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Differential expression of membrane-anchored proteoglycans in rabbit articular chondrocytes cultured in monolayers and in alginate beads. Effect of transforming growth factor-β1

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Abstract

Cell-surface proteoglycans (PGs) were extracted with Triton X-100 from rabbit articular chondrocytes cultured in monolayers and in alginate beads. They were first purified on DEAE-Trisacryl columns and the proportion of hydrophobic PGs was determined by both Octyl-Sepharose chromatography and partitioning in Triton X-114. These two methods revealed that the proportion of hydrophobic PGs was higher in monolayer culture system as compared to alginate beads (24 and 15%, respectively). Characterization of the PGs by Sepharose CL 6B gel filtration followed by electrophoresis indicated that the PGs isolated from monolayers were composed of three chondroitin sulfate (CS) PGs (core proteins of 180, 100 and 50 kDa) and a heparan sulfate (HS) PG (core protein of 60 kDa). In the alginate system, CSPGs with core proteins of 180, 45 and 32 kDa were observed, but no HSPG was present. In parallel, the effect of TGF-β on the distribution of membrane-associated PGs was studied. The results showed that the synthesis of cell-surface PGs was stimulated by TGF-β in monolayers whereas it was inhibited in alginate beads, but the amount of hydrophobic PGs was not altered by the growth factor. These data clearly indicate that TGF-β induces a differential expression of the PG families present at the cell surface. Taken together, the results reveal the complex regulation of cell-surface PG distribution, which obviously depends on the culture method used and suggest that rabbit articular chondrocytes may differentially respond to extracellular ligands according to their morphological state and environment.

Keywords: Membrane-anchored proteoglycan; Alginate bead; Articular chondrocyte; (Rabbit)

1. Introduction

Articular cartilage is a specialized connective tissue composed of sparsely distributed chondrocytes embedded in an extracellular matrix that consists largely of a viscoelastic gel of polyanionic proteoglycans (PGs) immobilized within a dense network of collagen fibrils. The most abundant PG species in cartilage are aggrecans which are high-molecular weight molecules (up to $3.5 \cdot 10^6$ Da) consisting of a large number of chondroitin sulfate (CS) chains covalently linked to an axial protein core. These PG monomers can interact specifically with hyaluronic acid to form large multimolecular aggregates [1]. In addition to aggrecans, two members of the small

Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; ECM, extracellular matrix; HS, heparan sulfate; IL-1, interleukin-1; PG, proteoglycan; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGF-β, transforming growth factor-β

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dermatan sulfate (DS) PG family, biglycan and decorin [2] are present in cartilage. Decorin is a multifunctional molecule that binds to a variety of macromolecules: collagens, fibronectin, heparin cofactor II and growth factors. For example, decorin binds to the surface of the collagen fibrils and inhibits collagen fibrillogenesis [3]. Decorin also binds transforming growth factor-beta (TGF-β) and inhibits its mitogenic activity [4]. The precise biological functions and significance of these inhibitory properties in normal cartilage are not yet understood. Biglycan also binds to collagen fibrils and TGF-β, thus competing with decorin.

Several populations or fragments of large aggregating or non-aggregating PGs, together with several species of small PGs have been described in cartilage by various authors (for review see [5]). Further, immunological evidences indicate the presence of another member of the CS/DS PG family [6]. However, little is known about the PGs present at the cell surface of articular chondrocytes. Membrane-associated PGs have been isolated from various other cell types. Plasma membrane-anchored PGs are a family of glycoproteins which include syndecan-1, -2, -3 and -4 [7], glypican [8], betaglycan [9], CD 44 [10] and the melanoma PG named NG2 [11]. Virtually all cells express one or more of these molecules, but the repertoire of cell surface PGs is cell-type specific and often changes during development (for review see [7]). Among them is the heparan sulfate (HS) PG syndecan, which is implicated in a variety of cellular events [7]. Its cell-surface localization and the capacity of HS chains to interact with numerous molecules including cytokines, extracellular matrix proteins, enzymes and proteinase inhibitors strongly suggest that HSPGs are involved in several aspects of cell behaviour [12]. In addition, HS at the cell surface is essential for the receptor binding activities of FGFs [13,14]. There are three known types of association between HSPGs and the plasma membrane: (1) direct intercalation of the core protein into the lipid bilayer; (2) intercalation through a glycosylphosphatidyl inositol (GPI) anchor covalently bound to the core protein; (3) specific or relatively non specific interactions between HS chains and other molecules associated with the plasma membrane. Therefore, the cell-surface PGs of chondrocytes are likely to play a prominent role in the pathological alterations of cartilage, such as those of osteoarthritis. They could release growth factors from endogenous reservoirs, and/or modulate the response of the cells to cytokines. They could also take a part in the activation process of chondrocytes during early events of osteoarthritis.

Some years ago, a study reported the presence of four different families of PGs associated to the cell surface of calf articular chondrocytes cultured in suspension [15]. In the present paper we extent this study to rabbit articular chondrocytes cultured in monolayers compared to tridimensional cultures as this method is more related to the in vivo situation [16]. It was of interest to compare the expression of cell-surface PGs in conditions where the chondrocytes are flattened and in close contact (confluency) to that of cells maintained round-shaped in a 3d-environment, isolated from each other and very slowly proliferating. This article focuses on the structure and the hydrophobicity of membrane-associated PGs. We show that the culture system can influence the type of membrane-anchored PGs present at the cell surface of articular chondrocytes, suggesting that the expression of different PG families depends on the differentiation state of the cells.

Among the factors involved in the regulation of chondrocyte metabolism is TGF-β [17,18], a multifunctional regulator of cellular activity belonging to a large family of polypeptides which regulate cell growth, cell differentiation and cell function [19]. It is well known that TGF-β affects chondrocyte metabolism in regulating cell proliferation [20] as well as extracellular matrix (ECM) synthesis [21–23]. It has been also established that this factor can have opposite effects to Interleukin-1 (IL-1) thus may help chondrocytes restore ECM during osteoarticular diseases [24,25]. It seems therefore interesting to study the effect of TGF-β on the nature and distribution of membrane-associated PGs.

2. Materials and methods

2.1. Reagents

Dulbecco-modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco BRL (Pontoise, France). CelPrep alginate (1.25%

w/v in 20 mM HEPES buffer) was from FMC (Rockland, ME, USA). Collagenase type I from Clostridium histolyticum and proteinase type XXV from Streptomyces griseus used for tissue dissociation were from Sigma (St. Louis, MO, USA). $H_2^{35}[SO_4]$ (25 Ci/mg; 0.9 TBq/mg), [^{125}I]iodine (100 mCi/ml) came from Amersham (Bucks., UK). DEAE-Trisacryl was supplied by IBF (France), Sepharose CL 6B and octyl-Sepharose CL 4B were from Pharmacia Fine Chemicals (France). Phosphatidylinositol-phospholipase C (PI-PLC) from *Bacillus cereus* was from Boehringer Mannheim (Germany), heparitinase was from Seigakaku (Japan), chondroitinase ABC and keratanase were obtained from Sigma. Other chemicals were of analytical-reagent grade and obtained from standard commercial sources. Human recombinant TGF-β was supplied from R and D Systems (MN, USA).

2.2. Chondrocyte cultures and metabolic radiolabeling of proteoglycans

Articular cartilage slices were taken from the shoulders and the knees of 3-week-old male rabbits. Chondrocytes were isolated with sequential digestion of the matrix using pronase (4 mg/ml; 1 h 30 min at 37°C) and bacterial collagenase (1 mg/ml; overnight at 37°C) and cultured in DMEM supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml) and 10% FCS (heat-inactivated for 30 min at 56°C)[26]. The cells were grown at 37°C in a 5% CO_2-95% atmosphere environment with medium change every 2–3 days.

2.2.1. Monolayer cultures

After enzymatic dissociation, cells were plated in 75 cm^2 Falcon flasks at $1.5 \cdot 10^6$ cells/flask. After reaching confluency (6–7 days), the primary cultures were used for experiments in order to avoid dedifferentiation of the chondrocytes. Such primary cultures were previously shown to produce both collagen type II [27] and PGs specific of cartilage [28]. At confluency, cultures were fed with sulfate-free medium containing $H_2^{35}[SO_4]$ (final concentration 20 μCi/ml) for a period of 24 h. For TGF-β treatment, the growth factor was added during the last 48 h of culture at the concentration of 2 ng/ml, which was shown to be the optimal concentration to stimulate PG synthesis [22].

2.2.2. Cell culture in alginate beads

The preparation of chondrocytes in alginate beads was performed as previously described [29]. Briefly, the cells were suspended in the sterile-filtered low viscosity alginate gel (1.2%) at the density of $1.5 \cdot 10^6$ cells/ml, then slowly expressed through a 22-gauge needle in a dropwise fashion into a 100 mM $CaCl_2$ solution. After 4 washes in 0.15 M NaCl and one wash in DMEM medium, the beads were finally cultured in complete culture medium. On day 12, cultures in alginate were incubated with $H_2^{35}[SO_4]$ (20 μCi/ml) for 24 h, in sulfate-free medium. The treatment with TGF-β was identical as that described for the monolayer cultures.

Following the 24-h labeling with $H_2^{35}[SO_4]$, the media from both monolayer and alginate cultures were harvested, but were not analyzed for PG content.

2.3. Extraction of proteoglycans

In the case of alginate cultures, the beads were rinsed carefully three times with PBS. Then three volumes of dissolving buffer composed of 55 mM sodium citrate, 0.15 M NaCl and 25 mM EDTA were added per volume of packed beads. The suspension was incubated at 37°C with gentle agitation until the beads were completely dissolved (approximately 10 min). The cells were then pelleted at $800 \times g$ for 10 min and the pellet washed twice with PBS.

The cell-associated matrix from both monolayer and alginate cultures were first extracted with chondroitin sulfate solution (1 mg/ml in PBS, in the presence of proteinase inhibitors (PI): 50 mM EDTA, 10 mM N ethyl maleimide (NEM), 10 mM benzamidinium chloride, 100 mM 6-aminohexanoic acid, 1 mM PMSF) 30 min at 4°C. After centrifugation for 10 min at $800 \times g$, the supernatant containing the PGs chased by chondroitin sulfate treatment was stored frozen until needed for analysis. The cell pellet was further treated for 15 min at 37°C with 10 mM Tris-HCl, pH 7.5, buffer containing 1% Triton X-100, in presence of PI. After centrifugation, the supernatant containing membrane-anchored material was

stored frozen in the presence of 4 M Guanidine · HCl and 10 mM NEM.

2.4. Qualitative assays for proteoglycans

2.4.1. PG purification and radioiodination

PGs from both fractions (CS chase and Triton X-100 extracts) were purified by anion exchange chromatography on a DEAE-Trisacryl column. Briefly, both extracts were first dialyzed against 10 mM Tris-HCl, 0.15 M NaCl, 0.5% Triton X-100, 8 M urea buffer, pH 6.8, containing PI for 48 h at 4°C. They were then applied to a DEAE-Trisacryl column (1×5 cm) equilibrated in the same buffer. After loading, the column was washed with 10 ml of buffer and eluted with a 0.15–1.2 M gradient of NaCl. Fractions (1 ml each) were collected and radioactivity was measured on all samples. The fractions containing ^{35}S-labeled PGs isolated on DEAE-Trisacryl column were pooled, dialyzed against water and lyophilized. In the case of membrane-associated PGs (Triton extract), the material was dissolved in 500 μl of 50 mM Tris-HCl, 4 M urea, 0.5% Triton X-100 (pH 7.5) containing PI and batch-adsorbed to 150 μl of packed volume DEAE-Trisacryl equilibrated in the same buffer. The gel was washed with 200 mM Tris-HCl buffer, 10 mM octylglucoside (pH 7.5) containing P.I.. Bound material was radioiodinated using chloramine-T, as described [30]. After extensive washes with 200 mM Tris-HCl, 10 mM octylglucoside and with 50 mM Tris-HCl, 4 M urea, 0.5% Triton X-100 containing PI to remove free label, the ^{125}I-PGs were eluted from the beads with the same buffer containing 1 M NaCl. Aliquots were frozen and stored at -20°C until analysis.

DNA content was determined using bisbenzimidazole (Hoechst 33258) as described [31].

2.4.2. Hydrophobic affinity chromatography

Before testing hydrophobicity of purified PG fractions from the Triton extract, Triton X-100 was exchanged for dialyzable octylglucoside detergent. PG samples were dialyzed against 10 mM Tris-HCl buffer, pH 7.2, containing 4 M urea and loaded onto a DEAE-Trisacryl column equilibrated with the same buffer. The column was washed with 20 ml of the same buffer containing 50 mM octylglucoside. Bound PGs were eluted with 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidinium chloride in which 75 mM octylglucoside was added. The samples were dialyzed against 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidinium chloride, mixed with 1.5 ml octyl-Sepharose CL 4B and put overnight on a shaker at 4°C [32]. Octyl-sepharose CL 4B was transfered in a small column and the gel was successively eluted with 15 ml each of: (i) 10 mM Tris-HCl, pH 7.2, 4 M guanidinium chloride; (ii) the same buffer containing 1% Triton X-100.

2.4.3. Triton X-114 partitioning

^{125}I-PGs were subjected to Triton X-114 phase partitioning to isolate hydrophobic PGs, as previously described [33]. Briefly, PGs were diluted into 100 μl of 50 mM Tris-phosphate (pH 7.0), 2% Triton X-114 and were layered over a cushion of 6% sucrose, 0.06% Triton X-114 in 50 mM Tris-Phosphate (pH 7.0). After incubation for 5 min at 35°C, the tube was centrifuged at $1000 \times g$ for 10 min at 25°C. The upper phase was removed, Triton X-114 was added to 0.5% and the mixture was layered again over the original sucrose cushion. After incubation and centrifugation as above, the pellet (10 μl) was retrieved and diluted to 100 μl in 50 mM Tris-phosphate, pH 7.0 (detergent-rich phase). The top phase was also saved and retreated with 2% Triton X-114, incubated and centrifuged. The top phase was collected (aqueous phase).

2.4.4. Gel filtration chromatography

Purified fractions of PGs from CS chase were dialyzed, lyophilized, treated or not with chondroitinase ABC and applied on a Sepharose CL 4B column (0.6×120 cm) equilibrated and eluted in sodium acetate pH 6.8 buffer containing 4 M GuaHCl and PI. The elution was performed at 4°C with a flow rate of 10 ml/h. Blue dextran and phenol red were added to mark the void and total volumes, respectively. Fractions of 1 ml were collected, aliquots of 100 μl were mixed with scintillation cocktail and counted for [^{35}S] dpm.

Samples from the Triton X-114 partitioning were applied in sodium acetate, pH 6.8, buffer containing 4 M GuaHCl and 0.5% Triton X-100 onto columns (0.6×100 cm) of Sepharose CL 6B equilibrated in the same buffer and eluted at 4°C at a flow rate of 12 ml/h. Blue dextran and phenol red were added to

mark the void and total volumes, respectively. Fractions (1 ml) were collected, aliquots of 100 μl were mixed with scintillation cocktail and counted for [^{125}I] dpm.

2.4.5. Enzymatic analysis of proteoglycans

Heparitinase digestions were carried out in 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.01% bovine serum albumine containing PI (50 mM 6-amino-hexanoic acid, 1 mM PMSF, 20 μg/ml leupeptin, 2.5 μg/ml pepstatin A). The enzyme was used at 10 mIU/ml for 2 hrs at 37°C. Chondroitinase ABC and keratanase were used at 0.1 U/ml for 24 h at 37°C. The digestions were carried out in 50 mM Tris-HCl, pH 8.0, 60 mM sodium acetate, 50 mM NaCl, 1 mM sodium fluoride, 0.05% BSA containing PI.

2.4.6. Gel electrophoresis

Enzyme-treated samples were analyzed by SDS-PAGE [34] under non-reducing conditions in 4–15% gradient gels. Molecular weights were determined using prelabeled protein standards (Amersham). Gels were dried and autoradiographed using Kodak X-OMAT AR films at −80°C.

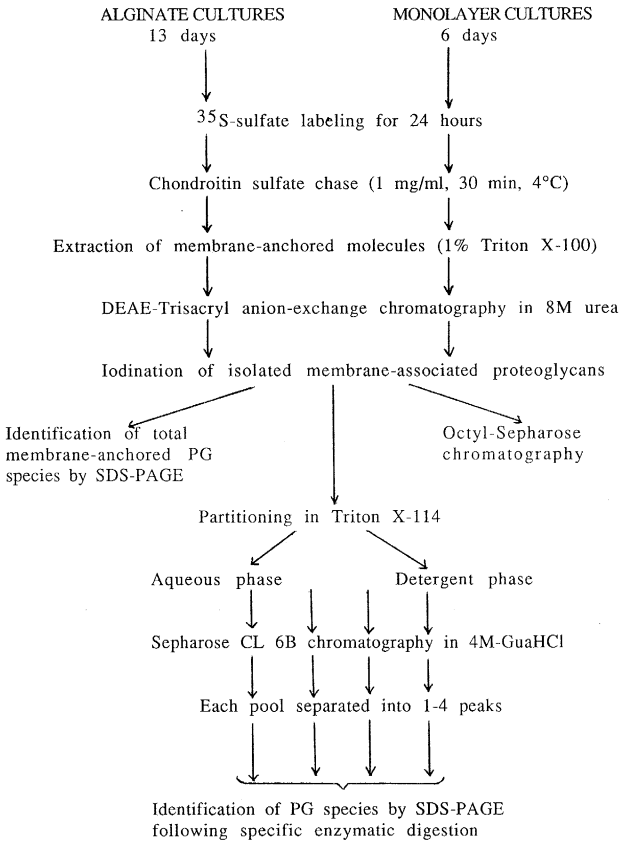

Fig. 1. Flow diagram outlining the experimental protocol used to isolate and characterize membrane-anchored proteoglycans from articular chondrocytes cultured in alginate beads and monolayers.

3. Results

A flow diagram summarizing the experimental protocol used is shown in Fig. 1.

Cells were cultured for 6 and 13 days respectively for monolayers and alginate beads before being metabolically labeled with ^{35}S-sulfate. Concerning monolayer cultures, the studies were performed when the cells reached confluency (6–7 days in culture), corresponding to an optimal matrix protein synthesis. Regarding alginate beads, the analyses were performed after 13 days. Indeed, previous data reported that the optimal PG synthesis occurs in alginate beads after 14 days of culture [35].

The PGs recovered in the citrate extract of alginate beads corresponded to those found in the culture medium when the chondrocytes are cultured in monolayers, e.g. mainly aggrecans, but also small DS/CSPGs. In order to discard most of the aggrecans present in the cell environment, articular chondrocytes were treated with chondroitin sulfate as described in Section 2. The PGs recovered by CS treatment were first isolated on DEAE-Trisacryl column before being submitted to gel-filtration on Sepharose CL 4B column (Fig. 2A and B). The results show that PGs of high molecular weight and susceptible to chondroitinase ABC but not to heparitinase, were recovered by this treatment, indicating that they likely correspond to aggrecans. It was shown that this treatment did not extract small PGs which were still present at the cell surface so that this preliminary extraction step was used to facilitate their recovery.

3.1. Isolation of membrane-associated PGs

PGs from both monolayers and alginate cultures, extracted with Triton X-100, were isolated on

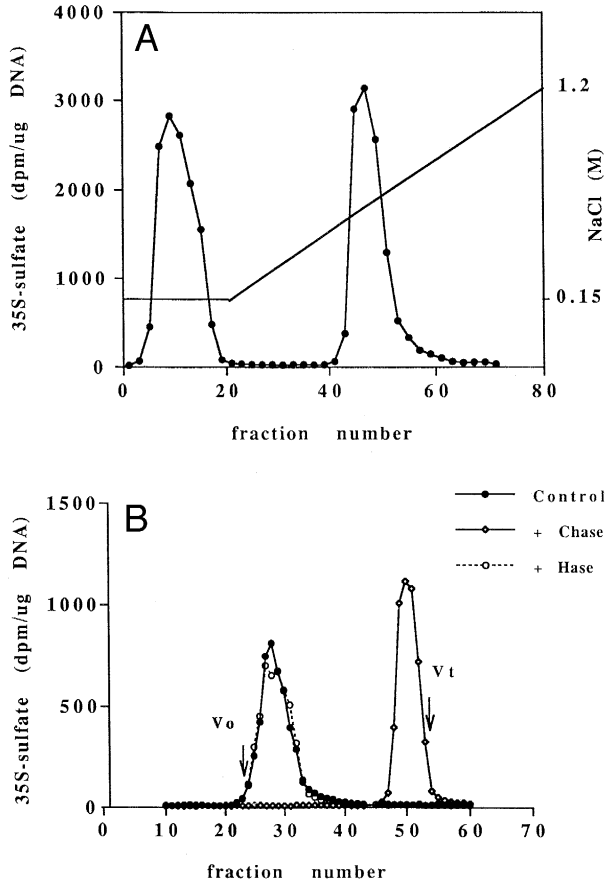

Fig. 2. Anion-exchange chromatography and characterization by gel filtration of PGs from monolayer cultures extracted with chondroitin sulfate chase. The PGs were first isolated on a DEAE-Trisacryl column (1×5 cm) (A) equilibrated in 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 8 M urea buffer containing PI. The PGs were eluted with a 0.15–1.2 M gradient of NaCl. Fractions (1 ml) were collected and radioactivity counts performed on all samples. The PG containing fractions were pooled, dialyzed against water and lyophilized before being digested by specific enzymes (Chase: chondroitinase ABC; Hase: heparitinase) and submitted to gel filtration on a Sepharose CL 4B column (0.6×120 cm) (B) equilibrated in sodium acetate, pH 6.8, buffer containing 4 M guanidine·HCl. Blue dextran and phenol red were added to mark void and total volumes respectively. Fractions were collected, aliquots of 100 μl were mixed with scintillation cocktail and counted for ^{35}S dpm.

DEAE-Trisacryl columns (Fig. 3A and B, respectively). A first peak of ^{35}S-radiolabeled material was observed at the beginning of the elution representing acid or neutral glycoproteins. The PGs from monolayers and alginate beads were both eluted around 0.55 M NaCl.

3.2. Electrophoresis of PGs from the Triton X-100 extract

An aliquot of PGs fraction purified on DEAE-Trisacryl was dialyzed against H_2O, lyophilized and than submitted to SDS-PAGE in 4-15% gradient. Fig. 4A and B showed that the families of membrane-associated PGs are quite different, depending on the type of cell culture used. Articular chondrocytes cultured in monolayer (Fig. 4A) synthesized both heparan and chondroitin sulfate PGs; after digestion by heparitinase, a core protein of ~60–65 kDa appeared while two PGs containing CS chains were also observed with core proteins of apparent molecu-

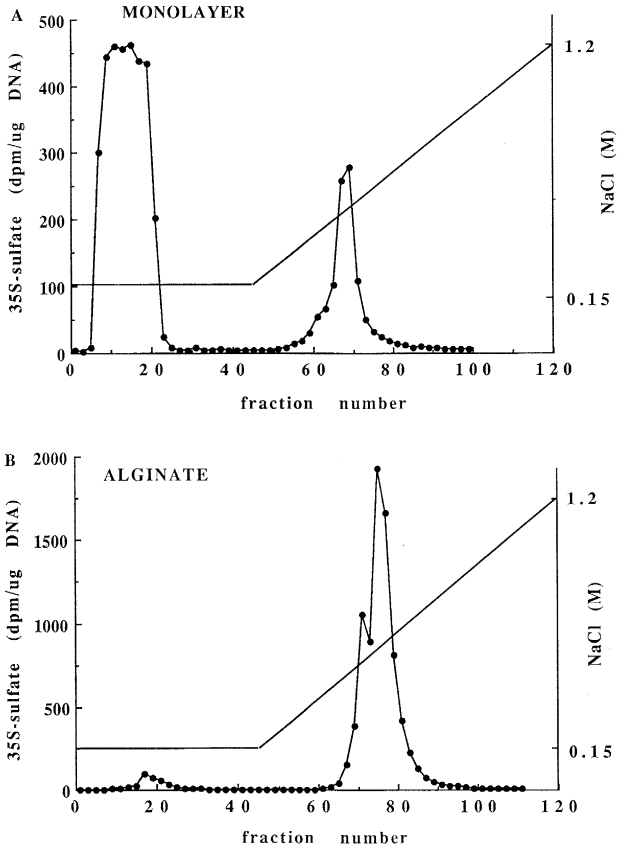

Fig. 3. Anion-exchange chromatography of cell-surface proteoglycans. After Triton X-100 extraction from monolayers (A) or alginate beads (B), the PGs were isolated on a DEAE-Trisacryl column (1×5 cm) equilibrated in 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 8 M urea buffer containing PI. The PGs were eluted with a gradient from 0.15 to 1.2 M NaCl. Fractions (1 ml) were collected and radioactivity counts performed on all samples.

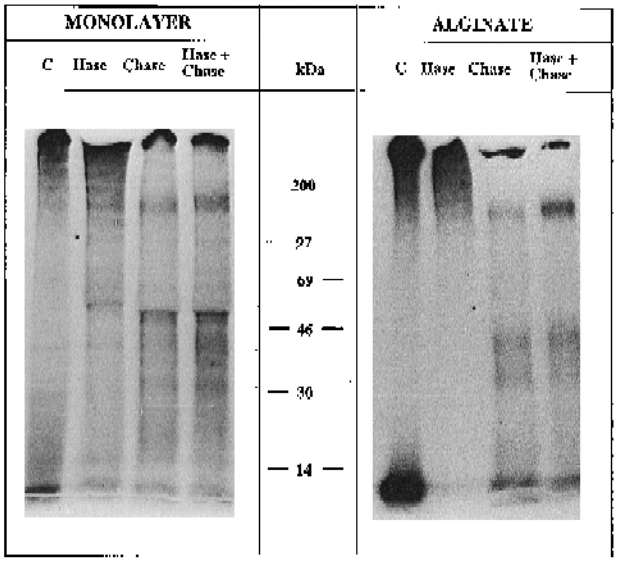

Fig. 4. Analysis of cell-surface proteoglycans by SDS-PAGE. After isolation on DEAE-Trisacryl column, the PGs from monolayers and alginate beads were radioiodinated and digested by specific enzymes (heparitinase: Hase; chondroitinase ABC: Chase) prior electrophoresis on a 4–15% gel under non-reducing conditions. Gels were dried and autoradiographed at −80°C.

lar weight of 180 and 55 kDa. The families of membrane-associated PGs in chondrocytes cultured in alginate beads are quite different (Fig. 4B): in these conditions, the cells synthesized three CSPGs, with high and low molecular weights: 180, 45 and 32 kDa. The high molecular weight CSPG seems to be the same as in monolayer culture, but the small PGs clearly have distinct properties. Therefore, we concluded that culture conditions are determinant for the types of membrane-associated PGs which are produced in vitro by articular chondrocytes.

PGs were also submitted to heparitinase and chondroitinase treatments together in order to see the presence of hybrid PGs such as betaglycan or syndecan. In alginate cultures, the results showed no difference in the pattern of bands obtained with both enzymes together, or with chondroitinase alone; it confirms that these PGs are not expressed at the protein level in our culture system.

In monolayer cultures, the 60 kDa-band observed after heparitinase alone is no longer detected after combined chondroitinase + heparitinase digestions. However, more intense radioactivity level is present around 55 kDa; that may correspond to two types of core proteins bearing CS or HS chains, co-migrating at the same distance.

3.3. Hydrophobic affinity chromatography of membrane-associated PGs

An aliquot of isolated PGs from the Triton X-100 extract was submitted to octyl-sepharose chromatography in order to evaluate the proportion of hydrophobic membrane-associated PGs. Fig. 5A and B show that the proportion of PGs retained on the column and finally eluted with Triton X-100 is different from monolayer to alginate system. In the first case (monolayer, Fig. 5A) 56% of the PGs are retained by octyl-sepharose whereas there are only 32%

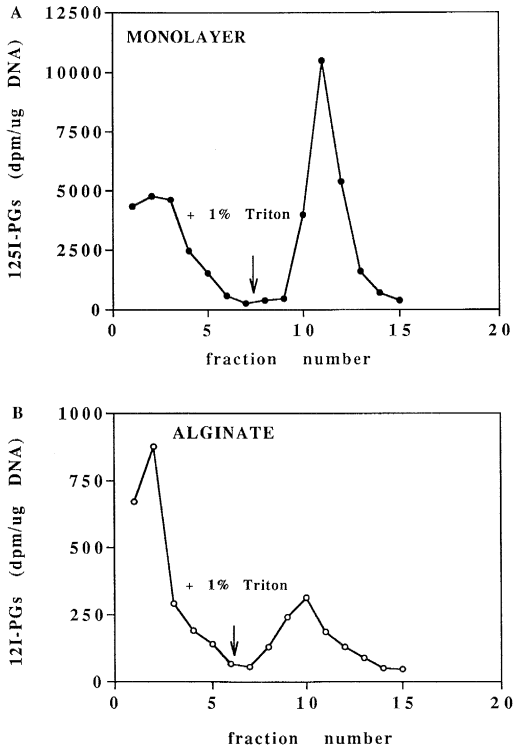

Fig. 5. Hydrophobic affinity chromatography of membrane-associated proteoglycans. The cell-surface PGs from monolayers (A) and alginate beads (B) isolated by DEAE-Trisacryl anion-exchange chromatography were radioiodinated and submitted to exchange of detergent with octyl-glucoside. PGs were then mixed with octyl-sepharose CL 4B gel, transfered in a small column and the gel was successively eluted with 10 mM Tris-HCl, pH 7.2, 4 M GuaHCl and the same buffer containing 1% Triton X-100. Fractions (1 ml) were collected, aliquots of 100 μl were mixed with scintillation cocktail and counted for [^{125}I] dpm.

found in the alginate system (Fig. 5B). This experiment has been reproduced four times, and the results indicated that the proportion of hydrophobic membrane-associated PGs were always higher when chondrocytes were cultured in monolayer than in alginate beads.

3.4. Partitioning of membrane-associated PGs in Triton X-114

Partitioning in Triton X-114 was used as an alternative method to determine the proportion of hydrophobic PGs in both culture systems. Table 1 shows that the proportion of PGs recovered in the detergent phase is higher in the case of chondrocytes cultured in monolayer as compared to the alginate beads system: 24.5% and 15.8% respectively (mean of 9 experiments). The means are statistically different as revealed by the calculation of the F value (test of Fisher). These results confirm the difference observed with octyl-Sepharose chromatography.

PGs recovered in the detergent phase were submitted to PIPLC digestion, and the proportion of hydrophobic PGs susceptible to this enzyme was always higher in the monolayer culture than in alginate system (not shown).

3.5. Gel-filtration chromatography of hydrophobic PGs

PGs recovered in the Triton X-114 detergent phase were then submitted to gel-filtration chromatography on Sepharose CL 6B column. Fig. 6A shows that the hydrophobic PGs from monolayer cultures were eluted in two peaks with Kav of 0.06 and 0.51. In the alginate system, the PGs were eluted in one peak only (K_{av} of 0.03), the second peak present in monolayers being absent in the case of tridimensional cultures (Fig. 6B). In both systems, the peak observed near the total volume of the column represent free ^{125}I-radioactivity, as revealed by SDS-PAGE (not shown).

Table 1
Partitioning of cell-surface proteoglycans in Triton X-114

Exp.	Monolayer	Alginate
1	18.9	10.5
2	16	9.6
3	27.6	25.9
4	33	24.6
5	28.6	8.6
6	31.2	17.6
7	11.7	9.3
8	30	16
9	23.9	20.3
Mean	24.5	15.8 *

Radioiodinated PGs were layered over a cushion of 6% sucrose containing Triton X-114 as described in Section 2. The results are expressed as the proportion of PGs recovered in the detergent phase (% of total cell-surface PGs). * Means are statistically different as revealed by the test of Fisher ($P < 0.025$).

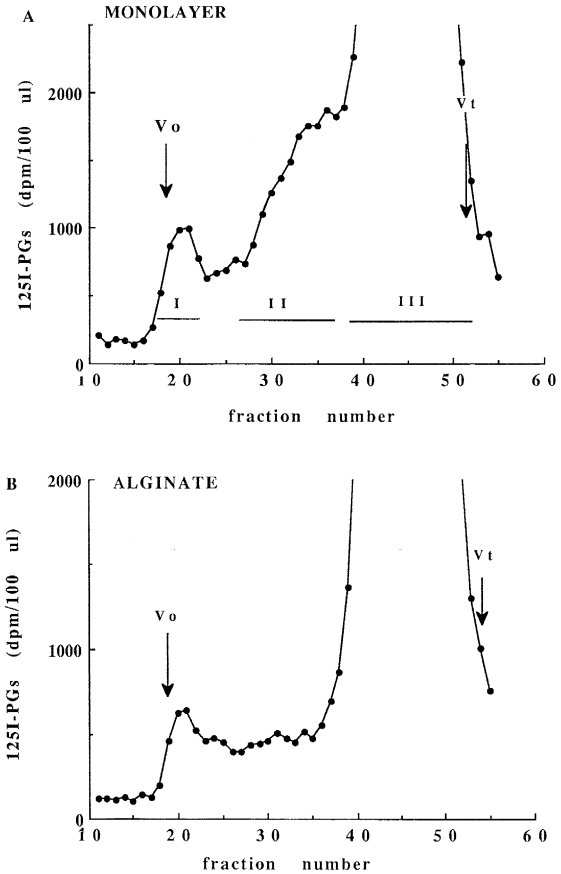

Fig. 6. Gel-filtration chromatography of hydrophobic proteoglycans. Samples from monolayers (A) and alginate beads (B) recovered in the detergent phase after partitioning in Triton X-114 were applied in sodium acetate pH 6.8 buffer containing 4 M GuaHCl onto columns (0.6×100 cm) of Sepharose CL 6B equilibrated in the same buffer and eluted at a flow rate of 12 ml/h. Blue dextran and phenol red were added to mark the void and total volumes, respectively. Fractions (1 ml) were collected, aliquots of 100 μl were mixed with scintillation cocktail and counted for [^{125}I] dpm.

The fractions corresponding to each peak were pooled, dialyzed, lyophilized in order to be digested by specific enzymes (heparitinase, chondroitinase ABC and keratanase) before being submitted to SDS-PAGE.

3.6. Characterization of hydrophobic PGs by SDS-PAGE following digestion by specific enzymes

In the case of monolayer cultures, the characterization of the three peaks by electrophoresis show that each peak is composed of different PG families; the first peak, which was eluted on Sepharose CL 6B with a K_{av} of 0.06 contain both HS and CSPG. The HSPG has a core protein of about 60–65 kDa (Fig. 7A, peak I) and the two CSPGs of 100 and 50 kDa. It is tempting to speculate that the HSPG could represent glypican, previously described in other system [8]. However, we could not confirm this hypothesis as no antibodies capable of recognizing glypican in the rabbit system is so far available. The CSPGs detected in our study may correspond to the PG100 [36] and decorin and/or biglycan that have been already described in cartilage.

The second peak, eluted at Kav 0.51 on Sepharose CL 6B, is composed of two proteins of 45 and 30 kDa, which were resistant to the enzymes tested, suggesting that they probably correspond to contaminant proteins or to core proteins lacking GAG chains (Fig. 7A, peak II) although these molecules have been first isolated by anion-exchange chromatography. The difference of migration between control and material digested with chondroitinase does not seem to be significant.

In the case of the alginate system, the two peaks were pooled together for the subsequent enzymatic digestions and electrophoresis. The results showed in Fig. 7B indicate that the PGs are composed of two CSPG families with core proteins of about 100 and 55 kDa.

Taking together, the results concerning hydrophobic PGs show that CSPGs with core protein of 100 kDa are present in both culture systems, and that the families of CSPGs with lower molecular weight core proteins vary according to the type of culture. Moreover, a HSPG with a core protein of 60–65 kDa is only observed in the case of monolayer cultures together with two proteins of 45 and 30 kDa.

3.7. Characterization of hydrophilic membrane-associated PGs

The same set of experiments was performed on PGs recovered in the Triton X-114 aqueous phase and the summarized results are given in Table 2.

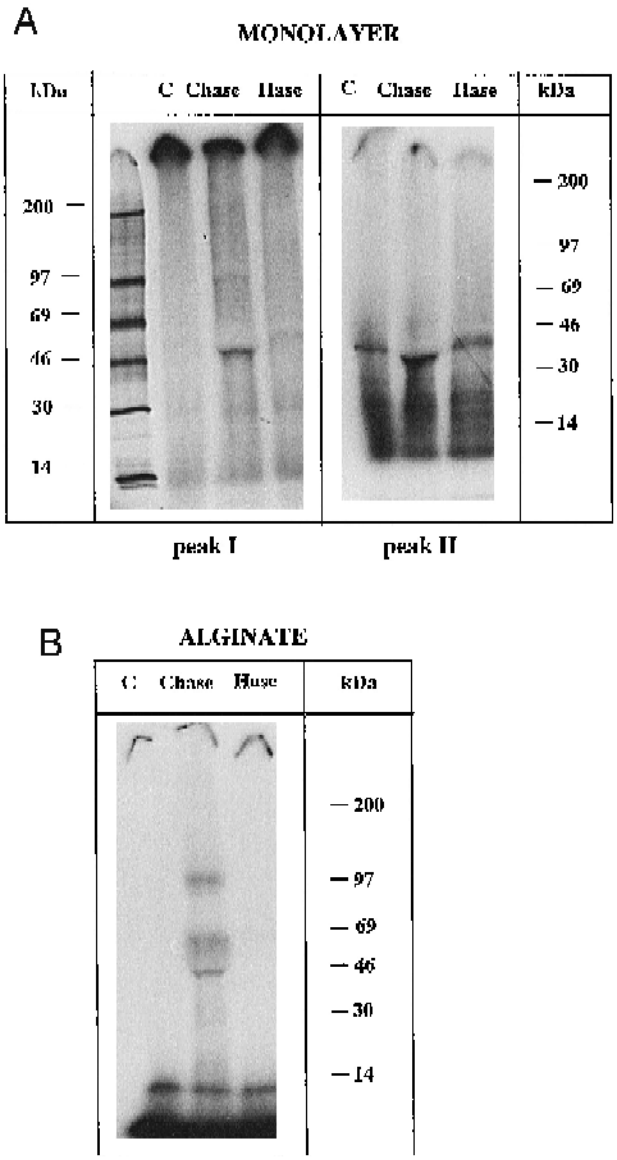

Fig. 7. Characterization by SDS-PAGE of hydrophobic cell-surface proteoglycans from monolayer cultures (A) and alginate cultures (B). Hydrophobic PGs recovered in the different peaks after gel-filtration on Sepharose CL 6B were digested by specific enzymes (heparitinase: Hase; chondroitinase ABC: Chase) prior electrophoresis on a 4–15% gel under non-reducing conditions. Gels were dried and autoradiographed at −80°C.

Table 2
Summary of the results obtained from the characterization of membrane-anchored proteoglycans in monolayers and alginate beads

	Hydrophobic PGs	Hydrophilic PGs
Monolayer	HSPG 60–65 kDa	CSPGs high mol. wt.,
	CSPGs 100 kDa	200 kDa
	45–50 kDa	core prot. 50, 30 and
	core prot. 45 and 30 kDa	20 kDa
Alginate	CSPGs 100 kDa	CSPGs 180 kDa
		100 kDa
	55 kDa	45 kDa

First, the PGs from monolayer cultures were eluted on Sepharose CL 6B, at K_{av} of 0, 0.48, 0.8 and 1. The elution profile of PGs from alginate system was slightly different: the second peak observed in monolayer cultures (Kav = 0.48) disappeared so that only three peaks were observed, eluted at K_{av} 0, 0.85 and 1.

These peaks were pooled, dialyzed, lyophilized, digested by specific enzymes before being submitted to SDS-PAGE.

In monolayer cultures, the PGs eluted at the void volume of the column represent high molecular weight material that did not enter the gel, probably aggregates of PGs and CSPG with core protein of about 200 kDa, corresponding to aggrecan. Two similar families of CSPG have been already described in a previous study [15]. The second peak eluted at K_{av} 0.48 contains molecules that are not susceptible to the enzymes tested, indicating that these molecules could be contaminant proteins of 50, 30 and 20 kDa. The third and fourth peaks contain mainly free radioactivity (not shown).

In the case of hydrophilic PGs from the alginate system, the first peak eluted near the void volume in Sepharose CL 6B is composed of three CSPGs with core proteins of 180, 100 and 45 kDa. Respectively, these PG families probably represent aggrecan or PG 100 and decorin or biglycan. The second peak of Sepharose CL 6B contains free radioactivity (not shown).

3.8. Effect of TGF-β on the nature and distribution of membrane-associated PGs

In parallel, we studied the effects of TGF-β on the nature of membrane-associated PGs. We demonstrated that the addition of the cytokine modified the proportion of hydrophobic PGs at the cell-surface, and that these modifications depend on the type of culture tested (Fig. 8).

First we observed that synthesis of membrane-associated PGs in monolayer was higher than in alginate system, the results being expressed on a DNA basis.

TGF-β enhanced the cell-surface PG synthesis in monolayers but has an inhibitory effect in alginate beads (respectively +106% and −37%). This latter result is different from that observed on aggrecan synthesis in the same conditions since this PG production is clearly stimulated under the effect of TGF-β (manuscript in preparation).

Interestingly, the amount of hydrophobic PGs was not affected by TGF-β, although the relative proportion was modified. Indeed in monolayer cultures, the total amount of membrane-anchored PGs is increased in the presence of TGF-β (+106%) but the amount

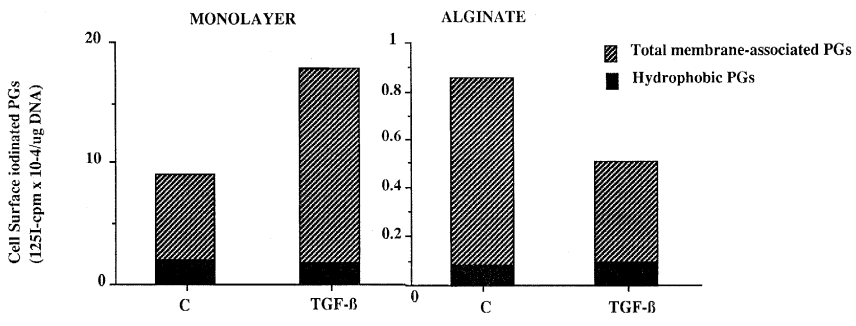

Fig. 8. Effects of TGF-β on the proportion of hydrophobic proteoglycans. TGF-β (2 ng/ml) was added for the last 48 h of culture in the monolayer and alginate bead systems. The proportion of hydrophobic PGs was assayed by partitioning in Triton X-114. The results of one typical experiment out of three are shown.

of hydrophobic PGs varies from 22 685 cpm/μg DNA for controls to 20 868 cpm/μg DNA for TGF-β-treated cultures, leading to a diminution of the relative proportion of hydrophobic PGs (10% in TGF-β-treated cells versus 22.3% in controls). In alginate beads, the overall synthesis of membrane-associated PGs was inhibited by TGF-β (-37%) but the amount of hydrophobic PGs was only slightly modified under TGF-β-treatment (737 and 977 cpm/μg DNA respectively in control and TGF-β-treated cells). In that case, the relative proportion of hydrophobic PGs was increased by the growth factor (9 and 19%, respectively for control and TGF-β-treated cultures).

These results demonstrate that TGF-β differentially regulates the synthesis of PG families in articular chondrocytes according to the type of cell culture used and the type of PG considered.

4. Discussion

It has been shown that articular chondrocytes cultured in gel beads composed of alginate retain their ability to synthesize cartilage-specific molecules such as aggrecan and type II collagen [16]. The advantage of the alginate bead system towards agarose culture is that the gel in which the chondrocytes are entrapped can be easily solubilized using a calcium chelator. We therefore decided to perform our studies with this culture system which seems to be better related to the in vivo situation than monolayers. Chondrocytes in monolayer cultures adhere to a plastic support, thus having a modified morphology as compared to cells in cartilage matrix, but they still express cartilage specific molecules (type II collagen and aggrecans) in primary culture. One disadvantage is that the cells dedifferentiate rapidly when subcultured [26,37]. However monolayers, in primary cultures have been widely used to study the synthesis of matrix macromolecules, because great numbers of cells can be obtained by this method, and the results are easily reproduced.

Previous studies have reported the characterization of typical cartilage PGs, e.g. aggrecan, decorin and biglycan in the alginate bead system [38]. However, a growing body of evidence suggests that cell-surface PGs could be also of great importance since they are primarily involved in the control of cell-cell and cell-matrix interactions.

Two preceding studies have focused on cell-surface PGs in chondrocytes; the first one was performed on suspension cultures of bovine articular cartilage chondrocytes and reported the presence of four different families of PGs at the cell surface [15]: (i) large PG typical for cartilage, apparently bound to hyaluronic acid at the cell surface, (ii) an intermediate-size CSPG, only found associated with the cells and apparently not related to the large PG, (iii) a small PG with DS side chains and (iv) a somewhat smaller PG containing HS side chains. The second study reveals that the major cell surface PG expressed by human chondrocytes at the mRNA level is amphiglycan [39].

It was therefore of interest to determine the identity of the different cell-surface PG families in two other cell culture systems: alginate beads and monolayers. The main problem in the study of cell-surface PGs in articular chondrocytes is that they only represent a small percentage compared to the bulk of aggrecans produced. Therefore, the first step in such investigation was to discard by a chase most of the large CSPGs forming a dense matrix around the cells.

Our results reveal for the first time that the cell-surface PG synthesis differs in a quantitative and qualitative manner between monolayers and alginate beads.

Quantitatively, it appears that the membrane-anchored PG production is higher in monolayers than in alginate beads. This result can be explained by the fact that in the alginate system the cells are embedded in a dense network of matrix molecules which could exert a retro-inhibition on their synthesis capacity.

Qualitatively, articular chondrocytes cultured in monolayers synthesized three CSPGs species (with core proteins of 180, 100 and 50 kDa), and a HSPG with a core protein of 60–65 kDa. The size of the core protein of this HSPG suggests that this molecule may be glypican [8]. The fact that the HSPG is recovered in the detergent phase after partitioning in Triton X-114 reinforced this hypothesis since glypican is a GPI-anchored PG. This HSPG is no more observed in the alginate system, indicating that the presence of the HSPG can be related to the morphological or differentiated state of the cells. It can be

hypothesized that confluent cells cultured in monolayers may express specific molecules related to cell-cell or cell-matrix adhesion. HSPGs are involved in such properties, in particular syndecan-4 which is associated with focal adhesion that contained $\beta 1$ or $\beta 3$ integrin subunits and those that formed on substrates of fibronectin, laminin, vitronectin or type I collagen [40]. This suggests that this HSPG may be expressed as a response of articular chondrocytes to a particular environment, namely monolayer culture.

This qualitative difference in the cell-surface PG synthesis is accompanied by a higher proportion of hydrophobic PGs in monolayers than in alginate beads as shown by two independent methods: octyl-Sepharose chromatography and Triton X-114 partitioning. This result is consistent with the presence of the HSPG in monolayers only, as this molecule was recovered in the detergent phase after partitioning in Triton X-114.

Some of the CSPGs identified at the cell surface are related to molecules already described: the high molecular weight CSPG expressed in both culture systems are probably aggrecans. In monolayer cultures, both CSPGs with core proteins of 200 kDa and higher molecular weight were observed, corresponding respectively to aggrecan monomers and PG aggregates. One previous study has already described these two PG families at the surface of bovine articular chondrocytes cultured in suspension [15]. Our data suggest that in monolayers, aggrecan is bound both in a free form to the cell surface of articular chondrocyte or associated to hyaluronic acid, composing aggregates of PGs of higher molecular weight. The CSPG with a core protein of 100 kDa present only in monolayer cultures may be related to the second pool of aggregating PGs as already described [36].

In parallel, the two small molecular weight PGs present in the alginate bead system (core proteins of 45 and 28 kDa) probably represent decorin and/or biglycan, as the mRNAs corresponding to these molecules were detected in the same culture model. These results are consistent with previous studies concerning both families of high molecular weight CSPGs and small PGs [15]. Furthermore, the heparan sulfate family expressed by our cells in monolayer cultures may be the same as the one described by these authors. We demonstrate that, as the HSPG is no more expressed in the alginate system, it could represent a dedifferentiated marker of the chondrocytes, and may differentially regulate the bioavailabality and activity of growth factors.

The results obtained in the presence of TGF-β demonstrate a differential regulation of cell-surface PG synthesis, depending on the type of culture studied and the PG families considered. The biosynthesis of membrane-associated PGs is increased in monolayers whereas it is inhibited in alginate culture. This inhibitory effect has been already reported [41] for chondrocytes cultured in another tridimensional culture system: agarose gels, but we recently demonstrate a stimulating effect of TGF-β on aggrecan synthesis in the alginate bead system. That TGF-β can differentially regulate the PG biosynthesis is consistent with other studies [42,43]. In one case, the authors revealed that TGF-β markedly stimulated the expression of biglycan and versican mRNAs but inhibited the expression of decorin mRNA in human skin fibroblasts [43]. The differential effect of TGF-β on cell-surface PG synthesis depending on the culture system demonstrates the importance of the cell environment for the response to extracellular factors, probably by the differential expression of growth factor receptors, depending on the morphological state of the cells [44].

In conclusion, our data show for the first time that the nature of cell-surface proteoglycans produced by articular chondrocytes in culture is highly dependent on the cell conditions, including cellular shape, cell to cell relationships and matrix environment. It is clear that their expression must be studied preferentially in tridimensional gel system as it better mimicks the in vivo situation. Great attention has to be paid in the future to this small fraction of PGs as they are likely to play a major role in both early activation process of chondrocytes and repair potentialities of cartilage in osteoarticular diseases.

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