RESEARCH ARTICLE

Effects of Extracellular Matrix on the Morphology and Behaviour of Rabbit Auricular Chondrocytes in Culture

Vega Villar-Suárez,[1] B. Colaço,[2] I. Calles-Venal,[1] I. G. Bravo,[3] J. G. Fernández-Álvarez,[1] M. Fernández-Caso,[1] and J. M. Villar-Lacilla[1]

[1]*Departamento Biología Celular y Anatomía, Facultad de Veterinaria, Universidad de León, 24071 León, Spain*
[2]*Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, 5000-911 Vila Real, Portugal*
[3]*Departamento Bioquímica y Biología Molecular, Universidad de León, 24071 León, Spain*

Isolated chondrocytes dedifferentiate to a fibroblast-like shape on plastic substrata and proliferate extensively, but rarely form nodules. However, when dissociation is not complete and some cartilage remnants are included in the culture, proliferation decreases and cells grow in a reticular pattern with numerous nodules, which occasionally form small cartilage-like fragments. In an attempt to reproduce this stable chondrogenic state, we added a cartilage protein extract, a sugar extract, and hyaluronan to the medium of previously dedifferentiated chondrocytes. When protein extract was added, many cartilaginous nodules appeared. Hyaluronan produced changes in cell phenotype and behaviour, but not nodule formation. Protein extract has positive effects on the differentiation of previously proliferated chondrocytes and permits nodule formation and the extensive production of type-II collagen. A comparison with incompletely dissociated chondrocyte cultures suggests that the presence of some living cells anchored to their natural extracellular matrix provides some important additional factors for the phenotypical stability of chondrocytes on plastic surfaces. In order to elucidate if it is possible that the incidence of apoptosis is related to the results, we also characterized the molecular traits of apoptosis.

INTRODUCTION

The last decade has seen increased interest in the development of bioengineered tissue implants for the replacement of locally damaged structural tissues, with special interest in articular cartilage because of its medical and economic importance.

Auricular cartilage has been much less studied, although it has some medical importance in plastic and reconstructive surgery and can constitute a simpler model for the study of chondrocyte culture in order to obtain a functional substitute. The lack of information includes basic data on auricular chondrocyte behaviour in culture.

Chondrocytes released from cartilage tissue and seeded on plastic surfaces lose their round shape and acquire a fibroblast-like phenotype. When chondrocytes of this shape are released from the plastic substratum and cultured in suspension, the dedifferentiation process reverses [1, 2, 3, 4]. The precise mechanisms controlling the dedifferentiation and redifferentiation processes are not known, but in articular chondrocyte redifferentiation, the differential expression of multiple genes is involved [5].

Extracellular matrix plays a major role in the regulation of cell differentiation in vivo. Some studies have demonstrated that when isolated chondrocytes are seeded onto articular cartilage disks in vitro, with or without living cells, they retain their phenotype and synthesize an appropriate cartilage matrix [6]. Recently, it has been reported [7] that the addition of hyaluronan to human articular chondrocyte cultures simultaneously promotes their proliferation and redifferentiation both on plastic surfaces and in a type-I/III collagen sponge. With the addition of hyaluronan, the cells form three-dimensional nodules as opposed to the monolayer control cells. Therefore, the authors suggest that the addition of hyaluronan ($100\,\mu$g/mL) in chondrocyte culturing might help

Correspondence and reprint requests to Vega Villar-Suárez, Departamento Biología Celular y Anatomía, Facultad de Veterinaria, Universidad de León, 24071 León, Spain, E-mail: vvillsua@uax.es

enhance chondrocyte redifferentiation and, consequently, improve bioengineered cartilage.

To perform experiments with rabbit auricular chondrocytes in monolayer culture, we added simple partially digested fragments or several extracellular matrix derivatives from auricular cartilage: a sugar extract and a protein extract. This report describes the effects that the auricular chondrocytes natural extracellular matrix, thus separated, and hyaluronan have on morphology, proliferation, and redifferentiation of cells in monolayer culture.

It is possible that the incidence of apoptosis is related to qualitative or quantitative differences in the extracellular matrix. In order to get a right cartilage implant, it is important to know if there is a significant percentage of apoptotic cells or not. Apoptosis IS an energy-dependent form of cell death that occurs physiologically during the cartilage development [8]. Since irreversible dedifferentiated chondrocytes cannot be distinguished from cells that still possess chondrogenic potential on the basis of morphological criteria alone, it is essential to identify discriminating molecular markers and to elucidate the signalling pathways involved in irreversible dedifferentiation as a potential means of preventing it. We investigated the presence of apoptosis in chondrocytes maintained in monolayer culture under different circumstances.

We used as apoptosis biochemical marker a mammalian gene product, p53. p53 is a tumor suppressor gene expressed in a wide variety of tissue types and is involved in regulating cell growth, replication, and apoptosis [9].

MATERIALS AND METHODS

Chondrocyte isolation and culture

Rabbit auricular cartilage was obtained from New Zealand white animals aged under two months, from which the perichondrium was carefully removed. Tissue samples were cut into pieces of approximately 1 mm^3. Dissociation was accomplished in the culture medium described below, without fetal calf serum, containing 2 mg/mL collagenase (type II, Sigma) and 0.1 mg/mL testicular hyaluronidase (type IV, Sigma) after 6–8 hours at 37℃.

Half of the cell suspension was filtered through a nylon mesh, centrifuged at 300 xg for 5 minutes, and cells resuspended in the culture medium. The other half, which included remaining small fragments of partially digested cartilage, was simply centrifuged and seeded with these small remnants of the dissociation process.

Cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (Sigma), 0.5 g/L glutamine (Sigma), and 0.5 mL/L of an antibiotic-antimicotic solution (Sigma) in 25-cm^2 tissue culture flasks at 37℃ in a water-saturated atmosphere containing 5% CO_2. The medium was changed every 2-3 days. For the filtered and unfiltered cell cultures, cell concentration in this phase was 10^4 cells/cm^2. The unfiltered ones, had 5–10 small cartilage remnants per flask.

Filtered cells were grown to confluence and then trypsinized, counted, and seeded on Thermanox plastic coverslips (Nunc) at a density of 2.5×10^5 cells/cm^2 on 24 well plates with medium with or without 100 µg/mL hyaluronan (from bovine vitreous humor. Sigma), crude cartilage protein extract, certain subfractions of it (see below), or sugar extract. These subcultured cells were maintained in culture for 4–7 days in order to test the primary effects of the additions.

Preparation of extracts

As control experiments, alliquots of cartilage-free extracts cultures were used. Each experiment was repeated 6 times.

Cartilage extracts were made from triturated rabbit auricular cartilage. The homogenate was centrifuged in PBS at 10 000 rpm for 5 minutes.

For cell-free *sugar extract* preparation, samples were processed according to [10, 11]. After centrifugation for 10 minutes at 50 000 xg: the supernatants were used for the preparation of the crude protein extract and the precipitate resuspended in Tris-HCl 100 mM, pH 7.6. After the addition of 0.1 g of type XXIII protease of *Aspergillus orizae* (Sigma) for each 6 g of resuspended precipitate, the sample was sonicated for 180 minutes to ensure homogeneity during proteolysis. Undigested proteins and peptides were precipitated by using a 5% concentration of tricloroacetic acid; after 30 minutes shaking, samples were centrifuged for 20 minutes at 75 000 xg and the precipitate discarded. Polysaccharides were precipitated by adding KAcO at a final concentration of 0.5 M and 1.5 volumes of isopropanol at −20℃. The samples were maintained overnight at −20℃ and then centrifuged for 20 minutes at 75 000 xg, frozen at −80℃, washed with 80% ethanol, and lyophilized.

Cell-free *crude protein extract* was obtained according to [12]. After centrifugation for 10 minutes at 50 000 xg, the nucleic acids present in the supernatant were precipitated with streptomycin sulphate at 0.75% (w/v) and, after 30 minutes shaking, removed by centrifugation at 50 000 xg for 20 minutes. The precipitate discarded, the supernatant was concentrated by ammonium sulphate precipitation (90% saturation). After 30 minutes shaking, proteins were collected by centrifugation at 75 000 xg for 20 minutes. The supernatant was then discarded and the precipitate obtained from the above centrifugation resuspended in PBS, and the salt removed by passage through a Sephadex G-25 (PD-10) column (Amersham-Pharmacia) equilibrated with PBS. All procedures were carried out at 4℃. The final protein concentration in the desalted extract was measured by Bradford's method using BSA as standard.

Crude protein extract was divided into three subfractions by passages through Centriprep (Amicon) with cutoff membranes of 100 and 30 kd: large (> 100 kd), medium (30–100 kd), and small (< 30 kd) [13]. Using this method, we assumed that large protein extract also

contained some medium and small proteins, medium protein extract included some small but no large proteins, and small protein extract comprised only small proteins. This was confirmed by SDS-PAGE.

Light microscopy

For light microscopy, cells were fixed with ethanol/acetic acid (99:1), dehydrated in ethanol, and stained with hematoxylineosin. The extracellular matrix was stained with toluidine blue and alcian blue 8 GX (Sigma) at pH 1.0.

Indirect immunofluorescence

Indirect immunofluorescence was performed incubating cultures fixed as above and washed, for 2 hours with primary antibodies against collagen II (mouse monoclonal anticollagen II, Sigma, 1:1 500) diluted in PBS. Samples were then washed twice in PBS and incubated for 1 hour with FITC-conjugated secondary antibodies raised in goat against mouse IGM (Sigma), that had been diluted 1:50, washed twice in PBS, and mounted in a nonfluorescing mounting medium (Sigma). Control experiments were carried out on normal auricular cartilage and on chondrocyte monolayer cultures with or without primary antibody.

Cell proliferation assay

Cell proliferation assay was performed by 5-bromo-2′ deoxy-uridine (BrdU) labelling. BrdU (Sigma) was incorporated into DNA in living cultures for 1 hour. BrdU replaces thymidine during the S phase of the cell cycle. Washed and fixed in paraformaldehyde, the cultures were incubated with mouse anti BrdU monoclonal antibody (Sigma), which was detected by a secondary antibody (biotinylated goat antimouse IgG, Sigma) and then conjugated with extravidin-peroxidase (Sigma), marking cells synthesizing DNA. The procedure was carried out according to the supplier's instructions. Monolayer cultures without BrdU incorporation were used as a negative control.

Apoptosis assay

To evaluate cell apoptosis, an imunohistochemistry for p53 was performed. The cells were washed with PBS and fixed with paraformaldehyde at 3%, following a microwave heating pretreatment in EDTA 0.1 mM, pH 8.0, for antigen retrieval.

After endogenous peroxidase activity was quenched, cultures were incubated overnight with primary anti-p53 antibodies (clone Pab 240 – Labvision), and were diluted 1:100 (concentration 2 μg/mL). The primary antibody was detected by using a secondary antibody (biotinylated goat antimouse IgG, Sigma) and then conjugated with extravidin-peroxidase (Sigma). Finally, staining was performed with AEC (Sigma) during 10 minutes. Among all reaction steps, extensive washing with PBS was

performed. As positive control, cells of bladder carcinoma were used.

Thermanox was then mounted for observation. The number of cells that stained positive for apoptosis were counted.

This assay was carried out for all samples: (a) cells filtered through a nylon mesh, (b) not filtered cultures, (c) cultures with addition of crude protein extract, (d) sugar extract, and (e) extracts of large, medium, and small proteins. All of them, before doing the apoptosis assay were maintained in culture for one month.

Scanning electron microscopy

For scanning electron microscopy, cells were fixed with 2% glutaraldehyde and 5% paraformaldehyde in 0.1 M PBS, pH 7.2, for 1 hour at room temperature, dehydrated in graded series of acetone, critical-point dried and sputter-coated, and finally examined in a JEOL JSM-6100 scanning electron microscope.

RESULTS AND DISCUSSION

Chondrocytes from two-month-old rabbits undergo a rapid change in phenotype, termed dedifferentiation, when isolated from cartilage tissue and cultured on tissue culture plastic. These dedifferentiated cells redifferentiate in suspension culture and synthesis of cartilage extracellular matrix molecules reinitiates, but cell proliferation decreases. In articular chondrocytes, this apparently simple process involves changes in the expression of multiple genes [5], indicating that this phenomenon is not so simple. To induce redifferentiation in articular chondrocytes, many authors [14, 15, 16, 17] have studied the effect of various scaffolds; extracellular matrix components [7], or cartilage-specific growth factors [18, 19, 20], applied in osteogenesis as well as bone and cartilage repair [21, 22].

Cells filtered through nylon mesh

Isolated and filtered auricular chondrocytes lost their characteristic round shape (Figure 1a) to acquire a fibroblast-like phenotype on plastic substrata (Figure 1b); cells had a typical spindle-shaped or triangular phenotype with long filopodia (Figure 3a); no special rearrangements of cells (nodules) or differentiated subpopulations (polygonal cells) were found. These dedifferentiated cells attached easily to the substratum to form a confluent and complete monolayer of flattened cells. Only rarely did any three-dimensional growth appear, with the formation of a nodule. No refractile matrix was visible between the cells even in longer culture periods. Metabolic changes in this dedifferentiated state include the switch of type-II collagen to type-I collagen synthesis and a decrease in extracellular matrix proteoglycans. Our cultures of auricular chondrocytes filtered through a nylon mesh reproduced these features.

Indirect immunofluorescence of type-II collagen showed weak or no labelling (Figure 4a). Incubation with BrdU and its detection revealed a high proliferation rate.
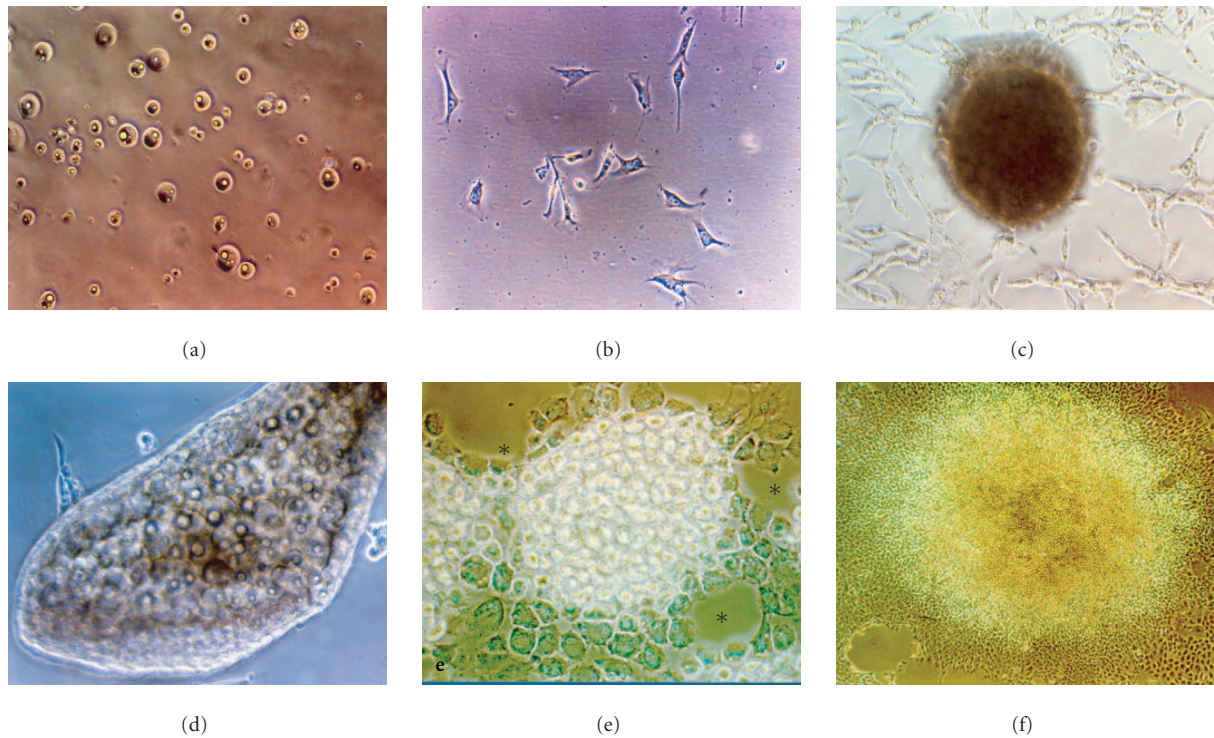
(a)   (b)   (c)

(d)   (e)   (f)

FIGURE 1. Phase-contrast microscopy. (a) Newly isolated chondrocytes filtered through nylon mesh, showing their characteristic round shape in suspension, OM OM × 50. (b) Mesh-filtered cells adopt a flattened fibroblast-like phenotype on plastic substrata, OM × 50. (c) Nodules formed by unfiltered cells, OM × 10. (d) Small cartilage-like masses occasionally formed by nodules from unfiltered cultures, OM × 50. (e) Hollow circular structures (asterisk). (f) In unfiltered cultures, many chondrogenic plates and nodules are surrounded by cells forming a radial pattern, OM × 10.
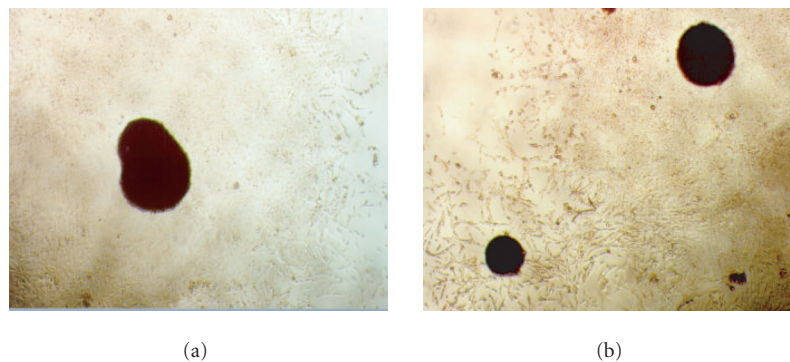

(a)   (b)

FIGURE 2. Phase-contrast microscopy. (a) Nodule surrounded by polygonal cells formed by previously dedifferentiated chondrocytes cultured with crude protein extract, OM × 10. (b) Very small nodules produced by the addition of small protein extract to previously dedifferentiated chondrocytes. Their cells soon detach from the aggregate to remain free in the culture medium, OM × 10.

A mean of 35% of cells were synthesizing DNA in monolayer culture (Figure 4b).

Cells not filtered through nylon mesh

However, when the seeded chondrocytes were not totally isolated and a few remnants of cartilage were included in the cell culture, fewer cells attached to the substratum and less proliferation occurred, so the cells did not cover all the available plastic surface even over longer culture periods of two months. At the same time, most cells showed clear characteristics of a differentiated state (Figures 1c, 1d, 1e, and 1f), usually ten to twenty nodules per culture flask. Occasionally, these nodules can develop small cartilage-like masses.

This behaviour in adult chondrocytes coincides with that reported by [23] in fetal mouse Meckel's cartilage
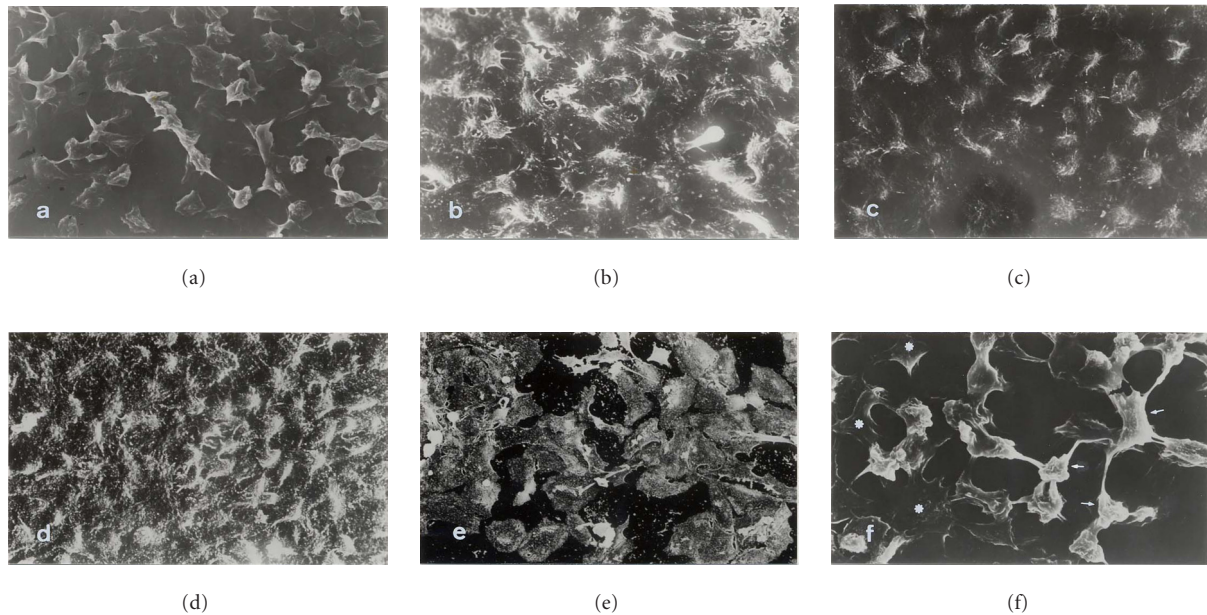
FIGURE 3. Scanning electron microscopy. (a) Subconfluent filtered cells showing a flattened fibroblast-like phenotype on plastic substratum, OM ×250. (b) Hyaluronan addition: chondrocytes grow more three-dimensionally owing to less flattening. Cells are round, with short processes, and are separated by many intercellular spaces, OM ×330. (c) Addition of sugar extract: cells became rounded with short cellular processes, forming a compact monolayer without intercellular spaces, OM ×350. (d) Addition of large protein extract: note cell-rounding, OM ×250. (e) Addition of small protein extract: cells form very small clusters with more intercellular spaces and the appearance of flattened cells, OM ×110. (f) Addition of small protein extract: two different cell morphologies are observed, one of flattened cells (asterisk) and one of widely separated rounded or star-shaped cells with incipient three-dimensional growth (arrows), OM ×350.

chondrocytes in vitro, but in our experiments we saw no signs of terminal transdifferentiation to osteocyte-like cells or extracellular matrix calcification. A similar differentiation programme has been observed on plastic substrata in a rat mesenchymal cell line [24].

Crude protein extract addition

This addition to previously proliferated chondrocytes does indeed replicate the features reported above, with a few differences: nodules are numerous but their size is small, no circular structures are present and no polymorphic cells are observed (Figure 2a).

The main difference is the presence of some small pieces of undigested cartilage containing living cells and extracellular matrix. The maintenance of the chondrogenic differentiation produced by these pieces may depend on cell-cell interactions, cell-matrix interactions, or both. It has been reported [6] that chondrocytes seeded in vitro onto articular cartilage disks, with or without living cells, retain their differentiated phenotype and synthesize cartilage extracellular matrix molecules.

We have observed that a protein extract of this nature can indeed replicate these conditions. Therefore, these results allow us to rule out direct cell-cell interactions between differentiated (cartilage remnants) and isolated chondrocytes as a significant mechanism.

Hyaluronan addition

Chondrocytes were mostly polygonal in optic microscopy but they did not form nodules. Scanning electron microscopy showed them to be relatively rounded (not flattened), with short processes and separated by many intercellular spaces (Figure 3b). However, as they did not form nodules, cultures of 4 to 7 days can be classified as slack monolayers of nonflattened cells with many intercellular spaces.

Indirect immunofluorescence clearly revealed the presence of type-II collagen (Figure 4c). BrdU incorporation detected very low levels of cells synthesizing DNA and, therefore, a very low level of proliferation (mean 2%) (Figure 4d). It is known that hyaluronan is produced by suspension culture articular chondrocytes, that its localization is adjacent to the cells [5], and that proteoglycans synthesized by chondrocytes interact extracellularly with hyaluronan molecules to form large and extremely hydrophilic aggregates; therefore, hyaluronan can increase proteoglycan retention and distribution [25, 26, 27].

Our results differ from those of other studies of articular chondrocytes with regard to proliferation and nodule formation. Ehlers et al [7] describe a redifferentiation effect caused by adding hyaluronan to the culture medium, which includes the formation of nodules growing three-dimensionally out of the monolayer,
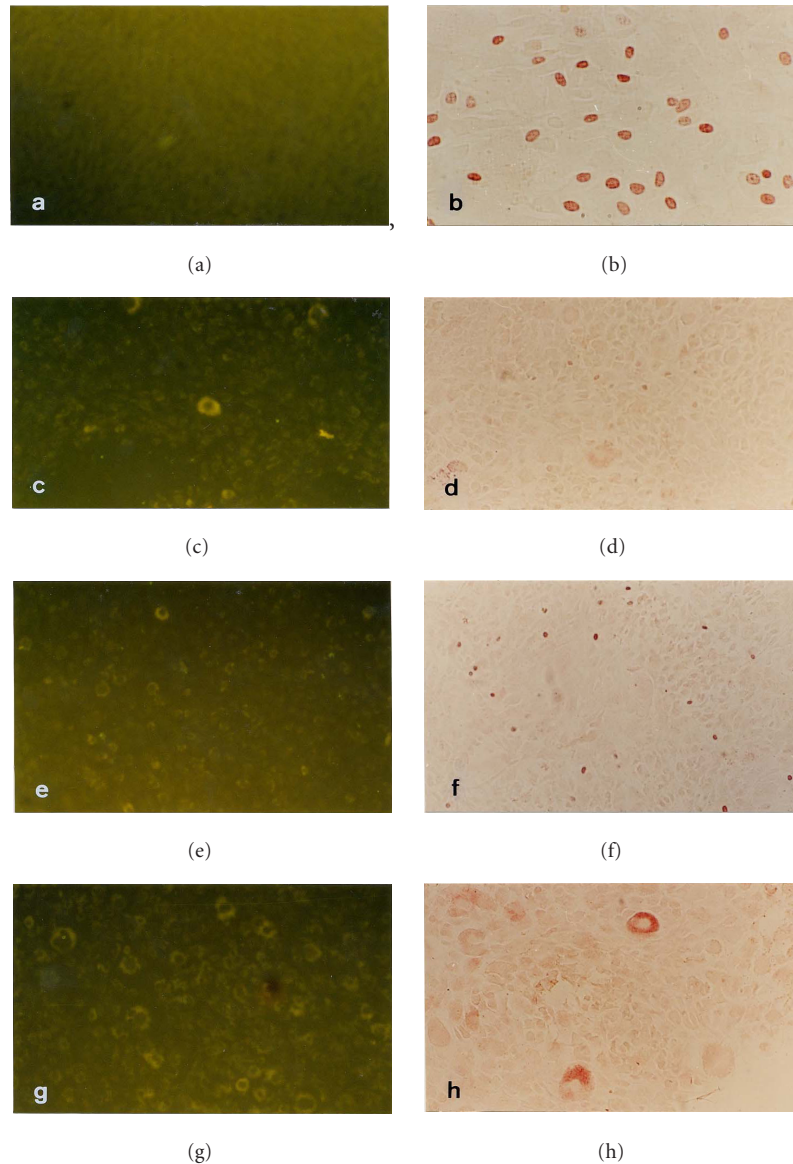
FIGURE 4. Detection of type-II collagen (left) and BrdU (right). (a), (b) Filtered cells. (c), (d) Addition of hyaluronan. (e), (f) Addition of sugar extract. (g), (h) Addition of small protein extract.

and an increase in proliferation at the critical dose of $100\,\mu$g/mL. Also it has been reported [28], that a hyaluronan derivative enhances proliferation. These differences can be first attributed to the distinct type of chondrocytes studied here and species differences. Others [29], report differences in quantity, association with other extracellular macromolecules, and molecular weight between elastic and hyaline cartilage hyaluronan in bovine animals.

However, although our results seem to differ from those of the studies mentioned, in embryo chondrogenesis, mesenchymal cell condensations are associated with a reduction in hyaluronan and an increase in chondroitin sulphate, so hyaluronan blocks condensation formation and its removal permits the development of these structures [30]. Our results are also in agreement with [31] which reports that the digestion of hyaluronan with hyaluronidase induced articular chondrocyte attachment to tissue culture plates, cell aggregation, and fibroblast-like morphology. Yang et al [32] also report that the addition of exogenous hyaluronan to the chondrocyte growth medium decreased cell-substratum adhesion.

Sugar extract addition

Polysaccharides are the least known of matrix components in functional aspects. Polysaccharides such as

agar, agarose, and alginate, have been successfully used to redifferentiate chondrocytes. In our case, proliferation is scarce, and type-II collagen detection suggests a certain redifferentiation. The sugar extract preparation was designed to contain most of the sugars present in the natural extracellular matrix. Thus, hyaluronan and sulphated glycosaminoglycans, such as chondroitin sulphate and heparan sulphate, including some fragments of the proteoglycan central core protein were present.

With this addition, cells became relatively rounded with short cellular processes, but they formed a compact monolayer without intercellular spaces (Figure 3c). Nodules were not found. Therefore, from a morphological point of view, the results of this addition are similar to those of hyaluronan addition, but in the latter case the cells formed a monolayer with very few intercellular spaces.

Type-II collagen was also detected in these cultures (Figure 4e) and cell proliferation was scarce (mean 6%) (Figure 4f). These results indicate that, in relation to intercellular adhesion, addition of the whole sugar fraction has the opposite effect of adding hyaluronan alone. Sulphated glycosaminoglycans may be responsible for this, but other nonproteoglycan polysaccharides may increase cell adhesion and neutralize the effects of hyaluronan. In this regard, Yang et al [32] suggest that imbalances in aggrecan or link protein concentration, or the degradation of hyaluronan disrupt the matrix network and cause the chondrocytes to aggregate or adhere to the plates.

Addition of extracts of large, medium, and small proteins

The experiments described above suggest that the so-called factors synthesized by chondrocytes in cells from nonfiltered cultures are present in the crude protein extract and are not directly related to the sugar fraction of the extracellular matrix. For this reason we divided the proteins of the crude extract into three groups in an attempt to obtain some information on their identity.

In general, the effects of these protein extracts are similar to those reported for crude protein extract addition (those of the large protein extract were identical) (Figure 3d), no circular structures were present and no polymorphic cells were observed.

Differences appeared mainly with small protein extract (Figure 2b), where effects included a very small nodule size, an increase in intercellular spaces in all areas, greater substratum adhesion permitting an almost complete occupation of its surface by cells, and the appearance of flattened cells in considerable quantities. Nodules were numerous, but redifferentiated cells soon separated from one another and detached from the aggregate to remain free in the culture medium. Therefore, these small proteins perhaps can induce to redifferentiate chondrocytes, but to a lesser extent, and the stability of the nodules was very limited.

In general, scanning electron microscopy of cultures with small protein extract added revealed considerable cell-rounding (Figure 3e). However, two different cellular morphologies were observed: one comprising separated flattened cells forming a monolayer, and one of very separated rounded or star-shaped cells with incipient three-dimensional growth (Figure 3f). These were multilayered, forming small aggregates very similar to the initial clusters found in the unfiltered cultures (Figure 2b).

Indirect immunofluorescence of collagen II showed considerable labelling in all the protein extract additions (Figure 4g). BrdU incorporation and its detection revealed low cell proliferation (mean 4.5% in the small protein extract addition) (Figure 4h).

Crude protein extract contains factors active in chondrocyte redifferentiation on plastic surfaces, while the polysaccharide fraction of the matrix seems only partially involved.

Apoptosis

Apoptotic staining is shown in Figure 5. In cells filtered and maintained a month in culture, 11% of the total cells counted (450) were positive (Figure 5a). In cells not filtered the apoptotic range was 97.5% positive and 2.5% negative (Figure 5b). The results obtained with the addition of crude matrix extract revealed weak or no labelling (Figure 5c). Cultures with sugar extract and hyaluronan addition showed a value of 8% positive cells (Figure 5d). The addition of large, medium, and small proteins results were very similar to not filtered cultures (3% positive) (Figure 5e).

In short, the differentiated chondrocyte phenotype is unstable and difficult to maintain in vitro. Although this difficulty is overcome by such culture conditions as suspension or pellet cultures, the problem persists on plastic surfaces. These cultures are possible with embryonic cells [23], or with mesenchymal stem cells [24], but for tissue engineering purposes, adult chondrocytes are more interesting. In this way, we report that adult auricular chondrocytes can be maintained in the differentiated state and redifferentiated on plastic surfaces.

Current knowledge suggests that the ability of grafted chondrocytes to survive and produce cartilage in vivo is related to their differentiation at the time of implantation. It is therefore essential to determine the culture conditions and their effects prior to implantation.

It is known that differentiated chondrocytes synthesize a cartilage-specific pericellular matrix, which consists primarily of type-II collagen and cartilage-specific proteoglycans. This matrix, as well as several specific substances, such as growth factors and adhesion molecules, are required for chondrocyte differentiation and survival [33]. Interactions between chondrocytes and their surrounding matrix play an essential role in maintaining the differentiated cell phenotype. They are mediated by specific surface receptors and integrins, the latter being largely of the ß1-family [34, 35, 36]. Some of them may act as
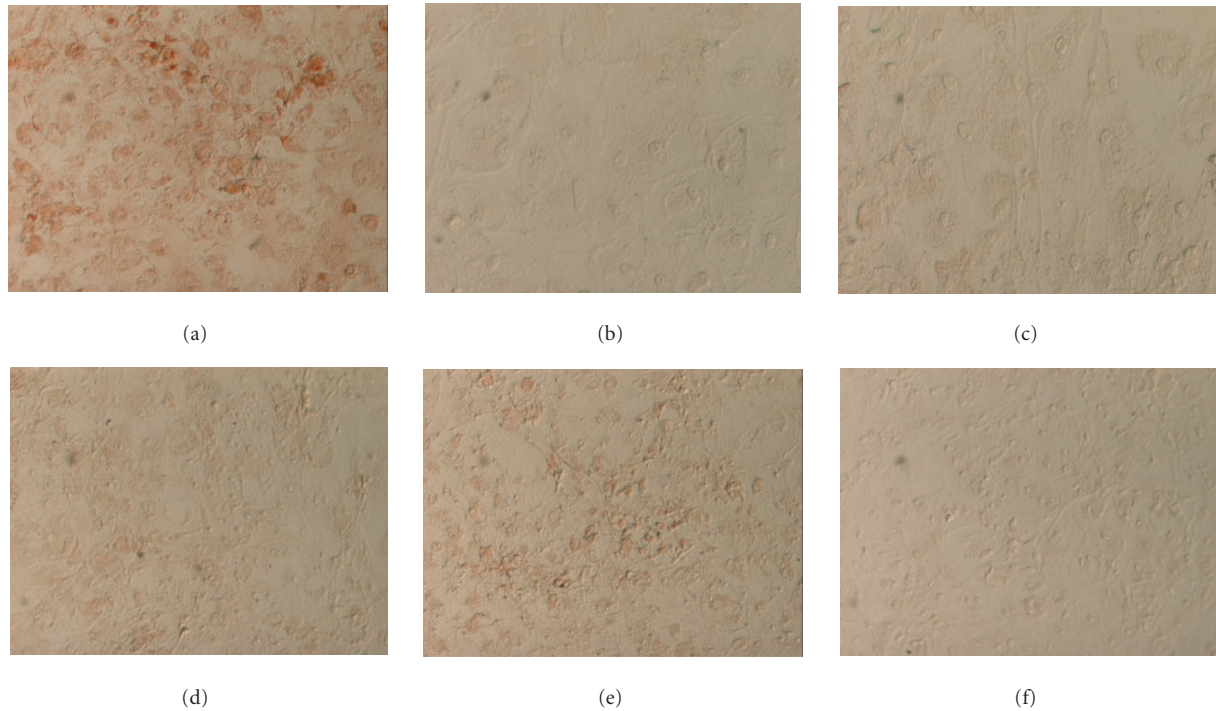
(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 5. Identification of p53 as a biochemical marker of apoptosis. (a) Cells filtered: 11% of the total cells counted (450) were positive. (b) In cells not filtered the apoptotic range was 97.5% positive and 2.5% negative. (c) Addition of crude matrix extract revealed weak or no labelling. (d), (e) Cultures with sugar extract and hyaluronan addition showed a value of 8% for positive cells. (f) The addition of large, medium, and small proteins results were very similar to those of not filtered cultures (3% positive).

TABLE 1. Role of extracellular matrix components on morphology (fibroblastic and round cells), proliferation (BrdU incorporation), and redifferentiation (colagen II production) of auricular cultured chondrocytes. Determination of apoptosis using p53 as a biochemical marker.

	Type-II collagen production	Cell proliferation (BrdU)	Morphology	Apoptosis
Filtered cells	+	+++++	Fibroblastic	11 %
No of filtered cells	+++++	++	Round	2.5 %
Cell-free sugar extract	++	+++	Fibroblastic	8 %
Hyaluronan	+++	++	Round	8 %
Cell-free crude protein extract	++++	++	Round	2 %
Cell-free small protein extract.mw < 30 kd	+++	++	Round	3 %

a specific receptor for type-II collagen in chondrocytes [37], and there are also indications that the integrin-type-II collagen interaction suppresses chondrocytes apoptosis [38]. We have previously shown that the differentiation was higher in cultures where collagen type-II was present (cells not filtered, extract protein addition), and also the apoptosis mean was very low. Now, our results have shown that in addition, the rate of apoptosis was decreased.

This study provides some data in this regard. Results indicate that hyaluronan and extracellular matrix sugars round off the shape of dedifferentiated chondrocytes in a first sign of redifferentiation, which is confirmed by the appearance of type-II collagen. Protein extracts also cause nodule formation and the extensive production of type-II collagen. However, comparison with incompletely dissociated chondrocyte cultures suggests that the presence of some living cells anchored to their natural extracellular matrix provides some very important factors for redifferentiation and phenotypical stability.

We summarize these results in Table 1, where we show the role of extracellular matrix components in morphology (fibroblastic and round cells), proliferation (BrdU incorporation) and redifferentiation (colagen II production) of auricular cultured chondrocytes. We have reported too the determination of apoptosis using p53 as a biochemical marker.

ACKNOWLEDGMENTS

REFERENCES

[1] Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 1982;30(1):215–224.

[2] Bonaventure J, Kadhom N, Cohen-Solal L, et al. Re-expression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp Cell Res.* 1994;212(1):97–104.

[3] Schulze-Tanzil G, de Souza P, Villegas Castrejón H, et al. Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* 2002;308(3):371–379.

[4] Villar-Suárez V, Calles-Venal I, Bravo IG, et al. Differential behavior between isolated and aggregated rabbit auricular chondrocytes on plastic surfaces. *J Biomed Biotechnol.* 2004;2004(2):86–92.

[5] Haudenschild DR, McPherson JM, Tubo R, Binette F. Differential expression of multiple genes during articular chondrocyte redifferentiation. *Anat Rec.* 2001;263(1):91–98.

[6] Chen AC, Nagrampa JP, Schinagl RM, Lottman LM, Sah RL. Chondrocyte transplantation to articular cartilage explants in vitro. *J Orthop Res.* 1997;15(6):791–802.

[7] Ehlers EM, Behrens P, Wünsch L, Kühnel W, Russlies M. Effects of hyaluronic acid on the morphology and proliferation of human chondrocytes in primary cell culture. *Ann Anat.* 2001;183(1):13–17.

[8] Horton WE Jr, Feng L, Adams C. Chondrocyte apoptosis in development, aging and disease. *Matrix Biol.* 1998;17(2):107–115.

[9] Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature.* 1991;352(6333):345–347.

[10] Heinegard D, Sommarin Y. Isolation and characterization of proteoglycans. In: Colowick SP, Kaplan NO, eds. *Methods in Enzymology. Vol 144.* Orlando, Fla: Academic Press Inc; 1987:319–371.

[11] Rosenthal RS, Dziarski R. Isolation of peptidoglycan and soluble peptidoglycan fragments. *Methods in Enzymology. Vol 235.* Orlando, Fla: Academic Press Inc; 1994:253.

[12] Humphries DE, Sugumaran G, Silvert JE. Decreasing sulfation of proteoglycans produced by cultured cells. *Methods in Enzymology. Vol 179.* Orlando, Fla: Academic Press Inc; 1989:428.

[13] Bollag DM, Edelstein SJ. *Protein Methods.* New York, NY: Wiley-Liss Inc; 1991, chap IV.

[14] Frenkel SR, Toolan B, Menche D, Pitman MI, Pachence JM. Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair. *J Bone Joint Surg Br.* 1997;79(5):831–836.

[15] Liu H, Lee YW, Dean MF. Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. *Biochim Biophys Acta.* 1998;1425(3):505–515.

[16] Ehlers EM, Fuss M, Rohwedel J, Russlies M, Kühnel W, Behrens P. Development of a biocomposite to fill out articular cartilage lesions. Light, scanning and transmission electron microscopy of sheep chondrocytes cultured on a collagen I/III sponge. *Ann Anat.* 1999;181(6):513–518.

[17] An YH, Webb D, Gutowska A, Mironov VA, Friedman RJ. Regaining chondrocyte phenotype in thermosensitive gel culture. *Anat Rec.* 2001;263(4):336–341.

[18] Kato Y, Iwamoto M, Koike T. Fibroblast growth factor stimulates colony formation of differentiated chondrocytes in soft agar. *J Cell Physiol.* 1987;133(3):491–498.

[19] Galera P, Redini F, Vivien D, et al. Effect of transforming growth factor-β1 (TGF-β1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation process. *Exp Cell Res.* 1992;200(2):379–392.

[20] Okazaki R, Sakai A, Nakamura T, Kunugita N, Norimura T, Suzuki K. Effects of transforming growth factor β s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. *Ann Rheum Dis.* 1996;55(3):181–186.

[21] Nathanson MA. In vitro proteoglycan synthesis in response to extracts of demineralized bone. *Clin Orthop Relat Res.* 1994;299:263–281.

[22] Wang J, Glimcher MJ. Characterization of matrix-induced osteogenesis in rat calvarial bone defects: I. Differences in the cellular response to demineralized bone matrix implanted in calvarial defects and in subcutaneous sites. *Calcif Tissue Int.* 1999;65(2):156–165.

[23] Ishizeki K, Chida T, Yamamoto H, Nawa T. Light and electron microscopy of stage-specific features of the transdifferentiation of mouse Meckel's cartilage chondrocytes in vitro. *Acta Anat (Basel).* 1996;157(1):1–10.

[24] Lunstrum GP, Keene DR, Weksler NB, Cho YJ, Cornwall M, Horton WA. Chondrocyte differentiation in a rat mesenchymal cell line. *J Histochem Cytochem.* 1999;47(1):1–6.

[25] Shimazu A, Jikko A, Iwamoto M, et al. Effects of hyaluronic acid on the release of proteoglycan from the cell matrix in rabbit chondrocyte cultures in the presence and absence of cytokines. *Arthritis Rheum.* 1993;36(2):247–253.

[26] Lindenhayn K, Perka C, Spitzer R, et al. Retention of hyaluronic acid in alginate beads: aspects for in vitro cartilage engineering. *J Biomed Mater Res.* 1999;44(2):149–155.

[27] Kikuchi T, Yamada H, Fujikawa K. Effects of high molecular weight hyaluronan on the distribution and movement of proteoglycan around chondrocytes cultured in alginate beads. *Osteoarthritis Cartilage.* 2001;9(4):351–356.

[28] Patti AM, Gabriele A, Della Rocca C. Human chondrocyte cell lines from articular cartilage of metatarsal phalangeal joints. *Tissue Cell.* 1999;31(6): 550–554.

[29] Wusteman FS, Gillard GC. Hyaluronic acid in elastic cartilage. *Experientia.* 1977;33(6):721–723.

[30] Hall BK, Miyake T. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int J Dev Biol.* 1995;39(6):881–893.

[31] Lee V, Cao L, Zhang Y, Kiani C, Adams ME, Yang BB. The roles of matrix molecules in mediating chondrocyte aggregation, attachment, and spreading. *J Cell Biochem.* 2000;79(2):322–333.

[32] Yang BB, Zhang Y, Cao L, Yang BL. Aggrecan and link protein affect cell adhesion to culture plates and to type II collagen. *Matrix Biol.* 1998;16(9):541–561.

[33] Dessau W, von der Mark H, von der Mark K, Fischer S. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. *J Embryol Exp Morphol.* 1980;57:51–60.

[34] Urist MR. *The Origin of Cartilage: Investigations in Quest of Chondrogenic DNA Cartilage: Development, Differentiation and Growth. Vol. II.* New York and London: Academic Press and B.K. Hall; 1983.

[35] Loeser RF. Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. *Arthritis Rheum.* 1993;36(8):1103–1110.

[36] Dürr J, Goodman S, Potocnik A, von der Mark H, von der Mark K. Localization of β1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. *Exp Cell Res.* 1993;207(2):235–244.

[37] Shakibaei M. Inhibition of chondrogenesis by integrin antibody in vitro. *Exp Cell Res.* 1998;240(1):95–106.

[38] Shakibaei M, Schulze-Tanzil G, de Souza P, et al. Inhibition of mitogen-activated protein kinase kinase induces apoptosis of human chondrocytes. *J Biol Chem.* 2001;276(16):13289–13294.