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Original article

Study of Human Chondrocyte Redifferntiation Capacity in Three-Dimensional Hydrogel Culture

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Abstract

Objective(s)
Articular cartilage tissue defects cannot be repaired by the proliferation of resident chondrocytes. Autologous chondrocyte transplantation (ACT) is a relatively new therapeutic approach to cover full thickness articular cartilage defects by *in vitro* grown chondrocytes from the joint of a patient. Therefore, we investigated the redifferentiation capability of human chondrocytes maintained in alginate culture.
Materials and Methods
The cartilage specimens obtained from 50 patients who underwent total knee and hip operations at the teaching hospital of Isfahan University of Medical Sciences, Isfahan Iran. Isolated primary chondrocytes were first grown in monolayer cultures for 1 to 6 passages (each passage lasting about 3 days). At each passage, monolayer cells seeded in alginate culture and investigated morphologically and immuno-cytologically for expression of cartilage-specific markers (collagen type II and cartilage-specific proteoglycans).
Results
The chondrocytes from monolayer passages P1 to P4 introduced in alginate cultures regained a chondrocyte phenotype. Cells were interconnected by typical gap junctions and after few days, they produced a cartilage-specific extracellular matrix (collagen type II and cartilage-specific proteoglycans). In contrast, cells from monolayer passages P5 and P6 did not redifferentiate to chondrocytes in the alginate cultures.
Conclusion
Chondrocyte culture was established for the first time in Iran. The alginate culture conditions promote the redifferentiation of dedifferentiated chondrocytes that have still a chondrogenic potential. This procedure opens up a promising approach to produce sufficient numbers of differentiated chondrocytes for ACT. Indeed, in some patients the harvested cells were used immediately and successfully for transplantation.

Keywords: Alginate, Autologous, Chondrocytes, Collagen Type II, Proteoglycan, Transplantation

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Introducton

The gain of human chondrocytes for autologous chondrocytes transplantation (ACT) and cartilage tissue repair presents a major problem as these cells dedifferentiate rapidly to fibroblast-like cells during expansion in monolayer cultures (1-4). Dedifferentiated chondrocytes in monolayer cultures change their morphology as well as their programme of extracellular matrix synthesis (2, 5-7). They down regulate the synthesis of cartilage-specific markers such as collagen type II and cartilage-specific proteoglycans (CSPG) and undergo changes in their cell surface receptors (2). After extended monolayer culture, dedifferentiated chondrocytes proliferate but appear to lose their chondrogenic potential irreversibly (7-9). ACT is a proven method for the treatment of cartilage defects through the regeneration of hyaline-like cartilage. The cartilage formed by implanted autologous chondrocytes, has biomechanical properties similar to those of natural articular cartilage (10, 11). During arthroscopic examination the surgeon should ideally take a biopsy from the non-weight-bearing area of the proximal part of the medial or lateral condyle. Using a small, straight, concave chisel, a bone chip roughly 8-10 mm long and extending down to the subchondral bone plate is chiselled out, gripped with toothed forceps and separated by a twisting movement. The biopsy should contain roughly 200-300 mg of cartilage. The biopsy material is immediately placed in the nutrient medium tube. ACT offers an effective treatment for clinically significant and symptomatic cartilage defects involving the medial and lateral condyles of the femur and also, the trochlea and patella (10). So as to give the implanted chondrocytes the stimulus to adapt themselves to their natural function, continuous passive motion is recommended at the earliest time point possible after surgery. Recent studies have shown that dedifferentiated human chondrocytes are able to redifferentiate toward the chondrogenic lineage upon transfer into a 3-dimensional culture system (e.g., alginate or agarose) (12). However, after prolonged monolayer culturing, dedifferentiated chondrocytes irreversibly lose their chondrogenic potential (9, 13, 14). The transplantation of irreversibly dedifferentiated autologous chondrocytes may lead to cartilage degeneration and joint failure. Alginate has been used for the investigation of factors that may influence the capacity of chondrocytes to redifferentiate (9, 15).

Alginate is a linear polysaccharide consisting of β-D-mannuronic acid and α-L-guluronic acid. It polymerizes and forms a gel in the presence of divalent cations, especially Ca^{2+}. The alginate culture system offers all the advantages known of the cultivation of chondrocytes in agarose culture (13, 16-20).

In this study, we investigated the morphological features of dedifferentiated chondrocytes, during redifferentiation in alginate cultures.

Materials and Methods

Materials

A polyclonal (AB746) and a monoclonal (MAB1330) antibodies against type-II collagen were purchased from Chemicon International, Inc. (Temecula, CA, USA). Growth medium [Ham's F-12/Dulbecco`s modified Eagle`s medium (50/50) containing fetal calf serum (10%), ascorbic acid (25 µg/ml), streptomycin (50 IU/ml), penicillin (50 IU/ml), essential amino acids (1%), glutamine (1%) and amphotericin B (2.5 µg/ml)] was obtained from Seromed (Munich, Germany). Alginate, collagenase, OCT embedding medium and trypsin/EDTA (EG 3.4.21.4) were purchased from Sigma (Munich, Germany), Epon was obtained from Plano (Marburg, Germany), pronase was purchased from Roche (Mannheim, Germany).

Methods

Chondrocyte cultures

Primary cultures of human chondrocytes were prepared from articular cartilage as previously described (21). The study was approved by the Scientific & Ethical Committee of Isfahan University of Medical Sciences (IUMS), Isfahan, Iran. The cartilage specimens obtained from 50 patients who underwent total knee and hip operations at the teaching

hospital of IUMS. The patient's age ranged from 25 to 70 years. Briefly, human femoral head articular cartilage was cut into small slices and incubated in Ham`s F-12 medium. Cartilage slices were digested first with 1% pronase (Roche, Mannheim, Germany) for 2 hr at 37 °C and subsequently with 0.2% (v/v) collagenase for 4 hr at 37 °C. Cells, diluted in growth medium, were distributed by repeated pipetting. These cells cultured in monolayers for 1-6 passages. Cells (2×10^6/ml) of each passage introduced into alginate beads as described in detail elsewhere (3). The redifferentiation of chondrocytes was assessed by electron microscopy. The analysis repeated three times for each passage. The cells investigated after 1, 4, 6, 8, 11, and 15 days. All of the media were purchased from Seromed Munich, Germany, unless otherwise stated.

Alcian blue staining

The alginate beads were fixed in with 3.7% formaldehyde and stained by 0.05% alcian Blue (in 3% acetic acid (pH 1.5) containing 0.3 M $MgCl_2$) for 24 hr (22). They were then washed in 5% acetic acid and gradually dehydrated in ethanol. The samples investigated under a light microscope.

Alkaline phosphatase/anti-alkaline phosphatase (APAAP) method

The alginate beads were immersed in OCT embedding medium and immediately frozen in liquid nitrogen. Glass plates bearing 10-µm-thick sections through alginate beads fixed with ethanol (10 min) and washed in PBS. The slides incubated first with serum (diluted 1:20), for 10 min, and then with the primary antibodies (diluted 1:30) (Chemicon, Temecula, CA, USA), in a moist chamber overnight at 4 °C. After rinsing, cells incubated first with the polyclonal primary antibody and then with mouse anti-rabbit IgG antibodies (diluted 1:50) (Chemicon,, Temecula, CA, USA) for 30 min. Cells were again rinsed before incubation with the dual-system bridge antibodies (diluted 1:50). Following rinsing, the sections incubated with the dual-system APAAP complex (diluted 1:50) for 30 min. The cells washed, air dried and evaluated under a light microscope (23).

Transmission electron microscopy (TEM)

After fixation in 1% glutaraldehyde (in 0.1 M phosphate buffer) and post-fixation in 1% OsO_4 solution (in 0.1 M phosphate buffer), the cell cultures were rinsed and dehydrated in an ascending alcohol series (2) and afterwards embedded in Epon. Sections prepared using a Reichert Ultracut and staind with 2% uranyl acetate/ 2% lead citrate, prior to examination under a TEM 10 transmission electron microscope (Zeiss, Jena, Germany).

Results

Light microscopy

Cell culture

The monolayer culture of the primary isolated chondrocytes (Figure 1) was passaged every three days up to passage P6. Cells of each monolayer passage were introduced into alginate culture. The cells in monolayer culture consisted of morphologically heterogeneous small fibroblast-like cells that had a polymorphic shape, varying from round or bipolar, to reticular or star-shaped (Figure 1).

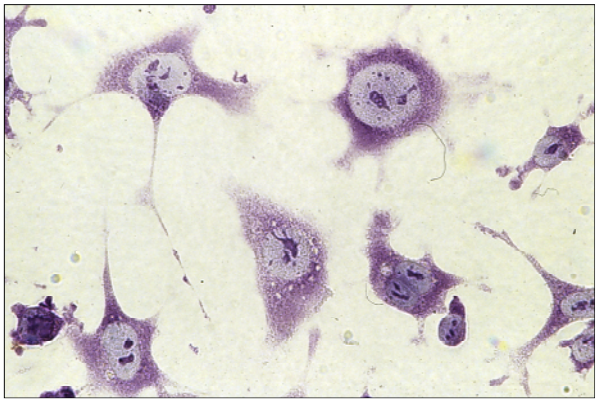

Figure 1. Light micrograph of second-passage chondrocytes grown in monolayer culture and stained with methylene blue. The cells increasingly assumed a polymorphic shape. Magnification: ×320.

Alkaline phosphatase/anti-alkaline phosphatase (APAAP) method

Cells from monolayer passages P1 to P6 were recultivated for 1, 3, 4, 5, 7 and 9 days in alginate culture and immunolabelled with anti-collagen type II (Figure 2A-F) antibodies, using the APAAP method. On the day seven,

collagen type II labelling was clearly positive in alginate cultures from P1 to P4 (Figure 2A-D). Cells from monolayer passages P5 and P6 remained collagen type II-negative for the entire period in alginate culture (Figure 2E-F).

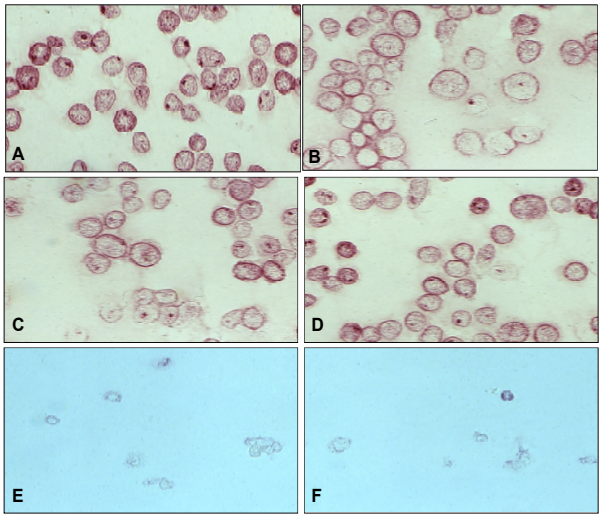

Figure 2A-F. Immunolabelling of chondrocytes (P1-P6) for type-II collagen according to the APAAP method. Immunolabelling was clearly observed around cells derived from passages P1-P4 (A-D) and was not detected around cells derived from passages P5 (E) and P6 (F). Magnification: ×160.

Transmission electron microscopy (TEM)

Cells from P1 to P6 monolayer cultures were recultivated in the alginate beads and their morphology was evaluated under the electron microscope. After 4 days in alginate, dedifferentiated cells derived from P1 to P4 had developed into chondrocytes. Typically, these cells had a rounded profile and contained a well-developed rough endoplasmic reticulum, a large Golgi apparatus, the usual complements of mitochondria, small vacuoles and granules. On the day 7, cells had formed a matrix, which was closely attached to the plasma membrane. The cartilaginous matrix consisted of fine collagen fibrils running singly and irregularly (Figure 3). Dedifferentiated cells derived from passages P5 and P6 did not redifferentiate into chondrocytes, when recultivated in alginate. These cultures showed mainly cell debris, dying cells and few mesenchymal or fibroblast-like cells (not shown).

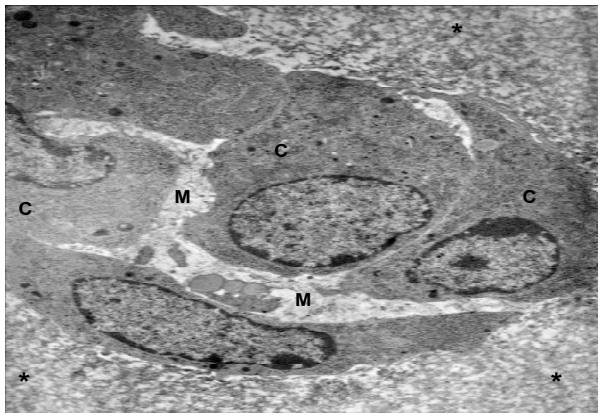

Figure 3. Electron micrograph of chondrocytes (C) that were derived from passage P2 and recultivated for 7 days in alginate (*). Chondrocytes derived from passage P2 have a typically round or oval profile and are surrounded by a matrix sheath (M) consisting of irregularly arranged fibrils. These redifferentiated chondrocytes contain a well-developed rough endoplasmic reticulum, a Golgi apparatus, the usual complement of mitochondria, vacuoles and granules. Magnification: × 5000.

Discussion

In the present study, human primary isolated chondrocytes were cultured and passaged up to six times (P1 to P6). When the dedifferentiated, fibroblast-like cells from these monolayer cultures were recultivated in alginate, only those derived from passages P1to P4 redifferentiated into chondrocytes. They alone synthesized type-II collagen and CSPG in alginate cultures. Cells derived from passages P5 and P6 could not redifferentiate and survive in alginate cultures.

The main aim of this study was to establish a chondrocyte culture system *in vitro* for the future human chondrocyte transplantation. Chondral or osteochondral defects are joint lesions that can cause pain and functional disability in young individuals, with a potential progression to early osteoarthritic changes (24). Because of the low capacity of intrinsic repair, several methods for inducing healing of the cartilage injuries have been proposed (25, 26).

The success of autologous cartilage transplantation (ACT) correlates with the capacity of implanted chondrocytes to produce a specific hyaline matrix containing collagen type II and cartilage-specific proteoglycans

(CSPG) (27). However, the main problem associated with ACT is that, during *in vitro* proliferation in monolayer culture chondrocytes rapidly dedifferentiate (2, 6, 28-30). Dedifferentiated chondrocytes develop a fibroblast-like appearance and produce matrix components normally not found in cartilage such as collagen type I (7, 31). Differentiated chondrocytes synthesize a cartilage specific pericellular matrix which consists primarily of type-II collagen and cartilage- specific proteoglycans. Interactions between chondrocytes and their surrounding matrix play a major role in maintaining the differentiated cell phenotype (6, 14, 20, 32). The results of monolayer chondrocyte culture and chondrocyte alginate bead culture showed that the production of proteoglycans, from passages 1 to 4 gradually increases, while from passages 5 to 6, the amount of these substances was tapering down (Data not shown). Therefore, indicating that the best passages for ACT are passages 2 to 4. Indeed, it has been described that the loss of chondrogenic potential in chondrocytes is from frequently passaged monolayer cultures (7). Our results are consistent with those by Schultze-Tanzil and coworker (8, 9) and they could show that the inhibition of key signalling proteins in the MAP kinase pathway, could lead to apoptosis and to the irreversible dedifferentiation of chondrocytes. Various authors showed that chondrocytes in alginate or agarose, synthesize a mechanically functional matrix (20, 33) similar to native articular cartilage (16, 17, 34). Previous studies suggest that alginate beads can be directly or indirectly used for repairing cartilage defects. Furthermore, reports suggest that alginate gives only minimal inflammatory reaction after *in vivo* implantation (35) and might be used for human application (36). It especially facilitates chondrocyte transplantation through preventing floating out of the cells during transplantation (10, 37). Dedifferentiated cells from monolayer passages P5 and P6 were not able to redifferentiate in alginate cultures. Loss of chondrogenic potential in chondrocytes from frequently passaged monolayer cultures has also been described by other others (7, 9, 13).

Conclusion

In conclusion, alginate cultures are a suitable *in vitro* system, like other three-dimensional culture systems to stimulate chondrocytes redifferentiation. In alginate cultures redifferentiated chondrocytes remain available in a differentiated state for days and can be used for autologous cartilage transplantation.

Acknowledgments

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