A porous PCL scaffold promotes the human chondrocytes redifferentiation and hyaline-specific extracellular matrix protein synthesis

N. Garcia-Giralt,[1] R. Izquierdo,[2] X. Nogués,[1] M. Perez-Olmedilla,[3] P. Benito,[1] J. L. Gómez-Ribelles,[3,4] M. A. Checa,[1] J. Suay,[3,4] E. Caceres,[1] J. C. Monllau[1]

[1]*URFOA-IMIM, Hospital del Mar, Universitat Autònoma de Barcelona, C/Doctor Aiguader 88, E-08003 Barcelona, Spain*

[2]*Departamento de Ingeniería de Sistemas Industriales y Diseño, Campus Riu Sec, Universitat Jaume I, Castellón 12071, Spain*

[3]*Centro de Biomateriales, Universidad Politécnica de Valencia, C. de Vera s/n, Valencia 46071, Spain*

[4]*Centro de Investigación Príncipe Felipe, Av. Autopista del Saler 16, Camino de las Moreras, 46013 Valencia, Spain*

Received 11 April 2007; revised 18 June 2007; accepted 20 July 2007
Published online 15 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31670

Abstract: The redifferentiation, proliferation, and hyaline-specific extracellular matrix (ECM) protein synthesis of chondrocytes cultured in a polycaprolactone (PCL) scaffold were analyzed. Gene expression of the type II collagen and aggrecan was assessed by real-time PCR in cells from PCL scaffolds, monolayer, and pellet cultures. The proliferative activity was assessed using Ki-67 immunodetection, and the chondrocytic differentiation was evaluated using S-100 immunodetection. The synthesis and deposition into scaffold pores of type II collagen and glycosaminoglycan were analyzed by immunohistochemistry and Alcian blue staining, respectively. All parameters were assessed throughout 28 days of cultures maintained in either fetal bovine serum-containing medium (FCM) or Insulin-Transferrin-Selenium-containing medium (ICM). Expression of the type II collagen gene was lower in FCM cultures than in ICM cultures for all culture systems ($p < 0.05$). Moreover, PCL scaffolds cultured in ICM were able to induce collagen gene expression more efficiently than pellet and monolayer cultures. Aggrecan gene expression did not vary significantly between mediums and three-dimensional system cultures, but in ICM cultures, the monolayer cultures had significantly higher levels of aggrecan gene expression than did either the PCL or pellet cultures. Chondrocytes cultured in PCL scaffolds or pellets with FCM did not proliferate to a great extent but did maintain their differentiated phenotype for 28 days. Levels of cartilage ECM protein synthesis and deposition into the scaffold pores were similar among PCL and pellet cultures grown in FCM and in ICM. In conclusion, chondrocytes seeded into PCL scaffolds, cultured in ICM, efficiently maintained their differentiated phenotype and were able to synthesize cartilage-specific ECM proteins.
 J Biomed Mater Res 85A: 1082–1089, 2008

Key words: scaffold; tissue engineering; biodegradable; PCL; chondrocyte; cartilage; redifferentiation; proliferation

INTRODUCTION

Since articular cartilage has a poor self-healing capacity, damage to chondrocytes and/or to the cartilage matrix can lead to severe joint deterioration. Hence, there is a pressing need to develop therapies for cartilage repair. Tissue engineering comprises the filling of cartilage defects with autologous chondrocytes grown in biodegradable scaffolds that support cell proliferation and/or appropriate matrix formation (i.e., protein synthesis). Arthroscopic implantation may be feasible with material of suitable mechanical properties for a given application. The scaffold must be able to withstand physiological loading until sufficient tissue regeneration occurs. Moreover, the material must be sufficiently porous to allow for effective transport of nutrients. Finally, it should be biocompatible, and degrade as the tissue matrix is produced, leaving only nontoxic degradation products. Artificial scaffolds can be composed of a variety of materials such as the biodegradable polymers, poly-

Correspondence to: N. Garcia-Giralt; e-mail: ngarcia@imim.es

Contract grant sponsor: Spanish Ministry of Science and Education; contract grant numbers: MAT2003-05391-C03, MAT2004-04980-C02

Contract grant sponsor: Catalan Departament de Universitats, Recerca i Societat de la Informació; contract grant number: 2005SGR 00762

Contract grant sponsor: Generalitat Valenciana; contract grant number: ACOMP06/028

caprolactone (PCL) or polylactic acid (PLA). These materials have been shown to support cell attachment, proliferation, and matrix production for a variety of cells, including chondrocytes, osteoblasts, and mesenchymal stem cells.[1–3] PCL is a semi-crystalline material that has good mechanical properties and degrades much more slowly than other polyesters.[4]

Solid scaffolds require *ex vivo* cell seeding, which permits distribution of cells throughout the scaffolds and allows for the synthesis of hyaline-specific extracellular matrix (ECM) proteins. Chondrocytes are frequently expanded in monolayer cultures before being seeded into a scaffold. This step induces the loss of the chondrocytic phenotype.[5] Redifferentiation is achieved by subsequently seeding the dedifferentiated chondrocytes in a three-dimensional environment.[6,7] In addition, Giurea et al.[8] found that retention of cells within PLA matrices was significantly improved by preincubation of the cells in the scaffolds before implantation. Because of the mechanical and degradation properties of PCL,[9] it can be used for long-term *in vitro* cell culture before implantation into the injury site. A PCL scaffold would thus maintain its architectural integrity and mechanical properties during the preimplantation period while chondrocytes are both redifferentiating and synthesizing new cartilage matrix.

Mature hyaline cartilage chondrocytes are characterized by their spherical cell morphology as well as expression of the genes for type II-collagen and aggrecan.[10] New scaffolds must therefore be evaluated for biological parameters including *in vitro* cell attachment, proliferation, gene expression, and matrix deposition.

We have previously described the physical properties of a porous, three-dimensional PCL structure in which the pores are fully connected throughout the foam structure after the filler material has been leached. The resulting motif consists of spherical cavities having a mean size of 130 ± 25 μm, with the same shapes as the original filler beads, and linked to each other by circular throats.[11] The scaffold has a high surface area to volume ratio, which provides a favorable environment for high-density accommodation of chondrocytes, similar to that in pellet cultures.

The aim of this study was to evaluate the capacity of chondrocytes cultured in this PCL scaffold to generate cartilage for future animal implantation. We characterized the scaffold in terms of chondrocytic redifferentiation and synthesis of hyaline-specific ECM proteins.

MATERIALS AND METHODS

Chondrocyte isolation

Human articular cartilage was obtained from osteoarthritic knee joints after prosthesis replacement. Human chondrocytes were obtained from osteoarthritic cartilage. Cartilage was extracted from the finest conserved region of the osteoarthritic knee although it cannot be considered as a normal cartilage.

The study was conducted in accordance with the 1975 Declaration of Helsinki, as revised in 1983, and approved by our local Ethics Committee. All patients submitted written informed consent before their inclusion in the study.

The cartilage was dissected from subchondral bone, finely diced, and then washed with supplemented [100 U penicillin, 100 μg streptomycin (Biological Industries) and 0.4% fungizone (Gibco)] Dulbecco's modified Eagle's medium (DMEM; Life Technologies). Cartilage digestive enzymes were prepared with this medium. For chondrocyte isolation, the diced cartilage was incubated for 30 min with 0.5-mg/mL hyaluronidase (Sigma-Aldrich) in a shaking water bath at 37°C. The hyaluronidase was subsequently removed and 1-mg/ml pronase (Merck, VWR International SL) was added. After 60-min incubation in a shaking water bath at 37°C, the cartilage pieces were washed with supplemented DMEM. After removal of the medium, digestion was continued by addition of 0.5 mg/mL of collagenase-IA (Sigma-Aldrich) in a shaking water bath kept at 37°C overnight. The resulting cell suspension was filtered through a 70-μm pore nylon filter (BD Biosciences) to remove tissue debris. Cells were centrifuged and washed with DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen SA). Finally, the cells were cryopreserved in liquid nitrogen with DMEM containing 20% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich) until use, or plated in tissue culture flasks for immediate chondrocyte culture.

Cell culture in PCL scaffolds

After isolation or thawing, cells were plated in culture flasks at high density in DMEM supplemented with 10% FBS and 50-μg/mL ascorbic acid (Sigma-Aldrich) at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed for every 3 days. After 7–14 days, adherent cells were harvested by incubation with trypsin–EDTA (Biological Industries) and seeded into material premoistened with Hanks' Balanced Salt Solution (Sigma-Aldrich) using an insulin syringe.

PCL samples were placed on a 96-well polystyrene culture plate (Nunc A/S). The samples fit snugly into the wells. After removing the Hanks' solution, porous scaffolds were injected with 500,000 cells in 50 μL of culture medium with 10% FBS and 50-μg/mL ascorbic acid. After cell injection, medium was gently added to ensure that the material was covered. To test only the cells attached onto the sample material, the biomaterials were changed to a new well after 1–2 days. Cells were cultured with DMEM supplemented with 10% FBS [FBS-containing medium, (FCM)] or 1% ITS [ITS containing medium (ICM); BD Biosciences] and 50-μg/mL ascorbic acid according to experimental conditions. The corresponding medium was changed for every 3 days. First or second passage cultures were used in all experiments.

Pellet preparation

After harvesting the cells from the culture flasks, resuspended cells were transferred to a 15-mL polystyrene centrifuge tube (1,000,000 cells per tube), to which culture medium was added until a total volume of 1 mL was reached. The cell suspension was centrifuged for 4 min at 1200 rpm. The resulting pellet was cultured with DMEM supplemented with 10% FBS or 1% ITS and 50-μg/mL ascorbic acid at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed for every 3 days.

Histology

The ability of chondrocytes to synthesize glycosaminoglycan (GAG) in the porous PCL scaffold was monitored by Alcian blue staining at 7, 14, and 28 days of culture, counterstained using Mayer's hematoxylin, and then analyzed by optical microscopy. Briefly, the scaffolds were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek) and cryosectioned (8-μm thick). Cryosections were air-dried and then fixed in acetone for 10 min at 4°C before staining, or were stored at −20°C until use.

Immunohistology

Immunohistological analysis was used to detect the synthesis of type II collagen, the expression of S-100 (a chondrocyte differentiation marker), and the expression of Ki-67 (a proliferation marker). At 7, 14, and 28 days of postseeding, scaffolds were embedded in OCT and cryosectioned (8-μm thick). Cryosections were air-dried overnight and then fixed in acetone for 10 min at 4°C, or were stored at −20°C until use. Sections were incubated for 1 h at room temperature with a 1:100 dilution of type II collagen antibody (Chemicon), 1:100 dilution of Ki-67 antibody (Dako Cytomation), or prediluted S-100 antibody (Dako Cytomation). Antigen–antibody complexes were detected calorimetrically using the EnVision Dual Link Kit (Dako Cytomation) and counterstained with Mayer's hematoxylin.

RNA extraction and real-time PCR

At 7, 14, and 28 days of culture, scaffolds were suspended in 0.5 mL of Tri Reagent (Molecular Research Center) and homogenized by vortexing. The RNA was then extracted according to Tri Reagent manufacturer's instructions. Total RNA was tested by agarose gel electrophoresis, and 7 μL was used to synthesize the DNA complementary strain according to the protocol of TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). The product was diluted by half with RNAse-free pure water, and 1 μL of the resulting solution was used to determine the gene expression of both aggrecan and type II collagen (COL2A1) using quantitative real-time PCR. Real-time PCR was conducted in a volume of 20 μL containing gene-specific Assay on Demand primers, TaqMan-MGB probe, and 10-μL TaqMan Universal PCR MasterMix 2× (Applied Biosystems) reacted in the following sequence: 2 min at 50°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C each cycle in 384-well plates with the ABI PRISM 7900 HT Detection System (Applied Biosystems). The results were analyzed using SDS TM software 2.1 (Applied Biosystems), and the expression levels were calculated against 18S expression and then normalized to an internal sample (relative quantification) using arbitrary units. All real-time PCR reactions for each sample were performed in triplicate. The sample used to normalize each experiment was one pellet at 28 days postseeding that had been cultured in the same experiment and was run in the same PCR plate. Real-time PCR for 18S was carried out under the same conditions, using an 18S endogenous control Assay on Demand (Applied Biosystems).

Data analysis

Quantitative data were analyzed with SSPS 10.0 software. Real-time PCR results were normalized using the endogenous control 18S and the same sample was used for relative quantification. Differences between PCL, pellet, and monolayer cultures were evaluated using the Mann-Whitney *U*-test. The *p*-values less than 0.05 were considered significant. All data were reported as mean ± SD.

RESULTS

Quantification of ECM protein gene expression

To assess the ability of porous PCL scaffolds to promote chondrogenic redifferentiation, we analyzed the expression levels of cartilage-associated genes at days 7, 14, and 28 in cells cultured in three-dimensional scaffold, and in monolayer and pellet cultures (controls) maintained in medium with or without FBS. In serum-free conditions, medium was supplemented with 1% ITS. Both FCM and ICM were supplemented with 50-μg/mL ascorbic acid. Expression of the type II collagen and aggrecan genes was assessed by real-time PCR.

Expression of the type II collagen gene was higher ($p < 0.05$) in all ICM culture systems than in all FCM culture systems at all times of measurement (Fig. 1). In FCM cultures, only the pellet systems induced redifferentiation throughout the time of culture [Fig. 1(a)]. The worst result was found in monolayer cultures, and the expression of the type II collagen gene was null in both PCL and monolayer cultures at 28 days. In contrast, in ICM cultures [Fig. 1(b)], the PCL scaffolds had higher increases in expression of the type II collagen gene than did the pellet and monolayer cultures, which behaved similarly at all times. This result indicates that PCL scaffolds induced chondrocyte redifferentiation more efficiently than did the pellet and monolayer cultures in ICM cultures [Fig. 1(b)].

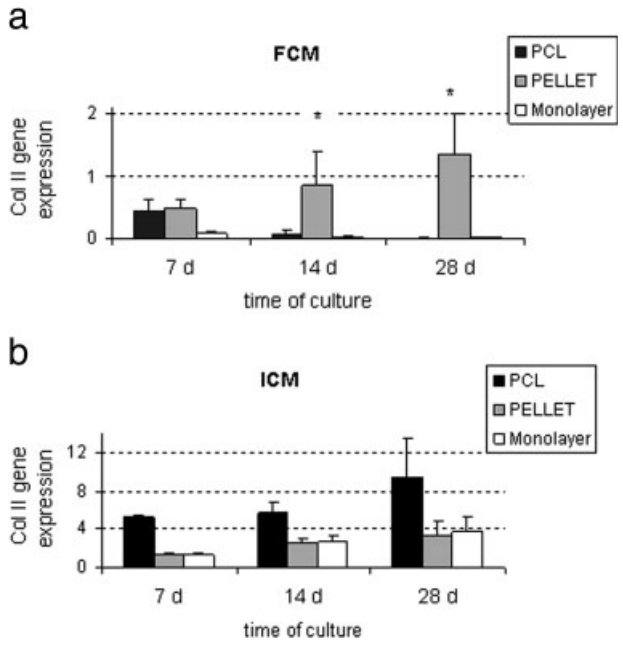

Figure 1. Relative quantification of the expression of the type II collagen gene. The specimens were cultured with (a) FBS-containing medium (FCM) and ascorbate, or (b) ITS-containing medium (ICM) and ascorbate. Note that the *y*-axis has a different scale in each figure. (*) Significant differences among culture systems were found ($p < 0.05$).

Expression of the aggrecan gene did not differ between the respective ICM and FCM cultures of PCL or pellet cultures (Fig. 2), although PCL cultures had half the level of expression than did the pellet cultures. For ICM cultures, monolayer systems always had significantly higher expression levels than did PCL or pellet systems [Fig. 2(b)]. In both culture media, the relative gene expression levels of the three specimen systems (monolayer > pellet > PCL) were constant at all times of measurement.

Analysis of chondrocyte proliferation and differentiation

We characterized the proliferation and differentiation of human chondrocytes cultured in PCL scaffolds, because these parameters determine the suitability of a biomaterial for tissue engineering. The results obtained for PCL systems were compared with those obtained for pellet cultures, which were used as a differentiated positive control. To assess the proliferation of cells cultured in PCL scaffolds and in pellets, we used Ki-67 detection at 7, 14, and 28 days of culture in FCM and in ICM (Fig. 3). For PCL and pellet cultures grown in FCM low proliferation was observed, and for those grown in ICM no proliferation was detected at any time.

Chondrocytic differentiation was evaluated using S-100 immunodetection (Fig. 4). All cultures tested positive at all times of analysis. The PCL chondrocytes were round and formed aggregates within the scaffold pores. The pellet chondrocytes were similar in appearance.

Analysis of the synthesis of hyaline-specific ECM proteins

In addition to quantifying gene expression, we also analyzed protein synthesis of type II collagen and GAG, and subsequent deposition of the proteins into the scaffold pores. Type II collagen was assessed by immunohistochemistry using a specific antibody (Fig. 5), and GAG was assessed by Alcian blue staining (Fig. 6). Pellet cultures were used as a three-dimensional positive control of hyaline-specific ECM proteins. Specimens were cultured in FCM or ICM and were analyzed at 7, 14, and 28 days of postseeding. Both Figures 5 and 6 show histological sections at 7 and 28 days of culture. Protein synthesis and deposition within scaffold pores were found to be similar between FCM and ICM cultures. Moreover, the amount of synthesized matrix in the scaffold was comparable with that in the pellet cultures.

DISCUSSION

Tissue engineering consists of extracting chondrocytes from cartilage by enzymatic digestion, allowing for the construction autologous transplant grafts

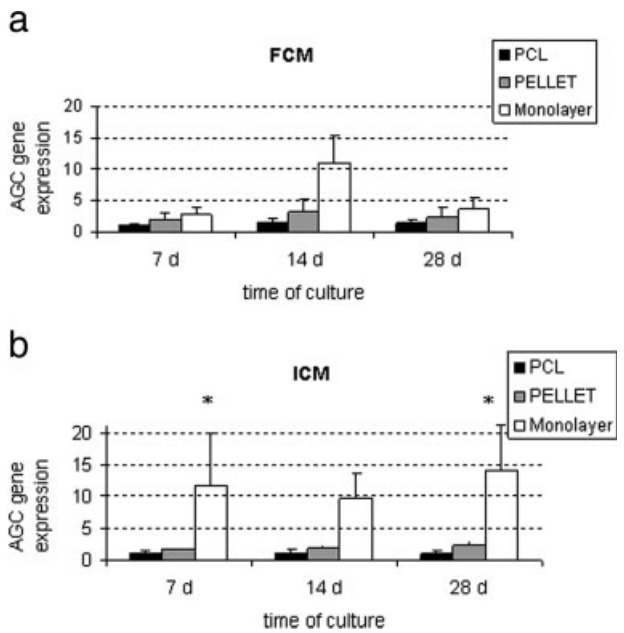

Figure 2. Relative quantification of the expression of the aggrecan gene. The specimens were cultured with (a) FCM and ascorbate or (b) ICM and ascorbate. (*) Significant differences among culture systems were found ($p < 0.05$).

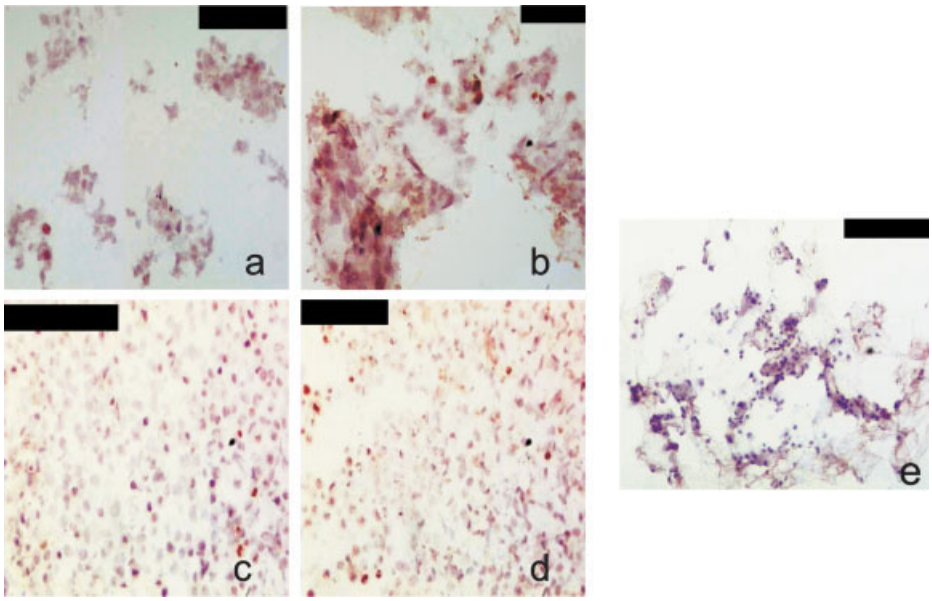

Figure 3. Immunohistochemical staining of Ki-67 on sections of human chondrocytes cultured into PCL scaffolds (a, b) or in pellet (c, d). Specimens were cultured in FBS-containing medium (FCM) at 7 (a, c) and 28 days (b, d) of culture or ITS-containing medium (ICM) at 14 days (e) of culture. Bar scale is 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

starting from minimal donor tissue. The free cells obtained are grown *in vitro* to a desired population level and then seeded in a scaffold, which in turn is transplanted into the defect site to restore normal function. The most favorable scaffold materials are biodegradable synthetic polymers, which offer a controllable degradation rate, high reproducibility, and are readily fabricated to obtain specific shapes and pore sizes. The current study expands upon previous work in which a new, porous PCL scaffold was

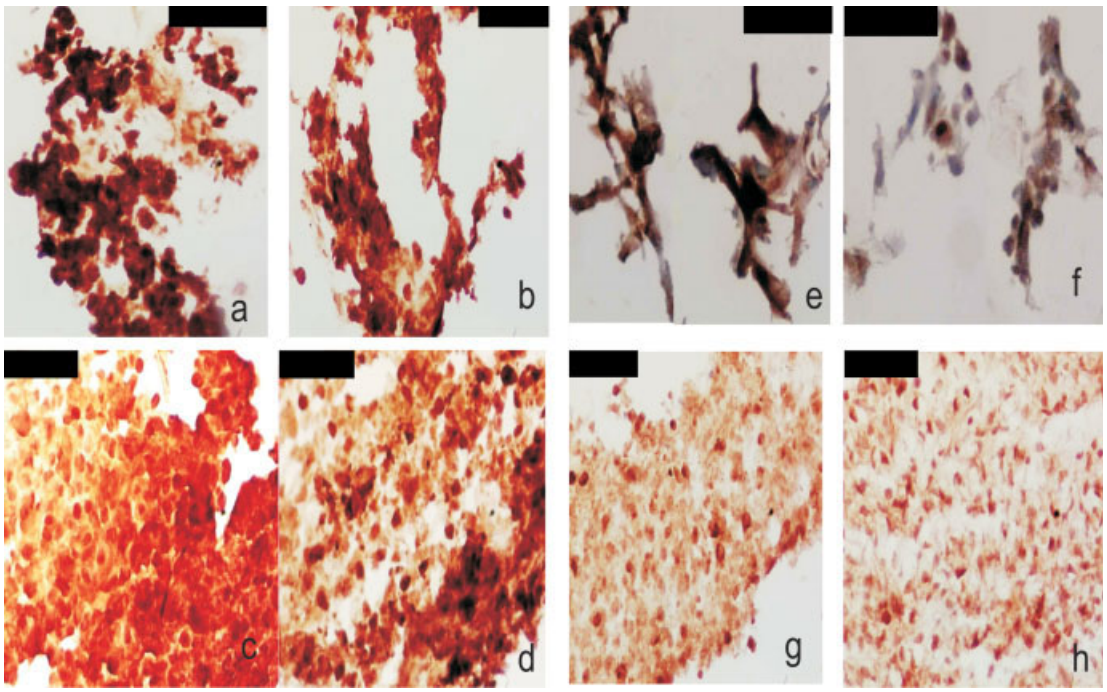

Figure 4. S-100 Immunohistochemical staining of sections of human chondrocytes cultured in PCL scaffolds (a, b, e, and f) or in pellets (c, d, g, and h). Specimens were cultured in FCM and ascorbate at 7 (a and c) and 28 days (b and d) of culture or in ICM and ascorbate at 7 (e and g) and 28 days (f and h) of culture. Bar scale is 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

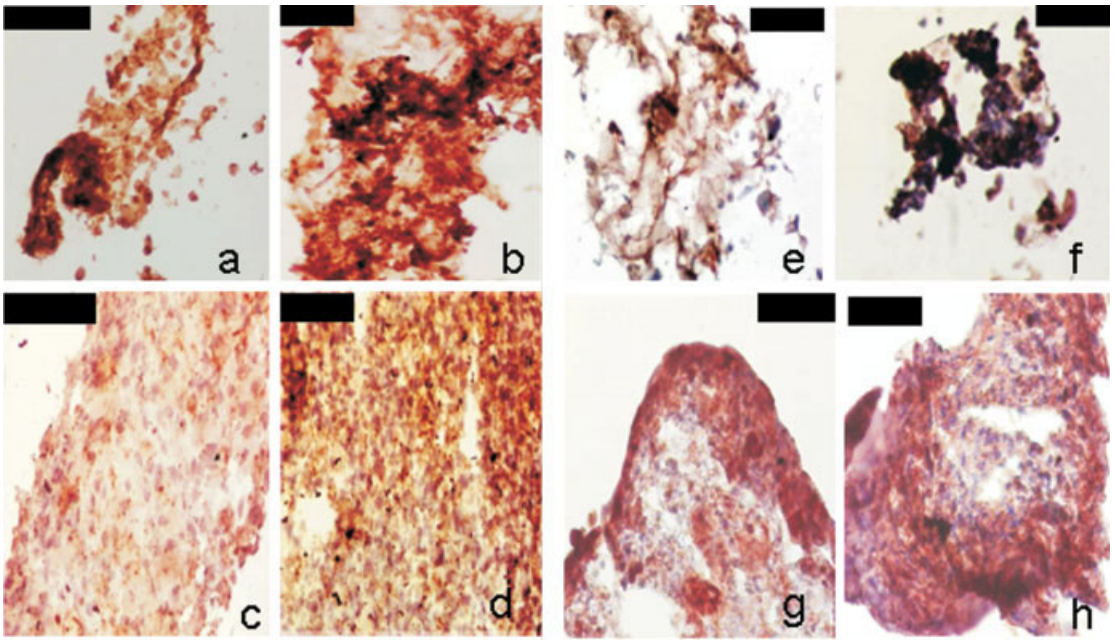

Figure 5. Type II collagen immunohistochemical staining of sections of human chondrocytes cultured in PCL scaffolds (a, b, e, and f) or in pellets (c, d, g, and h). Specimens were cultured in FCM and ascorbate at 7 (a and c) and 28 days (b and d) of culture or in ICM and ascorbate at 7 (e and g) and 28 days (f and h) of culture. Bar scale is 50 μm.

designed and tested for human chondrocyte adhesion and viability.[11] In the present study, this PCL scaffold was evaluated for cell proliferation and the synthesis of hyaline-specific ECM proteins. Pellet culture was used as a positive control for chondrocyte redifferentiation. Currently, the most common method to promote *in vitro* chondrogenesis of mesenchymal stem cells is to maintain them as a high-density pellet culture.[12] Pellets are formed by centrifugation, which compresses the cells into a high-density environment to promote cell–cell interaction, mimicking the cellular condensation observed in precartilage during embryonic limb bud development.[13] We performed staining and immunohistology of PCL and pellet cultures in parallel using the same methodology. Monolayer cultures were used as a negative control for chondrocyte differentiation, and as a positive control for proliferation. Numerous

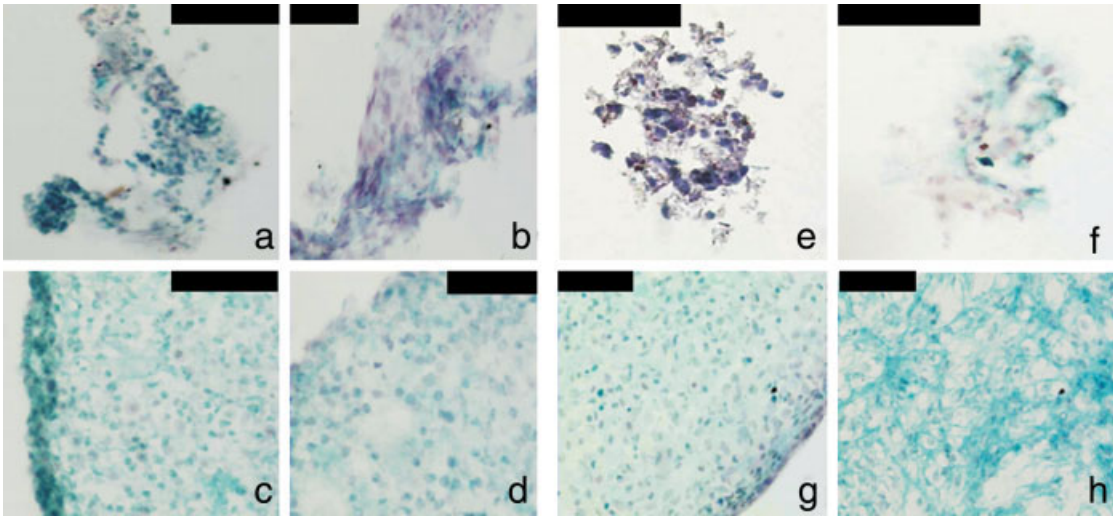

Figure 6. Alcian blue stained and hematoxylin counterstained histological sections of human chondrocytes cultured in PCL scaffolds (a, b, e, and f) or in pellets (c, d, g, and h). Specimens were cultured in FCM and ascorbate (a, b, c, and d) or ICM and ascorbate (e, f, g, and h), and GAGs were analyzed at 7 (a, c, e, and g) and 28 days (b, d, f, and h) of culture. Bar scale is 50 μm.

cells can be quickly generated with monolayer cultures. However, chondrocytes cultured in monolayers tend to dedifferentiate because of cytoskeletal modifications resulting from the two-dimensional culture environment.

Chondrocytes cultured in scaffolds are characterized by production of tissue-specific ECM proteins. Production of tissue-specific ECM proteins can be evaluated at the gene transcriptional level and at the protein synthesis level. A positive correlation between these two levels was found in previous studies.[14]

Our results show that a three-dimensional environment can significantly improve the redifferentiation capacity of human chondrocytes. However, for cultures grown in FCM, chondrocytes could only redifferentiate in a pellet culture system, as determined by measurements of type II collagen gene expression. Alternatively, all culture systems could redifferentiate when grown in ICM, wherein the greatest redifferentiation was found for PCL cultures. Although pellet cultures grown in FCM did have increases in the expression of the type II collagen gene, we observed much higher increases for those grown in ICM suggesting that the expression is enhanced by the presence of ITS.

Despite the gradual disappearance of gene expression in PCL-cultured cells over time, we observed by immunodetection the accumulation of type II collagen in the pores of PCL scaffolds grown in FCM at 28 days of postseeding. This suggests that whatever small amount of type II collagen mRNA that had been detected was translated into protein early on. This protein was in turn deposited into the pores, where it remained throughout the duration of the culture period. Since immunohistochemistry is not quantitative, we could not determine which culture system or medium provided the most efficient synthesis of cartilage ECM proteins. Only gene expression could be quantified at each time of measurement. Therefore, we can hypothesize that among the experimental conditions tested, PCL scaffolds cultured in ICM provided the best support for chondrocytes.

The expression of the aggrecan gene did not differ significantly among the FCM cultures, although the maximum gene expression was found in monolayer cultures, and pellet cultures had twice the level of aggrecan expression than did PCL cultures. The levels of aggrecan gene in ICM cultures were similar to those in the FCM cultures, but the monolayer cultures had significantly higher levels than did the three-dimensional cultures. Interestingly, aggrecan gene expression was independent of culture medium for PCL and pellet cultures. This result is in concordance with the fact that no differences in the levels of GAG deposited in the PCL scaffold pores were found by histological staining between cultures grown in FCM and those grown in ICM at 28 days of culture. These results are corroborated by the findings of Grunder et al.,[15] who studied cells cultured for 2 weeks in FCM. The authors found that type II collagen transcription was higher in cells embedded in alginate beads than in cells grown in a monolayer. In contrast, they did not observe any difference in expression of the aggrecan gene among the culture systems. Finally, they found that cells embedded in alginate beads had a very low rate of proliferation.

The gene expression results show that by culturing the monolayer-grown chondrocytes in PCL scaffolds in ICM, the dedifferentiated chondrocytes can redifferentiate by increasing their production of type II collagen. These results agree with those of previous studies using human chondrocytes encapsulated in alginate beads in ICM.[16]

Serum is a complex supplement containing proteins, growth factors, hormones, trypsin inhibitors, and so forth. Although the major constituents of serum are known such as albumin and transferrin, the exact composition and their effect on cell growth and physiology have not been determined. Therefore, serum can contain several factors that may interfere with normal cellular functions, such as differentiation.[17]

Using a serum-free medium chemically defined allows controlling the experiment parameters more accurately. However, cell cultures in the defined serum-free medium are not generally capable of proliferating or show minimal proliferation activity, likely because of the lack of mitogenic factors present in serum.

The studies of chondrocytes cultured in a three-dimensional environment with ITS presence and low concentrations or without serum show, in general, improved cell differentiation levels than serum containing cultures.[3,15,18]

Our results show that human primary chondrocytes cultured in either PCL scaffolds or pellets in FCM do not have a high rate of proliferation, but do efficiently maintain their differentiated phenotype beyond 28 days, as indicated by the synthesis of S-100 protein. However, chondrocytes in PCL are not able to synthesize type II collagen in long-term cultures with FCM. As reflected by S-100 production, the expression of chondrocytic phenotype after 28 days of postseeding was high in both FCM and ICM cultures, but the expression of the type II collagen gene was particularly striking in ICM cultures. No proliferation was detected for any of the ICM cultures, although ICM did favor the synthesis of cartilage ECM proteins.

Homicz et al.[19] demonstrated that human chondrocytes divided more rapidly in monolayers than in

alginate or resorbable polymer scaffold forms. Tsai et al.[20] obtained similar results for chondrocytes cultured on PCL polymer films, which proliferated at a lower rate than did chondrocytes cultured on polystyrene plates. In this study, the chondrocytes were cultured with 10% bovine calf serum, and the authors observed the same pattern of expression of the type II collagen gene as we did in our study: the expression of type II collagen in PCL substrates that had been observed on day 7 had totally ceased by day 14.

Cells grown in PCL scaffolds are rounded and proliferate slowly. These results suggest that disperse monolayer cells proliferate, whereas rounded three-dimensional cells differentiate. Therefore, the rounded chondrocytes of PCL and pellet cultures switch from a proliferative state to a nonproliferative state. This behavior was observed for both FCM and ICM cultures. The study performed by Li et al.[21] suggests that the suppression of cellular proliferation in PCL scaffolds and pellets results from the use of serum-free medium, and a high cell-density environment appears to favor mesenchymal chondrogenic differentiation.

CONCLUSIONS

We have demonstrated that chondrocytes seeded in PCL scaffolds and then cultured in medium supplemented with ITS and ascorbate efficiently maintained their differentiated phenotype and were able to synthesize cartilage-specific ECM proteins. Based on our experience, we conclude that PCL scaffolds are not a good culture substrate for promoting chondrocyte proliferation, regardless of the growth medium used. Moreover, FCM leads to a loss in expression of the type II collagen gene that is not compensated by chondrocyte proliferation. Therefore, for *in vivo* implant studies, we propose the use of monolayer-expanded chondrocytes cultured in an organized three-dimensional scaffold using serum-free medium supplemented with ITS.

References

1. Agrawal CM, Ray RB. Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. J Biomed Mater Res 2001; 55:141–150.
2. Ishaug SL, Yaszemski MJ, Bizios R, Mikos AG. Osteoblast function on synthetic biodegradable polymers. J Biomed Mater Res 1994;28:1445–1453.
3. Li WJ, Danielson KG, Alexander PG, Tuan RS. Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(epsilon-caprolactone) scaffolds. J Biomed Mater Res A 2003;67:1105–1114.
4. Pitt CG, Marks TA, Schindler A. Controlled Release of Bioactive Materials. New York: Academic Press; 1980, p 19.
5. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, Schlegel J. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002; 10:62–70.
6. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–224.
7. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P. Reexpression of hyaline-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 1994;212:97–104.
8. Giurea A, Klein TJ, Chen AC, Goomer RS, Coutts RD, Akeson WH, Amiel D, Sah RL. Adhesion of perichondrial cells to a polylactic acid scaffold. J Orthop Res 2003;21:584–589.
9. Williams JM, Adewunmi A, Schek RM, Flanagan CL, Krebsbach PH, Feinberg SE, Hollister SJ, Das S. Bone tissue engineering using polycaprolactone scaffolds fabricated via selective laser sintering. Biomaterials 2005;26:4817–4827.
10. Buckwalter JA, Mankin HJ. Articular cartilage: Tissue design and chondrocyte-matrix interactions. Instr Course Lect 1998; 47:477–486.
11. Izquierdo R, Garcia-Giralt N, Rodriguez M, Cáceres E, García SJ, Gómez Ribelles JL, Monleón M, Monllau JC, Suay J. Biodegradable PCL scaffolds with an interconnected spherical network for tissue engineering. J Biomed Mater Res A, 2007. Forthcoming.
12. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998;238:265–272.
13. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 2000; 8:309–334 [Review].
14. Saldanha V, Grande DA. Extracellular matrix protein gene expression of bovine chondrocytes cultured on resorbable scaffolds. Biomaterials 2000;21:2427–2431.
15. Grunder T, Gaissmaier C, Fritz J, Stoop R, Hortschansky P, Mollenhauer J, Aicher WK. Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. Osteoarthritis Cartilage 2004;12:559–567.
16. van Osch GJ, van der Veen SW, Verwoerd-Verhoef HL. In vitro redifferentiation of culture-expanded rabbit and human auricular chondrocytes for cartilage reconstruction. Plast Reconstr Surg 2001;107:433–440.
17. Jakob M, Démarteau O, Schäfer D, Hintermann B, Dick W, Heberer M, Martin I. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001;81:368–377.
18. Kisiday JD, Kurz B, DiMicco MA, Grodzinsky AJ. Evaluation of medium supplemented with insulin-transferrin-selenium for culture of primary bovine calf chondrocytes in three-dimensional hydrogel scaffolds. Tissue Eng 2005;11:141–151.
19. Homicz MR, Chia SH, Schumacher BL, Masuda K, Thonar EJ, Sah RL, Watson D. Human septal chondrocyte redifferentiation in alginate, polyglycolic acid scaffold, and monolayer culture. Laryngoscope 2003;113:25–32.
20. Tsai WB, Chen CH, Chen JF, Chang KY. The effects of types of degradable polymers on porcine chondrocyte adhesion, proliferation and gene expression. J Mater Sci Mater Med 2006;17:337–343.
21. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 2005;26:599–609.