Chondrocyte proliferation in a new culture system

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Abstract

Objective: This study has aimed to study different culture systems that might stimulate an increase in cell proliferation of normal and osteoarthritis chondrocytes from articular cartilage in rat model.

Material and Methods: Three culture systems using chondrocytes embedded in alginate beads were tested: chondrocytes cultured in Dulbecco's modified Eagle's medium (DMEM) as control, a co-culture system consisting of a monolayer of de-differentiated chondrocytes as a source of mitotic factors, and an enriched medium containing culture medium obtained from a monolayer of chondrocytes and DMEM. Normal and osteoarthritis chondrocytes were stained with 5-carboxyfluorescein diacetate succinimidyl ester and were cultured in each of the three systems. After 5 days of culture cell, proliferation was detected by flow cytometry. Chondrocyte phenotype was confirmed by collagen type II and MMP-3 expression. To determine possible molecules released into the medium by the cultured chondrocyte monolayer and which would probably be involved in cell proliferation, a study of mRNA and expression of transforming growth factor- β 1 (TGF- β 1), fibroblastic growth factor-2 (FGF-2), epidermal growth factor (EGF), platelet derived growth factor-A (PDGF-A) and insulin-like growth factor-1 (IGF-1) proteins was conducted.

Results and Conclusions: Chondrocytes in the co-culture system or in enriched medium showed an increase in proliferation; only when osteoarthritis chondrocytes were cultured in enriched medium

would they display a statistically significant increase in their proliferation rate and in their viability. When chondrocytes from the monolayer were analysed, differential mRNA expression of TGF- β 1 and IGF-1 was found during all passages, which suggests that these two growth factors might be involved in chondrocyte proliferation.

Introduction

Osteoarthritis is a disease of joints in which articular cartilage undergoes dramatic changes due to chondrocyte death and matrix degradation, leading to tissue breakdown. Adult articular cartilage shows a limited capacity to respond to injury and low potential for self-repair, resulting in the presence of fibrocartilaginous tissue that lacks structural and biomechanical properties of hyaline cartilage (1,2). Many strategies to address this problem have been proposed and tested, and one of the most explored since the 1960s is the use of cultured chondrocytes as a source of cells for transplantation, as a mean to restore articular tissue (3). However, chondrocytes are known to de-differentiate and adopt a fibroblast phenotype when cultured in monolayer (4–7). Different culture systems have been tested to mimic the microenvironment of chondrocytes inside their natural cartilage matrix, such as agarose gels (8–10), collagen gels (11–13) and the three-dimensional system using calcium alginate beads (14–16). The latter has proven effective in maintaining the chondrocyte phenotype (17), furthermore, this system offers the advantage of facilitating cell recovery by using calcium-chelating agents to dissolve the alginate beads, when necessary. Recently, Lee *et al.* reported a method to improve and maintain proliferation potential and phenotype of chondrocytes cultured directly within alginate beads (18).

All these studies show that articular chondrocyte proliferation can be stimulated by using the appropriate culture matrix. On the other hand, the importance of culture medium growth factors in stimulating chondrocyte

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proliferation is well known. Several studies have shown that addition of growth factors, such as fibroblast growth factor (FGF-2), transforming growth factor beta1 (TGF- β 1), and insulin-like growth factor-1 (IGF-1) to the culture medium, alone or in different combinations, can induce chondrocyte proliferation and/or differentiation and extracellular matrix synthesis (19–27). Additionally, exogenous bone morphogenetic protein-6 (BMP-6) in growth-plate chondrocytes induces progressive maturation and it is suggested that BMP-6 is an autocrine stimulator of chondrocyte differentiation (28). Yet, it has been reported that administration of increasing concentrations of TGF- β 1 to monolayer articular chondrocytes stimulate DNA synthesis, and when IGF-1 is added in combination with TGF- β 1, there is synergistic action, suggesting that both factors have autocrine/paracrine effects on cultured chondrocytes (28–31). We speculate that de-differentiated chondrocytes, under these conditions, might secrete certain growth factors that could enhance cell proliferation in chondrocytes encapsulated in alginate beads. Consequently, we tested a culture system consisting of normal or osteoarthritic chondrocytes encapsulated in alginate beads together with a monolayer of de-differentiated chondrocytes as a potential source of mitotic factors. Cell division was assessed in both normal and osteoarthritic chondrocytes using flow cytometry; furthermore, chondrocyte phenotype was determined by collagen type II and MMP-3 expression. The results show a significant increase in normal chondrocyte proliferation within co-cultured and enriched media, whereas in osteoarthritis chondrocytes, only the enriched medium-induced proliferation. These results suggest that chondrocytes cultured in monolayers release different types of factors into the culture medium that stimulate cell proliferation; therefore, the analysis of growth factors that participate in proliferation of chondrogenic cells was considered to be important. Evaluation of mRNA and protein expression of the growth factors IGF-1, EGF, PDGF-A, FGF-2 and TGF- β 1, in cells obtained from monolayers of de-differentiated chondrocytes after several passages, showed differential expression of these factors, particularly TGF- β 1 and IGF-1, which displayed a dramatic change in their mRNA expression.

Materials and methods

Animal model

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Center for Research and Advanced Studies (CINVESTAV) and was conducted in accordance with National Research Guidelines (NOM-062-ZOO-1999). Male Wistar rats

weighing approximately 130–150 g were used in this study. The experimental osteoarthritis model was induced by partial meniscectomy (32); surgery was carried out under general anaesthesia.

Normal and osteoarthritis chondrocyte isolation

Chondrocytes were isolated from normal and osteoarthritis articular cartilage from the knees of male Wistar rats. Cartilage was removed from animals that were subsequently sacrificed using overdose of anaesthesia. Articular cartilage was digested for 10 min in 0.02% bovine trypsin (Research Organics, Cleveland, OH, USA), as reported by Srivastava *et al.* (33), in phosphate-buffered saline/glucose (PBS/glucose: glucose 9.99 mM, KCl 2.95 mM, NaCl 130 mM and Na₂HPO₄ 5.25 mM, in MilliQ water, pH 7.2–7.4, and supplemented with 50 μ g/ml gentamicin), in the dark and was continuously shaken, at room temperature. Next, the cartilage was treated for 2 h in 0.02% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and in PBS/glucose at 37 °C as reported by Van Susante *et al.* (34), also in the dark and shaken. The cells obtained after digestion were filtered through a 50- μ m nylon mesh (PGC Scientific, Gaithersburg, MD, USA), and washed twice with PBS/glucose containing 1 mM ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich, St Louis, MO, USA), pH 7.2–7.4. The cell pellet was resuspended in culture medium – Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corporation, Grand Island, NY, USA) – containing 1 mM EGTA (pH 7.2–7.4), and viability was assessed using the vital dye exclusion test with trypan blue.

For chondrocyte natural fluorescence assessment, an aliquot of isolated chondrocytes was fixed in 4% paraformaldehyde (Electron Microscopy Science, Fort Washington, PA, USA) in PBS supplemented with 5% foetal bovine serum (FBS), for 2 h, washed twice and resuspended in 200 μ l of PBS/FBS 5%, then analysed by flow cytometry (Becton Dickinson FACSCalibur, San Jose, CA, USA).

Chondrocytes cultured in monolayer

In all experiments, only normal isolated chondrocytes were used to obtain the monolayer culture. Cell suspension was adjusted to 3.5×10^4 cells/ml and 1 ml was seeded in a culture flask (Cell Culture Flask, angle neck, Nalgene International, Rochester, NY, USA) at concentration of approximately 1400 cells/cm², then 10 ml of DMEM supplemented with 10% FBS were added and cultured at 37 °C/5% CO₂ to reach 40% confluence. Confluence observations were carried out using an inverted microscope (Olympus IM, $\times$ 20 objectives, Olympus, Tokyo, Japan).

Chondrocytes stained with CFSE

Chondrocytes were stained with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE), which binds irreversibly to cells' cytoplasmic proteins. When cells divide, CFSE labelling is distributed equally between daughter cells, which consequently display half the intensity of fluorescence; with this dye it is possible to identify 10 generations consecutively (35).

Normal and osteoarthritis isolated chondrocytes were resuspended in 1 ml of PBS/glucose/EGTA 1 mM, pH 7.2–7.4, and then incubated with 10 µl of CFSE, 0.5 mM (Molecular Probes, Eugene, OR, USA), in PBS/glucose for 10 min at 37 °C in a water bath and was slowly shaken every 2 min. Afterwards, cells were washed twice with culture medium DMEM/EGTA 1 mM supplemented with 10% FBS. An aliquot of freshly stained cells was analysed by flow cytometry, as previously described, to obtain the basal values of fluorescence intensity.

Chondrocytes encapsulated in alginate beads

The rest of normal and osteoarthritis chondrocytes stained with CFSE were encapsulated in calcium alginate as follows: cell suspension was adjusted to 2×10^6 cells/ml and 1 ml of this cell suspension was added to an equal volume of calcium alginate 2.4% (Sigma Chemical, St. Louis, MO, USA) in PBS/glucose, to provide a final concentration of 1×10^6 cells/ml in 1.2% alginate. The cell/alginate suspension was dropped through a 22 G needle on to a solution of CaCl₂ 102 mM (Research Organics), in NaCl 150 mM, and was incubated for 10 min at room temperature to induce cross-linking of the alginate gel. Chondrocytes encapsulated in alginate beads were washed twice in PBS/glucose and twice in DMEM.

Culture of chondrocytes encapsulated in alginate beads

Alginate beads containing normal and osteoarthritis chondrocytes were divided into six culture groups.

Normal chondrocytes

1. Control group: alginate beads were added to a culture flask that did not contain a chondrocyte monolayer, and were cultured in DMEM supplemented with 10% FBS.
2. Co-culture group: alginate beads were added to a culture flask containing a monolayer of de-differentiated chondrocytes, which had reached 40% confluence, and were cultured in DMEM supplemented with 10% FBS.
3. Enriched culture medium group: the alginate beads were cultured in enriched medium (5 ml DMEM, 4 ml of medium from the co-cultured chondrocytes and 1 ml of FBS).

Osteoarthritis chondrocytes

1. Control group: alginate beads were cultured in DMEM supplemented with 10% FBS only.
2. Co-culture group: alginate beads were co-cultured with the monolayer of de-differentiated chondrocytes in DMEM supplemented with 10% FBS.
3. Enriched culture medium group: alginate beads were cultured in enriched medium (5 ml DMEM, 4 ml from the co-cultured chondrocytes and 1 ml of FBS).

All groups were cultured for 5 days at 37 °C/5% CO₂. The culture medium was replaced every 24–48 h, as needed. All the experimental groups were repeated at least three times.

Proliferation of chondrocytes

After 5 days of culture, alginate beads of each group containing normal or osteoarthritis chondrocytes stained with CFSE were fixed in 4% paraformaldehyde in PBS for 2 h, washed twice in PBS/glucose, and then the chondrocytes were recovered by incubation of alginate beads in 55 mM sodium citrate, in PBS/glucose/EGTA 1 mM (pH 7.2–7.4) for 20 min, were washed twice and resuspended in PBS/glucose. Cell suspensions were centrifuged at 554 *g* for 10 min, resuspended in 200 µl PBS/EGTA 1 mM and analysed by flow cytometry for assessment of cell division.

Mitotic phases

Samples from chondrocyte cultures were placed on microscope slides using a cytospin (Cytospin 3, Shandon Lipshaw, Pittsburgh, PA, USA). The conventional haematoxylin and eosin technique was used for identification of the phases of mitosis.

Normal chondrocyte phenotype

Normal chondrocytes encapsulated in alginate beads were cultured for 14 days in order to allow synthesis of extracellular matrix within the beads and to be evaluated by Western blot analysis for collagen type II expression, following methodology previously described by Gomez-Camarillo & Kouri (36). Briefly, 25 µg of protein from alginate beads of co-culture and control cultures of normal chondrocytes was assessed using a whole rat cartilage as positive control and HeLa cells as negative control. Samples were loaded to each lane and electrophoresis was performed in 10% sodium dodecyl sulphate–polyacrylamide gel (SDS-PAGE), and then proteins were transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% skimmed dry milk in PBS/Tween 2% for 1 h at room temperature, then the membrane was incubated overnight at 4 °C with a monoclonal antibody against

collagen type II (1 : 200 in PBS/Tween 2%, Chemicon International Inc., Temecula, CA, USA). Immunoreactions were visualized after incubation for 1 h with horseradish peroxidase-labelled anti-mouse secondary antibody (1 : 3000 in PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA), using chemiluminescence of the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech, Buenos Aires, Argentina). As internal control, Western blot testing for actin expression was performed; a primary monoclonal antibody against actin (1 : 250 in PBS/2% Tween, Santa Cruz Biotechnology) was used for this purpose.

Osteoarthritis chondrocyte phenotype

Osteoarthritis chondrocytes were cultured in alginate beads for 7 days (an adequate time to detect extracellular matrix proteins by flow cytometry), and were evaluated for chondrocyte phenotype using immunodetection assessed by flow cytometry. Antibodies against $\alpha 1$ collagen type II subunit (1 : 250 in PBS, Chemicon International Inc.), and MMP-3 (1 : 300 in PBS, Santa Cruz Biotechnology) were used. Pre-incubation was performed with 0.2% immunoglobulin G-free bovine serum albumin (Sigma Chemical) in PBS/FBS 5% for 20 min at room temperature. Then, cells were washed with PBS/FBS 5% and were incubated for 30 min at room temperature with primary antibody, followed by Cy5-tagged anti-mouse secondary antibody (Collagen II) and fluorescein isothiocyanate-tagged anti-rabbit secondary antibody (MMP-3); incubation was accomplished for 30 min in the dark. Cells were then washed twice in PBS and assessed by flow cytometry. All procedures were performed at 4 °C.

Growth factors expression in chondrocyte monolayer culture

Monolayer-cultured chondrocytes from several serial passages, which reached 90% confluence, were washed twice in PBS and then centrifuged at 554 g for 5 min at 4 °C. Cell pellets were resuspended in 200 μ l of Radio Immuno Precipitation Assay (RIPA) buffer, containing a protease inhibitor cocktail, incubated on ice for 20 min, and total protein was quantified with modified Lowry protein assay reagent (37,38). Forty micrograms of protein from different serial passages of chondrocyte monolayer cultures were loaded in each lane; electrophoresis was performed using 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and detection by Western blot analysis was performed with a specific antibody for each growth factor in the following dilutions in PBS: IGF-1 (1 : 250), FGF-2 (1 : 250), PDGF-A (1 : 1000), TGF- β 1 (1 : 1000), EGF (1 : 250), all from Santa Cruz Biotechnology.

Real-time reverse transcriptase-polymerase chain reaction for growth factors mRNA in chondrocyte monolayer culture

Total RNAs of chondrocytes (passages 0–4) were extracted by TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. Validated polymerase chain reaction (PCR) primers were used for real-time mRNA expression analysis: IGF-1 (Cat. No. PPR06664A), FGF-2 (Cat. No. PPR06641A), PDGF-A (Cat. No. PPR06694A), TGF- β 1 (Cat. No. PPR06430A) and EGF (Cat. No. PPR43509A). These primers were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA), with a constitutive control: cyclophilin, its primers were 5'-ATGGTCAACCCACCGTGTT-3' and 5'-CGTGTGAAGTCACCACCC-3' (Gibco BRL). As negative control, real-time reverse transcriptase-PCR (RT-PCR) was performed in parallel without the RNA template. Components of the master mix were prepared on ice (0.25 μ l SYBR® Green One-Step enzyme mix InVitrogen, Carlsbad CA, USA, 6.25 μ l 2 $\times$ SYBR® Green Reaction Mix InVitrogen, Carlsbad CA, USA with ROX mix, 1 μ l forward and reverse primers, and 12.5 μ l of water); 220 ng of sample RNA were added to each tube/well to a total volume of 12.5 μ l. Reactions specimens were placed in a preheated thermal cycler as described below: 50 °C for 1-min hold, 95° for 5 min hold, 40 cycles of: 95 °C for 15 s, 55.9 °C (TGF- β 1, PDGF-A, EGF, cyclophilin) or 58.9 °C (IGF-1, FGF-2, cyclophilin) for 30 s, and final cycle of 95 °C, 15 s, 60 °C, 1 min, and 95 °C, 15 s. Data were collected and results analysed by 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Levels of mRNA were compared between different passages by normalization to cyclophilin, each condition was analysed in triplicate in the same session and standard deviations were calculated. Gene expression was quantified relatively using the $2^{-\Delta\Delta CT}$ method from ABI Prism 7700 Sequence Detection System.

Statistical analysis

Each experiment was repeated five times (for cell proliferation) and three times (in the case of phenotype determination); in addition, data were collected for each experiment using Flow Cytometry WinMDI program and were analysed applying Student's *t*-test.

Results

Monolayer cultures

After seeding, the chondrocytes became attached to the culture flask surface between 24 and 48 h. They adopted a

Figure 1. Comparative study of cell proliferation of normal chondrocytes cultured in different systems. Right panels show histograms obtained by flow cytometry where it is possible to observe the chondrocyte fluorescent intensity, in the left panels, graphic representation of flow cytometry data. The histograms were divided into seven zones (M1–M7): M1 maximum of fluorescence of chondrocytes after staining [zone where chondrocytes recently stained with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) were located], and M7 is the minimum of fluorescence. Each consecutive zone represents a 2-fold decrease in intensity of cell fluorescence, signifying that each zone represents new cell division. (a) Chondrocyte natural fluorescence was observed. (b) Chondrocytes recently stained with CFSE. (c) Chondrocytes after 5 days co-culture. (d) Chondrocytes after 5 days culture in DMEM (control). (e) Normal chondrocytes cultured in enriched medium showed highest proliferation index 58% in M2 (histograms were divided only in three zones due to homogeneity of cell behaviour).

Figure 2. Student's *t*-test comparing co-cultured chondrocytes (black column) and control chondrocytes (white column). Five-day cultures show that chondrocyte proliferation was significantly higher in the co-culture system; this was especially evident for flow cytometry histogram zones M5, M6 and M7.

fibroblast-like morphology, and reached 40% confluence between 72 and 96 h after seeding.

Normal chondrocyte proliferation

In the flow cytometry histograms, highest fluorescence intensity was located at the right edge of the *X*-axis. Normal chondrocytes from co-cultures and control cultures displayed loss of fluorescence intensity after 5 days shown by cell displacement to the left side of the *X*-axis (indicating chondrocyte proliferation); however, in the control cultures, cell division was lower. Chondrocyte division was higher by the 5th day for the co-culture group; at this time, the greatest displacement of chondrocytes was found towards the less fluorescent zone (left edge of *X*-axis) of the histogram. Differences between normal chondrocytes from co-cultures and control cultures in histogram zones M1 to M4 were not significant (Fig. 1). However, a significant difference was found in histogram zones M5 ($P = 0.003$), M6 ($P = 0.001$) and M7 ($P = 0.004$) (Fig. 2). Normal chondrocytes cultured in enriched medium had increase in cell proliferation; this indicates that contact of alginate beads and monolayer cells was not necessary for induction of proliferation (Fig. 1e).

Osteoarthritis chondrocyte proliferation

Osteoarthritis chondrocytes embedded in alginate beads cultured in DMEM/FBS 10% and in co-culture systems did not show any significant proliferation in relation to the zero time point ($P \leq 0.05$). In addition, after 5 days of culture, cell viability was reduced from 80% to 60%. However, when alginate beads containing osteoarthritis chondrocyte were cultured in enriched culture medium, significant proliferation was observed, which is directly related to

loss of fluorescence intensity ($P = 0.001$); furthermore, cell viability reached 90% (Fig. 3).

Flow cytometry histograms from osteoarthritis chondrocytes were divided only in three zones because cell division was more synchronized than that of normal chondrocytes, showing comparable behaviour during the different days studied.

Mitosis phases

After 5 days of culture, in normal and osteoarthritis chondrocytes embedded in alginate beads, it was possible to identify all phases of mitosis, verifying that cell division was completed (Fig. 4).

Normal chondrocyte phenotype

In both experimental and control cultures, chondrocytes embedded in alginate beads retained their phenotype. This was verified by Western blot analysis, which confirmed collagen II expression in alginate beads of both cultures after 14 days (Fig. 5).

Osteoarthritis chondrocyte phenotype

Osteoarthritis chondrocytes encapsulated in alginate beads were evaluated by flow cytometry before and after 7 days of culture in enriched culture medium. Cells were labelled with type II collagen and MMP-3 monoclonal antibodies. At time zero, osteoarthritis chondrocytes expressed a great amount of MMP-3, whereas collagen type II was only minimally expressed. After 7 days of culture, an increase in collagen type II expression (18%) and a diminution of MMP-3 (8%) were observed. These results indicate that providing a three-dimensional matrix support and supplementary nutrients within the enriched culture medium, osteoarthritis chondrocytes were able to express their normal phenotype again (Fig. 6).

Real-time RT-PCR and Western blot analysis to demonstrate expression of growth factors

Real-time RT-PCR results were performed for chondrocyte monolayer cultures, wherein the threshold cycle (C_T) is defined as the fractional cycle number at which fluorescence intensity exceeds threshold intensity. Values obtained indicate that the growth factors that displayed higher numbers of mRNA copies were IGF-1 and TGF- β 1 and, growth factors mRNAs of PDGF-A, FGF-2 and EGF were expressed to a lower degree.

Once the C_T values were standardized with the endogenous gene of cyclophilin in each passage, growth factor expression was assessed in chondrocytes of each culture

Figure 3. Osteoarthritis chondrocytes: five days culture in different media. Histograms were divided only in three zones due to homogeneity of the cell behaviour. M1 represents major fluorescence intensity, whereas M3 showed no fluorescence (right panels). (a) Chondrocytes cultured in DMEM supplemented with 10% of FBS. (b) Chondrocytes co-cultured with a monolayer of de-differentiated chondrocytes. (c) Chondrocytes cultured in enriched medium. In the left panels, graphic representation of statistical differences between fluorescence intensity at day 0 (grey columns) and 5 days after culture in different media (black columns).

and were compared. The growth factor that showed highest overexpression in level of mRNA in the third passages was TGF- β 1, which increased its number of copies in each passage, to its greatest indication of protein level in the fourth passage (Fig. 7). Additionally, even though the number of copies of mRNA of IGF-1 increased from passages 2 to 4, it was not possible to detect the protein by Western blot analysis. On the other hand, in tests of chondrocytes of each of the serial culture passages, only two to five of the growth factors displayed a high level of protein expression (EGF and PDGF-A), and, additionally, growth factors FGF-2 and TGF- β 1 overexpressed their proteins in passages 3 and 4, respectively (Fig. 8).

Discussion

Our results indicate that normal chondrocytes embedded in alginate beads and co-cultured with de-differentiated chondrocyte monolayer, as well as when cultured in enriched medium, displayed increased cell division compared with to controls grown in DMEM. These results led us to believe that certain molecules, such as growth factors, might be secreted into the culture medium by de-differentiated chondrocytes, enhancing local chondrocyte proliferation.

Osteoarthritis chondrocytes after 5 days of culture in the DMEM alone showed no statistically significant increase in cell proliferation compared to time zero, while

Figure 4. Mitotic phases in co-cultured chondrocytes (asterisks). A, Prophase; B, Metaphase; C, Anaphase; D, Telophase. Bars = 15 μm.

in the co-culture system there was little proliferation and cell viability was reduced, probably due to lack of nutrient availability and due to presence of excreted products caused by cell population growth in the monolayer. Thus, we believe that combination of DMEM with enriched medium (half-and-half), which showed significant increase in osteoarthritis chondrocyte proliferation and viability (85–90%), was the best procedure to achieve these parameters, perhaps because of presence of crucial growth factors in the enriched medium. However, it is important to note that these growth factors should be combining with molecules present in DMEM to accomplish cell proliferation.

Brittberg *et al.* reported successful treatments for damaged articular cartilage after transplanting autologous chondrocytes that originated from a monolayer culture system (39). However, as is well known, monolayer chondrocytes do not retain the chondrocyte phenotype. Thus, it is doubtful that the fibroblastic-like cells would be able to re-express the articular chondrocyte phenotype after the transplantation. On the other hand, Wakitani *et al.* cultured rabbit chondrocytes in collagen type I gel, and demonstrated that the newly formed tissue became hard after a prolonged culture (40).

Recent studies have shown important differences in chondrocyte phenotype depending on the physiological

Figure 5. Collagen II expression in co-cultured chondrocytes, evaluated by Western blotting. Primary antibodies recognized a 97-kDa protein and a 120-kDa protein; the protein with lower electrophoretic mobility possibly represents a native form of collagen II. Line 1: positive control, normal cartilage; Line 2: chondrocytes from co-culture; Line 3: chondrocytes from control culture; Line 4: negative control, HeLa cells.

balance between expression of type II collagen and MMP-3 (41). This balance has preferential expression of MMP-3 in osteoarthritis chondrocytes and this results in determines extracellular matrix breakdown. In addition, it is well known that inflammatory cytokines such as IL-1 during osteoarthritis induces synthesis of MMPs, such as MMP-3 and MMP-9, and aggrecanases by chondrocytes, which, in cartilage breakdown intervene; in both autocrine and paracrine manners. Furthermore, they also play a role in extracellular matrix breakdown (42,43). In addition, expression of MMP-3 and MMP-13 has been reported in pannus-like osteoarthritis cells (44).

Our results reveal that DMEM enriched with co-culture medium, induced chondrocytes synthesis of collagen II in tridimensional culture conditions, suggesting that a combination of growth factors with molecules present in DMEM might revert osteoarthritis chondrocytes phenotype to normal. These findings might be important for osteoarthritis cartilage regeneration.

On the other hand, analysis of five growth factors identified by real-time RT-PCR showed differential expression. In the chondrocyte monolayers after several culture passages conditions were as follows: IGF-1 analysis showed contradictory behaviour since even though it was not possible to identify the protein by Western blotting, (probably because of an inadequate sensitivity of our procedure or that the growth factor were secreted to the culture medium), expression of its mRNA displayed increases throughout the serial passages of chondrocyte culture. This might be related to post-transcriptional or post-traductional modification that could probably be associated with homeostasis in articular cartilage, since in adults IGF-1 stimulates synthesis of extracellular matrix proteins by chondrocytes, controlling its degradation and also associated with cell death. Biodisponibility of IGF-1 is regulated by two cell-binding proteins, insulin growth factor binding protein and

Figure 6. Immunodetection of collagen type II and MMP-3 in osteoarthritis chondrocytes, evaluated by flow cytometry. Dot plot, cells with collagen II and MMP-3 (marked) positive located to the right of the X axis, while the left side is the unmarked zone. (a) Control of immunodetection (without collagen type II antibody). Cells located to the left of the X axis; (b) osteoarthritis chondrocytes at time 0, cells located to the left of the X axis, indicating that the chondrocytes did not synthesize type II collagen; (c) osteoarthritis chondrocytes at 7 days of culture in enriched culture medium located to the right of the X axis, indicating that they express collagen II, typical of normal chondrocytes; (d) control of immunodetection (without MMP-3 antibody). Osteoarthritis chondrocytes are located to the left of the X axis; (e) osteoarthritis chondrocytes at time 0, cells located to the right of the X axis, indicating that the chondrocytes express MMP-3, the typical phenotype of osteoarthritis chondrocytes and (f) osteoarthritis chondrocytes at 7 days of culture in enriched culture medium located to the left of the X axis, indicating that the chondrocytes did not synthesize MMP-3.

fibronectin (45), which probably were not present in the culture system we used.

TGF- β 1 mRNA expression increased four fold in relation to the beginning of the culture; this was confirmed by protein expression in the fourth passage, suggesting that TGF- β 1 might play a role in de-differentiation of chondrocytes cultured in monolayers. Nevertheless, it is known that this factor is stored inactively within the cartilage (300–500 ng/g of cartilage), and is only expressed and activated after a proteolytic process (46,47). Probably a post-transcriptional and post-transductional situation might be present in our culture system; undoubtedly, this idea requires further study.

Low expression of FGF-2 mRNA in chondrocyte monolayers in several passages of the culture and protein

expression only in the third passage made us speculate that its absence might not be involved in the loss of chondrocyte phenotype and it might play a role in cell proliferation. Mandl *et al.* have shown that the addition of fibroblast growth factor-2 in serum-free medium has a role as a potent mitogen and reduces de-differentiation of human chondrocytes in monolayer cultures (48).

Growth factor PDGF-A showed low expression of its mRNA; however, it has high expression of the protein, suggesting its role during chondrocyte de-differentiation and proliferation. In the case of EGF, its mRNA expression increased only in the fourth passage; however, its protein was present in all the passages. This suggests that this factor might mediate the de-differentiation and proliferation of chondrocytes in our culture system.

Figure 7. Reverse transcriptase–polymerase chain reaction (RT-PCR) amplification of growth factors IGF-1, FGF-2, PDGF-A, TGF-β1 and EGF. Total RNA obtained from monolayers of de-differentiated chondrocytes, through passages P0, P1, P2, P3 and P4, subjected to RT-PCR utilizing primers described in the Material and Methods section. Levels of mRNA were compared between different passages by normalization to expression of mRNA of endogenous cyclophilin, each condition was analysed in triplicate in the same session and standard deviations were calculated. Gene expression was quantified relatively using the $2^{-\Delta\Delta CT}$ method. Each growth factor is indicated at the bottom, and fold increase is indicated in the Y axis.

Figure 8. Western blot analysis of growth factors IGF-1, FGF-2, PDGF-A, TGF-β1 and EGF. Samples of 40 mg of total protein from passages 0 to 4 of chondrocytes P0, P1, P2, P3 and P4 were loaded in each lane, electrophoresed in 12% SDS-PAGE, and processed for Western blotting with specific antibody for each growth factor, as described in the Material and Methods section. Actin was used as the reference gene and loaded control, and expression of growth factors was compared between the different passages. The autoradiographs are representative of three independent experiments.

We can conclude that the release of growth factors from chondrocyte monolayer cultures enriches the culture medium in which chondrocytes embedded in alginate beads retain their phenotype.

Therefore, use of chondrocyte culture in enriched media, as a source of growth factors, can be an alternative system to stimulate cell proliferation and to design new types of implant for treating damaged cartilage. Moreover, study of the supernatants emanating from the different passages of co-cultures, which is being accomplished, should add more information to this study.

However, these results in an experimental model in rat should be taken cautiously and require further study to clarify behaviour of the indicated growth factors; overall it needs to be carried out in human tissue to truly determine its feasible application.

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