Proteoglycans synthesized by an osteoblast-like cell line (UMR 106-01)

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The proteoglycans synthesized by an osteoblast-like cell line of rat origin (UMR 106-01) were defined after biosynthetic labelling with [³⁵S]sulphate and [³H]glucosamine. Newly synthesized labelled proteoglycans were characterized by differential enzymic digestion in combination with analytical gel filtration and SDS/PAGE. UMR 106-01 cells were found to synthesize three major species of proteoglycan: a large chondroitin sulphate proteoglycan of $M_r \sim 1 \times 10^6$, with a core protein of $M_r \sim 350\,000$ – $400\,000$; a small chondroitin sulphate-containing species of $M_r \sim 120\,000$ with a core protein of $M_r \sim 43\,000$; and a heparan sulphate proteoglycan of $M_r \sim 150\,000$, with a core protein of $M_r \sim 80\,000$. Over 70% of the newly synthesized intact proteoglycan species are associated with the cell layer of near-confluent cells; however, accessibility to trypsin digestion suggests an extracellular location. Chemical characteristics of the proteoglycans and preliminary mRNA hybridization indicate that the small chondroitin sulphate proteoglycan is probably PG II (decorin). The large chondroitin sulphate proteoglycan is most likely related to a hyaluronate-aggregating species from fibroblasts (versican), and the heparan sulphate proteoglycan bears striking similarities to cell-membrane-intercalated species described for a number of cell types.

INTRODUCTION

Proteoglycans (PGs) are important components of the non-collagenous macromolecules in bone. Purification and characterization of intact PGs from bone tissue has revealed two classes of small chondroitin sulphate (CS) PGs, variously termed PG I (or biglycan) and PG II (or decorin), both composed of relatively small core proteins (M_r 40 000–45 000) which are substituted with two and one glycosaminoglycan chains respectively [1]. PGs extracted from the bone matrix are synthesized by the resident osteoblasts; rat [2] and chick [3] calvaria and osteoblast-like cells derived from human [4], rat [5] and mouse [6] synthesize sulphated macromolecules, some of which have been partially characterized. The PGs synthesized and secreted by osteoblasts are thought to be involved in control of the mineralization processes required to form competent bone [7].

Additionally, there is evidence that PGs associated with the cell membrane [particularly the class of heparan sulphate (HS) PGs] are involved in both cell–cell and cell–matrix interactions [8], and they may also play a prominent role in the formation and maintenance of bone tissues. Because HS has not been identified in extracts of intact bone [1], the presence of this class of PG in osteoblast cultures has largely been ignored. However, although the matrix PGs PG I and PG II would accumulate in the osteoid of bone, cell-membrane-associated PGs, which have much shorter half-lives, would not be likely to constitute a significant proportion of the matrix. In order to understand the nature and potential function of PGs in bone, it is therefore important to study the spectrum of PGs synthesized by osteoblasts.

Several osteogenic-sarcoma cell lines, derived originally from a transplantable rat osteogenic sarcoma, have been widely studied as a model for bone-forming cells. In particular, the clone UMR 106 [9] and a subclone UMR 106-01 [10] have been shown to behave similarly to normal osteoblasts in their response to bone

agonists [11]. The present paper describes the characterization of the PGs synthesized by UMR 106-01 osteogenic-sarcoma cells, providing a basis for further studies of the regulation of PG metabolism by bone cells.

EXPERIMENTAL

Materials

Ultrapure guanidine hydrochloride, urea and ¹⁴C-labelled protein standards were from Bethesda Research Laboratories; 6-aminohexanoic acid, benzamidine hydrochloride and X-Omat AR film were from Eastman Kodak Co.; the buffer Hepes and the detergent CHAPS were from Calbiochem; Triton X-100 (Surfact-Amps X-100) was from Pierce Chemical Co.; chondroitinase ABC (*Proteus vulgaris*), chondroitinase AC II (*Arthrobacter aurescens*), endo- β -galactosidase (*Escherichia freundii*), keratanase (*Pseudomonas* sp. IFO-13309) and heparitinase (*Flavobacterium heparinum*) were from Seikagaku Kogyo, through ICN Biomedicals; electrophoresis chemicals were from Bio-Rad; and Sephadex G-50, Q-Sepharose, Sepharose CL-2B and prepacked Superose 6 were from Pharmacia Fine Chemicals. Na₂³⁵SO₄ (10–25 mCi/ml), D-[6-³H]glucosamine hydrochloride (20–38 Ci/mmol) and ENTENSIFY (fluorography kit) were from New England Nuclear; nick-translation kit was from Boehringer Mannheim; Hybond-N transfer membrane was from Amersham; Ready Safe liquid-scintillation cocktail was from Beckman; Falcon culture flasks (75 cm²) and plates (6 × 35 mm-diameter wells) were from Becton Dickinson Labware; Eagle's Minimal Essential Medium (MEM) and fetal-calf serum (FCS) were from Grand Island Biological Co.; and Centricon ultrafiltration devices (membranes with M_r -10 000 cut-off) were from Amicon Corp.

The clonal cell line UMR 106-01 was kindly provided by Professor T. J. Martin (St. Vincent's Institute of Medical Re-

Abbreviations used: PG, proteoglycan; CS, chondroitin sulphate; HS, heparan sulphate; MEM, Eagle's minimal essential medium; FCS, fetal-calf serum.

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search, Melbourne, Australia). We have recently observed apparent clonal divergence in the cell line known as UMR 106-01 when obtained from different sources. We therefore propose that the clonal variant described here be designated *UMR 106-01 (BSP)* to acknowledge the high secretion of bone sialoprotein [12]. Human fibroblast RNA was kindly given by S. Lamande (Department of Paediatrics, University of Melbourne, Australia).

Cell culture

Essentially, the procedures of Forrest *et al.* [10] were used to culture and passage UMR 106-01 cells. Cells were stored in liquid N₂ in 10% (v/v) dimethyl sulphoxide in FCS. Cultures were initially plated into 75 cm² culture flasks in MEM containing non-essential amino acids, Hepes (20 mM), gentamycin sulphate (50 µg/ml) and 10% (v/v) FCS, and maintained at 37 °C in 5% CO₂ in air. Subculturing to either 75 cm² flasks (for passaging) or to 35 mm-diameter wells (for experiments) was done by incubating cell layers with 0.25% (w/v) trypsin, 0.53 mM-Na₂EDTA in 0.15 M-NaCl/50 mM sodium phosphate, pH 7.4 (PBS), at 37 °C for 4–5 min. Cells were counted with a haemocytometer, and all experiments were carried out with pre-confluent exponential-phase cultures. Radioisotopes (50–100 µCi of [³H]glucosamine/ml and/or 50–100 µCi of [³⁵S]sulphate/ml) were added to 35 mm-diameter pre-confluent cultures in a final volume of 1.5–2.0 ml of MEM plus 10% FCS.

Isolation of proteoglycans

After each labelling protocol, fractions of medium were removed and the cell layers washed twice with PBS. Each cell layer was extracted with 2 ml of 4 M-guanidine hydrochloride containing 2% Triton X-100, 10 mM-*N*-ethylmaleimide, 5 mM-phenylmethanesulphonyl fluoride, 0.1 M-6-aminohexanoic acid, 5 mM-benzamidine hydrochloride, 50 mM-Na₂EDTA and 50 mM-sodium acetate, pH 5.8, for 24 h at 4 °C with constant stirring [13]. Identical cultures were used for cell counting. The macromolecular fraction of each extract was separated from unincorporated radioactive precursors, guanidine hydrochloride and other salts by chromatography on Sephadex G-50 (fine) columns (8 ml bed volume) equilibrated and eluted with 8 M-urea/0.2 M-NaCl/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0 [14]. Fractions containing labelled macromolecules were applied to 2 ml columns of Q-Sepharose anion-exchange resin pre-equilibrated in the same buffer. After sample application, the columns were washed with 3 column vol. of the same solvent, except that 0.5% CHAPS was substituted for the Triton X-100. This was done to exchange the Triton X-100 for a detergent with a higher critical micellar concentration (M. Yanagishita & R. J. Midura, unpublished work), so as not to interfere with subsequent concentration steps over ultrafiltration membranes. Bound material was eluted from the Q-Sepharose with 4 M-guanidine hydrochloride containing 0.5% CHAPS and 50 mM-sodium acetate, pH 6.0. Over 95% of the ³⁵S-labelled material was recovered in a volume of 3–4 ml. The ³⁵S-labelled material was concentrated to a final volume of 50 µl in a Centricon ultrafiltration device containing a membrane of *M_w*-10000 nominal exclusion. The concentrated sample was then injected on to a Sepharose 6 column equilibrated and eluted with 8 M-urea/0.2 M-NaCl/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0, at a flow rate of 0.4 ml/min. Effluent fractions (0.4 ml) were collected and samples analysed for radioactivity. Three ³⁵S-labelled pools were recovered (see Fig. 2 below), and each was applied directly to a 2 ml column of Q-Sepharose pre-equilibrated with 8 M-urea/0.5% Triton X-100/0.2 M-NaCl/50 mM-sodium acetate, pH 6.0. After application, the column was washed with 2 bed vol. of the same solvent, and then eluted with a continuous

NaCl gradient (0.2–1.2 M), with a total volume of 50 ml. Fractions (1.0 ml) were collected at a flow rate of 16 ml/h. The NaCl gradient was monitored by conductivity, and samples of each fraction were counted for radioactivity. ³⁵S-labelled peaks were pooled as described in the text.

Trypsin treatment of the cell layer

After radiolabelling, cell layers were washed twice with culture medium without serum before each treatment. Cell layers were incubated with trypsin (10 µg/ml) at 37 °C for 30 min, after which the cells were pelleted (300 g, 5 min at 4 °C) and washed twice with cold PBS. Cells were then extracted with 4 M-guanidine hydrochloride containing 2% Triton X-100 as described above. Extracts were analysed by direct application to Superose 6.

Analytical column chromatography

A Superose 6 column (1.0 cm × 30 cm) was eluted with 4 M-guanidine hydrochloride/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0. Calibration was with radiolabelled protein standards, with the plot of ln *M_r* versus *K_a* linear in the range *M_r* 25000–200000, and has been shown previously to give a good approximation of true hydrodynamic size of intact proteoglycans for decorin [15], corneal keratan sulphate proteoglycan [15] and biglycan [16]. Molecular-size estimates for glycosaminoglycan chains are based on the data of Wasteson [17] for Sepharose 6B and are used for comparative purposes. The accuracy of *M_r* determination on the Superose 6 column for CS chains was assessed by direct comparison with a Sepharose 6B column calibrated with standards obtained from Å. Wasteson (M. Yanagishita, unpublished work). For HS chain-size estimates, the calibration should hold for similarly sulphated disaccharides, as is the case for the UMR 106-01 HS PG (see the Results section). A Sepharose CL-2B column (0.7 cm × 90 cm) was eluted with 4 M-guanidine hydrochloride/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0, at a flow rate of 3 ml/h. An estimate of the PG size was based on comparison with elution position of the large aggregating PG from cartilage. Appropriate fractions were collected and analysed for radioactivity.

SDS/PAGE

Polyacrylamide gradient (4–20%) slab gels (0.15 cm × 14 cm × 16 cm) were prepared in the buffer system of Laemmli [18], with a stacking gel of 3% polyacrylamide. Electrophoresis was done overnight at 15 mA constant current. The gels were then fixed in 50% (v/v) methanol containing 10% (v/v) acetic acid. Fluorography was done with ENTENSIFY according to the manufacturer's instructions.

Enzymic treatments

Digestions with chondroitinase ABC (50 m-units), chondroitinase AC II (50 m-units), heparitinase (10 m-units), keratanase (500 m-units) and endo-β-galactosidase (5 m-units), in 50–100 µl reaction volumes, were done in 0.1 M-Tris/HCl, pH 7.2, containing 10 mM-Na₂EDTA, 0.2% CHAPS, 1 mM-phenylmethanesulphonyl fluoride, 0.36 mM-pepstatin and 10 mM-*N*-ethylmaleimide [19–21]. Excess enzyme was used in all cases, and digestion mixtures were incubated at 37 °C for 2 h. Reactions were terminated by addition of an equal volume of either 8 M-guanidine hydrochloride (for gel filtration) or 2-fold-concentrated SDS/PAGE sample buffer for SDS/PAGE.

Chemical analyses

Alkaline borohydride treatment was done in 0.05 M-NaOH at 45 °C for 24 h in the presence of 1 M-sodium borohydride [22]. Excess borohydride was destroyed by neutralization with acetic acid.

Disaccharide analyses

Chondroitinase ABC digests were eluted on Sephadex G-50 in pyridinium acetate, pH 6.0. CS disaccharide standards (20 μ g each) were added to the included digestion-product peak, followed by freeze-drying. Disaccharides were separated by Partisil 5-PAC chromatography as previously described [23]. Standards were detected by absorbance at 232 nm, and fractions were collected for determination of radioactivity.

RNA isolation and molecular probing

Total cellular RNA was extracted by lysing 3×10^8 cells in 4 M-guanidine hydrochloride containing 25 mM-sodium citrate, pH 7.0, 0.5% *N*-lauroylsarcosine and 0.1 M-2-mercaptoethanol, and purified by centrifugation through a CsCl cushion [24,25]. RNA (10 μ g/lane) was electrophoresed on 0.9% (w/v) agarose gels containing formaldehyde, followed by transfer to Hybond-N membranes. Northern blots were prehybridized and the membrane-bound RNA was hybridized to specific cDNA probes under stringent conditions. The cDNA clone for human PG I [26] was kindly given by Dr. L. Fisher (NIDR, NIH, Bethesda, MD, U.S.A.). The cDNA encoding glyceraldehyde-phosphate dehydrogenase has been described previously [27]. cDNA clones were 32 P-labelled by nick translation. Hybridizations were carried out at 42 °C for 16 h in 50% formamide containing 0.75 M-NaCl, 0.1% SDS, 4 mM-EDTA, 0.04% polyvinylpyrrolidone, 0.04% BSA, 0.04% Ficoll and 100 μ g of herring sperm DNA/ml. Membranes were washed with 0.30 M-NaCl/0.03 M-sodium citrate ($2 \times$ SSC) containing 0.1% SDS at room temperature and then with $0.1 \times$ SSC containing 0.1% SDS at 60 °C for 30 min. Membranes were dried and exposed for various times to Kodak XAR-5 X-ray film with intensifier screens at -70 °C.

RESULTS

Kinetics of [35 S]sulphate incorporation into macromolecules

Cultures of UMR 106-01 cells were inoculated into multiple 35 mm-diameter culture wells at low density ($\sim 1 \times 10^5$ cells/well) and maintained for several days in medium containing 10% FCS. Under these conditions the cells exhibited a doubling time of ~ 23 h. At 2 days before reaching confluence, cultures were incubated with [35 S]sulphate for up to 24 h. The amounts of 35 S-labelled macromolecules released into the medium and those present in 4 M-guanidine hydrochloride extracts of the cell layers were determined at various times after initiation of labelling (Fig. 1). The kinetics of the release of 35 S-labelled macromolecules into the medium was nearly linear over the duration of the experiment. The amount in the cell layer increased almost linearly for 8 h and then approached a plateau at 12–24 h. At 24 h the amount of labelled material in the medium was only 25–30% of the total. However, approx. 50% of the incorporated label in the medium was present in sulphated glycoprotein of $M_r \sim 70000$, and not in PG. This material was subsequently identified as bone sialoprotein II [12]. Approx. 90% of the cell-layer-associated 35 S-labelled macromolecules were PGs or glycosaminoglycans (see below). Since kinetics indicated that the incorporation of label approached steady-state levels at 24 h, this time period was used for subsequent experiments.

Isolation of PGs from the cell layer by gel filtration and anion-exchange chromatography

UMR 106-01 cells in culture were incubated with [35 S]sulphate for 24 h. The cell layer was then extracted with 4 M-guanidine hydrochloride containing Triton X-100. Labelled macromolecules were recovered in the excluded volume after elution from Sephadex G-50 and applied to Q-Sepharose. More than 95% of the 35 S-labelled molecules bound to the column, and were

eluted with 4 M-guanidine hydrochloride (results not shown). After concentration, this PG fraction contained $> 90\%$ of the original 35 S-labelled molecules in the cell layer. Chromatography on Superose 6 eluted with an 8 M-urea solvent revealed three pools (Fig. 2): pool I, K_a 0–0.31; pool II, K_a 0.37–0.60; and pool III, K_a 0.65–0.85. Each pool was applied to Q-Sepharose and eluted with a linear NaCl gradient. Pool I yielded a single peak (pool A, Fig. 3a) eluted at 0.72 M-NaCl. Pool II also gave a single major peak (pool B) with an earlier-eluted shoulder (Fig. 3b). Pool III, however, was more heterogeneous (Fig. 3c) and was divided into two fractions, eluted at ~ 0.39 M-NaCl (pool C) and 0.50 M-NaCl (pool D).

Samples of each pool A–D were digested with different glycosidases, and the digests were analysed by chromatography on Superose 6. The samples contained exclusively CS and HS ($> 95\%$), on the basis of susceptibility to chondroitinase ABC and heparitinase. There was no detectable keratan sulphate, as assessed by total resistance to keratanase and endo- β -galactosidase (results not shown). Additionally, there was no dermatan sulphate, as assessed by the equal effectiveness of

Fig. 1. Time course of incorporation of [35 S]sulphate into macromolecules

Cultures of UMR 106-01 cells were incubated with [35 S]sulphate for up to 24 h. At the times shown, the medium was removed and the cell layer extracted as described in the text. The incorporation of radiolabel into both medium and cell-layer fractions was determined by Sephadex G-50 chromatography. Each point represents the mean of triplicate cultures; the S.D. was less than the symbol size for all determinations.

Fig. 2. Superose 6 chromatography in 8 M-urea of cell-layer fraction

Cultures were labelled with [35 S]sulphate for 24 h, and the cell layer was extracted with 4 M-guanidine hydrochloride containing Triton X-100. Fractions were pooled as indicated by the numbered bars and further purified, as indicated in the text.

Fig. 3. Q-Sepharose anion-exchange chromatography of ^{35}S -labelled macromolecules isolated on Superose 6

Pools obtained in Fig. 2 were applied to columns of Q-Sepharose eluted with a linear NaCl gradient. Peaks A–D of ^{35}S -label were pooled and further analysed.

chondroitinase AC II and chondroitinase ABC. The following data are thus confined to analyses of samples incubated with either chondroitinase ABC or heparitinase.

The elution profile for a pool A on Superose 6 (Fig. 4a) showed two peaks, a minor one in the excluded volume and a major one with a peak elution position of $K_a = 0.17$ as well as a later-eluted shoulder of $K_a \sim 0.36$. Chondroitinase ABC digestion removed 30% of the label (Fig. 4b), leaving an HS-containing peak at $K_a = 0.17$ ($M_r \sim 150\,000$). After heparitinase digestion (Fig. 4c) about 70% of the label was eluted near the total volume of the column as a mixture of digestion products. Two CS-containing peaks were revealed after the digestion, one in the void volume and a second broad peak centred at $K_a = 0.36$ ($M_r \sim 120\,000$). Their presence was confirmed by the chondroitinase digestion (Fig. 4b), which removed most of the excluded component and the late-eluted shoulder observed in the undigested sample.

The major portion of pool B was eluted relatively late as a broad peak centred at $K_a = 0.56$ (Fig. 4d). The remainder was eluted as an excluded peak and a partially included broad peak, presumably a cross-contamination of pool materials.

Chondroitinase ABC digestion removed about 68% of the total radioactivity (Fig. 4e) and revealed HS-containing material distributed into two species: one at $K_a = 0.17$, which comprised 9% of the total and was eluted identically with the major HS PG in pool A (Fig. 4b), and one at $K_a = 0.64$, which consisted of free HS chains of $M_r \sim 17\,000$, on the basis of its resistance to treatment with alkali (see below). Heparitinase treatment revealed CS peaks (Fig. 4f): a major peak at $K_a = 0.53$ ($\sim 60\%$ of the total), and a small amount of the large, excluded CS PG described in pool A. The major CS peak in pool B was resistant to treatment with alkali, and thus represents CS chains of $M_r \sim 27\,000$ (see below).

Pool C, which was isolated on the basis of its small size (Fig. 2) and low charge density (Fig. 3c), was eluted from Superose 6 as a single major peak (Fig. 4g) at $K_a = 0.75$. Over 80% of this material was resistant to chondroitinase ABC digestion (Fig. 4h) and did not change elution position after treatment with alkali (results not shown); it therefore represents HS chains of $M_r \sim 11\,000$. Only 10% of pool C (less than 1% of the total cell-layer ^{35}S -labelled macromolecules) remained after heparitinase digestion (Fig. 4i), most likely representing the CS chains shown in Fig. 4(f).

Pool D, which was eluted on Q-Sepharose with a higher charge density than for pool C (Fig. 3c), distributed into at least three distinct peaks on Superose 6 (Fig. 4j). Chondroitinase ABC digested 35% (Fig. 4k) and revealed two HS-containing species: one at $K_a = 0.17$, identical with the elution position of the HS PG described above; and a second at $K_a = 0.75$, representing the HS chains of $M_r \sim 11\,000$. Heparitinase treatment (Fig. 4l) revealed a small amount of the large excluded CS PG as well as CS chains, again probably representing the tailing edge of the $M_r \sim 27\,000$ CS chains described above.

The different PG species in the cell layer and their proportions as a percentage of the total ^{35}S -labelled macromolecules are shown in Table 1. There are at least three distinct PG species: an HS PG of apparent $M_r \sim 150\,000$, which constitutes 70% of the cell-layer intact PG population; and two CS PG species, one of $M_r > 200\,000$ (excluded on Superose 6) and a smaller species of apparent $M_r \sim 120\,000$. The larger CS PG species was eluted from Sepharose CL-2B with a peak $K_a = 0.57$, corresponding to $M_r > \sim 1 \times 10^6$. Approx. 30% of the ^{35}S label was in free HS and CS chains, which are probably intermediates in the intracellular degradation pathways [28] for the intact PGs described above. They are present as two apparent populations: HS chains of $M_r \sim 17\,000$ and $\sim 11\,000$ and CS chains of $M_r \sim 27\,000$ and $\sim 17\,000$.

The PGs present in the medium compartment were also analysed (results not shown) and indicated that all three PG species were present, albeit in lesser amounts as well as different relative proportions. The sulphated glycoprotein represented 48% of the ^{35}S label, the intact HS PG 25%, and the remaining 25% comprised almost equal amounts of the large and small CS PGs. There were no detectable free chains in the medium compartment, providing further evidence of their intracellular localization.

Glycosaminoglycan analyses

Samples of pools A and B (Figs. 3a and 3b) enriched in the intact PG species were digested with chondroitinase ABC or heparitinase and the respective undigested glycosaminoglycans released by alkali treatment. The CS PG species in pool A (Fig. 4f) yielded a broad peak of CS chains at $K_a = 0.51\text{--}0.58$ ($M_r \sim 22\,000\text{--}28\,000$), as shown in Fig. 5(a). The intact HS PG from pool A (Fig. 4b) was substituted with a relatively monodisperse population of HS chains eluted at $K_a = 0.47$ ($M_r \sim 34\,000$) on Superose 6 (Fig. 5b). Pool B from Q-Sepharose

Fig. 4. Compositional analysis of proteoglycan species by differential enzyme digestion and molecular-sieve chromatography on Superose 6

Pools obtained in Fig. 3 were applied to a Superose 6 column before and after incubation with either chondroitinase (C'ase) ABC or heparitinase (H'ase). V_0 and V_t were at fractions 20 and 54 respectively.

Table 1. Distribution of ^{35}S -labelled macromolecules in the cell layer of confluent UMR 106-01 cells after 24 h labelling

Distribution is expressed as a percentage of total ^{35}S -labelled macromolecules eluted from Q-Sepharose in samples generated from a typical experiment; values in parentheses represent percentages of intact PG species.

Species	$10^{-3} \times M_r$	Distribution (%)
Large CS PG	> 1000*	9.2 (16)
Small CS PG	120†	8.3 (14)
HSPG	180†	40.9 (70)
CS chain	27†	13.7
	17†	2.8
HS chain	17†	5.1
	11†	9.0
BSP II‡		10.0

* Relative size estimated on Sepharose CL-2B.

† Relative size estimated on Superose 6.

‡ See Midura *et al.* [12] for identification.

chromatography (Fig. 3), which contained a small proportion of the large CS PG but no detectable amounts of the smaller CS PG (Fig. 4f), was incubated with heparitinase, followed by β -elimination of the glycosaminoglycan chains. CS chains were eluted as a single broad peak (Fig. 5c) at $K_d = 0.56$ ($M_r \sim 24000$), consisting of CS chains derived from the larger CS PG co-eluted with the $M_r \sim 27000$ chains contained in the original sample

(Fig. 4f). The HS chains in pool B were eluted as a very broad peak (Fig. 5d), consisting of at least two populations: HS chains of $M_r \sim 34000$ derived from the intact HS PG and the HS chains of $M_r \sim 17000$ already present in pool B (Fig. 4e).

In separate experiments, the large and small CS PG populations were isolated from cultures labelled with $[^{35}\text{S}]$ sulphate and $[^3\text{H}]$ glucosamine. The HS PG species were removed by prior incubation with heparitinase, followed by Q-Sepharose and Superose 6 chromatography (results not shown). Samples of each pool were digested with chondroitinase ABC and the disaccharide digestion products were resolved on a Partisil 5-PAC column [20]. Typical disaccharide compositions of the large and small CS PGs are summarized in Table 2 and are essentially identical, with about 40% 4-sulphated and 30% 6-sulphated, and the remainder unsulphated. The relatively high content of unsulphated disaccharide is probably responsible for the co-elution of the CS PGs with the HS PG species on the Q-Sepharose anion-exchange resin.

PG core-protein preparations

PGs were isolated from the cell layer of cultures labelled for 24 h with $[^{35}\text{S}]$ sulphate and $[^3\text{H}]$ glucosamine, by elution on Superose 6. The amount of ^3H in the glucosamine precursor in the PG fraction was approximately twice that of the ^{35}S . Samples were incubated with either heparitinase or chondroitinase ABC, and the intact PGs (CS PG in the heparitinase digestion; HS PG in the chondroitinase ABC digestion) were separated from digestion products by anion-exchange chromatography on Q-Sepharose (results not shown). The bound PGs were eluted with 4 M-guanidine hydrochloride containing 0.5% CHAPS. The intact PG preparations eluted

Fig. 5. Superose 6 chromatography of alkaline-borohydride-digested proteoglycans

Pools obtained in Fig. 3 were digested with either heparitinase (H'ase; *a* and *c*) or chondroitinase (C'ase; *b* and *d*), and the remaining intact glycosaminoglycan chains were released by alkaline borohydride. V_0 and V_i were at fractions 20 and 54 respectively.

from Q-Sepharose were further chromatographed on Superose 6 to separate the three PG populations. A sample of each was incubated with the appropriate glycosidase to remove the glycosaminoglycan chains, and then portions of both intact and treated were analysed by SDS/PAGE (4–20%) (Fig. 6*a*).

Table 2. Disaccharide analysis of CS PGs

Distribution of ^3H radioactivity is expressed as a percentage of total radioactivity co-eluting with disaccharide standards. Samples are representative of many similar analyses.

Species	Distribution (%)		
	$\Delta\text{di-OS}$	$\Delta\text{di-6S}$	$\Delta\text{di-4S}$
Large CS PG	28	30	41
Small CS PG	23	32	44

The intact HS PG migrated only a small distance into the resolving gel (lane 1), to a position equivalent to a globular protein of $M_r \sim 370\,000$. The HS PG core formed a single narrow band, at $M_r \sim 80\,000$ (lane 2). The large CS PG only partially entered the resolving gel (lane 3), and its core migrated only a short distance, being resolved as an apparent triplet, with the major band equivalent to a globular protein of $M_r \sim 400\,000$ (lane 4). The small CS PG revealed a broad band (lane 5), from which a predominant core protein (lane 6) at $M_r \sim 43\,000$ was revealed. The smaller CS PG preparation was heavily contaminated with [^{35}S]sulphate-labelled material, resolved as a doublet around $M_r \sim 70\,000$, which is seen in both lanes of intact and digested material. This material migrated to a position identical with that expected for bone sialoprotein II from these cells, and its prominence in the fluorograph is due to heavy sulphation of both tyrosine residues and oligosaccharides [26].

In order to confirm the identity of the small CS PG, a cDNA encoding PG I [26] was used to probe a Northern blot of RNA isolated from UMR 106-01 cells and human fibroblasts. Control experiments indicated that this clone of human origin cross-hybridized with rat mRNA. The resulting autoradiograph (Fig. 6*b*) indicates that there are undetectable levels of mRNA coding for PG I in UMR 106-01 cells (panel 1), even after extended exposure of the blot (results not shown). RNA was assessed by re-probing the filter with a cDNA encoding glyceraldehyde-3-

Fig. 6. (*a*) SDS/PAGE of intact proteoglycans and core proteins, and (*b*) RNA blot hybridization

(*a*) Samples of proteoglycan pools labelled with [^3H]glucosamine and [^{35}S]sulphate were prepared as described in the text. Lane 1, intact HS PG; lane 2, heparitinase digestion of HS PG; lane 3, intact large CS PG; lane 4, chondroitinase ABC digestion of large CS PG; lane 5, small CS PG; lane 6, chondroitinase ABC digestion of small CS PG. Arrows indicate core proteins generated by glycosidase digestions. M_r standards are indicated. (*b*) RNA from human fibroblasts (F) and UMR 106-01 cells (U) was electrophoresed on an agarose gel, transferred by Northern blotting to nylon membrane, and hybridized to cDNA probes encoding PG I (panel 1) and GAPDH (panel 2). RNA markers are indicated.

Fig. 7. Trypsin-accessibility of ^{35}S -labelled macromolecules

Duplicate cultures were labelled with ^{35}S sulphate for 24 h, after which one culture (●) was further incubated with trypsin; ○, intact control. Cells were extracted and the macromolecular fraction was eluted on Superose 6. V_0 is at fraction 19 and V_t at fraction 54.

phosphate dehydrogenase (panel 2), indicating that the UMR 106-01 RNA was of good quality despite the absence of a PG I signal. It can therefore be inferred that the small CS PG observed by SDS/PAGE and gel chromatography is most likely PG II (decorin).

Trypsin treatment of the cell layer

After labelling of UMR 106-01 cultures for 24 h, medium was removed and the cell layer washed twice before incubation with trypsin. Trypsin released approx. 60% of the ^{35}S -labelled macromolecules from the cell layer as assessed by Sephadex G-50 chromatography. ^{35}S -labelled PGs resistant to trypsin and those extracted from untreated cell layers were compared by molecular-sieve chromatography on Superose 6 (Fig. 7). It was apparent that trypsin removed most of the higher-molecular-sized material, representing the intact PG species, and caused very little decrease in the amounts of the smaller-molecular-sized material, representing the free glycosaminoglycan chains. These results indicate that almost all the intact PG species are located in trypsin-accessible sites, either on the cell surface or within a pericellular matrix, whereas the free CS and HS chains are probably located inside the cell, as has been described for other cells [14].

DISCUSSION

A clonal rat osteoblast-like cell line, UMR 106-01, synthesizes three predominant species of PG. Under tissue-culture conditions, the three species are located in both the cell layer and the medium compartment, with most of the newly synthesized PGs located in the cell-layer fraction of confluent cells. However, the distribution of the two CS PG species can be markedly altered if pulse-labelled immediately after passage of cells, with most of the intact CS PGs located in the medium compartment. This would suggest that in confluent cells there is an extensive pericellular matrix which serves to bind secreted PGs and maintains them in close proximity to the cells. The HS PG, on the other hand, is probably representative of a class of cell-membrane-intercalated species observed in a number of cells, and bears striking similarities to the membrane-intercalated HS PG of ovarian granulosa cells [29] and parathyroid cells [30].

It is a common observation in this laboratory [29] that the migration distance of intact PGs on SDS-PAGE is anomalous relative to globular protein standards. However, values determined for deglycosylated core proteins are more reliable, and, when combined with the hydrodynamic size determined for the intact PG on gel filtration, a relatively accurate representation of the PG characteristics is possible. The data obtained in the present study suggest that the HS PG species consists of a single core protein of $M_r \sim 80000$ substituted with two to four HS chains, as well as oligosaccharides to give an overall size of $M_r \sim 150000$ – 190000 . This is identical with that described for HS PGs isolated from rat ovarian granulosa cells [29] and a rat parathyroid cell line [30]. The secreted CS PG pool is composed of at least two distinct species: one with a large core protein of $M_r \sim 350000$ – 400000 substituted with up to 30 CS chains (M_r 25000 each); and a smaller species containing a core of $M_r \sim 43000$ substituted with one or two CS chains.

PGs from mineralized bone have been isolated and characterized. Fetal bone has been reported to contain two small PGs, PG I (biglycan) and PG II (decorin), which are distinct gene products [31]. However, PG I has been reported to be absent from adult bone [32]. In addition, there is little information about the factors which regulate the expression of these species in bone cells or the stage of differentiation at which they are synthesized. The biosynthesis of PGs associated with new bone formation has been studied after pulse-labelling with ^{35}S sulphate *in vivo* and *in vitro* [2–6], with the predominant PG described as a small CS/dermatan sulphate-containing species with core protein of M_r 40000–50000. However, Beresford *et al.* [4] did observe a significantly larger CS PG, representing a small component of human osteoblast-like cells with a core protein of $M_r \sim 340000$ – 390000 .

The spectrum of PGs synthesized by UMR 106-01 cells are consistent with an adult bone phenotype, despite differences in relative proportions which may be attributable to the transformed nature of these cells, in addition to species differences. The most striking observation in the present study is the high rate of synthesis of an HS PG by these cells, which has previously not been reported for either bone extracts or osteoblasts in culture, although it was suggested that human osteoblast-like cells may synthesize a hybrid PG containing both CS and HS chains [4]. The HS PG synthesized by UMR 106-01 cells is probably a plasma-membrane-intercalated species [29] containing a highly hydrophobic domain which imparts to it properties that make it difficult to recover in procedures optimized for bone matrix extraction. It is therefore not surprising that it has not been previously described by other workers investigating PGs synthesized by osteoblasts. Its role in the function of osteoblasts imbedded in an extracellular matrix is certainly worthy of investigation in terms of cell–cell, cell–matrix and cell–hormone/growth-factor interactions.

The UMR 106-01 cells are an ideal system for the analysis of the role of PGs in extracellular-matrix formation. The absence of detectable mRNA encoding PG I allows the interaction of PG II with matrix components to be studied in isolation. The present study provides a base on which to approach the roles of a variety of PG species in extracellular-matrix formation and regulation at the protein and molecular level.

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