Response of Human Chondrocytes Prepared for Autologous Implantation to Growth Factors

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ABSTRACT: This study investigated metabolism of autologous chondrocytes after initial expansion immediately before implantation. Chondrocytes cultured in either monolayers or alginate beads were treated with insulin-like growth factor-1 (IGF-1), osteogenic protein-1 (OP-1), or a combination. Proteoglycan synthesis and DNA content were tested in both cultures. Alginate beads also were analyzed with live/dead cell assay, safranin O/fast green stain for histology, and immunohistochemistry with antibodies against collagen type II and VI, aggrecan, decorin, and fibronectin. In monolayers, autologous chondrocytes changed their morphologic appearance. In alginate, they maintained chondrocytic phenotype. Growth factors, especially combined, promoted cell survival and induced chondrocyte proliferation. OP-1 stimulated the largest cartilage-specific matrix and the most accumulation of collagen type II and fibronectin, although the overall matrix synthesized by autologous chondrocyte implantation cells was smaller than that produced by normal chondrocytes. The clinical implications of this study suggest a significant promise for anabolic growth factors in cartilage repair as a potential modifying therapy for the enhancement of chondrocytic phenotype of autologous chondrocytes.

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INTRODUCTION

Autologous chondrocyte implantation (ACI) is a widely used method for treating localized articular cartilage lesions by inducing chondrogenesis within the defect. The majority of the literature focuses on the clinical outcomes of such procedures,[2,25,27] leaving the biological aspect largely undefined. Despite encouraging clinical follow-up data, there are still some concerns whether chondrocytes that undergo a number of passages maintain their phenotype, proliferative activity, and ability to synthesize and properly assemble newly formed extracellular matrix. Also in question is whether these cells need to be stimulated with growth factors to remain metabolically active to produce appropriate matrix.

However, there are only limited studies that focus on the biology of autologous chondrocytes prepared for implantation. In these reports, ACI cells were examined immediately as leftovers at the time of implantation (after initial expansion)[15,30] or placed in different culture systems,[9,14-16] or biopsies of newly formed cartilage were followed at a certain time point for structural, histologic and immunohistologic, and molecular biology analyses.[1,24] Zheng et al[30] examined the cellular phenotype and apoptotic index of ACI cells and found that although they were of chondrocytic lineage and only 11% displayed characteristics of apoptosis, they expressed both type I and II collagens, suggesting changes in their phenotype. In contrast, ACI cells cultured in suspension maintained the differentiated phenotype of mature chondrocytes and produced type II collagen only.[15] The presence of type II collagen and its metabolic products was suggested as the evidence for cartilage turnover and remodeling in biopsy samples of ACI surgery, although these samples looked like fibrocartilage.[24]

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Single attempts were made to optimize autologous chondrocytes with growth factors, serum, or both[9,13,16,29]; however, the results were inconclusive. Transforming growth factor-β (TGF-β) stimulated proliferation of bovine chondrocytes only in the primary culture, whereas ascorbic acid had the same effect in the third passage.[9] Despite the desirable increase in cell number, the expression of collagen type II was lost after the third passage.

In a similar study with human autologous chondrocytes cultured in pellets,[16] TGF- β1 stimulated type II collagen and glycosaminoglycan production in the absence of serum, whereas serum alone induced type I collagen. Likewise, the presence of serum in monolayer cultures of autologous chondrocytes from aged human patients inhibited both cell proliferation and chondrogenesis, whereas serum-free conditions resumed growth rates but proper extracellular matrix deposition was only partially restored.[13] Transplantation of rabbit chondrocytes transfected with adenovirus containing bFGF gene induced a more pronounced regeneration of the articular cartilage defect.[29] However, taken together, these reports do not provide a definitive evaluation of ACI cells or their morphology, phenotype, and optimal culture conditions.

This study was undertaken to analyze the phenotype of autologous chondrocytes after initial expansion in 2 types of culture, monolayers and alginate beads; to test their anabolic response to selected growth factors; and to compare this response between monolayers and alginate beads. Two growth factors were chosen for this study, insulin-like growth factor-1 (IGF-1) and osteogenic protein-1 (OP-1), which is also called bone morphogenetic protein-7 (BMP-7), because they are produced endogenously by human adult articular chondrocytes and are able to stimulate the synthesis of cartilage extracellular matrix components.[6,8,19] In addition, OP-1 was shown to exhibit not only pro-anabolic but also anticatabolic activities,[7,11] and when combined with IGF-1, OP-1 revealed a synergistic effect on chondrocyte proliferation and cartilage matrix synthesis, while synergistically inhibiting the catabolic effects of interleukin (IL)-1β and fibronectin fragments.[17-19]

MATERIALS AND METHODS

Culture Medium and Chemicals

Dulbecco's modified Eagle medium, Ham's F12 medium, fetal bovine serum, penicillamine/streptomycin/fungizone, and gentamicin were purchased from Invitrogen Corp (Carlsbad, Calif). Low-viscosity alginate (Keltone LV) was obtained from Kelco (Chicago, Ill). The Live/Dead Viability/Cytotoxicity Kit was obtained from Molecular Probes (Eugene, Ore). Safranin O and fast green dyes were purchased from Fisher Scientific (Pittsburgh, Pa). Anti-fibronectin monoclonal antibody was purchased from Chemicon International Inc (Temecula, Calif); anti-aggrecan, anti-decorin, anti-type II collagen, and anti-type VI collagen monoclonal antibodies were purchased from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, Iowa). Recombinant human OP-1 (rhOP-1) was provided by Stryker Biotech (Hopkinton, Mass). Recombinant human IGF-1 was obtained from Chiron Corp (Emeryville, Calif). Peroxidase-labeled goat anti-mouse IgG was purchased from Organon Technika Corp (Durham, NC). All other chemicals used for this study were purchased from either Sigma (St Louis, Mo) or Fisher Scientific.

Patients

After institutional review board approval was obtained for the study, all patients provided informed consent. Cartilage biopsy specimens were obtained from a low load-bearing area of each affected knee by arthroscopic surgical procedure in 8 patients ranging in age between 23 and 43 years. From the biopsy samples, chondrocytes were isolated and cultured in monolayer for 7 to 21 days by Genzyme Laboratories (Genzyme Tissue Repair, Cambridge, Mass). Several weeks after the index surgery, a suspension of expanded cells was returned for implantation. Before a second surgical procedure, an aliquot of initially expanded extra cells (at least 1.2×10^7 cells per patient) was collected for further biochemical analysis. Chondrocytes from each individual were analyzed separately. At least 3 samples from different patients were used for each experiment.

Cell Culture

After initial expansion at Genzyme Laboratories, for the purpose of this project, cells were placed in high-density monolayer cultures (2×10^6 cells per well in a 12-well plate) or resuspended in 1.2% sodium alginate solution at 2×10^6 cells/mL for formation of alginate beads. This cell density resulted in an average encapsulation of 20,000 cells per bead. Both cultures were maintained in the presence of 10% fetal bovine serum for up to 7 (monolayer) or 14 days (alginate bead), with changes of media every other day.

Chondrocytes were treated with OP-1, IGF-1, or a combination of OP-1 and IGF-1 (100 ng/mL of each growth factor). In addition, chondrocytes in monolayer cultures also were treated with a dose of 200 ng/mL of OP-1 alone or in combination with IGF-1 (100 ng/mL) to study metabolic activity. Cell survival (LIVE-DEAD Cell Assay), proliferation (DNA levels), and proteoglycan synthesis (sulfate incorporation) were measured after 48 hours for monolayers and after 2, 7, 10, and 14 days for alginate beads.

Cell Survival Assay

Cell survival was measured as described previously[10] using calcein AM to stain live cells and ethidium bromide

homodimer-1 to stain dead cells. These reagents were obtained from Molecular Probes. At least 100 cells were counted in triplicate for each data point.

Proteoglycan Synthesis

Sulfate incorporation was measured during the final 4 hours of culture at each time point by pulse-labeling of chondrocytes with 10 μCi/mL ^{35}S-sulfate (Perkin Elmer, Boston, Mass). The culture medium pool and cell-associated matrix pool were separated and collected. Cell-associated matrix was digested with papain.[23] In each medium and dissociative extract, ^{35}S-labeled proteoglycans were quantified by liquid scintillation counting following rapid filtration, as described previously.[21] The concentration of ^{35}S-proteoglycans synthesized was normalized to DNA content.

Histology and Immunohistochemistry on Alginate Beads

To visualize the matrix deposited by chondrocytes, alginate beads were fixed in 4% paraformaldehyde in 0.1 M of cacodylate buffer (pH 7.4) containing 10 mM of calcium chloride for 4 hours at 20°C, washed overnight at 4°C in 0.1 M of cacodylate buffer (pH 7.4) containing 50 mM of barium chloride, dehydrated through serial alcohols and xylene, embedded in paraffin, sectioned at 6 μm, and processed for histology and immunohistochemistry as described previously.[5] For histology, the beads were stained with safranin O and fast green.[26]

For immunohistochemistry, before incubation with the primary antibodies, bead sections were digested with keratanase (*Pseudomonas* sp EC 3.2.1.103 [0.01 U/mL]), keratanase II (*Bacillus* sp KS 36 (0.0001 U/mL]), and chondroitinase ABC (*Proteus vulgaris* EC 4.2.2.2 [0.01 U/mL]) in 100 mM of Tris/50 mM of sodium acetate buffer (pH 6.5) at 37°C for 90 minutes to increase the penetration of antibodies into the matrix. All 3 proteinases were obtained from Seikagaku (Tokyo, Japan). For negative controls, the primary antibodies were replaced with either normal serum or secondary antibody alone. All primary antibodies were applied at a 1:100 dilution. To detect aggrecan, decorin, fibronectin, and collagen type II and VI, horseradish peroxidase-conjugated secondary antibody was used. Evaluation and documentation of the results was performed with a Nikon Eclipse 600 microscope (Tokyo, Japan) with a Spot 2 camera; photographs were taken with the MetaMorph software program (Universal Imaging Corp, Downingtown, Pa).

Morphometric Analysis

Safranin O-stained sections underwent morphometric analysis under 400× magnification to quantify the size of the newly deposited matrix using a Nikon Eclipse E600 microscope connected to a PC running MetaMorph Imaging series 6.1 software. With this software, the area occupied by each chondrocyte and its newly synthesized matrix was determined by selecting the area of the "cavity" within the alginate bead created by the cell and matrix, forming a better representation of the actual size of the newly synthesized matrix before shrinkage artifact from histologic preparation. The method has been described in detail previously.[4] The control and all experimental groups were subjected to the morphometric analysis. The data were collected for 43 to 70 cells/cell clones with their surrounding matrix for each group in a blind manner and normalized to the number of cells within the selected area. The size of the cell per se was not subtracted from the size of the matrix because it was difficult to distinguish between the cell-associated matrix and the cell itself.

Statistical Analysis

The statistical significance of results was determined by analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software, Inc, San Diego, Calif) and 1-way ANOVA. Post-hoc testing was completed using a 2-tailed *t* test. The level of significance was set at $P < .05$.

RESULTS

Chondrocyte Morphology and Survival

Autologous chondrocytes exhibited significant culture-dependent differences in behavior and response to growth factors. Cells plated in high-density monolayers changed their appearance, became elongated, and revealed a fibroblast-like morphology within the first hours of culture (Figure 1). Treatment with growth factors was not able to prevent chondrocyte dedifferentiation (data not shown). The same chondrocytes encapsulated in alginate beads did not change their shape and remained rounded throughout the entire culture period (up to 2 weeks) (Figures 2 and 3). No significant cell death was observed in either culture system within the first days; however, by day 14 in alginate beads, the number of living cells in the fetal bovine serum control group was reduced by 24% ($P < .05$) (Figure 2A). In the presence of growth factors (all 3 treatment groups), the cell survival rate was similar among the groups and remained at the initial, day 0 control levels (Figure 2). After it was found that chondrocytes in monolayer cultures changed their phenotype, the majority of experiments were performed on cells cultured in alginate.

Chondrocyte Proliferation

Cell proliferation induced by growth factors also was culture-type dependent. In monolayers, the greatest cell proliferation was observed after 48 hours in the combined OP-1 and IGF-1 treatment group at the maximum dose (200 ng/mL; approximately 55%, $P < .01$) (Figure 4A). In the other experimental groups of monolayer culture, the level

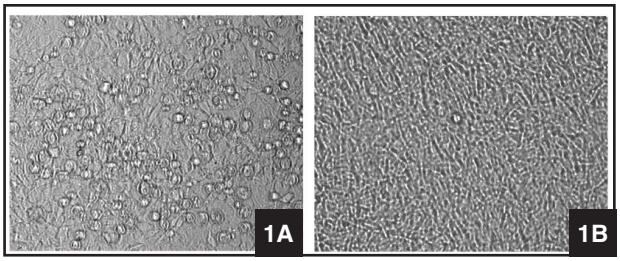


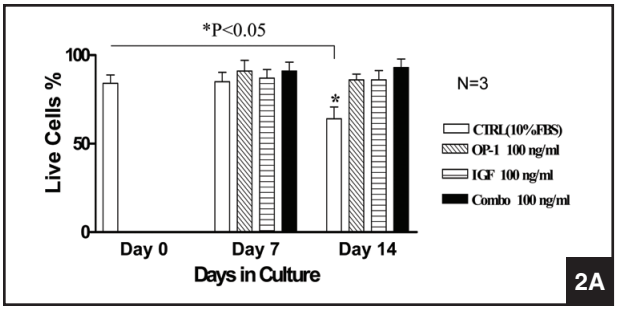
Figure 1. Photomicrographs showing changes in the appearance of autologous chondrocytes cultured in high-density monolayers after 4 hours (A) and 48 hours (B) of culture (original magnification ×200).

of cell proliferation did not vary substantially between treatments and was above the values of serum control by approximately 35% ($P < .01$). In autologous chondrocytes cultured in alginate beads, the highest cell proliferation was observed in the combined OP-1 and IGF-1 group (100 ng/mL of each growth factor), which became evident by day 7 and reached its maximum by day 14 ($P < .01$ compared with the corresponding control group) (Figure 4B). Individually, OP-1 and IGF-1 also affected cell proliferation, but this was apparent only by day 10. Under treatment with individual growth factors, cell numbers were increased by 30% to 40% ($P < .01$), whereas combined OP-1 and IGF-1 increased cell proliferation from 2-fold on day 7 ($P < .01$) to 3-fold on day 14 ($P < .01$) compared with serum control.

Proteoglycan Synthesis

Importantly, growth factors alone or in combination induced not only cell proliferation but also proteoglycan synthesis by each cell; however, the effect varied among culture types and treatment groups. In monolayer cultures, OP-1 induced a statistically significant increase in proteoglycan synthesis after 48 hours only at a dose of 200 ng/mL ($P < .05$). A dose of 100 ng/mL led to some stimulation in proteoglycan synthesis but did not reach statistical significance. In chondrocytes cultured in alginate, the effect of OP-1 (100 ng/mL) on proteoglycan synthesis was relatively constant and constituted approximately 2-fold ($P < .01$) at days 7, 10, and 14. The effect of IGF-1 on proteoglycan synthesis was observed only in chondrocytes cultured in alginate beads and only at days 10 and 14 (2-fold increase, $P < .05$). Combined OP-1 and IGF-1 treatment resulted in the strongest anabolic response. In monolayers, proteoglycan synthesis was increased by approximately 3-fold ($P < .05$) and did not differ between the 100 and 200 ng/mL doses of growth factors (Figure 5A). In alginate beads, the effect of combined treatment gradually increased from day 7 (approximately 2.85-fold, $P < .01$) to days 10 and 14 (approximately 5-fold, $P < .01$) (Figure 5B).

Of note, the timing of response to growth factors varied between cultures. In monolayers, growth factors increased cell proliferation and proteoglycan synthesis after 48 hours of treatment (Figure 5A). In alginate cultures, the effect of growth factors became apparent only by day 7. No noticeable changes were detected after 48 hours.



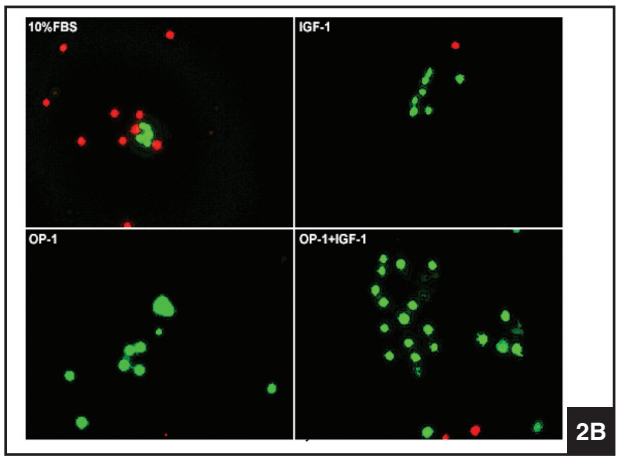


Figure 2. Graphs showing the effect of growth factors on the survival of autologous chondrocyte implantation cells cultured in alginate. Graph showing living cells at days 7 and 14 compared with the control at day 0; the number of live cells was calculated per 100 cells and expressed as the percentage to total cell number (A). Fluorescent micrographs showing live/dead cells that were cultured for 14 days in alginate beads and treated with serum, insulin-like growth factor-1 (IGF-1) (100 ng/mL), osteogenic protein-1 (OP-1) (100 ng/mL), or combined IGF-1 (100 ng/mL) and OP-1 (100 ng/mL); red dots represent dead cells and green dots represent living cells (original magnification ×200) (B). (Abbreviations: FBS, fetal bovine serum; CTRL, control; Combo, combined IGF-1 and OP-1.)

Histology

Staining of alginate beads with safranin O and fast green allowed visualization of newly synthesized matrix. Nonspecific binding of safranin O dye to the negatively charged alginate matrix produced intense red stain, whereas matrix synthesized by chondrocytes appeared with a lighter intensity of red (Figure 3, upper 2 rows). Although all cultures were maintained in the presence of 10% serum, chondrocytes on the periphery of the beads showed higher metabolic activity and deposited more newly synthesized matrix than chon-

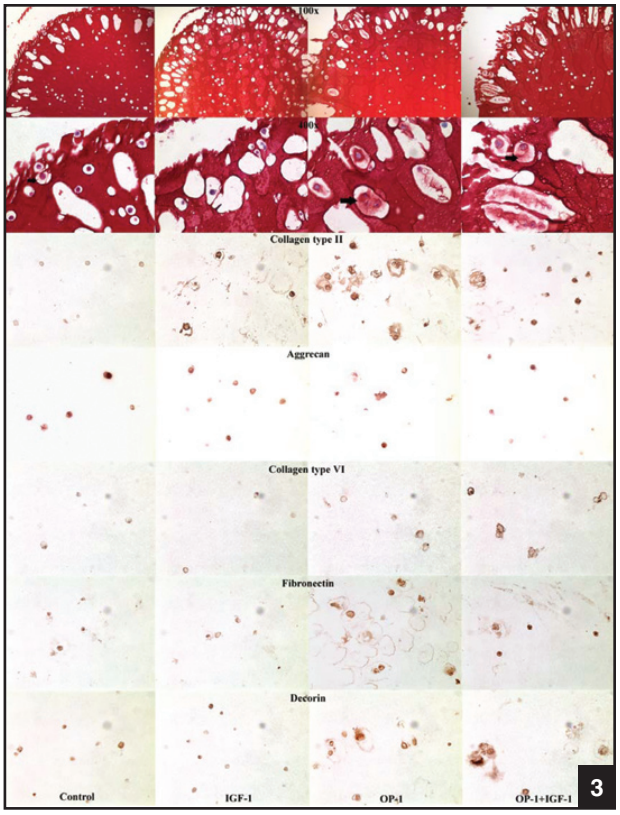
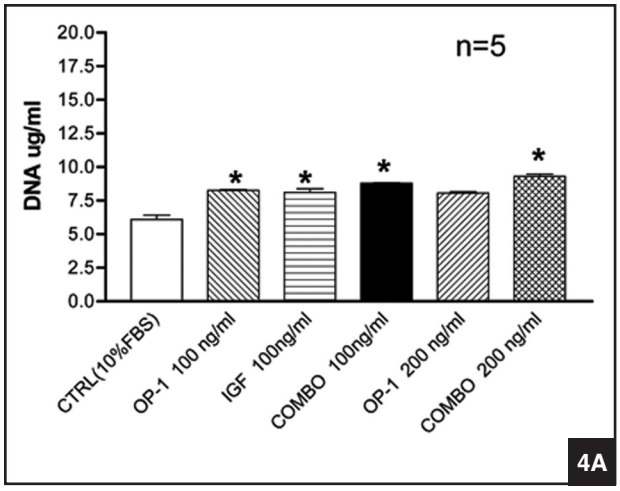


Figure 3. Collage showing autologous chondrocyte implantation cells cultured for 14 days in the presence of serum, insulin-like growth factor-1 (IGF-1) (100 ng/mL), osteogenic protein-1 (OP-1) (100 ng/mL), or combined OP-1 (100 ng/mL) and IGF-1 (100 ng/mL) and evaluated for histology with safranin O staining (upper 2 rows) or immunohistochemistry with anti-type II collagen, anti-aggrecan, anti-type VI collagen, anti-fibronectin, and anti-decorin antibodies. All stains were developed by the application of horseradish peroxidase-conjugated secondary antibodies that produced brown color. Histology images were taken under original magnification ×100 (upper row) or ×400. All images with the antibody stains were taken under original magnification ×400.



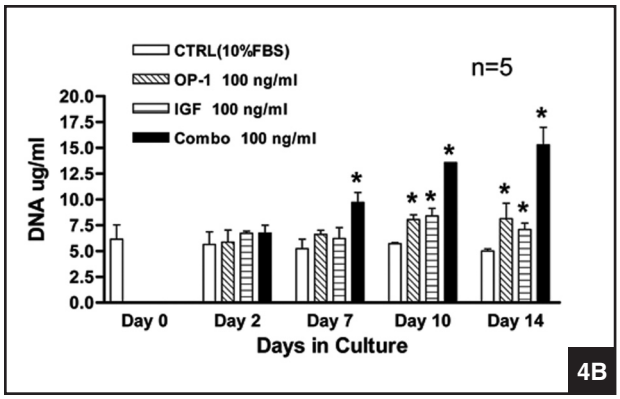


Figure 4. Graphs showing chondrocyte cell number, reflecting proliferation, during culture in the presence of serum, insulin-like growth factor-1 (IGF-I), osteogenic protein-1 (OP-1), or combined OP-1 and IGF-1. Human autologous chondrocytes were cultured for 48 hours in high-density monolayers (A) or in alginate beads (B). Cell numbers at the end of culture were measured in triplicate samples using a DNA assay (mean±SD) (*$P < .01$ versus serum control). (Abbreviations: CTRL, control; Combo, combined IGF-1 and OP-1.)

drocytes in the middle part of the bead (arrows indicate new matrix formation). This phenomenon has not been observed previously (at least to such extent) in normal chondrocytes cultured in alginate.[5] In the serum control group (Figure 3), barely visible matrix was observed around chondrocytes. The histologic appearance of alginate beads cultured in the presence of growth factors indicated no apparent difference in the amount of matrix deposited in the presence of OP-1 alone or combined OP-1 and IGF-1 (Figure 3). In both groups, growth factors caused considerable matrix formation. In the IGF-1 alone group, no significant matrix deposition was noticed. The apparent shrinkage of the matrix within the areas occupied by chondrocytes is believed to be caused by the artifacts produced in the fixation and processing procedures.

Morphometry

To quantify the amount of matrix deposited by chondrocytes in alginate, morphometric analysis was applied in which the size of the matrix surrounding each cell or clone of cells in square micrometers was divided by the number of chondrocytes residing within this area. Compared with serum control, IGF-1 alone did not induce significant matrix accumulation (Figure 5), whereas OP-1 appeared to have the strongest effect. The size of the matrix produced by OP-1 was significantly different compared with IGF-1 treatment or serum control (1.6-fold compared with IGF-1 and 1.85-fold compared with control, $P < .001$). Of note, the matrix synthesized by chondrocytes stimulated with the combination of growth factors was smaller than with OP-1 alone, although it was larger than the matrix produced by chondrocytes treated with IGF-1 alone or serum ($P < .001$) (Figure 6). The dif-

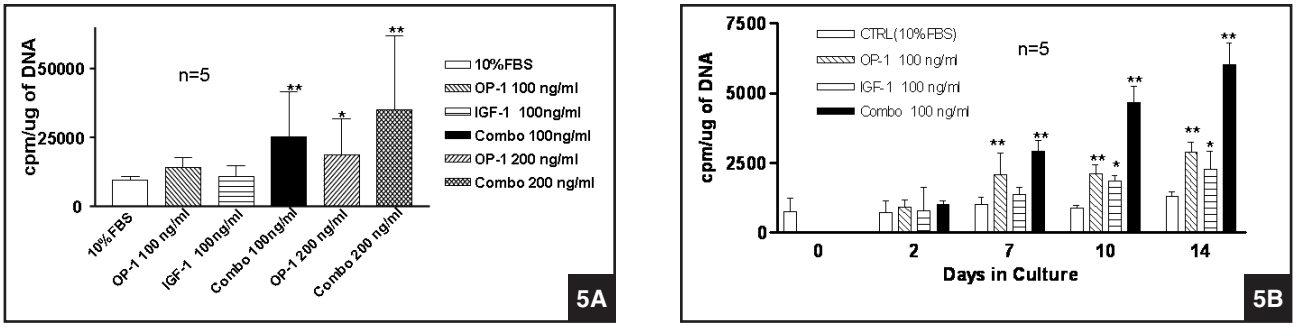


Figure 5. Graphs showing chondrocyte proteoglycan synthesis after culture in the presence of serum, insulin-like growth factor-1 (IGF-1), osteogenic protein-1 (OP-1), or combined IGF-I and OP-1. Human autologous chondrocytes were cultured in high-density monolayers for 48 hours (A) or in alginate for 14 days (B). Proteoglycan synthesis was measured in triplicates during the last 4 hours of culture using sulfate incorporation and was normalized for cell numbers by DNA assay (mean±SD) (*$P < .05$ versus control, **$P < .01$ versus control). (Abbreviations: FBS, fetal bovine serum; CTRL, control; Combo, combined IGF-1 and OP-1.)

ference in the size of the matrix synthesized by OP-1 and combined treatment was statistically significant ($P < .02$). Also of note, although growth factors induced chondrocyte proliferation, as shown in this study and in a previous study,[19] they also induced matrix production per cell.

Immunohistochemical Analyses

Histologic and immunohistochemical analysis of alginate beads confirmed the morphology of differentiated chondrocytes in cells prepared for ACI and showed that by day 14 of culture, these cells produced extracellular matrix proteins specific for articular chondrocytes: type II and VI collagens, aggrecan, decorin, and fibronectin, although the amount of deposited proteins was relatively low compared with normal cells[4,8] and varied among treatments (Figure 3). There were no apparent differences in the amount of synthesized matrix proteins between serum control and IGF-1 treatment groups, with the exception of type II collagen. Staining with anti-type II collagen antibody revealed a more abundant presence of type II collagen in the IGF-1 beads than in the control beads. Type II collagen was primarily localized in the cells, with small amounts being deposited on the edges of the lacunas in the territorial matrix and in the interterritorial matrix.

The strongest stain for type II collagen was detected in chondrocytes treated with OP-1, and it was more intense than in cells treated with the combination of OP-1 and IGF-1. Collagen type II was localized in the cells and in the territorial and interterritorial matrices. Staining with anti-aggrecan antibody was similar in all of the experimental groups, with a lesser staining under the combined treatment; aggrecan was primarily localized intracellularly or around the cells.

Overall, type VI collagen was synthesized by chondrocytes at low levels and was barely detected in serum control and IGF-1 beads. Higher levels of type VI collagen were found in the OP-1 group, whereas the most stain was observed in chondrocytes treated with combined OP-1 and IGF-1. Type VI collagen was localized primarily in the pericellular matrix.

Fibronectin was barely seen in beads cultured in the presence of 10% serum or IGF-1. Again, as in the case with type II collagen, the most fibronectin synthesis was stimulated by the OP-1 alone treatment; combined growth factor therapy caused a lower production of fibronectin. It was localized intracellularly and in the interterritorial matrix.

Decorin was detected at the background levels in the control and IGF-1 stimulated beads. Both OP-1 alone and combined OP-1 and IGF-1 induced more decorin accumulation than serum or IGF-1 alone; the intensity of stain and the pattern of distribution was similar in these 2 groups. Decorin was detected in the cells and in the interterritorial matrix.

DISCUSSION

Little is known about the phenotype and anabolic activity of human autologous chondrocytes after initial expansion and before implantation. Our findings demonstrated autologous chondrocytes after culture at Genzyme Laboratories still maintained differentiated chondrocytic phenotype and were metabolically active by responding to growth factors. In addition, these cells demonstrated good proliferating activity in either culture system under treatment with growth factors, suggesting they likely remain active after implantation into the joint. Importantly, the growth factor responses induced in autologous chondrocytes in this study appear less pronounced, but similar in nature to those of primary cells,[4,19] since in an earlier study we showed that the highest cell proliferation, proteoglycan synthesis, and cell survival were stimulated in primary chondrocytes treated with a combination of OP-1 and IGF-1.

In the current study, chondrocyte proliferation was observed not only under the combined treatment but also under treatment with each growth factor separately. Because ACI cells undergo multiple divisions during the preparation

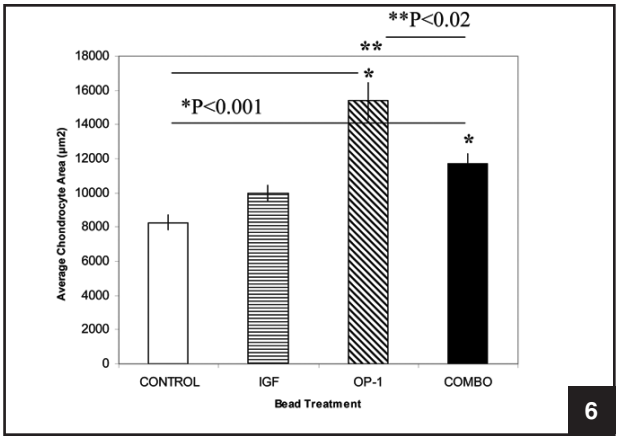


Figure 6. Graph showing morphometric analysis of the matrix deposited by autologous chondrocytes cultured in alginate (presented as an average size of the matrix produced by chondrocytes; comparison is made between treatment groups). The differences were statistically significant between the following groups: osteogenic protein-1 (OP-1) versus control ($P < .001$), OP-1 and insulin-like growth factor-1 (IGF-1) versus control ($P < .001$), and OP-1 versus OP-1 and IGF-1 ($P < .02$). (Abbreviation: Combo, combined IGF-1 and OP-1.)

steps performed at Genzyme Tissue Repair, the cells apparently become more sensitive to culture and growth factors and demonstrate a higher mitotic activity. Proliferation of ACI cells in response to other growth factors, for instance TGF-β, also was reported for monolayers in the presence of 10% fetal calf serum,[9] whereas in the pellet cultures, TGF-β-stimulated proliferation was significantly higher in the absence of serum than in its presence.[16]

We found that autologous chondrocytes maintained their phenotype only when encapsulated in alginate. Seeding of chondrocytes in high-density monolayers resulted in cell dedifferentiation characterized by changes in cell shape; the chondrocytes became elongated and revealed a fibroblast-like morphology. The presence of serum, mini-insulin-transferrin-selenious acid, or growth factors was not able to prevent or even delay these changes. This phenomenon has not been described in the literature, and perhaps the explanation may lie in the mechanisms that control cell-cell interaction. When ACI cells are seeded on collagen membrane,[14] in pellets,[16] or in alginate (as in the current study), they do not undergo observed phenotypic changes, suggesting that cell-matrix interaction provides a more natural environment for cells and warrants the stability of their chondrocytic phenotype.

Contrary to previous studies with primary chondrocytes,[4,19] we found a synergistic effect of combined growth factor therapy only on chondrocyte proliferation and new proteoglycan synthesis, whereas morphometry and immunohistochemistry results indicated a higher matrix formation, collagen type II and fibronectin accumulation in beads treated with OP-1 rather than with combined OP-1 and IGF-1. Of note, an overall amount of matrix deposited by autologous chondrocytes under either treatment was at least 10-fold smaller than that deposited by primary articular chondrocytes,[4] suggesting a reduced synthetic activity of ACI cells that underwent initial expansion with subsequent culture. Evidently, matrix proteins other than proteoglycans contribute the most to the overall matrix deposition by autologous chondrocytes prepared for implantation in the presence of growth factors.

Apparent contradictions between metabolic data and matrix analysis could be explained by the following. First, IGF-1 is known for its mitogenic activity and the ability to primarily induce proteoglycan synthesis.[12,20,22,28] Therefore, when combined with OP-1, the effect is synergistic with regard to cell proliferation and proteoglycan synthesis. This is supported by our earlier findings in which OP-1 had a stimulatory activity on IGF-1 signaling[17-19] and by our current hypothesis that OP-1 could restore the responsiveness of chondrocytes to IGF-1. Second, the ability of IGF-1 to stimulate other matrix components may be weak, especially in ACI cells, and hence their synthesis could be driven only by OP-1. Third, all of the experiments were performed in the presence of fetal bovine serum, which according to Cartwright and Shah[3] may itself contain up to 100 ng/mL of IGF-1. Therefore, the effect of added recombinant IGF-1 on matrix synthesis may depend on availability of specific receptors. Altogether, further studies are needed to define the most optimal culture conditions for autologous chondrocytes before implantation.

We also anticipated beads treated with the combination of growth factors would show a correlation between the levels of proteoglycan synthesis and aggrecan accumulation. However, immunohistochemical analysis with anti-aggrecan antibody revealed the opposite: there was less aggrecan detected under the combined treatment with OP-1 and IGF-1. We could speculate that this is due to a higher turnover rate of these cells and that elevated proteoglycan synthesis is perhaps accompanied by the enhancement in proteoglycan degradation. In addition, proteoglycans other than aggrecan may contribute to the increased proteoglycan synthesis, which is supported by staining with the anti-decorin antibody. It showed more decorin core protein in the combination group. The differences in responses induced by combined treatment may as well suggest distinct signaling mechanisms that control anabolic effects in primary chondrocytes and cells prepared for autologous chondrocyte implantation, especially after they underwent initial expansion.

Because it is critical for successful healing of cartilage defects to have not only a sufficient amount of proliferating cells but also metabolically active cells that synthesize extracellular matrix specific for articular cartilage, the results of our study suggest a therapeutic potential for growth factor therapy in the treatment of chondrocytes prepared for au-

tologous implantation. However, more studies must be performed to characterize autologous chondrocyte cells, their responses to cyclic load and synovial fluid environment, and the conditions of transplantation. Alternative culture systems (monolayers versus alginate or pellet) should be considered for better preservation of the phenotype in the autologous chondrocytes. Finally, we believe the dedifferentiation of autologous chondrocytes may occur in vivo and lead to the formation of fibrocartilage rather than hyaline cartilage; thus, these issues should be the subject of further investigations.

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