Costal versus articular chondrocytes in alginate three-dimensional cultures

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Abstract
Given the difficulties in accessing articular cartilage as a source of chondrocytes to be used in fabricating cartilage constructs, alternative sources are required. The present study examined chondrocytes from costal cartilage for their suitability in cartilage tissue engineering. Chondrocytes isolated from rat knee and rib hyaline cartilage were separately mixed with alginate and placed in a calcium chloride solution as two mm beads. The beads were incubated over 2 months, during which time the structural features, proliferation rates, and gene expression levels were determined by microscopy, [3-(A, 5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazulium bromide] (MTT) assay, and real-time PCR analysis, respectively. The majority of both articular and costal chondrocytes were observed to be organelle-rich round to oval cells embedded in lacuna-like cavities within the alginate beads. The propagation patterns of both cell types were similar, undergoing proliferation during the first 40 days and almost ceasing propagation over the remaining 20 days of the culture period. The levels of aggrecan and type II collagen (cartilage specific) gene expression in costal and articular chondrocyte cultures were comparable; expression levels were very low during the initial days of culturing but were significantly upregulated by study termination (day 60). Interestingly, in contrast to cultured articular cells, the level of collagen I expression was negligible in costal cultures ($p<0.05$). Collectively, these data suggest that the costal chondrocytes could provide a beneficial and more accessible source of chondrocytes for three dimensional (3D) cartilage constructs.

Keywords: Costal and articular chondrocytes; Alginate; Proliferation; Gene expression; Ultrastructure

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INTRODUCTION

Tissue engineering has recently made considerable progress in providing reconstructions or replacement tissues for injuries to the skin, aorta, muscles, bladder, breast, bones, tendons and cartilage (Bujan *et al*., 2005; Butler and Orgill, 2005; Lu *et al*., 2005; Qin *et al*., 2005; Sahoo *et al*., 2005; Shinoka *et al*., 2005; Sodian *et al*., 2005; Tare *et al*., 2005; Turhani *et al*., 2005; Patrick, 2004). Among these, cartilage repair using tissue engineering should in theory be more straightforward given its avascular nature and single cell type (chondrocytes) construction. (Cao *et al*., 1997; Cohen *et al*., 1993; Vacanti *et al*., 1991).

Tissue engineering approaches need an appropriate cell source to provide harvested cells. In addition, several appropriate techniques are subsequently required to seed the carrier scaffold with cells to construct a 3D tissue implant that is to be transplanted in the defective site. Traditionally, cells for cartilage engineering are primarily obtained from articular knee cartilage (Arøen *et al*., 2004; Mandl *et al*., 2004; Brittberg *et al*., 2003; Cancedda *et al*., 2003; Dozin *et al*., 2002; Glowacki, 2000; LeBaron and Athanasiou, 2000; Brittberg, 1999; Brittberg *et al*., 1996; Benya *et al*., 1978). Since articular cartilage is a weight-bearing tissue providing smooth lubricated surfaces for joint movement, tissue removal impairs its function and imposes additional defects. Moreover, the non-weight-bearing region in the joint cartilage would be considered as the alternative chondrocyte source, but tissue removal from joint requires an additional manipulation of the structure and more importantly the amount of harvestable specimen from that location may be limit-

ed. This necessitates alternative chondrocyte donor locations.

Several options can be considered as possible donor sites. For the intended purpose, fibro cartilage and elastic cartilage are inappropriate because they differ from hyaline (articular) cartilage with respect to their structure, function, cell arrangement, and the presence or absence of perichondrium (Dehaven and Arnoczky, 1994). Chondrocytes from other hyaline sources could theoretically be used in this context. In this regard, hyaline cartilage at the costal end of thoracic cage could provide a viable alternative. To date, there is very limited information available on the suitability of costal chondrocytes for cartilage tissue engineering (Isogai *et al*., 2006).

The objectives of the present study were to examine chondrocytes from rat costal cartilage in terms of structure, proliferation, and gene expression in an alginate 3D culture and compare those characteristics with those of articular chondrocytes. The eventual goal of this research was to assess the ability of costal chondrocytes to provide a viable alternative to articular chondrocytes for manufacturing 3D cartilage scaffolds useful for cartilage defect implants.

MATERIALS AND METHODS

Chondrocyte isolation and culture

Cell harvesting: All animal experiments were approved by the Ethics Committee at the Royan Institute, Tehran, Iran. Ten male Wistar rats aged 4-6 weeks were sacrificed by cervical dislocation and used as a source of cells. Articular chondrocytes were harvested by dissecting tissue from the femoral condyles and tibial plateaus of the right knee of each animal. Costal cartilage was obtained from the anterior end of the upper ribs near their sternal attachments. Harvested tissues were placed in DMEM (Dulbecco's modified Eagle's medium, Gibco, Germany) supplemented with 15% (v/v) fetal bovine serum (FBS), 100 IU/ml of penicillin (Gibco, Germany) and 100 IU/ml of streptomycin (Gibco, Germany). Under sterile conditions, cartilage tissue was minced into 1-2 mm pieces, washed with phosphate buffer solution (PBS) and supplemented with 2 ml of enzymatic solution consisting of 0.2% (w/v) type I collagenase (Sigma, Germany) and 0.1% (w/v) pronase (Sigma, Germany) at a 1:1 ratio. Digestion continued at 37°C for 24 h.

Monolayer culture: Chondrocytes were collected and plated in 75 cm^2 culture flasks at 5×10^4 cells/cm^2. After culturing to confluency at 37°C and 5% CO_2, cells were detached by trypsin/EDTA and subcultured. To obtain sufficient cells for each experiment, several additional passages were performed.

Three dimensional cultures in alginate gel: Approximately 5×10^6 chondrocytes (passage-5) from each source were uniformly and separately suspended in a 1 ml of 1% alginate gel (Fluka, Germany). The mixture was then loaded into a 5 ml sterile syringe with a 22 g needle. Alginate beads, approximately 2 mm in diameter, were then formed by drop-wise addition of 7 µl of alginate cell suspension (containing approximately 3.5×10^4 cells) into a $CaCl_2$ suspension (102 mM). Each 5-6 beads were washed with PBS and cultured in 24-well culture plates for a period of 60 days, with the medium being changed twice weekly. At the end of the cultivation period, the chondrocyte structures were examined by light and electron microscopy. Moreover, cultures were quantified throughout the experiment for chondrocyte proliferation rates and gene expression levels by the [3-(A, 5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazulium bromide] (MTT) assay and real time RT-PCR analysis, respectively.

Analytical technique

Microscopy: Cell-alginate constructs were fixed with a solution consisting of 2.5% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde buffered with 0.1 M sodium cacodylate pH 7.4, for 24 h at 4°C. Post-fixation was performed for 1 h at 4°C in 1% (w/v) osmium tetraoxide in 0.1 M sodium cacodylate. Ascending concentrations of ethanol and several washes with pure acetone were used to dehydrate and clear the specimens, respectively. Specimens were subsequently embedded in araldite resin, polymerized at 60°C for 24 h, and finally cut into either 300-500 nm semi-thin sections for toluidine blue staining, or 70-90 nm ultra thin sections for staining with lead citrate and uranyl acetate. Ultra-thin sections were observed by transmission electron microscopy (TEM) (Zeiss EM900, Germany). Some beads were also prepared for paraffin embedding.

Cell proliferation: To quantify and compare the expansion rates of monolayer cultures, 5×10^4 chondrocytes

(passage-5) were cultivated in plastic culture dishes for 7 days to determine the fold-increase in cell numbers for both culture types. A MTT assay was used to count viable cells. To compare the proliferation capacities of the cells in 3D cultures, chondrocytes were cultivated in alginate as previously described for a period of 60 days, during which cells were counted using the MTT assay at different time points (20, 40 and 60 days).

To quantify the cells in alginate beads, cells were released by dissolution in 55 mM sodium citrate (pH 7.4); for monolayer culture quantification, the medium was discarded. Subsequently, 300 ml of fresh DMEM and 20 µl of MTT solution (5 mg/ml) were added to the chondrocytes. Cells were then incubated at 37°C for 2 h before removing the supernatant to which 200 µl of dimethyl sulfoxide (DMSO, Sigma, Germany) was subsequently added. Absorbance was recorded at 540-630 nm and compared to a standardized curve for cell quantification.

Real-time polymerase chain reaction (*RT-PCR*): Real-time PCR was performed to assess the expression ratios of a set of chondrocyte related genes and their up- or down-regulation at different time points for both alginate and monolayer cultures. RNA was extracted from chondrocytes cultivated in alginate at 20, 40 and 60 days using the RNX-PlusTM solution (RN7713C; CinnaGen Inc., Tehran, Iran) and assayed by a spectrophotometer (S2100 Diode Array Spectophotometer, WPA, UK) at 260 nm. Prior to reverse transcription (RT) reactions, samples of the isolated RNA were treated with 1U/ml of RNase-free DNaseI (EN0521; Fermentas, Germany) per mg of RNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311; Fermentas, Germany) and 1X reaction buffer with $MgCl_2$ for 30 min at 37°C to eliminate residual DNA. To inactivate DNaseI, 1ml/25mM EDTA for each mg of RNA in treated sample was added and incubated at 65°C for 10 min.

Standard RT reactions were performed with 2 mg of total RNA using a random hexamer as a primer and a RevertAid™ first strand cDNA synthesis kit (K1622; Fermentas, Germany) according to the manufacturer's instructions. For every reaction sequence, one RNA sample was prepared without RevertAid™-MuLV reverse transcriptase (RT-reaction) to provide a control for DNA contamination in the subsequent PCR. To minimize variation in the RT reactions, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Afterwards, relative real-time PCR was applied for quantitative measurement of gene expression; primer efficiency was determined using standard curves acquired from serially diluted positive templates for all genes in this study.

Reaction conditions for the PCR were 40 cycles of a two phase PCR (denaturation at 95ºC for 15 s; annealing at 60ºC for 30 s) after an initial denaturation step (95ºC for 10 min). Reaction mixtures included 2 µl of cDNA as a template, 12.5 µl of Power SYBRR Green PCR Master Mix (ABI, USA), and 0.5 mM each of the forward and reverse primers (Table 1). Each sample employed a target gene and a reference gene as an internal control.

Table 1. Primers used in real-time PCR.

Gene name	Sequence	Annealing Temperature
Collagen II	F: 5´ ACTTTGACCGAGCCCAGCGG3´ R: 5´ CAATGTCAACAATGGGAAGG 3´	60ºC
Aggrecan	F: 5´ GCCGAGGGCAACAGCAGGTT 3´ R: 5´ CCTTCCCATTGTTGACATTG 3´	60ºC
Collagen I	F: 5´ ACTGGAGAGGTGGGCACCC 3´ R: 5´GTCTTGGAGGAAACTT TGCT 3´	60ºC
GAPDH	F: 5´ TGAAGATTGTCAGCAATGCC 3´ R: 5´ AACATCATCTGCTTCTACTG 3´	60ºC

Statistical analysis: The Student t-test was used for comparing the obtained values. All measurement tests were performed in triplicate. All values are stated as mean ± standard deviation. A P value of < 0.05 was considered to be statistically significant.

RESULTS

Cell Morphology: In the monolayer culture, costal chondrocytes appeared to be morphologically polygonal to round in shape, while those from the articular cartilage tended to be slightly elongated in appearance (Fig. 1 A, B). Articular chondrocytes were observed to be slightly smaller than costal chondrocytes during primary culture. Following subsequent passages, both cell types adopted a fibroblastic morphology (Fig. 1 C-F).

In the case of alginate cultures, cells from both chondrocyte sources acquired a round to oval cell morphology after being cultivated in the 3D scaffolds (Fig. 2 A-D). Isogenic groups, consisting occasionally of

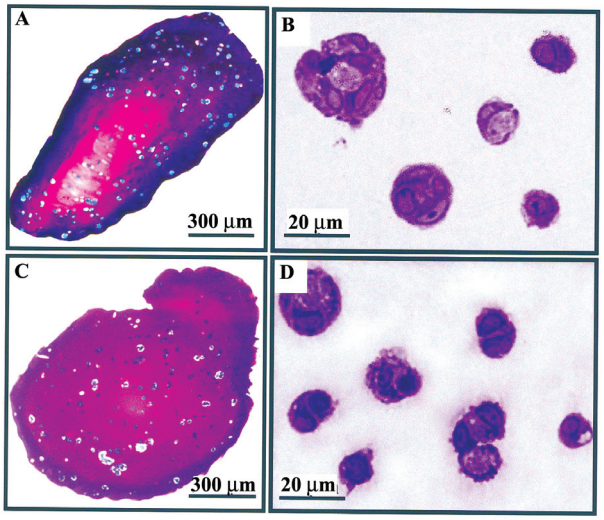

Figure 2. Toluidine blue staining of the sections prepared from the alginate beads. A: a representative image of 7 micrometer sections of whole alginate bead prepared from costal chondrocytes. B: the central region of the same section in higher magnification. C: a representative image of 7 micrometer sections of whole alginate bead prepared from articular chondrocytes. B: the central region of the same section in higher magnification. In sections, cells can be observed within the lacuna-like cavities of alginate as either individual cells or as isogenic groups. The purple stain is indicative of the metachromatic properties of matrix deposited among the cells.

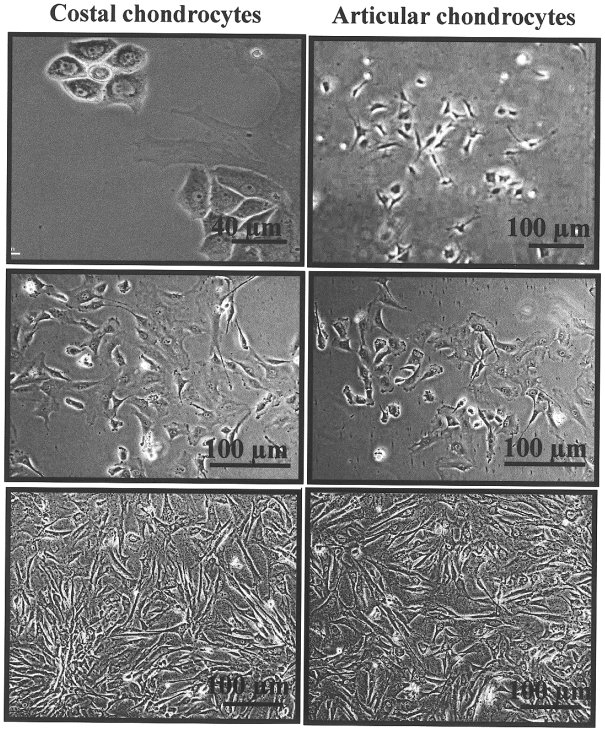

Figure 1. Monolayer culture of chondrocytes. A: Costal chondrocytes possessed round to polygonal morphology at primary culture. B: Articular chondrocytes had polygonal to elongated morphology at primary culture. Cells assumed elongated morphology at passage 3 (C and D) and passage 5 (E and F).

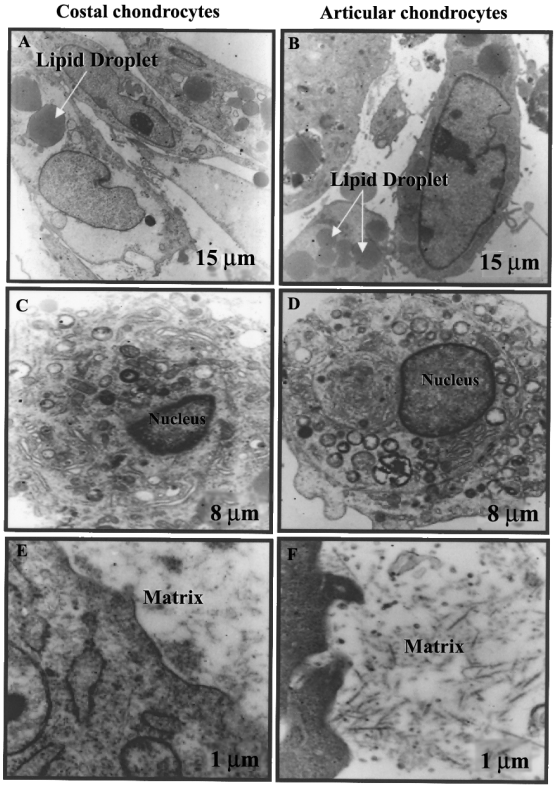

Figure 3. Transmission electron microscopic images of the cultivated cells. A-B: Costal and articular chondrocytes had euchromatin nuclei surrounded with cytoplasm containing lipid droplets. C-D: Secretory organelles, including well developed RER, GC, and secretory vacuoles were abundant in the cytoplasm of both cells. E-F: Deposited matrix around articular chondrocytes was fibrillar compared to the homogeneous matrix of costal chondrocytes.

elongated cells, were found in some regions, indicating clonal proliferation within the alginate constructs. Toluidine blue staining indicated a metachromatic matrix near chondrocytes within the alginate gel for both cell sources (Fig. 2). Collectively, there were no observable morphology differences between chondrocytes from the two cell sources for 3D cultured cells.

TEM images indicated that chondrocytes from both sources possessed an indented euchromatic nucleus surrounded by an organelle-rich cytoplasm (Fig. 3 A, B). Cytoplasmic organelles included lipid droplets, well developed rough endoplasmic reticulum (RER), Golgi complex (GC) and secretory vacuoles (Fig. 3 C, D). These morphologic features collectively imply that the cells were actively engaged in matrix synthesis and deposition. The only apparent difference identified between costal and articular chondrocytes was the appearance of the deposited matrix, which was slightly fibrillar among articular chondrocytes and somewhat homogenous among the costal chondrocytes (Fig. 3 E, F).

Proliferation: The MTT assays indicated that there were no significant proliferation differences between chondrocyte sources in the monolayer culture up to day 3 (Fig. 4 A). After day 4, costal chondrocytes tended to be more proliferative than their articular counterparts ($p < 0.05$). Regarding the 3D cultures, costal chondrocytes proliferated at a slightly more rapid rate than articular chondrocytes. The difference was statistically significant on day 20 ($p < 0.05$), but on the subsequent days, there were no statistic differences between the two cell types. By day 40, both cell types proliferated to twice their initial population, with proliferation appearing to become slower thereafter (Fig. 4 B).

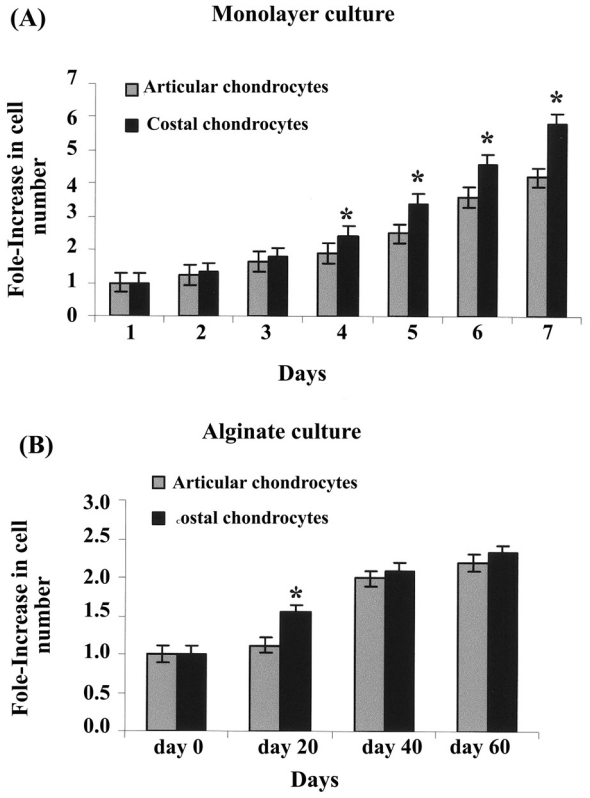

Figure 4. Proliferation rates of costal and articular chondrocytes. In monolayer cultures A: proliferation rates were similar up to day 3, after which costal cultures proliferated significantly more rapidly (upper graph, * indicates p<0.05). In 3D culture, B: although the difference on day 20 was significant (* indicates p < 0.05) on the subsequent days, there were no significant differences between the two cell types. Within the alginate, cells grew to twice the seeding number by day 40, after which the number remained almost unchanged.

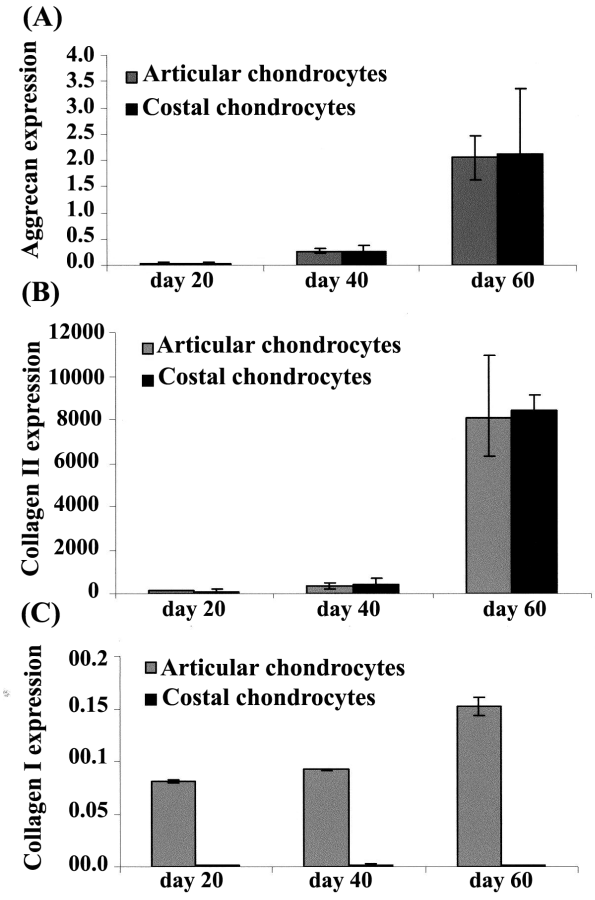

Figure 5. Real-time PCR data of costal and articular chondrocyte cultures. A: Expression levels of aggrecan were similar. B: Collagen II followed a pattern similar to aggrecan, with no differences identified. C: With collagen I expression, articular chondrocytes expressed some levels of this gene, while costal cultures failed to express collagen I during the entire culture period.

Real-time PCR: The level of aggrecan mRNA production by costal chondrocyte cultures in 3D alginate scaffolds was comparable to that of articular chondrocyte cultures; both cultures demonstrated negligible production during the initial 20 days, followed by a slight increase after 40 days and a significant increase when approaching the termination of culturing at day 60 (Fig. 5 A). Type II collagen expression followed a similar pattern during the culture period (Fig. 5 B). In contrast, articular chondrocyte cultures demonstrated increasing type I collagen expression (Fig. 5 C) throughout the culture period, while costal cultures had negligible expression at each time point ($p < 0.05$).

DISCUSSION

In the present study, cartilage constructs fabricated using costal chondrocytes cultured on 3D alginate scaffolds were compared to similar constructs substituted with articular chondrocytes. Costal chondrocytes possessed morphological features, proliferation capacity and expression levels of genes typical of hyaline cartilage that were comparable to articular chondrocytes. Despite the fact that costal chondrocytes failed to produce type I collagen mRNA, which is produced at minimal levels in the hyaline cartilage, costal chondrocytes were able to propagate more rapidly than articular chondrocytes in the monolayer culture.

Ultra-structural characterizations in the present investigation revealed that while alginate culture of both chondrocyte types were similar in terms of fine cell structures, some differences were identified in the deposited matrix. The matrix deposited among articular chondrocytes had a fibrillar appearance, compared to a homogeneous matrix accumulated among costal chondrocytes. This corresponded with real-time PCR results, which indicated differences in the expression of type I collagen in articular chondrocyte cultures.

Currently, there are few reports on the cartilage regeneration capacity of costal cartilage *in vivo*. Sceparowicz *et al*. (2006) compared costal and articular chondrocyte transplantations in treating cartilage defects in rats. They concluded that both chondrocyte sources produced hyaline-like repair tissue, with no differences identified. Other studies have indicated the effective repair capabilities of either costal chondrocyte or osteochondral based grafts *in vivo* (Mori *et al*., 2003; Popko *et al*., 2003; Sato *et al*., 2003).

Data from this study demonstrated some advantages in using costal over articular chondrocytes for cartilage construct fabrication. In this regard, the only comprehensive study that has been conducted (Isogai *et al*., 2006) has compared chondrocytes from different cartilaginous sites in a bovine model including costal, auricular, nasoseptal and articular chondrocytes. Cells were cultivated on a copolymer containing poly-L-lactic acid (PLLA) and poly-caprolactone (PCL), followed by their subcutaneous implantation in athymic mice for up to 20 weeks. They concluded that chondrocytes obtained from different sites may elicit distinct responses during their respective tissue-engineered neocartilage development. Furthermore, the highest expression of type II collagen mRNA was observed in costal chondrocyte cultures, in agreement with results of this study. The important point of this research was that the negligible expression of type I collagen in costal chondrocyte culture was also demonstrated, which adds further weight to the potential value of costal chondrocytes as a cellular material for tissue-engineered cartilage.

In a study by van Susante *et al*. (1995), where an alginate gel had been used as a carrier for articular chondrocytes, it was concluded that the number of chondrocytes diminishes as the time in culture increases. Mierisch *et al*. (2003) has reported similar findings following an *in vivo* study, where rabbit articular chondrocytes were cultivated in alginate and implanted in osteochondral defects in rabbit's knees. Examination of the repair processes indicates that cell density diminishes at the defect site. While this study was performed *in vitro*, the present findings suggest positive implications on the proliferation of chondrocytes from both sources when scaffolded using the alginate gel. Gagne *et al*. (2000) has similarly found that human articular chondrocytes cultivated in alginate undergo significant proliferation, depending on the initial cell seeding (Gagne *et al*., 2000). These discrepancies could stem from comparing results obtained from different species, different chondrocyte sources and different experimental designs.

According to the proliferation and gene expression patterns in the alginate culture of the present study, the cultivation period could be subdivided into the expansion phase (days 0-40) during which proliferation appears to be predominant and differentiation phase (days 40-60) during which proliferation slows down;

instead, the cartilage-specific collagen II and aggrecan genes were upregulated. Previous investigation has shown that proliferation and differentiation are two contrasting phenomena; the induction of differentiation typically is associated with the progressive loss of proliferative potential that leads to terminal differentiation (Potten and Lajtha, 1982). In the culture conditions of this study, the expression of aggrecan and collagen II genes were very high on day 60 as compared to those measured on days 20 and 40. This has occurred because by the end of culture period, the cells cultivated in alginate have received inductive influences from the alginate matrix enough to express the differentiated phenotype and slow down the proliferative activity. The other point in the results of this study that needs to be explained is the issue of the relatively high expression level of collagen I in the alginate cultures of articular versus costal chondrocytes. The reason is not clear and this point needs further investigation.

In summary, costal chondrocytes appear to provide a suitable source of harvestable cellular material for subsequent use in cartilage tissue engineering. Since chondrocyte monolayer multiplication is a necessary step prior to 3D cultivation due to the limited number of cells harvested in the hyaline cartilage, costal cells may provide certain advantages in future applications. Given that the similarities to articular chondrocytes have now been identified, additional investigation into the utility of costal chondrocytes as a harvesting source for tissue engineering applications is certainly warranted.

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References

Arøen A, Løken S, Heir S, Alvik E, Ekeland A, Granlund OG, Engebretsen L (2004). Articular cartilage lesions in 993 consecutive knee arthroscopies. *Am J Sports Med*. 32: 211-215.

Benya PD, Padilla SR, Nimni ME (1978). Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* 15: 1313-1321.

Brittberg M (1999). Autologous chondrocyte transplantation. *Clin Orthop Relat Res*. 367: 147-155.

Brittberg M, Nilsson A, Lindahl A, Ohlsson C, Peterson L (1996). Rabbit articular cartilage defects treated with autologous cultured chondrocytes. *Clin Orthop Relat Res*. 326: 270-283.

Brittberg M, Peterson L, Sjögren-Jansson E, Tallheden T, Lindahl A (2003). Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. *Bone Joint Surg Am*. 3: 109-3115.

Buján J, Pascual G, Corrales C, Gómez-Gil V, Rodríguez M, Bellón JM (2005). Muscle-derived stem cells in tissue engineering: defining cell properties suitable for construct design. *Histol Histopathol*. 20: 891-899.

Butler CE, Orgill DP(2005). Simultaneous in vivo regeneration of neodermis, epidermis, and basement membrane. *Adv Biochem Eng Biotechnol*. 94: 23-41.

Cancedda R, Dozin B, Giannoni P, Quarto R (2003).Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol*. 22: 81-91.

Cao Y, Vacanti JP, Paige KT, Upton J, Vacanti CA (1997).Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. *Plast Reconst Surg*. 100: 297-302.

Cohen S, Baño MC, Cima LG, Allcock HR, Vacanti JP, Vacanti CA, Langer R (1993). Design of synthetic polymeric structures for cell transplantation and tissue engineering. *Clin Mater*. 13: 3-10.

DeHaven KE, Arnoczky SP (1994). Meniscus repair: basic science, indications for repair, and open repair. *Instr Course Lect*. 43: 65-76.

Dozin B, Malpeli M, Camardella L, Cancedda R, Pietrangelo A (2002). Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: olecular and cellular aspects. *Matrix Biol*. 21: 449-459.

Gagne TA, Chappell-Afonso K, Johnson JL, McPherson JM, Oldham CA, Tubo RA, Vaccaro C, Vasios GW(2000). Enhanced proliferation and differentiation of human articular chondrocytes when seeded at low cell densities in alginate *in vitro*. *J Orthop Res*. 18: 882-890.

Glowacki J (2000). In vitro engineering of cartilage. *J Rehabil Res Dev*. 37: 171-177.

Isogai N, Kusuhara H, Ikada Y, Ohtani H, Jacquet R, Hillyer J, Lowder E, Landis WJ (2006). Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. *Tissue Eng*. 12: 691-703.

LeBaron RG, Athanasiou KA (2000). Ex vivo synthesis of articular cartilage. *Biomaterials* 21: 2575-2587.

Lu SH, Sacks MS, Chung SY, Gloeckner DC, Pruchnic R, Huard J, de Groat WC, Chancellor MB (2005). Biaxial mechanical properties of muscle-derived cell seeded small intestinal submucosa for bladder wall reconstitution. *Biomater*. 26: 443-449.

Mandl EW, Van der Veen SW, Verhaar JA, van Osch GJ (2004). Multiplication of human chondrocytes with low seeding densities accelerates cell yield without losing redifferentiation capacity. *Tissue Eng*. 10: 109-118.

Mierisch CM, Wilson HA, Turner MA, Milbrandt TA, Berthoux L, Hammarskjöld ML, Rekosh D, Balian G, Diduch DR (2003). Chondrocyte transplantation into articular cartilage defects with use of calcium alginate: the fate of the cells. *J Bone Joint Surg Am*. 85: 1757-1767.

Mori R, Kataoka H, Kuriwaka M (2003). Articular cartilage restoration with costal cartilage previously fused with bone. Ochi M. *Clin Orthop Relat Res*. 406: 262-274.

Patrick CW (2004). Breast tissue engineering. *Annu Rev Biomed Eng*. 6: 109-1030.

Popko J, Szeparowicz P, Sawicki B, Wołczyński S, Wojnar J (2003). Rabbit articular cartilage defects treated with cultured costal chondrocytes (preliminary report). *Folia Morphol* . 62: 107-112.

Potten CS, Lajtha LG (1982). Stem cells versus stem lines. *Ann N Y Acad Sci*. 397: 49-61.

Qin TW, Yang ZM, Wu ZZ, Xie HQ, Qin J, Cai SX (2005). Adhesion strength of human tenocytes to extracellular matrix component-modified poly(DL-lactide-co-glycolide) substrates. *Biomater*. 26: 6635-6642.

Sahoo SK, Panda AK, Labhasetwar V (2005).Characterization of porous PLGA/PLA microparticles as a scaffold for three dimensional growths of breast cancer cells. *Biomacromol*. 6: 1132-1139.

Sato K, Mio F, Hosoya T, Ito Y (2003). Two cases with osteochondritis dissecans of the capitulum humeri treated with costal osteochondral graft transplantation. *J Shoulder Elbow Surg*. 12: 403-407.

Shin'oka T, Matsumura G, Hibino N, Naito Y, Watanabe M, Konuma T, Sakamoto T, Nagatsu M, Kurosawa H (2005). Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. *J Thorac Cardiovasc Surg*. 129: 1330-1338.

Sodian R, Fu P, Lueders C, Szymanski D, Fritsche C, Gutberlet M, Hoerstrup SP, Hausmann H, Lueth T, Hetzer R (2005).Tissue engineering of vascular conduits: fabrication of custom-made scaffolds using rapid prototyping techniques. *Thorac Cardiovasc Surg*. 53: 144-149.

Szeparowicz P, Popko J, Sawicki B, Wołczyński S (2006). Is the repair of articular cartilage lesion by costal chondrocyte transplantation donor age-dependent? An experimental study in rabbits. *Folia Histochem Cytobiol*. 44: 201-206.

Tare RS, Howard D, Pound JC, Roach HI, Oreffo RO (2005). Tissue engineering strategies for cartilage generation-micromass and three dimensional cultures using human chondrocytes and a continuous cell line. *Biochem Biophys Res Commun*. 333: 609-621.

Turhani D, Cvikl B, Watzinger E, Weissenböck M, Yerit K, Thurnher D, Lauer G, Ewers R (2005). In vitro growth and differentiation of osteoblast-like cells on hydroxyapatite ceramic granule calcified from red algae. *J Oral Maxillofac Surg*. 63: 793-799.

Vacanti CA, Langer R, Schloo B, Vacanti JP (1991). Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. *Plast Reconstr Surg*. 88: 753-759.

van Susante JL, Buma P, van Osch GJ, Versleyen D, van der Kraan PM, van der Berg WB, Homminga GN (1995). Culture of chondrocytes in alginate and collagen carrier gels. *Acta Orthop Scand*. 66: 549-56.