Iranian Biomedical Journal 13 (3): 153-160 (July 2009)

Quantitative Analysis of the Proliferation and Differentiation of Rat Articular Chondrocytes in Alginate 3D Culture

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Received 24 December 2008; revised 10 May 2009; accepted 17 May 2009

ABSTRACT

Background: While articular chondrocytes are among those appropriate candidates for cartilage regeneration, the cell dedifferentiation during monolayer culture has limited their application. Several investigations have indicated the usefulness of alginate, but the topic of proliferation and differentiation of chondrocytes in alginate culture has still remained controversial. **Methods:** Rat articular chondrocytes were released by enzymatic digestion, plated at 5×10^4 cells/cm^2 and culture-expanded. Passaged-5 cells were then cultivated in alginate as 2-mm beads for a period of two months during which the expansion rate and the level of cell differentiation were determined by [3-(A, 5- dimethylthiazolyl- 2-yl)-1, 5-diphenyl tetrazolium bromide] assay and real-time PCR analysis respectively and compared with those of chondrocytes in monolayer culture. **Results:** Average population doubling time in alginate cultures (10.04 ± 0.9 days) tended to be significantly ($P<0.05$) higher than that in monolayer cultures (2.94 ± 0.3 days). The period of alginate culture could be subdivided into expansion phase (Days 0-40); during which proliferation appeared to be high and differentiation phase (Days 40-60) during which the expression of cartilage-specific genes including collagen II and aggrecan tended to be upregulated. During both the proliferation and differentiation phases, the expression of collagen I was low. At chondrocytes monolayer cultures, the proliferating cells appeared to have a very low expression level of cartilage-specific genes and a high expression level of collagen I gene during the entire culture period ($P<0.05$). **Conclusion:** It seems that alginate provides conditions in which rat articular chondrocytes are able to undergo proliferation and differentiation in certain time point of cultivation period. *Iran. Biomed. J. 13 (3): 153-160, 2009*

Keywords: Articular chondrocyte, 3D culture, Monolayer culture, Cell proliferation, Cell structure

INTRODUCTION

In contrast to other tissues, cartilage lacks blood and lymphatic vessels, being nourished by diffusion process. The lack of the blood vessel also caused the damages of cartilage tissue to be regenerated very poorly because the regeneration of tissue is normally mediated by specific repair mediators that reach into damage site through circulation [1, 2]. In most disorders associated with hyaline cartilage including osteoarthritis and trauma in which a considerable loss of tissue is occurred cartilage regeneration requires intervention [1]. In such circumstances, therapies like subchondral bone abrasion, allograft and auto graft transplantation are routinely being used. Other valuable approach would be the cell-based treatment strategy using chondrocytes isolated from articular cartilage of non-weight bearing area [3].

Autologous chondrocyte transplantation is among those methods that have been gained considerable attention because of the great progress in tissue engineering field and appropriateness of chondrocytes as a valuable cellular material for cartilage regeneration [4, 5]. Using a small biopsy from non-weight-bearing articular cartilage, chondrocytes can easily be isolated and expanded in culture [6]. One limitation associated with chondrocyte use in cell therapy is considered to be their scarce number in tissue. Since cell-based treatment of cartilage defects requires a large quantity of cells, it seems that in any attempt to regenerate cartilage defect, chondrocytes *in vitro* expansion is an inevitable step [7]. Previous studies

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have indicated that chondrocytes possess a good proliferative capacity when being cultivated as monolayers, but under this circumstance, they may undergo dedifferentiation with consequences of loosing the morphologic features as well as secretive function [8-10]. Some previous investigations have shown that 3D cultures of chondrocytes in a number of porous scaffolds may help the cells to maintain specialized phenotype [11, 12]. Alginate as a natural polysaccharide obtained from brown seaweed exists in solution form and has the property of becoming gel upon exposure to calcium ions. This composition exhibits the advantages of being highly flexible which render it as an appropriate chondrocyte carrier into cartilage defects [13-15]. In former investigations, chondrocytes have been cultivated in alginate in a variety of experimental design, but its impact on proliferation and differentiation of chondrocytes has remained a controversial issue [16-20].

In the present study, the subject of alginate effects on proliferation and differentiation of articular chondrocytes were investigated using the cells from rat knee joints. To thoroughly investigate the issue, alginate culture period was extended up to two months during which the proliferation rate of the cells as well as the expression levels of the cartilage specific genes by the cultivated cells were determined by [3-(A, 5- dimethylthiazolyl- 2-yl)-1, 5-diphenyl tetrazolium bromide] (MTT) assay and real-time PCR analysis, respectively. Moreover, in this study, chondrocytes morphology in alginate cultures was examined by light, transmission and scanning electron microscopic observations. In all these evaluations, chondrocytes in alginate were compared to the cells in monolayer culture.

MATERIALS AND METHODS

Isolation of chondrocytes from articular cartilage. In this study, 10 male Wistar rats, 4-6 weeks old, were studied. The use of animal in the experiments was approved by the Ethic Committee of Royan Institute (Tehran, Iran). The animals were sacrificed by cervical dislocation; their right knee joints were washed with 70% ethanol and shaved. Joint cavity was then exposed by an incision through the skin and articular capsule. The cartilage on the surface of tibia and femur was removed and placed in DMEM (Gibco, Germany) supplemented with 15% fetal bovine serum (FBS), 100 IU/ml penicillin (Gibco, Germany) and 100 μg/ml streptomycin (Gibco, Germany). The specimens were then minced into 1-2 mm pieces under sterile laminar hood and subjected to enzymatic digestion in 2-ml solution consisting of 0.2% type I collagenase and 0.1% pronase in 1:1 ratio at 37°C for 24 hours. Released chondrocytes were collected by centrifugation and plated in 75-cm^2 culture flasks at 5×10^4 cells/cm^2. Upon confluency, the cells were lifted by trypsin/EDTA and subcultured. Several additional passages were performed to obtain sufficient cells for below described experiments.

3D cultures in alginate gel. Passaged-5 chondrocytes (about 5×10^5) were uniformly suspended in 1 ml of 1% alginate gel (Fluka, Germany) and the mixture was loaded into a 5-ml sterile syringe with a 22-g needle. Alginate beads (approximately 2mm) were then made by dropping alginate-cell suspension through the injection needle into $CaCl_2$ suspension (102 mM). The beads were washed with PBS and cultured in DMEM medium supplemented with 15% FBS in an atmosphere of 5% CO_2 and 37°C for a period of two month. During the culture period, the medium was changed three times a week. At the end of cultivation period, the chondrocyte structure was examined by light and electron microscopy. Moreover, at different time points, the cultures were quantified in terms of the chondrocytes expansion rate as well as the expression of cartilage specific gene markers by the cultivated cells.

Light and electron microscopy:

Light and transmission electron microscopy (LM and TEM). To prepare the culture, specimens were first fixed by solution consisted of 2.5% glutaraldehyde and 1% paraformaldehyde buffered with 1% sodium cacodylate (pH 7.4) at 4°C for 24 hours. Post fixation was performed in 1% w/v osmium tetroxide in 1% sodium cacodylate at 4°C for 1 h and followed by embedding in molted agar. The specimens were then cut into about 1 mm pieces under stereomicroscope and subjected to ascending concentrations of ethanol for dehydration and pure acetone for clearing purpose. The cells were embedded in araldite resin, polymerized at 60°C for 24 hours and finally, cut into either 300-500 nm thick (semi thin) sections which were stained with toluidine blue or 70-90 nm thick (ultra thin) sections which were stained with lead citrate and ammonium acetate. Ultra-thin sections were observed by

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transmission electron microscope (Zeiss, EM900, Germany).

Scanning electron microscopy (SEM). The fixation of the specimens was performed as described for TEM. After dehydration with ascending concentrations of ethanol (30-100 grades), the specimens were lyophilized and observed with a scanning electron microscope (Zeiss, DSM 940A, Germany).

Assays of proliferation rates. To quantify and compare the expansion rate of the cells, passaged-5 chondrocytes (4×10^4) were cultivated either as 3D culture in alginate or monolayer culture on the surface of plastic culture dish for 7 days. At the end of cultivation period, cell population doubling time (DT) was determined for the cultures using DT = culture time/population doubling number (PDN). PDN was calculated using PDN = Log NI/N0 × 3.31 where N0 is the number of seeded cells and N1 the number of harvested cells. To count the cells MTT (Sigma, Germany) assay was used.

MTT Assay. Alginate beads were dissolved in sodium citrate to release the chondrocytes from the gel. To perform MTT, 300 ml fresh DMEM and 20 µl yellow solution of MTT (5 mg/ml) were added on the chondrocytes. The cells were then incubated at 37°C for 2 hours which was resulted in the formation of formazan crystal due to action of succinate-tetrazolium reductase belonging to mitochondrial respiratory chain on MTT. Supernatant was then removed and 200 µl dimethyl sulfoxide (DMSO, Sigma, Germany) was added on crystals. This caused the crystals to be dissolved and create a purple color. Absorbance of the color was recorded by Elisa-reader at 540-630 nm at dark room. Using the standard curve plotted for the known number of cells, the number of chondrocytes in alginate was determined.

Proliferation of chondrocytes in long-term culture within alginate gel. To precisely examine the cell proliferation in alginate, chondrocyte expansion rate was quantified using MTT assays at different time points including days 20, 40 and 60.

Real-time PCR:

Reverse transcription (RT)-PCR and real-time PCR. Relative real-time PCR was performed to assess exact expression ratio of a set of chondrocyte-like related genes and the rate of their up or down regulation in chondrocytes during different time points (day 20, 40 and 60) at alginate as well as monolayer culture. Total RNAs were extracted from chondrocytes cultivated in alginates on different time points of 20, 40 and 60 days and the chondrocytes in monolayer cultures on corresponding time points using the RNX^{TM} (-Plus) (RN7713C, Cinnagen Inc., Tehran, Iran). RNA concentration was assayed by spectrophotometer in length wave of 260 nm. Before RT reaction, samples of the isolated RNA was treated with 1 U/ml of RNase-free DNase I (EN0521, Fermentas, Opelstrasse 9, Germany) per 1 mg of RNA in order to eliminate residual DNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311, Fermentas, Germany) and 1 × reaction buffer with $MgCl_2$ at 37°C for 30 min. To inactivate the DNase I, 1 ml of 25 mM EDTA per 1 mg of RNA was added and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 mg total RNA using Random hexamer as a primer and a RevertAid^{TM} First Strand cDNA Synthesis Kit (K1622, Fermentas, Germany) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without Revert Aid^{TM}M-MuLV Reverse Transcriptase (RT-reaction) to provide a control for DNA contamination in the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. RT followed by quantitative PCR (Q-PCR) that is at present the most sensitive method for transcript abundance measurement. Q-PCR utilizes optical measurement of generated amplicons to survey PCR amplifications. After that we applied relative real-time PCR for quantitative measurement of Gene expression. At first, the efficiency of primers (Table 1) was determined by using standard curve acquired from data of serially diluted positive template which showed expression of all genes in this study. We had 6 serial dilutions with diluting coefficient of 1/5. We used these dilute templates for real-time PCR in order to plot standard curve and acquire efficiency of used primers. Standard curve was calculated by plotting the log value of the starting concentration versus the threshold cycle (Ct). The efficiency was ≈ 90-100%. In real-time assay, dissociation curve shows purification of PCR product. Reaction conditions were 40 cycles of a two-phase PCR (denaturation 95ºC for 15 s; annealing at 60ºC for 30 s) after an initial denaturation step (95ºC for 10 min). Reaction mixtures for real-time PCR included 2 µl cDNA as

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Table 1. Primers used in real-time PCR.

Gene name	Direction	Sequence	Annealing temperature	Product size
Collagen II	Forward	F: 5' CCAGAACATCACCTACCAC3' R: 5' CCCTCATCTCCACATCATTG 3'	60	145
Aggrecan	Forward	F: 5' ATGGTGACAAGGACGAGTTC 3' R: 5' CTCACCCTCCATCTCTTCAG 3'	60	101
Collagen I	Forward	F: 5' GAATATGTATCACCAGACGCAG 3' R: AGCAAAGTTTCCTCCAAGAC 3'	60	190
GAPDH	Forward	F: 5' TTCAACAGCAACTCCCATTC 3' R: 5' GCCATATTCATTGTCATACCAG 3'	60	102

template, 12.5 Power SYBR^R Green PCR Master Mix (ABI, USA), 0.5 mM of each forward and reverse primers. For each sample, we had a target gene and also a reference gene as internal control. The efficiency-calibrated model is a more generalized ΔΔCt model. In this model, Ct is the sign of the first cycle that amplification curve begins to rise. In this method, we should consider both Ct of target gene and also Ct of reference gene or housekeeping gene. ΔCt for each target gene is then calculated by subtracting the Ct number of target gene from that of housekeeping gene for each sample. ΔΔCt for each gene was calculated by subtracting the ΔCt of target sample from that of control sample.

Statistical analysis. Mean values ± SD (standard Deviation) were calculated for data obtained from the assays for chondrocytes proliferation as well as that obtained from real-time PCR. The data was analyzed with student's *t*-test using SPSS software version 13. A P value <0.05 was considered to be significant.

RESULTS

Primary culture. Some chondrocytes adhered and survived on plastic culture surfaces. These became the origin of a monolayer that covered the surfaces of the culture dish in 1 week. At this time, the cells were lifted and subcultured at 5×10^4 cells/cm^2. Passaged-5 cells were used to establish 3D culture in alginate gel. The cells during subcultures possessed rapid expansion rate reaching confluence in a period of 3-4 days. Chondrocytes in primary cultures appeared to be polygonal (Fig. 1A); while later at subcultures they observed as elongated spindle-shaped cells in morphology (Fig. 1B and 1C).

Electron microscopy:

Light and TEM. According to the semi-thin sections prepared from alginate culture and stained with toluidine blue, chondrocytes were observed to be located in lacunae-like cavities in alginate where

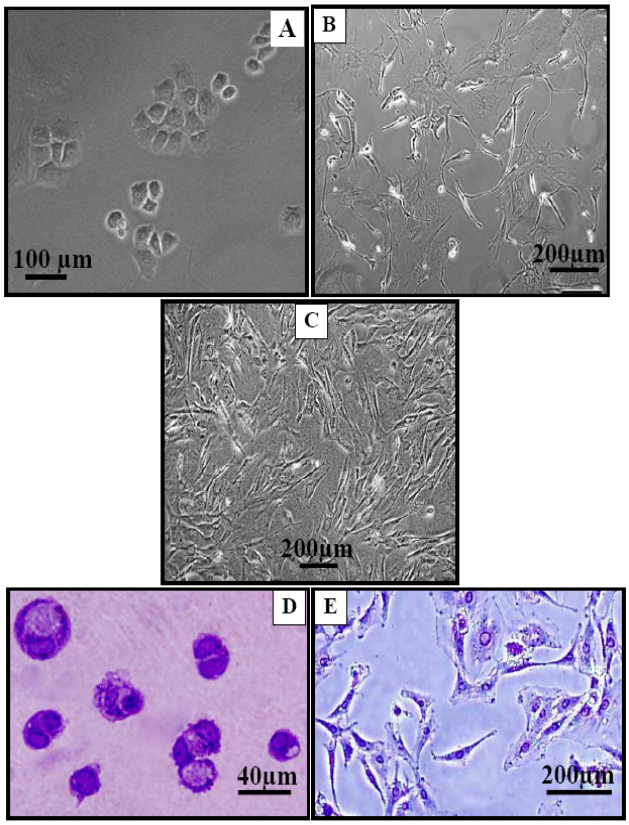

Fig. 1. (A) Chondrocytes in monolayer culture appeared as polygonal cells; **(B)** passaged-2 and **(C)** passaged-5 cells were morphologically elongated cells; **(D)** chondrocytes in alginate appeared as round to ovoid cells located in lacuna-like cavity. The cells were surrounded by methachromatic matrix; **(E)** monolayer culture showed no methachromatic matrix by toluidine blue staining. A-C and E, images of phase contrast invert microscope and D, image of light microscope.

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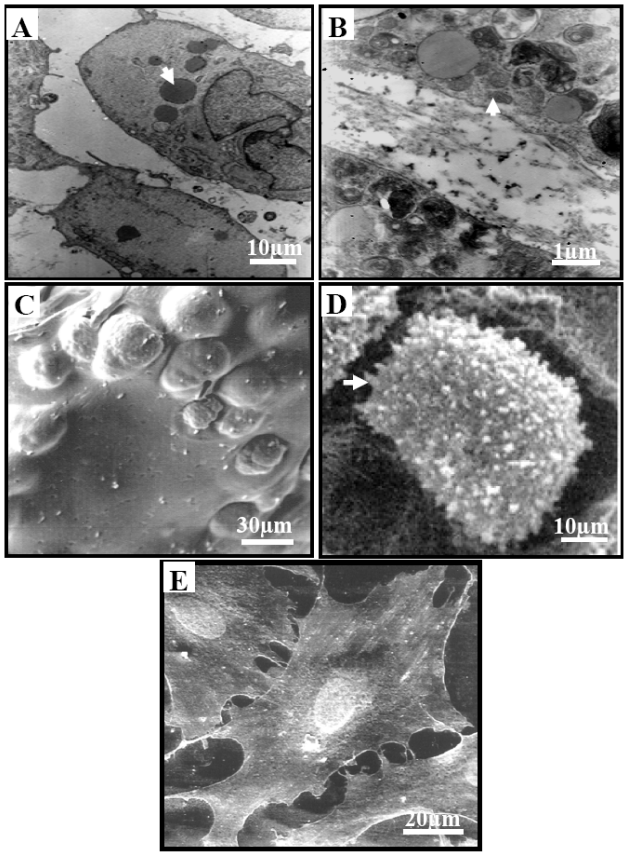

Fig. 2. (A) Chondrocytes in alginates possessed large euchromatin nucleus with lipid droplets (arrow) in their cytoplasm; **(B)** these cells also contained plenty of secretory vesicles (arrow); **(C)** chondrocytes with round to ovoid morphology were embedded in alginate; **(D)** their surface was covered with abundant microvillus (arrow) and **(E)** chondrocytes in monolayer were observed as elongated flattened cells without any surface projections. A-B, TEM images and C-E, SEM Images.

they were surrounded by their own deposited methachromatic matrix. In these sections, isogenic cells can also be visible (Fig. 1D). There was no methachromatic matrix in chondrocytes monolayer culture (Fig. 1E). According to the ultra-thin sections, chondrocytes in the alginate had a large euchromatin nucleus and a cytoplasm containing some lipid droplets, mitochondria and secretory vesicles (Fig. 2A and B).

Scanning electron microscopy. SEM images indicated that the chondrocytes in alginate possess a round to ovoid morphology and embedded in the lacuna-like cavity within the alginate (Fig 2C). The cell surfaces seemed to be covered with a plenty of microvilli which could be considered as an indication of their active state (Fig. 2D). Chondrocytes in monolayer observed to be in flattened elongated morphology without any microvilli on their surfaces. There also appeared to be no matrix deposited by the cells in monolayer cultures (Fig. 2E).

Chondrocyte proliferation at monolayer and alginate cultures. The expansion rate of chondrocytes in monolayer culture was significantly higher than that in alginate cultures. At monolayer culture, chondrocytes tended to be doubled in 2.94 days while at alginate cultures, the cells possessed a DT value equal to 10.04 days (Fig. 3A). This difference was statistically significant ($P<0.05$).

Proliferation of chondrocytes at long-term culture in alginate. According to our findings, while chondrocytes in monolayer cultures appeared to be in proliferation during the whole culture period, those in alginate tended to undergo proliferation up to day 40 after which expansion seemed to be ceased (Fig. 3B).

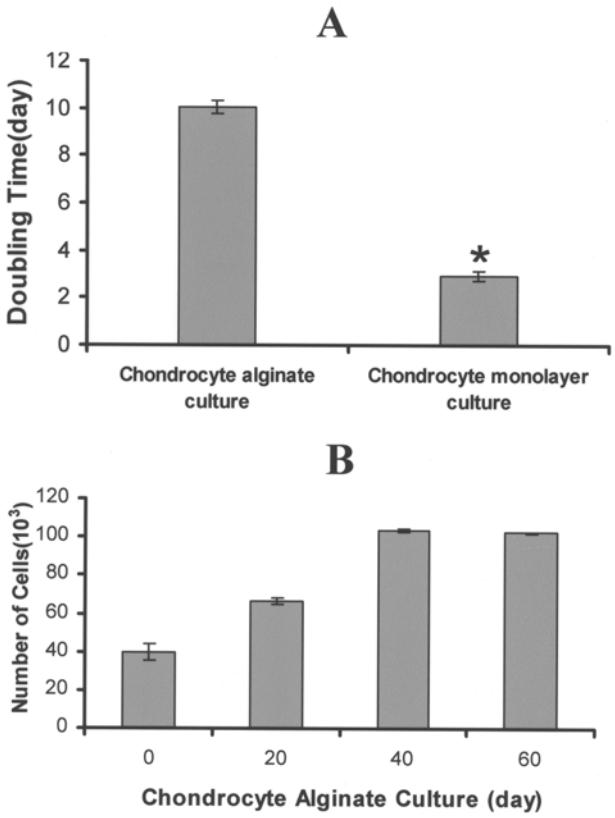

Fig. 3. (A) Population doubling time of chondrocytes in alginate compared with that of chondrocytes in alginate culture. *A statistically significant, lower ($P<0.05$) value than condrocytes in monolayer culture. **(B)** Proliferation of chondrocyte in alginates at different time points. The cells stopped expansion after the day 40 of culture period.

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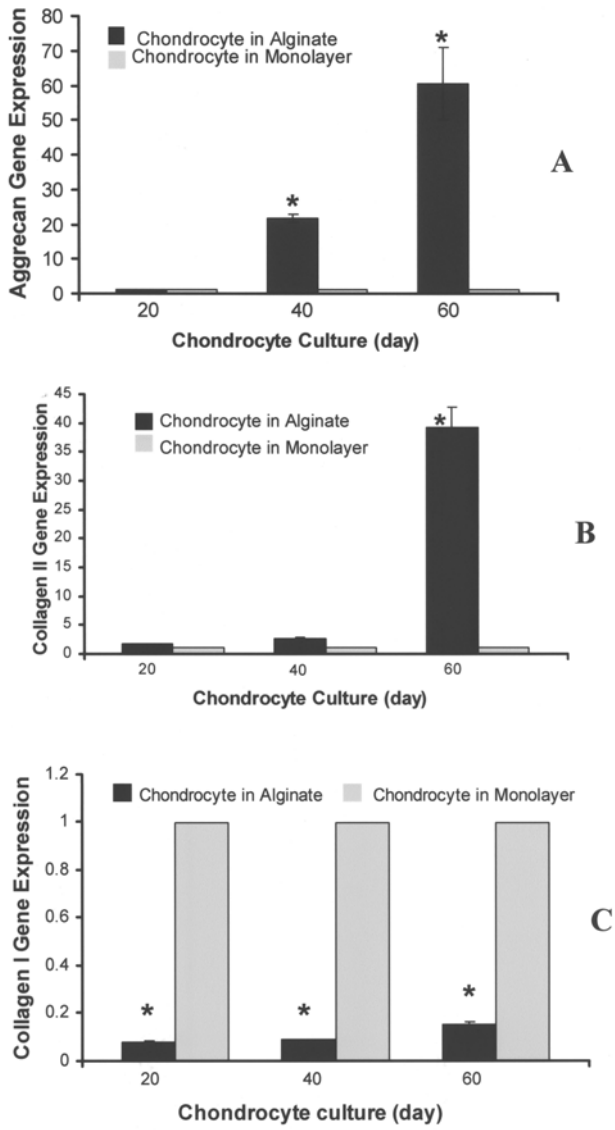

Fig. 5. Graph indicates the expression level of aggrecan **(A)** and Collagen II **(B)** genes during cultivation period at two culture systems.*A statistically significant, higher ($P<0.05$) value than at monolayer culture. C) Expression level of collagen I in chondrocytes in alginate was remained in minimal level compared with chondrocytes in monolayer during the entire period of culture. * A statistically significant, lower ($P<0.05$) value than at monolayer culture.

Real-time PCR. Real-time PCR analysis indicated that at monolayer culture, the expression level of aggrecan (Fig. 4A) and collagen II (Fig. 4B) genes were low during the entire culture period. In this culture, collagen I gene was expressed in a high level during the whole period of culture (Fig. 4C). At alginate culture, expression level of the aggrecan (Fig. 4A) and collagen II (Fig. 4B) genes tended to be low during the first two thirds (40 days) of culture period (expansion phase) when the cells were in proliferation. These genes were significantly upregulated during the last one third of culture period (differentiation phase). In this culture, collagen I expressed in a low level during the whole culture period (Fig. 4C). All differences between monolayer and alginate cultures were statistically significant ($P<0.05$).

DISCUSSION

In this study, we found both mitogenic and differentiating properties of alginate on articular chondrocytes in 3D cultures. These results would be of great importance for who are involved in the field of clinical applications of chondrocytes which occur in a scarce quantity of cartilage tissue. Therefore, any application of chondrocytes especially as the cellular material in regenerative medicine requires their *in vitro* expansion while maintaining the cell differentiated phenotypes.

Our study was not the first report on chondrocyte proliferation in alginate. A few studies have previously been conducted on this subject but the results were apparently contrasting [16-18]. Van Susante *et al.* [16] have reported that the number of chondrocytes cultivated in alginate gels has been gradually lost during the culture period. This is in disagreement with our results on the increase of chondrocyte number in the alginate gel. To explain this, it should be mentioned that the alginate construct being studied in the present study was very smaller in size than that in Van Susante *et al.* [16] investigation. Indeed, insufficiency of nutrient diffusion into the central part of large constructs could lead the central cells to die. In other study, rabbit MSC have been cultivated in alginate at 10×10^6 cells/ml in a 50 µl beads and then implanted into osteochondral defects [18]. The results have indicated the progressive loss of the cell density in defect site, reaching to 15% of initial density after 4 weeks. This result would be predictable considering that cartilage tissue is avascular and could not support a large number of transplanted cells in terms of their nutritional needs. The results by Gagne *et al.* [17] have emphasized the significance of cell density and its relationship with cell proliferation. These authors have examined the proliferation of chondrocytes in alginate at different densities including 10^4, 10^5 and 10^6 cells/ml and have came with this conclusion that cultivating the cells at lower cell density leads to their more proliferation. In the present study, we produced a chondrocytes/

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alginate construct using a density of 5×10^5 cells/ml and the cultures were ended up with significant proliferation of the chondrocytes and this is not in disagreement with Gagne *et al.* [17] findings.

The subject of differentiating effects of alginates has been studied under different conditions. In this context, it has been shown that collagen II has been only produced at chondrocyte cultures in alginates under low oxygen tension [20]. In the present study, chondrocyte/alginate constructs were incubated at conventional high O_2 tension and still high expression of cartilage specific genes including collagen II gene was observed. This result, however, is not in disagreement with that of aforementioned study because in that experiment, the constructs have been cultivated for up to 2-3 weeks while in the present study, the cultures were extended up to 8 weeks. Under our culture conditions, the level of cartilage differentiation was not significantly raised until the 6th week of culture period. The others have reported the direct relationship between cartilage matrix production and the initial cell seeding density in a short culture period of about 1-2 weeks [19]. Our study differs from those previous investigations in that we extended the culture period up to day 60 during which the expression level of cartilage specific genes were investigated at several time points. Our data indicated that the expression level of two fundamental genes of cartilage remained in a low level up to day 40 and after that, the expressions tended to be upregulated.

According to the results obtained by TEM, chondrocytes in alginates appeared as ovoid cells containing lipid droplets and secretory vesicles. This finding is in accordance with Häuselmann *et al.* [21] reports on ultra structural characteristics of bovine articular chondrocytes in alginate gel. Furthermore, in this study, cell surface characteristics of chondrocytes in alginate were further investigated using scanning electron microscopy. Abundant microvilli developed on the surfaces of chondrocytes in alginate but not in monolayer culture along with that of their TEM profiles were all indicative of the cell involvement in matrix secretion. This morphology, in turn, would be the result from active interaction of chondrocytes with alginate molecules.

Former studies have indicated that mass transfer into scaffold deeper parts would be impaired due to the insufficient penetration of nutrients and oxygen in static 3D culture conditions. This would result in the death of some cells in scaffold central part [22, 23]. In this study, to examine the chondrocytes expansion rate in outmost accuracy, MTT assay was used. This assay is based on mitochondrial enzymatic activity of living cells excluding the dead cells from cell count.

Taken together, it seems that, during the early days of chondrocytes 3D cultures, alginate gel mostly stimulated the cell proliferation. In contrast, the differentiating effects of alginate appeared at the later days of culture period during which chondrocytes proliferation seemed to be ceased. These findings are in accordance with the notion that differentiation and proliferation are two contrasting phenomenon [24]. Furthermore, at monolayer culture, plastic surfaces failed to provide sufficient microenvironment to maintain the chondrocytes differentiated state; rather this culture condition was in favor with chondrocytes proliferation. According to our findings, in monolayer cultures, there was some evidence of the expression of type I collagen gene which is the dominant component of fibro cartilage and bone matrix.

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[DOR: 20.1001.1.1028852.2009.13.3.1.5]
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