In vitro biosynthesis by articular chondrocytes of a specific low molecular size proteoglycan pool

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Received 20 November 1982

Proteoglycans synthesized by articular and epiphyseal chondrocytes in culture were compared. Proteoglycans extruded by the two types of cells into the culture medium are of identical M_r. On the other hand, the proteoglycans of cells or pericellular matrix synthesized by the articular chondrocytes are characterized by an heterogeneous fraction of low-M_r which is not present in the material derived from epiphyseal chondrocytes. There are at least two components in this fraction: the first seems to be a precursor of aggregated proteoglycans, the other may represent a component of cell coat. Stimulation of the cell cultures with vitamin D metabolites and somatomedin enhances proteoglycan biosynthesis but no modification is observed in the proteoglycan M_r.

Proteoglycan *Sulphation* *Chondrocyte*

1. INTRODUCTION

The chemical composition, M_r, aggregation ability and rheological properties of proteoglycans from calcifying and non-calcifying cartilages, either normal or pathological, have been extensively investigated in the last few years in an attempt to understand better the mechanism of calcification [1,2].

Proteoglycan production is also the phenotype expression of cultured chondrocytes, the amount and species of proteoglycans synthesized being under the influence of various factors (extracellular concentration of proteoglycans, hormones, drugs, metabolite concentration...) [3–9]. No comparison has been made so far, however, between the characteristics of proteoglycans synthesized in vitro by mature chondrocytes derived from epiphyseal, pre-osseous cartilage and those synthesized by chondrocytes of articular, hyaline non-calcifying cartilage.

The in vitro biology of the two types of cells in primary culture is quite different. For example, they reach confluence after different periods of time and the distribution of cells in culture flasks is different, articular chondrocytes being organized as a monolayer [10] and epiphyseal chondrocytes as multilayered colonies [11].

Finally, the electrophoretic properties of proteoglycans subunits synthesized and secreted by each type of cultured chondrocytes are different [12].

Cultures of the two types of chondrocytes provide useful model systems for use in the investigation of the biochemistry of calcification.

Here, we demonstrate that the two types of cells synthesize different pools of proteoglycans and that the low-M_r proteoglycans specific of articular chondrocytes represent at least two different types of molecules. One of these is a precursor of aggregated proteoglycans, the other may be a component of articular chondrocytes glycocalix.

2. MATERIALS AND METHODS

2.1. *Preparation and labelling of chondrocyte cultures*

Procedures for isolation and culture of chon-

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Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

drocytes from articular and growth plate cartilage of Fauve de Bourgogne young rabbits have been described in [10] and [11], respectively. Thin slices of articular and growth plate cartilage were gently scrapped from the upper end of the tibiae under sterile conditions, and digested separately with 0.2% hyaluronidase, 0.5% trypsin (Worthington) and 0.5% clostridial collagenase (Boehringer) in Gey's balanced salt solution. A pure chondrocyte suspension was obtained and incubated to large culture flasks (75 cm^2) each containing 12 ml Dulbecco's medium (Gibco-Flobio) supplemented with 10% fetal calf serum (FCS) 0.1 unit/ml penicillin and 0.1 μg/ml streptomycin.

The final concentration was 3.5×10^5 cells/75-cm^2 flask. The flasks were gassed with 10% CO_2 in air and maintained at 37°C. The medium was then changed 3-times/week until the cells stopped dividing. The stationary phase was reached after 9 or 21 days in culture for articular chondrocytes and growth plate chondrocytes, respectively. At that phase of the cell cycle each type of chondrocyte, in triplicate flasks, was deprived of FCS for 24 h and the medium was then replaced with fresh serum-free medium containing $Na_2{}^{35}SO_4$ (1.5 μCi/ml) (Amersham-France, Les Ulis). Cells were further incubated at 37°C for 20 h for long-term labelling, or for 15 min, 30 min and 60 min for pulse-labelling in the presence or absence of 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ (10^{-11} M) (gifts from Dr M. Garabédian, CNRS ER 126, Paris) or a somatomedin peptide with Insulin-like activity referred to as ILAs (10 ng/ml) (purified by H. Guyda and B. Posner at McGill University, Montreal), or 2% FCS. Cells and media were then separately studied.

Media from long-term labelling were treated as described under proteoglycan extraction and purification. Media from short-term pulse experiments were discarded. In all cases, cells were gently washed and chased with fresh medium containing 1 mM non-radioactive sulphate. This medium was removed prior to extracting the proteoglycans from the cell pellet.

2.2. *Proteoglycan extraction and purification*

At confluency, the medium from two 75-cm^2 flasks was pooled (28 ml) and proteoglycans were extracted by adding 7 ml 5% cetylpyridinium chloride (CPC) in the presence of protease inhibitors (10 mM Na_2 EDTA, 5 mM benzamidine, 0.1 mM 6-aminohexanoic acid). The whole purification process was then performed essentially as in [13]. Briefly after centrifugation at 3600 × *g*, the material was washed with 5 ml 0.1 M Na_2SO_4 and dissolved in 2 ml of 1.25 M $MgCl_2$. After centrifugation, the supernatant was reprecipitated with 8 ml ice-cold ethanol and left at 4°C overnight. The pellet was then stored lyophilized.

Proteoglycans were also obtained from cell monolayers or colonies by dissociative extraction with 3 M guanidinium hydrochloride in 50 mM Tris–HCl, pH 7.4 (5 ml/75-cm^2 Falcon flask) for 48 h at 0°C in the presence of protease inhibitors. The extracts were clarified by centrifugation at 3000 × *g* for 10 min, and then proteoglycans were precipitated with CPC at a final concentration of 1%. The CPC precipitates were then treated as in [13].

For some preliminary experiments proteoglycans were extracted from articular and epiphyseal cartilage by gentle soaking of the tissue slices in 4 M guanidinium hydrochloride 0.05 M Tris–HCl (pH 7.4) containing protease inhibitors, for 48 h at room temperature. The extracts were then extensively dialyzed against distilled water and made up with 0.5 M sodium acetate (pH 6.8) at the moment of gel filtration.

2.3. *Gel filtration*

Lyophilized proteoglycans were dissolved in 0.5 M sodium acetate (pH 6.8) and aliquots of 100–200 μl (20–100 μg of uronic acid) were applied to columns of Sepharose 2B or CL 6B (0.5 × 65 cm) preequilibrated in the same buffer in the absence or presence of an excess of non-radioactive carrier.

Proteoglycan elution was performed in associative condition with 0.5 M sodium acetate (pH 6.8) at a flow rate of 2.5 ml/h. Fractions of 0.4 ml were collected and the radioactivity and uronic acid content of each fraction were determined by scintillation counting and the carbazole assay as in [14], respectively.

2.4. *Analytical procedures*

Uronic acid content of samples or fractions was measured as in [14]; hexosamines were determined by a micro-modification of the Elson–Morgan

reaction as in [15], hexoses by the method in [16] and protein by the method in [17].

Conventional polyacrylamide slab gel electrophoresis was performed on 10% acrylamide gels in 0.1 M Tris–glycine buffer (pH 8.9).

Gels were stained for proteins with Coomassie Blue as described in [18] and for glycosaminoglycans with Toluidine Blue in 0.1 M acetate buffer (pH 4.5) after fixation in 10% trichloroacetic acid.

Statistical analysis was done by calculating the mean ± standard deviation (SD). The significance of differences was evaluated by Student's *t*-test.

3. RESULTS

3.1. *Pattern of proteoglycans synthesized by articular and growth plate chondrocytes at different stages of the culture*

Gel filtration was the experimental approach selected for studying the characteristics of proteoglycans synthesized by the two types of cells.

Preliminary experiments were carried out with proteoglycans extracted with 4 M guanidinium hydrochloride from articular and epiphyseal cartilage of young rabbits. Their gel permeation profiles were compared with those of the proteoglycans purified from the culture media of the corresponding cells maintained in culture. Under associative conditions the elution patterns of proteoglycans synthesized in vivo (fig.1A) were superimposable on those of proteoglycans synthesized in vitro only when cells in culture were in the stationary phase (fig.1C). The aggregating ability of a proteoglycan extract from dividing cultured chondrocytes appears completely abnormal (fig.1B). For this reason, the experiments presented here were always carried out on cultures of articular and epiphyseal chondrocytes at the 9th, 10th and 21st day of incubation, respectively.

Two pools of proteoglycans were considered:

(i) Proteoglycans of culture media; and

(ii) Cell proteoglycans, a composite fraction containing proteoglycans localized at the level of chondrocyte coat, in intracellular organels and in the pericellular matrix.

Proteoglycans purified from culture media exhibit nearly identical elution patterns in associative conditions from Sepharose 2B (fig.2A and B, right side) independently of the origin of the chondrocytes.

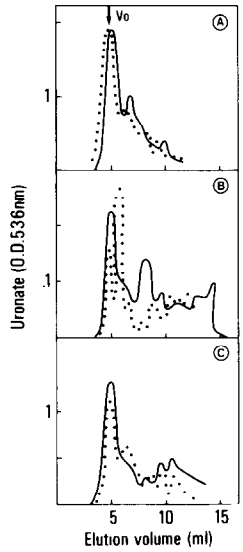

Fig.1. Elution profiles of hexuronic acid from a Sepharose 2B column under associative conditions after application of proteoglycans purified from: (A) articular (---) and epiphyseal (——) cartilage of young rabbits; (B) culture media of articular and epiphyseal chondrocytes at the 6th and 13th day of culture, respectively; (C) culture media of articular and epiphyseal chondrocytes at the 9th and 21st day of culture, respectively. Proteoglycans were extracted with 4 M guanidinium hydrochloride, 0.05 M Tris–HCl (pH 7.4) and purified as described in detail in section 2. Aliquots containing 20–100 μg uronic acid were chromatographed on a Sepharose 2B column (see section 2). V_o is the void volume of the column.

On the contrary, cell proteoglycans are not identical (fig.2A and B, left side). In fact, in the case of the material derived from articular chondrocytes, besides the peaks of aggregated proteoglycans and proteoglycan subunits, there is a retarded fraction eluted with the total volume of the column.

A similar profile is obtained when articular chondrocytes proteoglycans are purified without treatment of cell fraction with 3 M guanidinium hydrochloride by direct precipitation with 1% CPC and ethanol [13].

This peak is never present in the material derived from growth plate cells.

Treatment of cultures of the two types of cells by

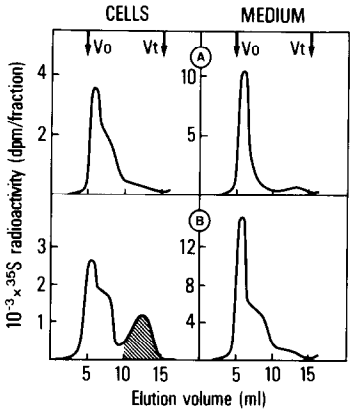

Fig.2. Sepharose 2B elution profiles of ^{35}S-labelled proteoglycans purified from cell fraction and culture media of confluent cultures of epiphyseal (A) and articular (B) chondrocytes. Confluent cultures of epiphyseal and articular chondrocytes were labelled with $Na_2{}^{35}SO_4$ (1.5 μCi/ml for 20 h) as described in the text. Proteoglycans were then extracted separately from the medium and from the cell pellet with 3 M guanidinium hydrochloride (see section 2). Samples containing about 3000–8000 dpm for cell pellet and 150 000–200000 dpm for culture medium of ^{35}S-labelled proteoglycans were applied to a column of Sepharose 2B and chromatographed as described in the text. Recovery of radioactivity from the column was greater than 85% in each case. V_0 is the void volume; V_t is the total volume of the column.

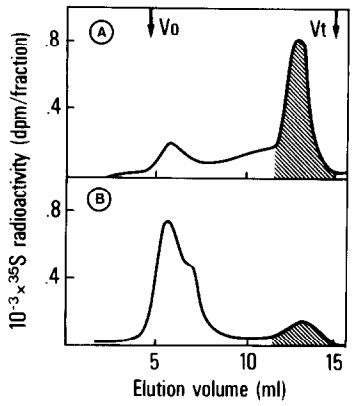

Fig.3. Sepharose 2B elution patterns of ^{35}S-labelled proteoglycans from cell fraction of articular chondrocytes. Confluent cultures of articular chondrocytes were labelled with $Na_2{}^{35}SO_4$ (1.5 μCi/ml) with a 15-min pulse. Proteoglycans were extracted with 3 M guanidinium hydrochloride from the cell layer. Samples of cellular extracts containing 4000 dpm of ^{35}S-labelled proteoglycans were applied to Sepharose 2B columns in the absence (A), or in the presence (B) of non-radioactive carrier. Recovery of radioactivity from the column was always greater than 85%. V_0 is the void volume; V_t is the total volume of the column. Shaded areas represent low-M_r proteoglycans.

either vitamin D metabolites ($1,25\text{-}(OH)_2D_3$ or $24,25\text{-}(OH)_2D_3$) somatomedin (ILAs) or FCS, does not modify the hydrodynamic properties of produced proteoglycans (not shown).

3.2. *Pulse-labelling of proteoglycans synthesized by cultured articular chondrocytes*

Culture chondrocytes were incubated in the presence of [^{35}S]sulphate for different periods of time (15 min, 30 min or 60 min) and proteoglycans extracted from the cellular pool were analyzed by chromatography on Sepharose 2B column in the absence or presence of non-radioactive carrier.

After 15 min labelling, most of the radioactivity extracted from the cellular pool and chromatographed in the absence of carrier is eluted as the retarded fraction previously described (fig.3A). When chromatographed in the presence of carrier, the elution profile is mainly composed of a large peak of proteoglycan aggregates and subunits with, to a smaller extent, the retarded peak (fig.3B). A similar profile was obtained after 30 min and 60 min labelling.

In this last case, the relative percentage of the remaining retarded peak compared with the total radioactivity extracted from the cellular pool and applied to the column is about 10–15%.

After labelling for 20 h the relative percentage of the retarded peak is about 30% of the applied radioactivity. A chase of 1 h or 4 h drastically reduces the amount of retarded material to 10–15% of the total applied radioactivity. The elution profile is then similar to fig.3B.

Again, no modification of these elution profiles is observed when the chondrocytes are treated with $1,25\text{-}(OH)_2D_3$, $24,25\text{-}(OH)_2D_3$ (10^{-11} M), ILAs (10 ng/ml), or fetal calf serum (2%) whereas the total [^{35}S]sulphate uptake in the cellular extract as well as in the medium is increased as shown in table 1.

Table 1

[^{35}S]Sulphate uptake in proteoglycans extracted from the cellular pool of cultured articular chondrocytes

Pulse labelling	[^{35}S]Sulphate uptake in cellular proteoglycans (dpm/μg DNA)	
	15 min ($\times 10^{-2}$)	20 h ($\times 10^{-4}$)
Control	3.17 ± 0.03	2.14 ± 0.50
FCS	4.05 ± 0.02[c]	5.26 ± 0.26[b]
24,25-$(OH)_2D_3$	4.00 ± 0.03[c]	3.80 ± 0.47[a]
1.25-$(OH)_2D_3$	4.12 ± 0.02[c]	4.24 ± 0.16[b]
ILAs	4.07 ± 0.02[c]	4.20 ± 0.50[a]

[a] $0.01 < p < 0.05$
[b] $0.001 < p < 0.01$
[c] $p < 0.001$

Confluent articular chondrocytes were incubated with 1.5 μCi/ml $Na_2{}^{35}SO_4$ for 15 min or 20 h. The cells were rinsed 3-times with buffer. Proteoglycans were then extracted with 3 M guanidinium hydrochloride and precipitated with CPC and finally dissolved in 1.25 M $MgCl_2$. Samples were then counted in a Packard Tricarb and the radioactivity was expressed as dpm/μg DNA. In the control group, the number of identical flasks is $n = 5$, in each treated group $n = 4$

3.3. *Characterization of the material of the retarded peak*

The material contained in the retarded fraction, when rechromatographed on Sepharose CL 6B (fractionation limits for polysaccharides 10^6–10^4 M_r), appears with the inner volume of the column as a composite peak (fig.4). At least two components are present in the fraction: the fast moving one has a K_{av} of 0.36, the slowest a K_{av} of 0.74. If compared with the K_{av} of globular proteins as M_r standards, within the limits of this comparison, one may extrapolate for the fast component an M_r of 300000, and for the other an M_r of 16000.

The chemical analysis of this fraction indicates that it is composed of proteins and carbohydrates: expressed/mg proteins it contains 0.14 mg uronate, 0.15 mg hexosamines and 2.45 mg hexoses. The ratio between hexosamines and uronate is close to 1 and suggests the presence of chondroitin sulphates but there is an abnormal elevation of neutral sugars. These quantitative results

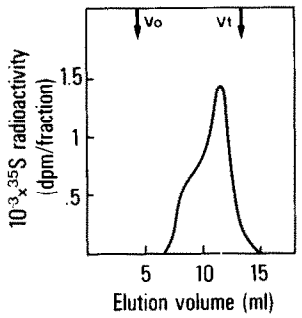

Fig.4. Chromatography on Sepharose 6B column, of ^{35}S-labelled proteoglycan material eluted as a retarded peak on Sepharose 2B column. Confluent articular chondrocytes were labelled with $Na_2{}^{35}SO_4$ for 20 h as described in the legend of fig.2. ^{35}S-Labelled proteoglycans were extracted from the cells with 3 M guanidinium hydrochloride (see section 2). Radioactivity (10000 dpm) was applied to Sepharose 2B columns and the ^{35}S-labelled material eluted as a retarded peak was then chromatographed on Sepharose 6B column. Recovery of radioactivity from the column was 90%. V_o is the void volume; V_t is the total volume of the column.

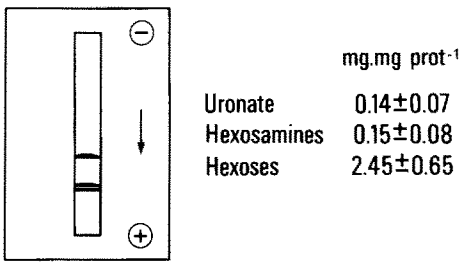

Fig.5. Schematic representation of polyacrylamide slab gel electrophoresis. Staining was performed with Coomassie Blue for proteins and with Toluidine Blue for glycosaminoglycans.

are not consistent with the hypothesis of a single type of molecule. Similarly, electrophoresis experiments reveal that this fraction is not homogeneous. Three bands appear on 10% polyacrylamide gels which stain with Coomassie Blue and metachromatically with Toluidine Blue, pH 4.5 (fig.5). The low spec. act. of the tracer precludes a correct quantification of the radioactivity present in each band.

4. DISCUSSION

It is well known that in cell and tissue cultures the biosynthetic activity of chondrocytes is markedly influenced by the composition of the extracellular environment. Matrix depletion [3,19,20], the presence of variable amounts of hyaluronic acid [12], proteoglycans, glycosaminoglycans [4] in the medium may modulate proteoglycan production by the cells and the proteoglycan size [21].

In fact, the results reported in fig.1 show that hydrodynamic size of proteoglycans freely diffused in culture medium, are strictly correlated to those of proteoglycans synthesized in vivo and secreted into the matrix only when cells in cultures are in the stationary phase. It is an important point to have cell cultures producing constantly the same type of macromolecules especially if some extrapolation in vivo will be made from the data obtained in vitro.

The results illustrated in fig.2B point out the existence of a pool of sulphated macromolecules of low-M_r specific of articular chondrocytes.

This retarded fraction may contain – at least in part – either a definitive material, possibly a precursor of other macromolecules or complexes, or a degradative product of cell metabolism.

Part of our data may support both hypotheses since stimulating protein synthesis with vitamin D metabolites, somatomedin or FCS increases the total [^{35}S]sulphate uptake, whereas no difference in the elution pattern of proteoglycan is observed. But other data such as pulse-chase experiments give evidence for the first hypothesis. After short-pulses of [^{35}S]sulphate the shifting of the label in the presence of carrier from the region of low hydrodynamic size to the region of aggregated material, demonstrates that a part of the material of the retarded peak is a 'precursor' of aggregated proteoglycans (fig.3). There are other reasons to exclude the possibility that this fraction is a degradative product. Proteoglycans derived from chondrocytes of growth plate, where degradative enzymes are more active, as shown in [22,23], do not contain this fraction. Furthermore, the whole purification procedure is carried out in the presence of inhibitors of hydrolytic enzymes.

In addition, the retarded fraction does not seem to be homogeneous. Gel sieving chromatography reveals that it is a composite fraction; acrylamide gel electrophoresis reveals 3 bands, all of which stain with the dyes of proteins and those of glycosaminoglycans. Chemical analysis shows that there is a uronate/hexosamine ratio characteristic of chondroitin sulphate (close to the unity) but that an abnormally high quantity of neutral sugars (hexoses) is also present. All these results suggest the existence of at least two pools of molecules in the retarded fraction observed in articular chondrocytes in vitro. The first, characterized by a uronate/hexosamine ratio of 1, may be the precursor of high-M_r proteoglycans. The other, characterized by a high hexose/protein ratio, is probably a glycoprotein component of the cell coat and/or structural glycoprotein from the extracellular matrix.

The difference between the elution pattern of labelled cellular proteoglycans of a 15–60 min pulse (fig.3) and that of a 20-h pulse (fig.2B) clearly indicates that a long pulse period, without renewal of the culture medium, causes an accumulation of low-M_r macromolecules (retarded peak) which represents about 35% of total radioactive compounds.

Since it was shown [24] that proteoglycans are secreted by the cells in the monomeric form at the same time as link proteins and hyaluronic acid, one may assume that the aggregated form of proteoglycans is present in the pericellular matrix and in the culture medium, while the low-M_r proteoglycans remain associated with articular chondrocytes. During a prolonged stimulus (20-h pulse) an inhibition of the secretory vacuoles is probably favoured by the modification of the medium and pericellular environment.

The renewal of culture medium for various times of chase promotes the secretion of proteoglycans to the pericellular matrix where aggregation occurs immediately. Since the amount of total radioactivity extracted from the cell pellet does not change for the various times of chase, one may suggest the transformation of 'small molecules into aggregates of proteoglycans'. The persistence of 10–15% of the radioactivity in low-M_r molecules indicates that a portion of the total proteoglycans is never exported from the cells. The possibility may be considered that this 10–15% of radioactivity is associated with some components of the glycoprotein coat of articular chondrocytes.

The fact that the retarded peak of proteoglycans has never been demonstrated in growth plate chondrocytes may be because:

(i) The pathways of extrusion and assembling of proteoglycans from the two types of cells are different;
(ii) The kinetics of extrusion are different;
(iii) Proteoglycans synthesized in vitro by the two types of cells are different, as in the case of rat chondrosarcoma described in [24].

In all these cases, the differences may have some relevance in the physiological functions that these cells exert in vivo; i.e., the biosynthesis of a calcifiable matrix.

These differences may also be the expression of a regulatory role of the proteoglycans on cell–cell recognition.

The presence of a fraction of low-M_r in chondrocytes of the articular cartilage may be responsible for their monolayer distribution in culture flasks and the lack of this fraction in epiphyseal chondrocytes may permit their organization in colonies.

ACKNOWLEDGEMENTS

We thank Dr A. Maroudas for reading this manuscript and for helpful advice, and A. Affouard for secretarial assistance. This research was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM) no. 81 40 27 and from the Consiglio Nazionale delle Ricerche (progetti internazionali).

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