing 1% serum was used as culture media;in group B the medium contained group A+100µl/ml SF; in group C, the medium contained groupA+150 µl/ml SF; the culture media in group D contained group A+200 µl/ml SF. Medium were changed twice a week. The level of differentiation was determined by analyzing the expression of cartilage specific genes by reverse transcription polymerase chain reaction (RT-PCR) on days 1,7, 14 and 21 of treatment.

Quantitative Reverse Transcription (RT)-PCR:
The cells of 4 groups in monolayer and micromass culture were cultured for three weeks and then collected. The total cellular RNA was extracted using easy-Blue Total RNA Extraction kit. The quantity of RNA was determined by absorption at 260 nm. After quantification of RNA, 2µg of RNA was synthesized into cDNA under the effect of reverse transcriptase. Then, the cDNA was taken as a template for PCR amplification of collagen2a1 and sox9. At the same time the β-actin gene was taken as the internal reference. RT was performed by a 60min incubation at 42ºc, followed by a 10min incubation at 72ºc to inactivate the RT. PCR amplification conditions for the resulting cDNA was performed by 35 cycles of 94ºc for 30s, 60ºc for 30s, and 72ºc for 40s.The reaction products were resolved by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. The RT-PCR primers were designed based on human sequences in Gen Bank. These sequences are listed in Table1.

TABLE 1. Primer sequences for RT-PCR

Gene		Primer sequences
SOX9	Forward	5'-GGAGGCAGAGGAGGCCACGGAG-3'
	Reverse	5'-ACTCGTTGACATCGAAGGTCTC-3'
COL2A1	Forward	5'-GAGCTGCTGGCCGCGTTGGACC-3'
	Reverse	5'-CCTGCTTGCCGGGCTCACCCGA-3'
β-ACTIN	Forward	5'-CACTCTTCCAGCCTTCCTTC-3'
	Reverse	5'-AGTCCGCCTAGAAGCATTTG-3'

Results
Chondrogenic differentiation of hMSCs in monolayer culture:
Human MSCs cultured in monolayer and treated with SF exhibited a more rounded shape than the cells from the control group. The expression of chondrocyte-specific genes that occurred following SF treatment was found to be dose-dependent and time-dependent.

RT-PCR analyses: We checked the expression of well-known cartilage-specific genes. An expression analysis for the known chondrogenic markers Sox9 and Col2a1 using RT-PCR in differentiated hMSCs was performed. In this manner, we showed that Sox9 and Col2a1 were induced during the course of hMSCs differentiaton into chondrogenic lineage. Treatment with 150 and 200 µg/ml SF(group C and D) increased type II collagen expression on days 14 and 21, respectively, when compared with the cells cultured in the control groups. Sox9, an early marker of chondrogenic differentiation, was detected as early as the day 14 of treatment with 150 and 200 µg/ml SF(group C and D). In these groups, Sox9 expression was increased on day 21 in contrast with day 14. (Fig1.B).
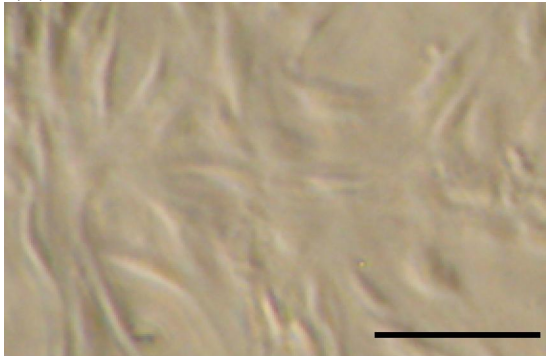
Chondrogenic differentiation of hMSCsin micromass culture
hMSCs were pelleted into micromasses and then differentiated in medium with 1% serum in the presence of SF, immediately after centrifugation, the cells appeared as a flattened pellet at the bottom of the tube. One day later, the pellet had a thickened lip. Between day 2 and 7, the pellet became spherical without any increase in size, it changed from white and opaque to a glistening transparent structure. Also, the weight of the pellets increased about 4-fold (Fig 2A).

RT-PCR analyses: As shown in Fig.3, marked expression of Sox9 was detected in group B after 2 weeks (Fig.3A), although the expression level was less than that at 3 weeks.For group C and D, Sox 9 expression, was detected on day 7 and then increased slightly on days 14 and 21 (Fig. 3B). Expression patterns of COL2A1 mRNAs were slightly expressed in pellets in groups, C and D after 2 weeks

but in group B expression of COL2A1 was detected on day 21. As for the control experiment, the expression of these genes was undetectable in non-treated pellets. As an internal calibration, β-actin was consistently expressed in various pellets and culture intervals. These results were consistent with the previous observations that expression of Sox9 is an early event in cartilage differentiation.
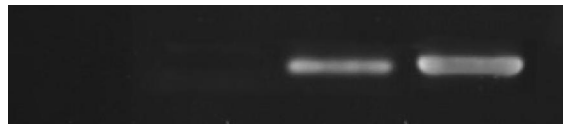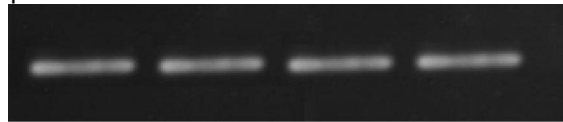
(A)

(B)
SOX9
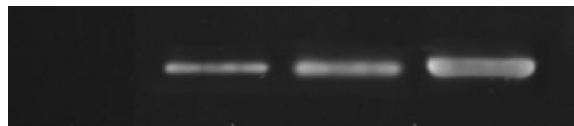
COL2A1

β-ACTIN

21   14   7   0
Days

Fig. 1: Effect of chondrogenic differentiation medium on hMSCs in monolayer culture (group B, C and D). (A) Morphology of hMSCs cultured in control medium (a) and in chondrogenic differentiation medium on day21 (b) Bar = 250 μm for a and b. (B) Effect of synovial fluid on the expression of chondrocyte-specific marker gees in hMSCs. RT-PCR analysis of SOX9,and COL2A1 genes was performed at the indicated time points. β-ACTIN was amplified as a control for total RNA concentration used in the RT-PCR.
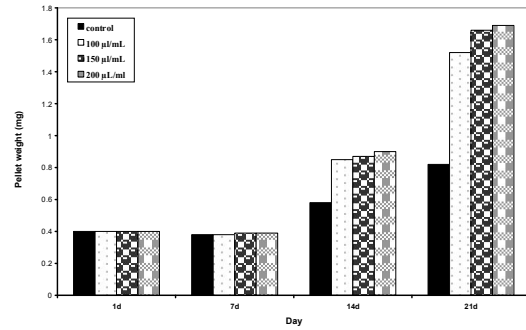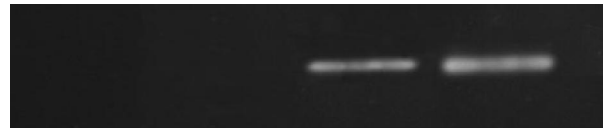

Fig. 2. Wet weight of pellets at days 1, 7, 14 and 21.Data are expressed as mean± SD (n=3).
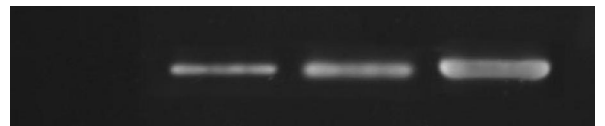
(A)
Sox9

COL2A1

β-ACTIN

21   14   7   0
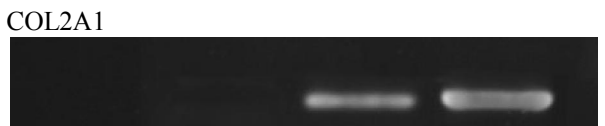**Days**

(B)
Sox9

COL2A1

β-ACTIN

21   14   7   0
Days

Fig 3. Effects of chondrogenic differentiation medium on hMSCs in micromass culture. (A) RT-PCR analysis of SOX9, and COL2A1 genes was performed at the indicated time points in group B, and (B) groups C and D.

Discussion

Understanding chondrogenesis will obviously require examining the process in several different experimental systems, any one of which will have some limitations(12). One approach to resolving the complexities of chondrogenesis is to examine simplified systems in vitro. We analyzed cartilage differentiation by human adult stem cells from bone marrow. Bone marrow is considered to be a suitable donor tissue for cartilage regeneration since it can be obtained from a comparatively small amount of iliac crest biopsy and contains multipotentmesenchymal stem cells. MSCs have the ability to differentiate along multiple connective tissue lineages including bone, cartilage, tendon, ligament, muscle, and fat (13). Several investigators have reported in vitro chondrogenic differentiation of MSCs in a pellet micromass culture with the defined medium containing bioactive factors such as TGF-β and dexamethasone.(14). Here, we demonstrated that synovial fluid of the human's knee has a noteworthy potential to induce chondrogenic differentiation in monolayer and micromass cultures of MSCs. The system used here has the advantage that SF contains TGF-β, dexamethasone and further factors that also promote and enhance the development of cartilage from MSCs. The hormone prolactin is one such factor of human SF that modulates the growth and chondrogenic differentiation of human MSCs (15). Pellets induced with high concentration of SF appear to be considerably larger in size than those induced with low concentraton of that(Fig2). SF can acts as a model for finding effective combinations of differentiation factors and deserves further research on its components and their potentials to induce chondrogenesis (16,17). To sum up, MSCs can be induced into cartilage by synovial fluid in a dose-depended manner.

Acknowledgment:

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