Evidence for redifferentiation of human chondrocytes grown on a hyaluronan-based biomaterial (HYAFF®11): molecular, immunohistochemical and ultrastructural analysis

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Abstract

Association of biomaterials with autologous cells can provide a new generation of implantable devices for cartilage repair. Such scaffolds should provide a preformed three-dimensional shape and prevent cells from escaping into the articular cavity. Furthermore, these constructs should have sufficient mechanical strength to facilitate handling in a clinical setting and stimulate the uniform spreading of cells and their phenotype redifferentiation. The aim of this study was to verify the ability of HYAFF®11, a recently developed hyaluronic-acid-based biodegradable polymer, to support the growth of human chondrocytes and to maintain their original phenotype. This capability was assessed by the evaluation of collagen types I, II and aggrecan mRNA expression. Immunohistochemical analyses were also performed to evaluate collagen types I, II and proteoglycans synthesis. A field emission in lens scanning microscopy was utilized to verify the interactions between the cells and the biomaterial.

Our data indicate that human chondrocytes seeded on HYAFF®11 express and produce collagen type II and aggrecan and downregulate the production of collagen type I. These results provide an in vitro demonstration for the therapeutic potential of HYAFF®11 as a delivery vehicle in a tissue-engineered approach towards the repair of articular cartilage defects. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Articular cartilage is a complex, highly organized tissue whose functional biomechanical properties are associated with its structural architecture [1]. Under healthy conditions, it functions as a load-bearing, wear-resistant material in synovial joints. Despite its durability and ability to maintain itself, mature articular cartilage is vulnerable to injuries and diseases (e.g. osteoarthritis and rheumatoid arthritis) that lead to irreparable tissue damage [2,3] causing disability and pain, due to its poor regenerative ability [4–6]. The repair tissue is often fibrocartilaginous and, over time,

tends to deteriorate into fibrous tissue with poor mechanical properties. The limited ability of injured cartilage to self-repair, combined with its tendency to deteriorate and to frequently cause clinical symptoms, has prompted the investigation of new methods for joint surface reconstruction. Conventional treatment using debridement and various methods of penetrating subchondral bone produced various outcomes since the resulting repaired tissue is fibrocartilage which lacks the appropriate biochemical and biomechanical properties of normal hyaline articular cartilage [7]. Consequently, new approaches in articular cartilage repair like transplantation of heterologous or autologous chondrocyte and periosteum, perichondrium or osteochondral grafting, have been explored [8–10]. Human cultured autologous chondrocyte transplantation is already being carried out in Europe and in the United States, after

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Brittberg et al. first performed this method in patients with deep cartilage defects in the femoral–tibial articular surface of the knee joint [11]. This technique, consisting in the injection of cultured chondrocytes into the area of the defect, involving a tight fixation with periosteal flap harvested from the proximal medial tibia, has been criticized [12,13]. To overcome the difficulties related to the surgical procedure and to find a method of maintaining chondrocytes in the area of the defect without outflow of the cells in the articular cavity, new approaches using different biomaterial carriers as scaffolds onto which cells are seeded, have been studied. Fibrin [14], polymers of polyglycolic and polylactic acids, alginate and collagen gels [15–17] and Gelfoam (a purified gelatin sponge) [18] are some examples of three-dimensional matrices tested in different animal models. However, not all these systems present an appropriate stability to create cartilaginous structures and, moreover, they are not always suitable to support the growth of chondrocytes expressing the original phenotype.

In particular, chondrocytes secrete cartilage-specific molecules including collagen types II, IX, XI and type X in the hypertrophic zone, and the proteoglycan aggrecan [19]. When grown in monolayer cultures, they lose their differentiated phenotype and become dedifferentiated cells which synthesize molecules normally found in fibroblasts like collagen types I, III and V and the proteoglycan versican [20].

The aim of this study was to verify the ability of a new biomaterial—a hyaluronan-based biopolymer referred to as HYAFF[®]11—in supporting chondrocyte growth and differentiation and thus suitable for use in chondrocytes transplantation procedures. To this end, a series of experiments were carried out using RT-PCR to verify the mRNA expression of collagen types I, II and aggrecan in chondrocytes grown on the biomaterial at 1 h, 1 day, 7 and 14 days after seeding. Moreover, the production of collagen types I, II and total proteoglycans was assessed by immunohistochemical analysis. Field emission in lens scanning microscopy (FEISEM) was utilized to highlight interactions between the cells and the biomaterial at very high magnification.

2. Materials and methods

2.1. Biomaterial

The biomaterial used in this study was a non-woven mesh of hyaluronan benzyl-ester: HYAFF[®]11 [21] derived from the total esterification of sodium hyaluronate (80–200 kDa) with the benzyl alcohol on the free carboxyl groups of glucuronic acid. The fibers were produced by extrusion and had a diameter of approximately 10 μm under dry conditions. The biomaterial was sterilized by γ-irradiation and kindly provided by FAB

S.r.l. (FIDIA Advanced Biopolymers, Abano Terme, Italy).

2.2. Chondrocyte culture and seeding on biomaterial

Human articular cartilage was obtained from the knees of seven patients (mean age 27 yr) with a history of trauma; four out of the seven patients were evaluated by RT-PCR and three by immunohistochemistry.

Chondrocytes were isolated by enzymatic digestion with 0.25% trypsin (Biochrom KG, Seromed[®], Berlin, Germany) at 37°C for 15 min and with 300 U/ml collagenase II (Worthington, Lakewood, NJ, USA) at 37°C for 4 h in Hams's F12 (Biochrom KG).

Digested material was centrifuged at 1000 rpm for 10 min, and pellets were resuspended in complete medium: Hams's F12 supplemented with 10% fetal calf serum (FCS), 1% pennicillin–streptomycin, 1% L-glutamine, 50 μg/ml L-ascorbic acid (Sigma, St. Louis, MO, USA), 1 ng/ml transforming growth factor $\beta 1$ (Calbiochem, CA, USA), 1 ng/ml insulin (Sigma), 1 ng/ml epidermal growth factor (Sigma), and 10 ng/ml basic fibroblast growth factor (Calbiochem) [22].

Cells were first expanded in monolayer cultures for three to four passages for about three weeks in Hams's F12 medium. After this expansion phase, 1×10^6 cells were seeded on HYAFF[®]11 ($1 \times 1 \, cm^2$) in 150 μl of culture medium in Petri dishes (Becton Dickinson, Plymouth, UK). The cells were allowed to adhere for 8 h at 37°C and then 2 ml of Hams's F12 medium was added. The medium was changed twice a week. For mRNA analysis, cultures were harvested 1 h, 1 day, 7 and 14 days after seeding. Moreover, 1×10^6 cells for each patient were collected after the expansion phase right before seeding on HYAFF[®]11.

For immunohistochemical evaluation, the scaffolds carrying chondrocyte cells were harvested at 1 h, 1 day, 7, 14, 21, 28, 40, 50 and 60 days. In one case, freshly isolated chondrocytes were utilized as control to verify the expression and production of collagens and proteoglycans.

2.3. Proliferation test

For each donor, cell proliferation rates within the biomaterial were determined at all the experimental time points in triplicate by 3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide (MTT) (Sigma) mitochondrial reduction, based on the Mosmann original protocol [23].

2.4. FEISEM

Sterilized silicon wafer chips of $3 \times 5 \, mm^2$ utilized as FEISEM specimen holders were coiled up with a thin layer of sterile HYAFF[®]11 and on each device 2×10^5

cells were deposited. The samples were then cultivated for 1 day, 4, 7 and 14 days in Hams's F12 complete medium (Biochrom KG) at 37°C and 5% CO_2. At the end of the growth period, the specimens were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 45 min, post-fixed in 1% osmium tetroxide in Veronal buffer for 30 min, dehydrated in an increasing ethanol series and critical point dried (Critical point dryer CPD 030, Bal-Tec AG, Lichtenstein). Before the FEISEM analysis, all the samples were coated with a 1.5 nm thick Platinum–Carbon film (Pt 80%; C 20%) by means of a multievaporation device Balzers MED 010 (Bal-Tec). The observations were performed with FEISEM Jeol JSM 890 (Jeol Ltd., Tokyo, Japan) at 7 kV accelerating voltage and 1×10–11 A probe current.

2.5. RNA isolation and RT-PCR analysis

Total RNA was extracted from cells cultured on HYAFF®11 scaffolds using the single-step guanidinium thiocyanate–phenol–chloroform method (RNAzolB, Biotecx Laboratories, Houston, TX, USA). Briefly, the scaffolds (four for each patient at each experimental time point) were collected at the different experimental times and were placed in Microcon 100 filtration devices (Millipore, Bedford, MA, USA) and centrifuged at $1500 \times g$ for 5 min in order to remove the liquid medium. Cells were lysed directly in the culture scaffold by addition of 0.5 ml of RNAzol B reagent and total RNA was subsequently isolated according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA per sample with 45 min incubation at 42°C, using Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT, USA) and oligo-(dT) priming. Amplification was performed in a Gene Amp PCR System 9600 thermocycler (Perkin Elmer) for 23–40 cycles of 15 s/95°C denaturation and 30 s/60°C annealing-extension, using recombinant Taq DNA Polymerase (Perkin Elmer) and the specific primer pairs reported in Table 1.

The parallel amplification of cDNA for the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

To enable semiquantitative comparison between samples, serial three-fold dilutions of cDNA (corresponding to 100–1.2 ng of total RNA) were subjected to increasing PCR cycles (23–40) in order to define the linear amplification range for each primer set. All cDNA samples (20 ng equivalent of total RNA) were then amplified at the optimal cycle number for each gene of interest (aggrecan: 26 cycles; collagen I: 29 cycles; collagen II: 35 cycles) and for the housekeeping gene GAPDH (23 cycles). The resulting bands were visualized on a 2% agarose gel stained with ethidium bromide and compared with a 123 bp DNA ladder (Life Technologies Ltd.) to confirm the predicted size. Gel photographs were taken under UV-transillumination using Kodak Digital Science DC120 camera (Kodak, Rochester, NY, USA) and relative levels of PCR products were quantified by densitometry using 1D Image Analysis Software (Kodak). Results were expressed as a percentage of the signal obtained from a parallel amplification for GAPDH in the same RT product.

2.6. Immunohistochemistry

Immunohistochemical staining for collagen type I, II and proteoglycans was performed in engineered cartilage tissue of the three patients studied. Specimens were embedded in OCT, snap-frozen in liquid nitrogen and stored at −80°C. Engineered cartilage tissue was sectioned into 5 μm cryostat sections, air-dried and stored at −80°C. Sections were transferred at room temperature, air dried for 15 min and fixed in acetone at 4°C for 10 min. Air-dried fixed samples were rehydrated and incubated at room temperature for 30 min with monoclonal antibodies (MoAb) anti-human collagen types I, II and anti-human proteoglycans (Chemicon International, Temecula, CA, USA), that were diluted 1:10, 1:10, 1:50, respectively, in 0.04 M pH 7.6 TBS

Table 1
Primer sequences used for reverse transcriptase–polymerase chain reaction

RNA template	Primer sequences	Length (base pairs)	References
Col I A1	5′-AAC GGC AAG GTG TTG TGC GAT G 3′-AGC TGG GGA GCA AAG TTT CCT C	296 bp	Kolettas et al., J Cell Sci 1995;108:1991–9
Col II A1	5′-AAC TGG CAA GCA AGG AGA CA 3′-AGT TTC AGG TCT CTG CAG GT	621 bp	Bonaventure et al., Exp Cell Res 1994;212:97–104
Aggrecan	5′-ATG CCC AAG ACT ACC AGT GG 3′-TCC TGG AAG CTC TTC TCA GT	501 bp	Bonaventure et al., Exp Cell Res 1994;212:97–104
GAPDH	5′-TGG TAT CGT GGA AGG ACT CAT GAC 3′-ATG CCA GTG AGC TTC CCG TTC AGC	190 bp	Blanco et al., J Immunol 1995;154:4018–26

containing 2% bovine serum albumin (BSA) (Sigma). An enzymatic pre-treatment with hyaluronidase 0.1% (Sigma) at 37°C for 5 min was performed only for the immunostaining of human collagen type II.

Slides were washed three times with 0.04 M pH 7.6 TBS and then incubated with goat anti-mouse and anti-rabbit immunoglobulins labelled with dextran molecules–alkaline phosphatase conjugated (Envision, Dako, Carpinteria, CA, USA) at room temperature for 30 min. After three washes with 0.04 M pH 7.6 TBS, the reaction was developed using new fucsin kit (Kit New Fucsin Substrate System, Dako, Carpinteria, CA, USA) in the presence of 5 mM Levamisole (Sigma) to block endogenous alkaline phosphatase.

Slides were counterstained with hematoxylin, mounted in glycerol gel and stored at 4°C for subsequent analysis. Negative controls were performed either omitting the primary antibody (for all the antibodies tested) or using an isotype-matched control. Specific controls were performed using irrelevant monoclonal antibodies.

2.7. Statistical analysis

Statistical computations were performed using CSS Statistica-Statistical software (Statsoft Inc. Tulsa, OK, USA). All values are expressed as the mean $\pm$ SE. Quantitative parameters were compared by means of the Mann–Whitney U-test.

3. Results

3.1. Cell growth and proliferation on HYAFF[®]11

Human chondrocytes cultured on HYAFF[®]11 had a spherical appearance as first observed by light micro-scopy. They gave the biomaterial a greater stability compared to the biomaterial alone, as shown by others [24], due to the production of a newly synthesized extracellular matrix. This was confirmed by MTT testing, which is directly related to chondrocyte activity, and which showed increased values over time, demonstrating a cell proliferation inside this scaffold (Fig. 1).

3.2. FEISEM

At the ultrastructural analysis, performed by FEI-SEM, only a few cells were detectable on the biomaterial surface at 24 h after seeding and all of them appear adherent to the HYAFF[®]11 fibers. At this time, a tapered cell shape was evident with the major axis parallel to the substratum fibers. A marked swelling of the chondrocyte shape is clearly evident in the nuclear region. The free membrane surface of this area presented closely packed microvilli, both in cylindrical and wing-like (ondulopodia) forms. An intense secretion of filamentous fibrils of 70–90 nm diameter characterized by a periodicity of globular structures every 50–90 nm. (Figs. 2 and 3) was also evident on both the free and the adherent surfaces of the cell. In particular, such filaments on the adherent surface constitute a diffuse link between the cell and the biomaterial.

Four days after seeding, all of the HYAFF[®]11 fibers were completely covered by chondrocytes. The tapered cell shape was still maintained but a reduction of most of the wing-like microvilli relative to the global amount of membrane specialization was detectable in about 50% of the cells. On the same elements, the presence of the filamentous secretion appears remarkably reduced but only by the free cell surface (Fig. 4).
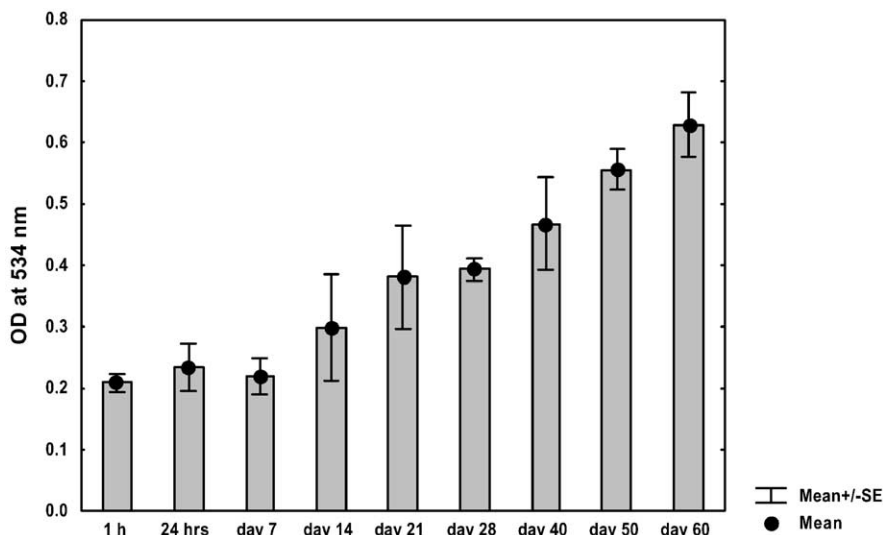

Fig. 1. Proliferation of human chondrocytes on HYAFF[®]-11 at all the experimental time points evaluated as described in Section 2. Data are expressed as mean optical density (OD) $\pm$ SE of one representative patient.
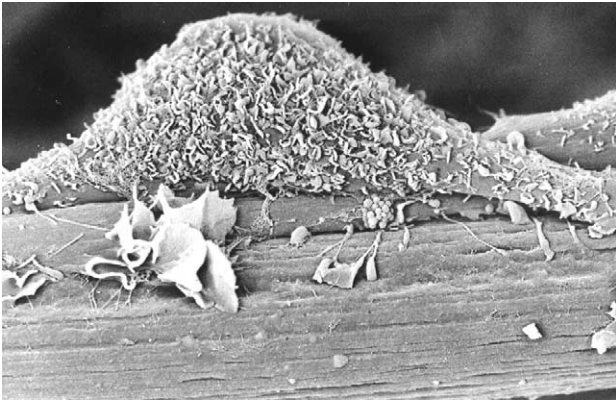
Fig. 2. Field emission in lens scanning microscopy (FEISEM). Image of chondrocytes 24 h after seeding on HYAFF®-11. The round cell shape near the nucleus is evident (×4000).
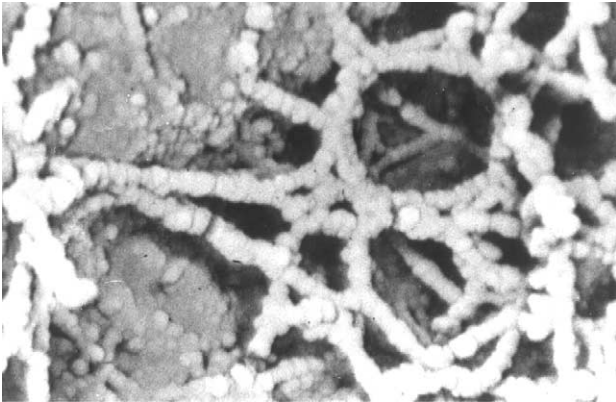

Fig. 3. The same image as Fig. 2. At higher magnification the presence of a filamentous secretion is evident. The surface arrangement of this secretion is characterized by the repetitive and regular alignment of globular structures (×120,000).
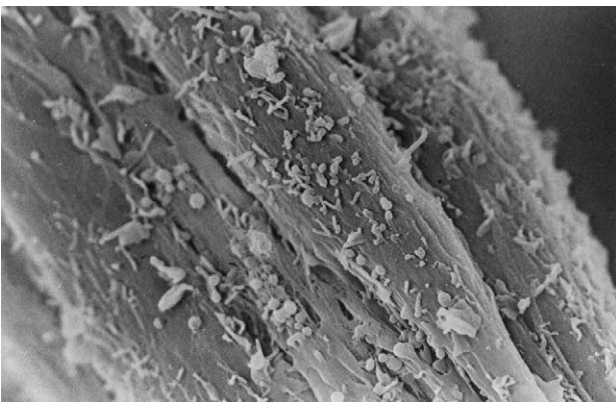

Fig. 4. Chondrocytes on HYAFF®-11 4 days after the seeding. The complete masking of the substratum by the cells is clearly detectable. A reduction of membrane specialization is also evident (×3500).

Seven days after seeding, a progressive increase of cell elements was easily detectable and the cells frequently exhibited the phenomenon of bridging across
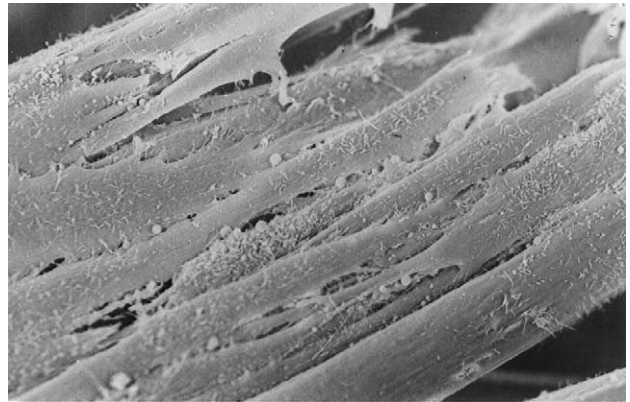

Fig. 5. FEISEM analysis of 7 days seeded chondrocytes. The cell shape appears flattened on the substrate and the amount of microvilli is greatly reduced on most of the cells. The bridging of chondrocytes between different HYAFF®-11 fibers is also evident.

different fibers of the substratum and wide overlapping of the membrane. At this stage of growth, about 75% of cells showed a flat shape with no major axis, the nuclear region was not recognizable and the secretion on the free surface was totally absent. The chondrocytes with such morphology appeared completely depleted of wing-like membrane specialization and even the tubular microvilli showed a reduction in amount. Only 25% of the cells still presented tapered shape and ondulopodia (Fig. 5). At 14 days of growth, the three-dimensional structure of HYAFF®11 was no longer recognizable, being entirely covered by flat and over-lapped chondrocytes widely crossing the biomaterial fibers without interruption. The microvilli distribution pattern resulted in few rarefied and short tubular elements.

3.3. mRNA analysis

In order to investigate temporal changes in collagen types I, II and aggrecan mRNA expression, scaffold cultured cells were analyzed by semiquantitative RT-PCR at the different time points. In all four patients under study, collagen I mRNA decreased from the initial hours to days 7 and 14 (Fig. 6). On the contrary, collagen II mRNA had a very low expression until day 7 but increased from day 7 to 14 (Fig. 7), closely resembling the expression noted in normal cartilage (Fig. 8). Aggrecan mRNA displayed individual trends in the four different subjects (Fig. 9).

3.4. Immunohistochemistry

To verify the retention of the chondrocyte phenotype, the neosynthesis of collagen type II was also confirmed by immunohistochemistry together with the analysis of collagen type I and proteoglycans (Fig. 10). In parti-
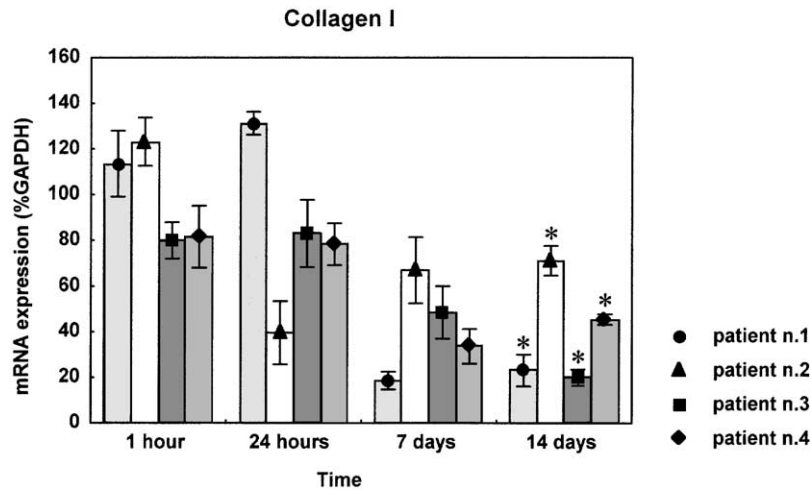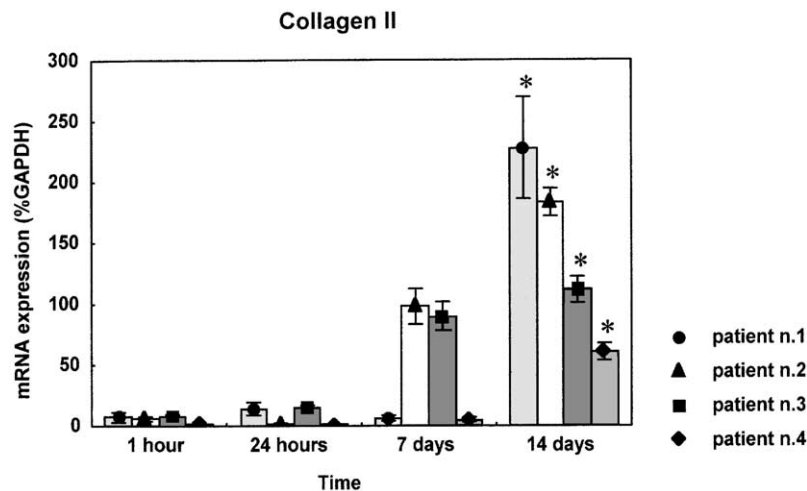
Fig. 6. Time course of collagen I mRNA expression analyzed by semi-quantitative RT-PCR in chondrocytes cultured on HYAFF®-11. Results for each of the four patients are from quadruplicate experiments and values are expressed as the mean±SE. *$p<0.05$ vs. 1 h.

Fig. 7. Time course of collagen II mRNA expression analyzed by semi-quantitative RT-PCR in chondrocytes cultured on HYAFF®-11. Results for each of the four patients are from quadruplicate experiments and values are expressed as the mean±SE. *$p<0.05$ vs. 1 h.

cular, collagen type II, which was produced by freshly isolated chondrocytes, was reexpressed by the cells that had been grown first in monolayer cultures and then seeded and grown onto the biomaterial. However, this expression was evident only starting from day 28 after seeding with a progressive increase up to 60 days; at this experimental time point, every cell was positive. On the contrary, collagen type I, which was not present in freshly isolated chondrocytes and which was still detectable in the earlier experimental time points in chondrocytes grown onto the biomaterial, became completely undetectable at the end of the culture period (i.e. by day 14). Proteoglycans, largely expressed by freshly isolated chondrocytes, were not produced until day 7 in culture onto the scaffold. Proteoglycans content substantially increased from day 14 to 60. Interestingly,

the size of HYAFF®11 fibers was maintained until day 60 probably due to the production of extracellular matrix that protects the biomaterial from degradation in vitro.

4. Discussion

Here, we report that human chondrocytes grown in a monolayer culture, once seeded onto a three-dimensional hyaluronan-based scaffold (HYAFF®11) re-express their differentiated characteristics. Phenotype instability of mature chondrocytes has been in fact consistently observed when they are isolated from the native cartilage matrix and grown in vitro in monolayer culture: a number of studies have shown that the use of

different biomaterials as carriers for the cells can restore their original characteristics [16–18,25–28]. HYAFF®11 is a biocompatible, biodegradable polymer, derived from the esterification of sodium hyaluronate which is an important component of the extracellular cartilage matrix playing a major role in a number of biological processes. Moreover, it has been shown in some animal species, that hyaluronan may influence the differentiation of chondrocytes in vitro [29] and that hyaluronic-acid-based polymers support osteogenesis and chondrogenesis in vivo [30]. Our results confirm that HYAFF®11 is a biomaterial capable of permitting a redifferentiation process which allows chondrocyte cells to express and produce collagen type II, which is a well-documented marker of hyaline articular cartilage always found in
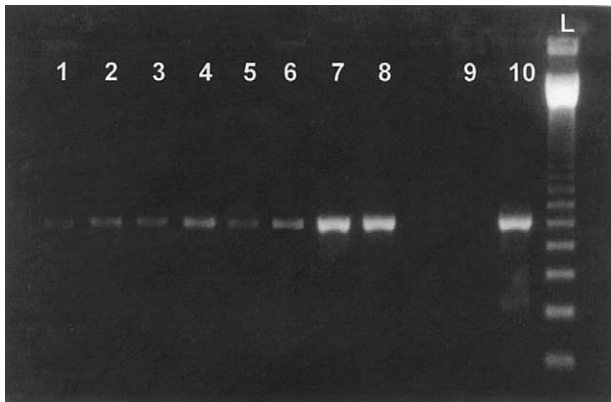

Fig. 8. Collagen II mRNA expression as detected by RT-PCR in chondrocytes cultured on HYAFF®-11 at different experimental times. Representative results from one patient are visualized on a 2% agarose gel stained with ethidium bromide. Lanes 1–2: 1 h of culture; lanes 3–4: 24 h of culture; lanes 5–6: 7 days of culture; lanes 7–8: 14 days of culture; lane 9: negative control (human bone marrow stromal cells); lane 10: positive control (freshly isolated chondrocytes); L: 123 bp ladder.

freshly isolated chondrocytes. This is evidenced by an increase in collagen type II mRNA expression which is followed by the production of the secreted protein, as detected by immunohistochemical analysis. On the other hand, collagen type I, which is mainly expressed by fibroblastic cells and usually found in monolayer cell cultures, is gradually reduced in chondrocytes grown on HYAFF®11 as evidenced by a decrease of mRNA expression and by the decrease of the synthesis of collagen type I protein. Although mRNA for aggrecan is detectable in all the samples evaluated, its expression is largely dependent on patient variability, suggesting that this proteoglycan should not be selected as a marker of chondrocyte differentiation. The expression of collagen types I and II mRNA, which, respectively, decrease and increase until day 14, permits to select an in vivo experimental approach using chondrocytes cultured onto the HYAFF®11 matrix. The choice of utilizing scaffolds cultured for 14 days could be motivated by the strategy of introducing cells which are not fully differentiated into the defect; these cells would be expected to complete their differentiation process in vivo. The results obtained by immunohistochemistry analysis confirmed that, at the time of transplantation, the cells were still undergoing an active differentiation process. In fact, when we analyzed the production of the proteins by means of this technique, we were not able to detect collagen type II during the experimental time points evaluated (i.e. up to day 14). This is probably due not only to the differences in the sensitivity of the two methods employed (i.e. PCR vs. immunohistochemistry), but probably also to a delay between collagen type II mRNA expression and the production of collagen type II protein. In fact, the positivity of cells for collagen type II becomes evident only in the latest stages of the cultures.

The ultrastructural analysis, performed by FEISEM, supports the results obtained by molecular and
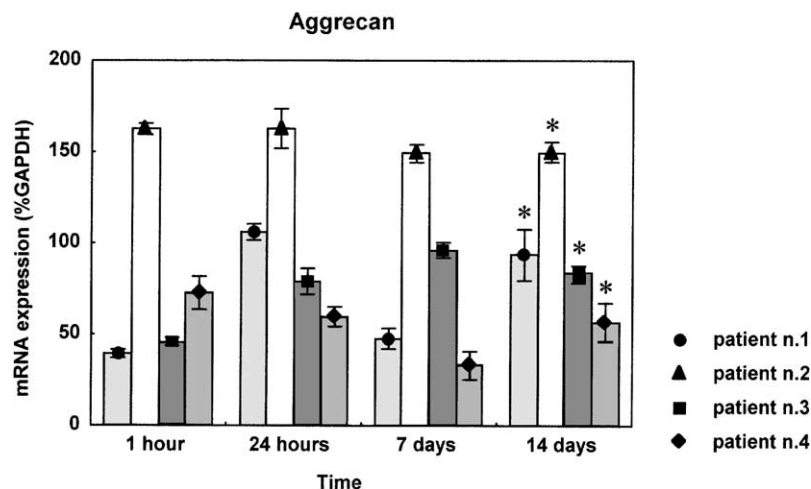

Fig. 9. Time course of aggrecan mRNA expression analyzed by semi-quantitative RT-PCR in chondrocytes cultured on HYAFF®-11. Results are from quadruplicate experiments and values are expressed as the mean±SE. Individual trends are displayed for each of the four patients.
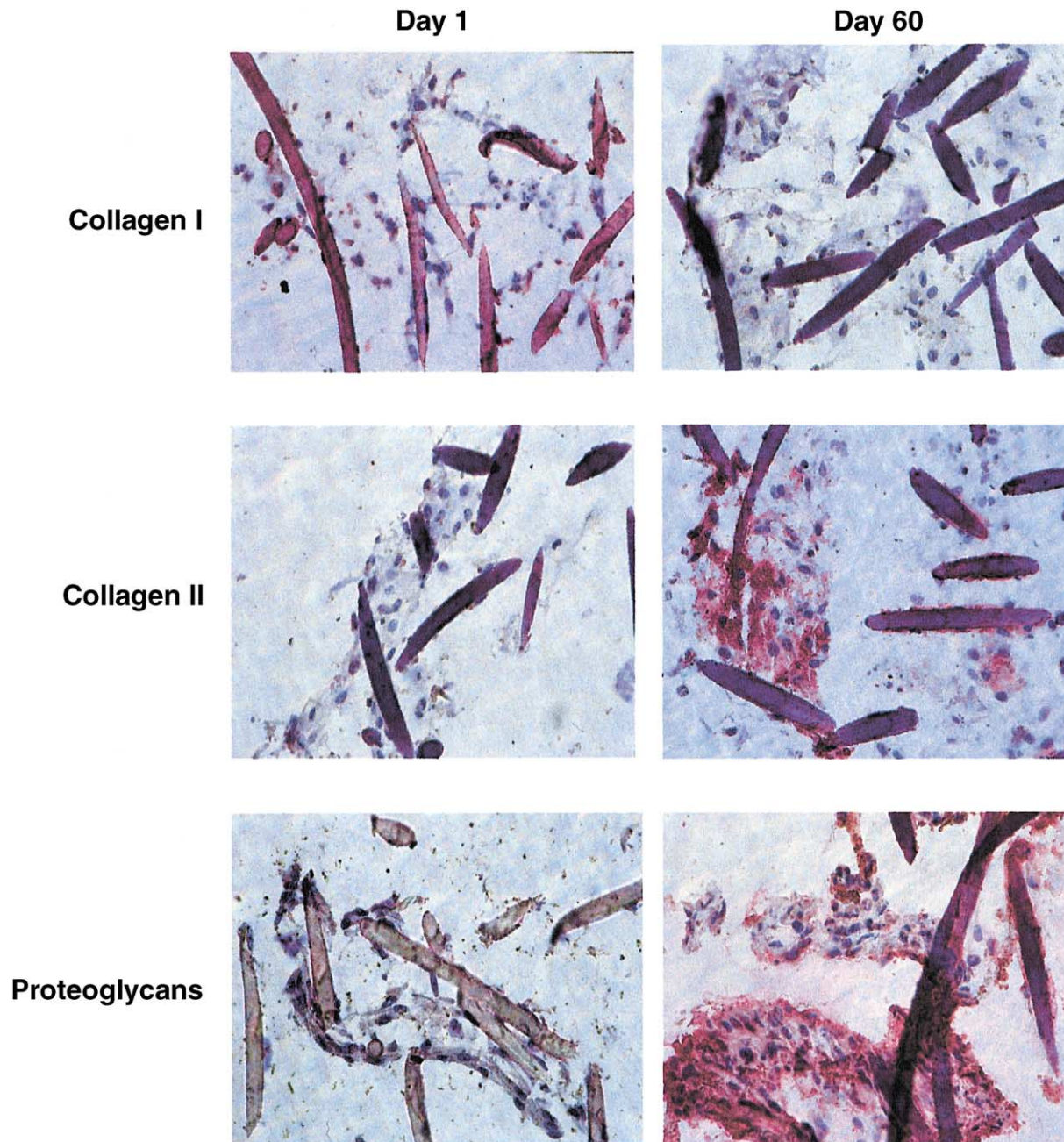
Fig. 10. Collagen I (upper panel), collagen II (middle panel), proteoglycans (lower panel) immunostaining of chondrocytes seeded on HYAFF®-11 at 1 day and at 60 days. The images are from one representative patient. Collagen types I, II and proteoglycans were developed using new fucsin (red color is positive stain).

immunohistochemical evaluations and confirms that the cell shape does not correlate with the expression of the chondrocyte phenotype [31,32]. In our experimental conditions, ultrastructural observations demonstrated that chondrocytes were able to completely colonize the HYAFF®11 scaffold in a couple of weeks and that the synthesis of collagen microfibrils took place at early time points during the process. During this time period, it was possible to observe a deep modification of the ultrastructural features of the cell shape, membrane

specialization and spatial orientation of the collagen secretion. Taken together, the data suggest that the redifferentiation of the chondrocytes was induced by the interaction with HYAFF®11 scaffold.

5. Conclusion

In this study, we showed that three-dimensional HYAFF®11 scaffold, a non-woven biomaterial based

on a hyaluronan benzyl ester, could be a suitable delivery vehicle for human chondrocytes. The cells are able to reexpress their differentiated phenotype once they are grown in this three-dimensional configuration. TheseobservationsprovidearationaleforusingHYAFF®11 scaffolds as a delivery vehicle for chondrocytes in the tissue-engineered clinical approach to the treatment of articular cartilage defects.

Acknowledgements

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