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Isolation and characterization of proteoglycans synthesized by mouse osteoblastic cells in culture during the mineralization process

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Proteoglycans in mineralized (0.5 M-EDTA/4 M-guanidinium chloride-extractable) and non-mineralized (4 M-guanidinium chloride-extractable) matrices synthesized by a mouse osteoblastic-cell line MC3T3-E1 were characterized at different phases of mineralization *in vitro*. Cell cultures were labelled with [^{35}S]sulphate and either [^{3}H]glucosamine or ^{3}H-labelled amino acids. At the mineralization phase a large majority of proteoglycans were extracted with 4 M-guanidinium chloride (G extract), and at least five species of labelled proteoglycans were identified; dermatan sulphate proteoglycans (DSPG, apparent M_r approx. 120000 and 70000), heparan sulphate proteoglycans (HSPG, apparent M_r approx. 200000 and 120000) and DS chains with very little core protein. DSPGs weakly bound to an octyl-Sepharose CL-4B column and HSPGs bound more tightly, whereas DS chains did not bind to the column. Amounts of labelled proteoglycans extracted with 0.5 M-EDTA/4 M-guanidinium chloride (EDTA extract) were much less than those in the G extract. Although the predominant species in the EDTA extract were comparable with the DS or DSPGs in the G extract, none of them bound to octyl-Sepharose CL-4B, indicating their lack of hydrophobicity. At the non-mineralizing phase a large chondroitin sulphate proteoglycan (M_r greater than 600000) was found in the matrix in addition to the five proteoglycan species similar to those at the mineralization phase. Although DS chains at the early phase were similar in size to those at the mineralization phase, the ratio of 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose to 2-acetamido-2-deoxy-3-*O*-(β-D-gluculo-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose was less than that at the mineralization phase. These results agree with those of previous studies performed *in vivo* and suggest that alteration in the synthesis of proteoglycans is involved in the mineralization process. They also suggest that at the osteoblastic mineralization front proteoglycans undergo partial degradation and lose their hydrophobicity.

INTRODUCTION

Chondroitin sulphate proteoglycans (CSPGs) in mineralized bone tissue of human, bovine and other species have been isolated and characterized [1,2]. These reports showed that bone proteoglycans differ from the major proteoglycan in cartilage in terms of molecular size, antigenicity and interaction with hyaluronic acid. In addition, Fisher *et al.* [3,4] have demonstrated that bone proteoglycans are composed of two distinct small CSPGs. Their functional roles have not yet been fully clarified, although proteoglycans are generally thought to be important ground materials in bone.

It has been shown that the amount of sulphated glycosaminoglycans apparently decreases as endochondral ossification proceeds [5]. Inhibition of hydroxyapatite formation and growth *in vitro* by either proteoglycans or CS has been demonstrated [6], resulting in the widely held belief that these anionic sulphate-containing macromolecules inhibit mineralization. There has been, however, no direct evidence that proteoglycans inhibit bone mineralization *in vitro* as well as *in vivo*. Metabolism of bone proteoglycans has also not been elucidated yet, although Prince *et al.* [7] have demonstrated by a radiolabelling study performed *in vivo* that a part of newly synthesized CSPGs and/or their degradation products are deposited in the mineralizing matrix of rat calvaria. In order to approach these problems, it is important to use a simple system *in vitro* that resembles the bone mineralization process *in vivo*.

An osteoblastic-cell line, MC3T3-E1, derived from newborn-mouse calvaria, developed by Kodama *et al.* [8], has many osteoblastic characteristics such as high alkaline phosphatase activity [8], synthesis of type I collagen [9] and response to parathyroid hormone [10] or 1,25-dihydroxyvitamin D_3 [11] through their specific

Abbreviations used: CSPG, chondroitin sulphate proteoglycan; DSPG, dermatan sulphate proteoglycan; HSPG, heparan sulphate proteoglycan; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluculo-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-2S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-2,4-bis-S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-2,6-bis-S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; ΔDi-4,6-bis-S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulpho-D-galactose.

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receptors. In addition, this cell line is able to mineralize *in vitro* under normal growth conditions [12]. This process has been considered to resemble matrix vesicle-mediated intramembranous ossification *in vivo* [12]. Although the proteoglycans synthesized by osteoblasts in culture have been characterized [13–15], analyses of proteoglycans at different phases of the mineralization process have hitherto not been performed.

In the present study, proteoglycans in the non-mineralized matrix and in the mineralized matrix synthesized by MC3T3-E1 cells were separately isolated from consecutive extracts with 4 M-guanidinium chloride and 0.5 M-EDTA/4 M-guanidinium chloride as described previously [1,16] and characterized at different phases of mineralization.

MATERIALS AND METHODS

Materials

Purest-grade guanidinium chloride and urea were obtained from Wako Pure Chemicals, Osaka, Japan; $Ba(NO_2)_2$ was from Nakarai Chemicals, Kyoto, Japan; Pronase E was from Kaken Seiyaku, Tokyo, Japan; Sephadex G-50 (fine grade), DEAE-Sephacel, octyl-Sepharose CL-4B, Sepharose CL-2B, Sepharose CL-4B and Sepharose CL-6B were from Pharmacia Fine Chemicals; cellulose-coated t.l.c. plastic sheets (without fluorescent indicator) were from Merck; aqueous solutions of $Na_2{}^{35}SO_4$, D-[1,6-^{3}H]glucosamine hydrochloride, L-[G-^{3}H]leucine, L-[G-^{3}H]serine and $^{45}CaCl_2$ were from Du Pont/NEN; ^{14}C-labelled proteins for the M_r standard kit and Amplify were from Amersham; fetal-calf serum was from Irvine Scientific; alpha modified minimum essential medium and Ham's F-12 medium were from GIBCO; chondroitinase ABC (chondroitin ABC lyase) (*Proteus vulgaris*), chondroitinase AC (chondroitin AC lyase) (*Arthrobacter aurescens*), chondro-4-sulphatase (*P. vulgaris*), chondro-6-sulphatase (*P. vulgaris*) and unsaturated disaccharide standards were from Seikagaku Kogyo, Tokyo, Japan. Other chemicals were purchased at the purest grade. MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Tohoku Dental College, Koriyama, Japan).

Osteoblastic-cell culture

The MC3T3-E1 cell line was derived from C57BL/6 newborn-mouse calvaria by non-enzymic treatment by Kodama *et al.* [8]. The cells were grown in alpha modified minimum essential medium containing 2.9 mM-phosphate, 25 mM-Hepes, 100 i.u. of penicillin/ml, 100 μg of streptomycin sulphate/ml and 10% (v/v) fetal-calf serum. The sulphate concentration of this medium was approx. 0.8 mM and the phosphate concentration was adjusted to that in normal mouse serum (approx. 3.0 mM). The L-ascorbic acid content was 5 μg/ml. Approx. 2×10^5 cells were seeded in 100 mm-diam. plastic dishes or 75 cm^2 plastic culture flasks containing 15–20 ml of the medium described above, and the medium was changed three times a week. The fourth day after seeding was designated as phase 1 (early phase with no mineralization), and the 32nd day after seeding as phase 2 (mineralization phase), when mineralized plaques were visible. Deposited calcium phosphate plaques were stained by the method of von Kossa with $AgNO_3$ [17]. In order to quantify mineralization, 100000 c.p.m. of $^{45}CaCl_2$ was added to the cultures for 48 h, and ^{45}Ca accumulation into matrix/cell layers was measured [18]. At day 4 ^{45}Ca accumulation into matrix/cell layers was only 16 ± 3 c.p.m./μg of DNA (mean $\pm$ S.E.M., $n = 5$), whereas at day 32 it increased markedly to 529 ± 6 c.p.m./μg of DNA (mean $\pm$ S.E.M., $n = 5$). By using von Kossa staining it was found that mineralization occurred mostly in the intercellular matrices (T. Matsumoto, Y. Takeuchi & E. Ogata, unpublished work).

Radioisotopic labelling of cultures

On an appropriate day, medium was changed to 10 ml of a mixture of alpha modified minimum essential medium and Ham's F-12 medium containing 10% (v/v) fetal-calf serum and antibiotics.

Cells were radiolabelled by incubation in the medium with 20–40 μCi of [^{35}S]sulphate/ml and 20 μCi of D-[^{3}H]glucosamine/ml for 48 h. For core-protein analysis, cells were incubated with either 50 μCi/ml of L-[^{3}H]serine or 100 μCi of L-[^{3}H]leucine/ml as well as with 40 μCi/ml of [^{35}S]sulphate for 48 h.

Extraction of proteoglycans

After the labelling, incubation media were collected and made 4 M with respect to guanidinium chloride by the addition of solid guanidinium chloride. Matrix/cell layers were washed twice with 6 ml of ice-cold phosphate-buffered saline (0.14 M-NaCl/8 mM-sodium phosphate buffer, pH 7.4) and then subjected to extraction with 6 ml of 4 M-guanidinium chloride containing 0.1 M-6-aminohexanoic acid, 50 mM-disodium EDTA, 50 mM-sodium acetate and 0.5% (w/v) Triton X-100, pH 6.0 (4 M-guanidinium chloride buffer) at 4 °C for 24 h with continuous stirring [19]. The extracts were then centrifuged at 30000 *g* for 30 min at 4 °C. The precipitates were re-extracted in the same solution. After centrifugation, the combined supernatants were designated as 4 M-guanidinium chloride extract (G extract). Subsequently, proteoglycans in the residues were further extracted at 4 °C for 72 h with 4 M-guanidinium chloride buffer containing 0.5 M-tetrasodium EDTA, pH 7.4 [16]. Residues were spun down at 12500 *g* for 40 min at 4 °C. The supernatants contained material that was 0.5 M-EDTA-extractable but resistant to extraction with 4 M-guanidinium chloride alone (EDTA extract). When cultures were labelled with $^{45}CaCl_2$ as described above, the EDTA extract contained approx. 20-fold the radioactivity of the G extract calculated on the basis of protein content. Benzamidine hydrochloride and *N*-ethylmaleimide were added to each sample before extraction to give final concentrations of 5 mM and 10 mM respectively.

Isolation of proteoglycans

Proteoglycans were isolated from each extract by the procedures described previously [20] with some modifications. In brief, unincorporated radioactive precursors were removed from each extract by chromatography on Sephadex G-50 (8 ml bed volume for each 2 ml of sample) eluted with a buffer containing 7 M-urea, 0.15 M-NaCl, 50 mM-sodium acetate and 0.5% (w/v) Triton X-100, pH 6.0 (7 M-urea buffer). Each excluded fraction (3.5 ml) was applied to DEAE-Sephacel (bed volume 2 ml) equilibrated with the 7 M-urea buffer, and then eluted with a linear NaCl gradient from 0.15 M to 1.0 M in the same

solvent. Peaks of labelled proteoglycans were pooled separately, and the solvent was changed to 4 M-guanidinium chloride buffer without Triton X-100. Each pooled sample was applied to octyl-Sepharose CL-4B (bed volume 2 ml) equilibrated with 4 M-guanidinium chloride buffer without Triton X-100. After being washed with 10 ml of the same solution, the column was eluted with a continuous gradient of Triton X-100 from 0 to 0.5% [21]. The total elution volume was 50 ml at a flow rate of 3.0 ml/h. Peaks of radioactivity were pooled separately. Samples were desalted and stored at −20 °C until further analysis.

Gel-filtration column chromatography

Sepharose CL-2B, CL-4B and CL-6B columns (0.7 cm × 100 cm) were prepared in and eluted with 4 M-guanidinium chloride buffer containing 0.5% Triton X-100. For each column, effluent fractions of approx. 0.9 ml were collected. Columns were calibrated with hyaluronic acid for the void volume and glucuronic acid for the total volume.

Chemical analyses and enzymic digestions

Alkaline borohydride treatment was done in 50 mM-NaOH/1 M-$NaBH_4$ at 45 °C for 24 h [22]. The reaction was stopped by neutralization of the solution with acetic acid.

The HNO_2 treatment was done at pH 1.5 by the method of Shively & Conrad [23].

Hexuronic acid concentrations were determined by the method described by Bitter & Muir [24].

Samples dissolved in 0.1 M-Tris/HCl/0.1 M-sodium acetate buffer, pH 7.3, were digested for 2 h at 37 °C with chondroitinase ABC (0.1 unit for each sample) [25] or chondroitinase AC (0.2 unit for each sample) [26]. Chondro-4-sulphatase or chondro-6-sulphatase digestion was done in 50 mM-Tris/HCl/50 mM-sodium acetate buffer, pH 7.3 [25]. Disaccharides derived from chondroitinase ABC digestion were analysed by cellulose t.l.c. [27].

SDS/PAGE

Isolated proteoglycans that were radiolabelled with both [^{35}S]sulphate and [^{3}H]leucine were dissolved in a small volume of 0.1 M-Tris/HCl buffer, pH 7.3, containing 5 mM-calcium acetate with 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 0.36 mM-pepstatin and 1 mM-phenylmethanesulphonyl fluoride as proteinase inhibitors. Each sample was digested with 0.1 unit of chondroitinase ABC at 37 °C for 40 min. An equal volume of gel sample buffer as described below was added, followed by boiling of the mixture for 10 min [13]. Gel sample buffer contained 4 M-urea, 3.1% (w/v) dithiothreitol, 4% (w/v) SDS, 20% (v/v) glycerol and 0.0024% Bromophenol Blue in 0.16 M-Tris/HCl buffer, pH 6.8. Linear-3–15%-polyacrylamide-gradient slab gels (130 mm × 100 mm × 1 mm) and stacking gels of 3% polyacrylamide were formed. The Laemmli buffer systems [28] were used with some modifications of 50 mM-Tris/HCl and 0.38 M-glycine as electrode buffers. Samples were electrophoresed at 15 mA/gel for 4–5 h at 4 °C. Gels were fixed in 200 ml of 30% (v/v) ethanol/10% (v/v) acetic acid for 30 min and then immersed in 200 ml of Amplify for 30 min. After drying under heat and vacuum, gels were placed in contact with Dupont Cronex 4 X-ray films.

RESULTS

Distribution of labelled macromolecules

Virtually all labelled macromolecules ([^{35}S]sulphate and [^{3}H]glucosamine as precursors) associated with matrix/cell layers were extracted in the G extract at phase 1. At phase 2 the G extract also contained most of labelled macromolecules, and EDTA extract had only about 1% of the ^{35}S radioactivity of the G extract. Macromolecules labelled with [^{3}H]glucosamine, [^{3}H]serine or [^{3}H]leucine were relatively rich in the EDTA extract (approx. 5–10% of the radioactivity of G extract), suggesting that the EDTA extract was scarce in proteoglycans compared with the G extract.

Incubation media contained approximately two-thirds and one-half of labelled macromolecules in G extracts at phase 1 and phase 2 respectively.

Isolation of proteoglycans by DEAE-Sephacel chromatography

Representative DEAE-Sephacel chromatographies of the G extract and the EDTA extract at phase 2 are shown

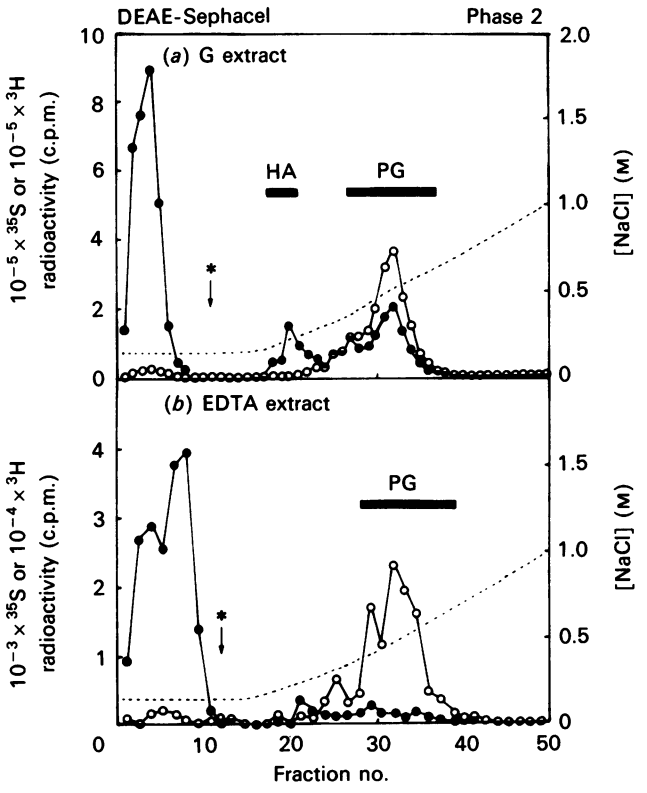

Fig. 1. DEAE-Sephacel chromatography of both G extract (*a*) and EDTA extract (*b*) at the mineralization phase (phase 2)

Cultures at phase 2 were labelled with [^{35}S]sulphate and [^{3}H]glucosamine for 48 h. Extracted macromolecules were applied on to a DEAE-Sephacel column and were eluted with a gradient of NaCl (------); ○, ^{35}S radioactivity; ●, ^{3}H radioactivity. The start of the gradient is indicated by an asterisk (*). Peaks containing hyaluronic acid (HA) and proteoglycans (PG) were pooled as indicated by the bars for further analyses. The proportion of ^{3}H radioactivity recovered in PG fractions was 21% in the G extract and 5% in the EDTA extract.

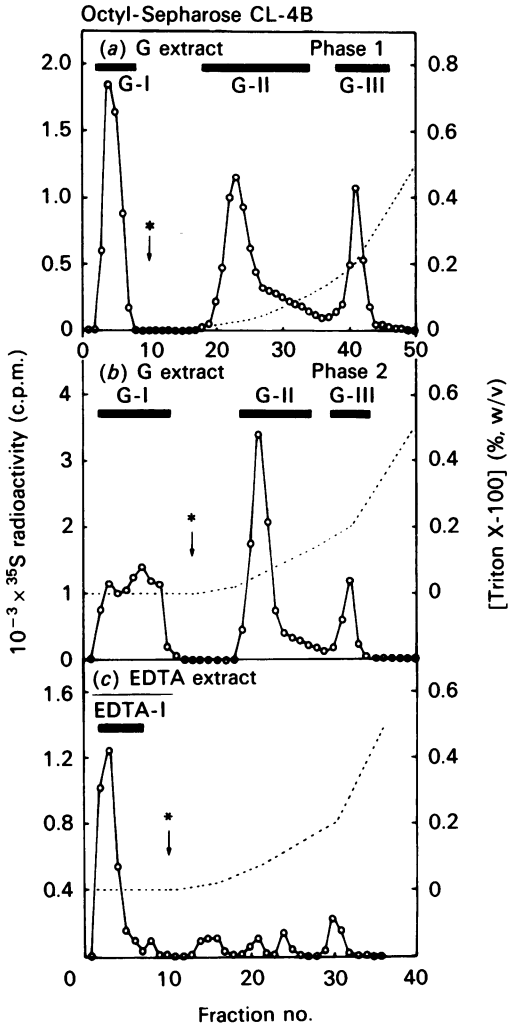

Fig. 2. Octyl-Sepharose CL-4B chromatography of proteoglycan fractions isolated by DEAE-Sephacel chromatography

Proteoglycan fractions indicated in Fig. 1 were chromatographed on octyl-Sepharose CL-4B and were eluted with a gradient of 0–0.5% Triton X-100 (------). The start of the gradient is indicated by an asterisk (*). Elution profiles of G extract at phase 1 (*a*), G extract at phase 2 (*b*) and EDTA extract (*c*) are shown. Each peak was pooled as indicated by the bar for further analyses.

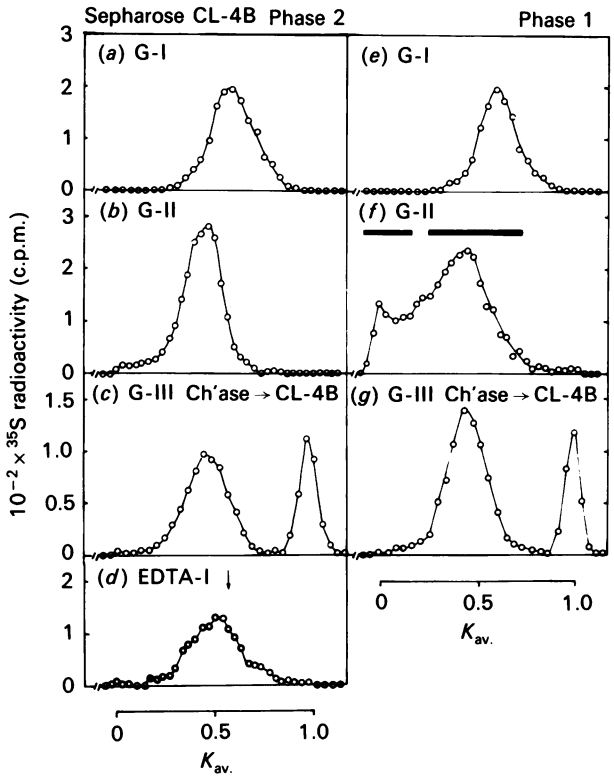

Fig. 3. Sepharose CL-4B chromatography of the ^{35}S-labelled proteoglycans eluted in 4 M-guanidinium chloride buffer at phase 2 (*a–d*) and at phase 1 (*e–g*)

(*a*) and (*e*) show the profiles of labelled materials in fraction G-I from octyl-Sepharose CL-4B chromatography at phase 2 and at phase 1 respectively; (*b*) and (*f*) show those in fraction G-II, and the profile of fraction EDTA-I is shown in (*d*). Fractions G-III at both phases were applied on the column after chondroitinase ABC (Ch'ase) digestion in order to remove DS or CS (*c* and *g*). Peaks at the total volume represent degradation products after chondroitinase ABC digestion. Fractions of G-II at phase 1 were pooled separately as indicated by bars for further analyses. The arrow in (*d*) indicates the peak position of fraction G-I.

in Fig. 1. The G extract yielded three major peaks of ^{3}H radioactivity (Fig. 1*a*). Most glycoproteins labelled with [^{3}H]glucosamine did not bind to the column in 0.15 M-NaCl. The second ^{3}H radioactivity peak was eluted at 0.25 M-NaCl. At least two-thirds of ^{3}H-labelled macromolecules in this fraction were excluded on Sepharose CL-2B chromatography, free from ^{35}S radioactivity and susceptible to chondroitinase ABC digestion (results not shown). These results indicated that most of the ^{3}H radioactivity in the second peak is in hyaluronic acid. The third ^{3}H radioactivity peak was co-eluted with the ^{35}S radioactivity and contained proteoglycans. This peak was collected as indicated by a bar in Fig. 1(*a*) for further analyses. The proportion of ^{3}H radioactivity recovered in the proteoglycan peak was 5% in the EDTA extract, and that in the G extract was about 21%. The profile for G extract of phase 1 matrix, which contained no mineralized plaque, was very similar to that for phase 2 (results not shown).

Separation of proteoglycans by hydrophobic-interaction chromatography

Octyl-Sepharose CL-4B chromatographic analysis of proteoglycan fractions in G extract isolated by DEAE-Sephacel chromatography generated three peaks of ^{35}S radioactivity. Representative elution profiles at both phases 1 and 2 are shown in Fig. 2. Unbound fractions (G-I) at phase 1 and phase 2 contained respectively 49% and 43% of the total ^{35}S radioactivity. Second peaks (G-II) were eluted at between 0.02% and 0.12% Triton X-100, which comprised 34% and 46% of the total ^{35}S radioactivity at phase 1 and at phase 2 respectively. Third peaks (G-III) were eluted at about 0.2% Triton X-100, which comprised 17% at phase 1 and 10% at phase 2 of ^{35}S-labelled proteoglycans.

Labelled proteoglycans extracted from EDTA extract gave a very different profile from those in the G extract

Table 1. Characteristics of glycosaminoglycan chains in each fraction separated by octyl-Sepharose CL-4B chromatography

Proteoglycans isolated by octyl-Sepharose CL-4B were treated with alkaline borohydride (OH^-/BH_4^-), followed by chromatography on a Sepharose CL-6B column. A portion of each sample was digested by either chondroitinase ABC or chondroitinase AC and was analysed by Sepharose CL-6B chromatography to determine susceptibility to each enzyme. For the last column, after chondroitinase AC digestion the proportion of ^{35}S radioactivity recovered in fractions of total volume from the Sepharose CL-6B column to the chondroitinase ABC-sensitive ^{35}S radioactivity was determined. Abbreviation: N.D., not determined.

		Distribution of ^{35}S radioactivity (%)	
	OH^-/BH_4^-, then Sepharose CL-6B chromatography ($K_{av.}$)	Chondroitinase ABC-sensitive	Chondroitinase AC-sensitive materials in chondroitinase ABC-sensitive
Phase 1			
G-I	0.41	78	51
G-II large	0.40	82	90
G-II small	0.43	69	55
G-III	0.50	25	N.D.
Phase 2			
G-I	0.41	82	42
G-II	0.41	88	53
G-III	0.53	30	N.D.
EDTA-I	0.49	82	55

(Fig. 2*c*). Approx. 75% of the ^{35}S radioactivity did not bind to the column (EDTA-I), indicating that most proteoglycans accumulating in EDTA extract during 48 h of labelling lack hydrophobic regions. This difference was not due to exposure of samples to the 4 M-guanidinium chloride buffer containing 0.5 M-EDTA because labelled proteoglycans in the G extract isolated by DEAE-Sephacel chromatography yielded an identical chromatogram with Fig. 2(*b*) following an incubation in the 4 M-guanidinium chloride/0.5 M-EDTA buffer at 4 °C for 72 h (results not shown).

Characterization of proteoglycans and glycosaminoglycans

Each peak of ^{35}S radioactivity isolated from octyl-Sepharose chromatography at phase 2 was analysed by Sepharose CL-4B chromatography (Figs. 3*a*–3*d*). Unbound fractions (G-I) were eluted at $K_{av.} = 0.56$ as a symmetrical peak (Fig. 3*a*). Fraction G-II was eluted at $K_{av.} = 0.43$ and had a small ascending shoulder (Fig. 3*b*). Analysis of fraction G-III material after chondroitinase ABC digestion showed that approx. 70% of the radioactive material resisted the enzymic digestion and was eluted at $K_{av.} = 0.46$ (Fig. 3*c*). This chondroitinase ABC-resistant material was degraded by HNO_2 treatment (results not shown), indicating the presence of heparan sulphate. Fraction EDTA-I ($K_{av.} = 0.52$) was eluted slightly faster than fraction G-I, and the elution profile was broad (Fig. 3*d*). All fractions except for G-III were mainly sensitive to chondroitinase ABC and partially susceptible to chondroitinase AC digestion (Table 1), indicating that they were mostly composed of dermatan sulphate proteoglycans (DSPGs). For phase 1 samples fractions G-I and G-III gave nearly the same results (Figs. 3*e* and 3*g*) as for the same fractions from phase 2. The intermediate fraction (G-II) was somewhat different between both phases. Fraction G-II at phase 1 contained an additional peak excluded from Sepharose CL-4B (G-II-large) (Fig. 3*f*). The G-II-large component contained approx. 30% of the total ^{35}S radioactivity in fraction G-II. This peak was rechromatographed on Sepharose CL-2B and was eluted at $K_{av.} = 0.47$ (results not shown). The M_r of the G-II-large component was estimated to be approx. 640000, based on the calibration of a Sepharose CL-2B column by Ohno *et al.* [29]. The G-II-large

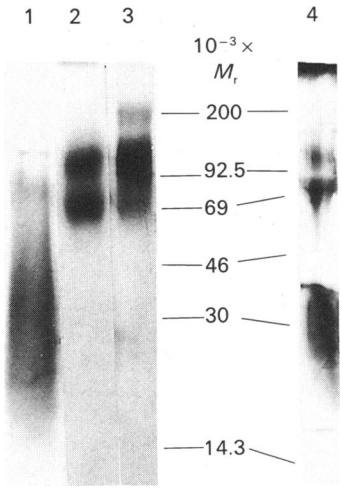

Fig. 4. Fluorography of SDS/PAGE gel analysing ^{35}S-labelled proteoglycans at phase 2

After an isolation by octyl-Sepharose CL-4B chromatography as shown in Fig. 2, portions of fractions G-I (lane 1), G-II (lane 2), G-III (lane 3) and EDTA-I (lane 4) were analysed by SDS/PAGE. The positions of M_r markers are indicated.

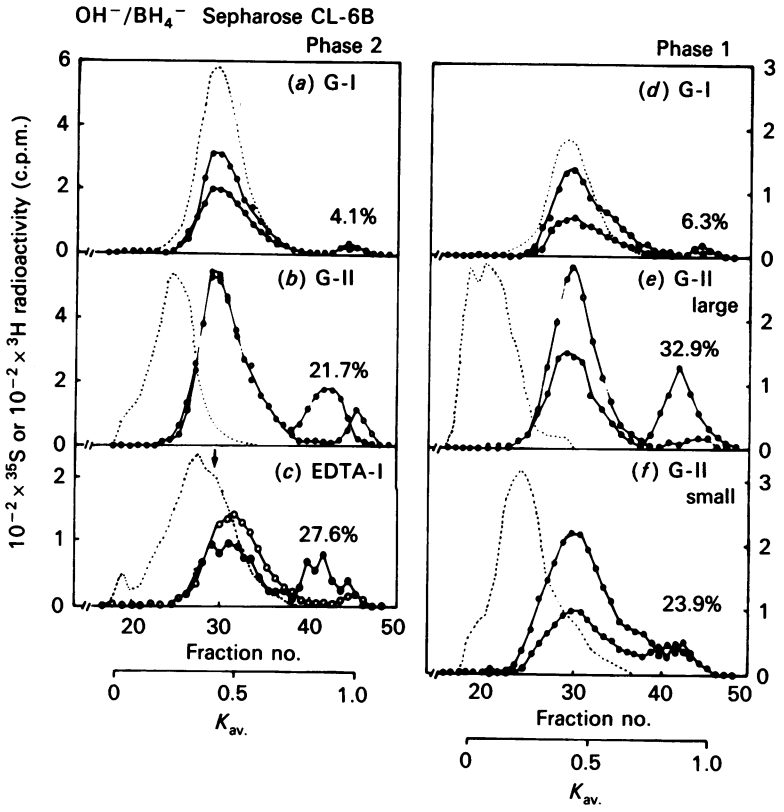

Fig. 5. Sepharose CL-6B chromatography of proteoglycans labelled with [^{35}S]sulphate and [^{3}H]glucosamine after alkaline borohydride treatment

Proteoglycans at phase 2 (*a–c*) and at phase 1 (*d–f*) were treated with alkaline borohydride (OH^-/BH_4^-). Fractions G-I (*a*), G-II (*b*) and EDTA-I (*c*) at phase 2 and fraction G-I (*d*) at phase 1 were isolated by octyl-Sepharose CL-4B chromatography. Fraction G-II large component (*e*) and fraction G-II small component (*f*) at phase 1 were fractionated by Sepharose CL-4B chromatography after octyl-Sepharose CL-4B chromatography: ○, ^{35}S radioactivity; ●, ^{3}H radioactivity. ^{35}S radioactivity profiles for untreated samples are also indicated (·····). The proportion of ^{3}H radioactivity recovered in oligosaccharide peak is indicated in each panel. An arrow in (*c*) indicates the peak position of fraction G-I at phase 2.

component was almost completely digested by chondroitinase AC, indicating a CSPG (Table 1). When the cells were labelled with [^{3}H]glucosamine in addition to [^{35}S]sulphate, the ^{3}H radioactivity was completely co-eluted with the ^{35}S radioactivity in each chromatogram, suggesting that contamination of glycoproteins in fractions G-I, G-II, G-III and EDTA-I was minimal, if any.

^{35}S-labelled materials at phase 2 were also analysed by SDS/PAGE (Fig. 4). Fraction G-I (lane 1) gave a smear with M_r less than 70000 and a prominent intensity centred at M_r 30000. Fraction G-II (lane 2) yielded two bands of similar darkness and a small amount of radioactivity remained at the top of the separating gel. These species could not be efficiently separated by gel-filtration column chromatography. Two major bands of fraction G-II had apparent M_r 120000 and 70000. Fraction G-III (lane 3) appeared as a broad band with apparent M_r 120000 with two minor species of M_r 200000 and 70000. Fraction EDTA-I (lane 4) yielded three bands, one of which was similar to fraction G-I and others were comparable with the 120000-M_r and 70000-M_r species of fraction G-II. After chondroitinase ABC digestion, both the 200000-M_r and the 120000-M_r species in fraction G-III migrated to the identical position with the undigested sample, whereas the 70000-M_r species were degraded (see Fig. 6, lane 3, below), indicating that fraction G-III had two heparan sulphate proteoglycans (HSPGs) of M_r approx. 200000 and 120000 and a DSPG of M_r 70000. An analysis of ^{35}S-labelled proteoglycans at phase 1 by SDS/PAGE gave a similar result to that at phase 2, and the large CSPG did not migrate into the separating gel (results not shown).

Glycosaminoglycan chains labelled with both [^{35}S]-sulphate and [^{3}H]glucosamine were released by alkaline borohydride treatment, and their M_r values were estimated by Sepharose CL-6B chromatography (Fig. 5 and Table 1) [30]. Glycosaminoglycan chains of fractions G-I and G-II at both phases had very similar molecular size, and they were eluted at $K_{av.}$ = 0.40–0.43, corresponding to CS chains with M_r 40000–45000 (Figs. 5*a* and 5*b* and Figs. 5*d*–5*f*). HS chains of fraction G-III at both of phases 1 and 2 were eluted at $K_{av.}$ = 0.50 and 0.53 on the same column, corresponding to M_r 30000 and 27000 respectively (results not shown). The elution position of fraction G-I at phase 2 did not change significantly after alkaline borohydride treatment, and little ^{3}H radioactivity was eluted near the column total volume (Fig. 5*a*). This suggests that fraction G-I at phase 2 is a single glycosaminoglycan chain with very little, if any, core protein and oligosaccharide components. Similarly, frac-

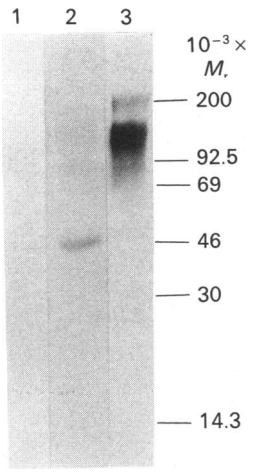

Fig. 6. Fluorography of SDS/PAGE gel analysing core proteins of proteoglycans at phase 2

Proteoglycans were labelled with both [^{35}S]sulphate and [^{3}H]leucine for 48 h at phase 2. Fractions G-I (lane 1), G-II (lane 2) and G-III (lane 3) were digested with chondroitinase ABC and electrophoresed on a linear-3–15%-polyacrylamide-gradient gel.

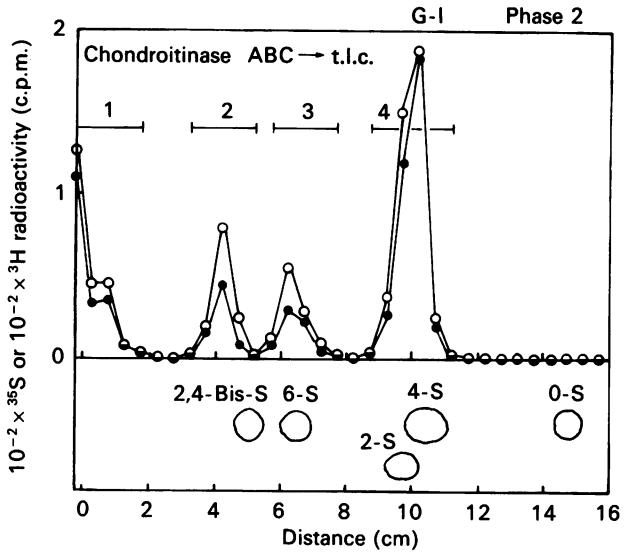

Fig. 7. Determination of labelled disaccharides of the DS by t.l.c.

Unbound fraction (G-I) from octyl-Sepharose CL-4B chromatography at phase 2 was subjected to chondroitinase ABC digestion, followed by t.l.c.: ○, ^{35}S radioactivity; ●, ^{3}H radioactivity. Spots represent migration positions of authentic ΔDi-2,4-bis-S, ΔDi-6S, ΔDi-2S, ΔDi-4S and ΔDi-0S. Peak 2 fractions were collected as indicated for further analysis.

tion G-I at phase 1 is probably a single chain as well (Fig. 5*d*). The hydrodynamic size of fraction G-II at phase 2 became smaller after alkaline borohydride treatment, and the sample contained 22% of the ^{3}H radioactivity eluted at $K_{av.} = 0.88$, indicating the presence of oligosaccharides (Fig. 5*b*). The large CSPG and DSPGs of fraction G-II at phase 1 contained oligosaccharides that accounted for as much as 32% and 24% of total ^{3}H radioactivity respectively (Figs. 5*e* and 5*f*). Fraction EDTA-I was composed of a glycosaminoglycan chain of M_r 31000 whose elution position was slightly later than those of fractions G-I and G-II ($K_{av.} = 0.49$ compared with 0.40–0.43), and it contained oligosaccharides (Fig. 5*c*), in contrast with fraction G-I.

Fractions G-I and G-II at both phases contained small amounts of chondroitinase ABC-resistant material (Table 1). The ^{35}S radioactivity in these species was eluted at $K_{av.} = 0.50$ and 0.22 respectively, which corresponded to HS chains and the HSPG in fraction G-III (results not shown).

Core protein–oligosaccharide complexes of DSPGs

Proteoglycans labelled with [^{35}S]sulphate and [^{3}H]-leucine as precursors were purified by the same procedures as described above. Samples of fractions G-I, G-II and G-III at phase 2 were subjected to chondroitinase ABC digestion, and then the digests were analysed by SDS/PAGE followed by fluorography (Fig. 6). The digest of fraction G-I gave no distinct bands (lane 1). Core protein–oligosaccharide complexes of fraction G-II yielded a single band with apparent M_r 45000 (lane 2). It is possible that DSPGs that were electrophoresed into two bands have core proteins with the same molecular size. Lane 3 shows materials released from fraction G-III after chondroitinase ABC treatment. Although the 70000-M_r DSPG was degraded by the enzyme, any core protein that might be present could not be detected. The 200000-M_r and the 120000-M_r species were not affected. An analysis by SDS/PAGE of core protein released from the DSPG in fraction EDTA-I was not possible because of low radioactivity in this fraction.

Disaccharide composition of CSPG and DSPGs

Disaccharides of each sample were analysed by t.l.c. after chondroitinase ABC digestion. In each chromatogram four migration positions of ^{35}S radioactivity were identified, namely a peak remaining at origin (peak 1) and three peaks that had migrated (peaks 2, 3 and 4) (Fig. 7). Peaks 3 and 4 were identical with those of ΔDi-6S and ΔDi-4S standards respectively. Radioactivity remaining at origin was resistant to chondroitinase ABC digestion, consistent with the presence of heparan sulphate and oligosaccharides on core proteins. Further analysis of peak 2 material by using the same t.l.c. after chondro-4-sulphatase and chondro-6-sulphatase digestion indicated that more than 80% of this material is ΔDi-4,6-bis-S (results not shown). The disaccharide composition of each glycosaminoglycan chain is summarized in Table 2. Small amounts of over-sulphated disaccharides were detected in each sample. Although the predominant disaccharide was ΔDi-4S in each case, the ratio of ΔDi-4S to ΔDi-6S varied among samples; it was significantly higher in the phase 2 samples than in the phase 1 sample. The CSPG in phase 1 also had lower content of ΔDi-6S than DS in the same phase. The relationship between DS chains in fraction G-I and the DSPGs is as yet unclear; however, identical glycosaminoglycan chain sizes and similar disaccharide compositions suggest that the DS chains are degradation products of DSPGs.

Proteoglycans secreted into the medium

Proteoglycans secreted into the medium during labelling were characterized by using the same procedures as

Table 2. T.l.c. analyses of disaccharide units susceptible to chondroitinase ABC

Proteoglycans isolated by octyl-Sepharose CL-4B chromatography were digested by chondroitinase ABC. Digests were run in the t.l.c. to determine the composition of the disaccharide units. Over-sulphated ΔDi was composed mostly of ΔDi-4,6-bis-S. The proportion of radioactivity of [^{3}H]glucosamine recovered in each peak to the total ^{3}H radioactivity after chondroitinase ABC digestion is shown. DS of the G extract was obtained from peaks not bound to octyl-Sepharose (G-I). DSPGs and CSPG were obtained from peaks weakly bound to octyl-Sepharose (G-II). DS/DSPGs of the EDTA extract were obtained from a peak not bound to octyl-Sepharose (EDTA-I).

	Distribution of ^{3}H radioactivity (%)		
Species	ΔDi-4S	ΔDi-6S	Over-sulphated ΔDi
G extract at phase 1			
DS	54	30	16
CSPG	71	20	9
DSPGs	55	29	16
G extract at phase 2			
DS	71	14	15
DSPGs	72	10	18
EDTA extract at phase 2			
DS/DSPGs	58	22	20

described above. Major components in the medium were DSPGs, which were almost identical with those in G extracts (results not shown). Although the CSPG (approx. 5–15%) and HSPGs (approx. 5%) were detected in the medium, any proteoglycans specific for the medium were not identified. No significant differences between two phases of cultures were found except for a relatively large content of the CSPG in the medium at phase 1 (results not shown).

DISCUSSION

In the present study proteoglycans synthesized by an osteoblastic-cell line MC3T3-E1 were characterized at different phases of culture period with or without mineralization. Proteoglycans associated with matrix/cell layers were extracted with sequential dissociative solution conditions, 4 M-guanidinium chloride buffer followed by 4 M-guanidinium chloride buffer containing 0.5 M-EDTA. This extraction procedure has been shown to extract matrix proteins associated with non-mineralized matrix separately from those in mineralized matrix [1,16]. The EDTA extract of matrix synthesized by MC3T3-E1 cells at the mineralization phase contained 20 times more calcium than did the G extract. Thus the EDTA extract is preferentially composed of mineralized matrix-associated proteoglycans compared with the G extract.

Proteoglycans in the G extract synthesized by MC3T3-E1 cells at mineralization phase consist of at least five distinct species: two DSPGs, DS chains and two HSPGs. The relative [^{35}S]sulphate incorporation into DSPGs, HSPGs and DS chains were approx. 45%, 20% and 35% respectively after 48 h labelling. At the early phase without mineralization, an additional large CSPG was identified. The DSPGs bound to octyl-Sepharose CL-4B with relatively weak hydrophobic interaction. They were separated into two bands by SDS/PAGE (apparent M_r 120000 and 70000), although gel-filtration chromatography could not completely separate these two molecules. Core protein–oligosaccharide complexes obtained by chondroitinase ABC digestion of the DSPGs had the same apparent M_r 45000. All DSPGs and DS chains contain glycosaminoglycans with similar molecular size and disaccharide composition. Octyl-Sepharose CL-4B column chromatography successfully separated DS chains from DSPGs in the G extract. It is possible that DS chains that did not bind to the octyl-Sepharose CL-4B column were derived from DSPGs, since both glycosaminoglycan chains are very similar. In this respect, cellular degradation of DSPGs in osteoblastic cells may be similar to that shown in rat ovarian granulosa-cell cultures [31]. The HSPGs in the G extract were separated into two M_r species (apparent M_r 200000 and 120000), and had glycosaminoglycan chains of M_r 30000. HSPGs showed stronger binding to octyl-Sepharose than did DSPGs, suggesting that these have hydrophobic domains that intercalate into the plasma membrane. The HSPGs, which were probably co-purified with DSPGs, in osteoblasts may be related to TGF-β1 receptors, as reported for fibroblasts [32,33]. These results in the phase 1 study are similar to those obtained by Beresford *et al.* [13] and Ecarot-Charrier & Broekhuyse [14], who used an osteoblast primary culture system without mineralization.

Very few ^{35}S-labelled proteoglycans were present in the EDTA extract, which mainly contained DS chains and unique DSPGs without hydrophobic interaction with octyl-Sepharose. They were separated by SDS/PAGE but not by gel-filtration chromatography, and their average molecular size was smaller than that of DSPGs in the G extract. Metabolic radiolabelling of bone proteoglycans *in vivo* has demonstrated three classes of proteoglycans similar to those in this study: a large CSPG excluded from Sepharose CL-6B and a small CSPG ($K_{av.}$ = 0.24) in non-mineralized bone matrix and a smaller CSPG ($K_{av.}$ = 0.36) in mineralized matrix [7]. Similar observations were reported by Tian *et al.* [19]. Thus it is possible that mineralization of the MC3T3-E1 cell culture system reflects that of bone in terms of metabolism of proteoglycans as well as previous studies in morphology [12]. Although the relationship between the small CSPG/DSPGs in the non-mineralized matrix

and the smaller CSPG/DSPGs in mineralized matrix is as yet uncertain, our results suggest that the hydrophobic DSPGs give rise to less hydrophic and smaller species by a partial cleavage of a hydrophobic domain.

Scott and co-workers and others have reported that proteoglycans occupy the trans-fibrillar loci of collagen fibrils in soft connective tissues such as tendon and skin (see ref. [34] for review). It has also been suggested by an electron microscopy that core proteins of proteoglycans interact with collagen fibrils [34]. Since the occupied region of collagen fibrils is suggested to be an initiation site of mineralization, proteoglycans possibly inhibit the mineralization process [34]. In contrast with soft connective tissues, proteoglycans in bone have been demonstrated to be located in the interfibrillar space and have not occupied any bands of collagen fibrils [34]. Although proteoglycans in bone (CSPGs) have different characteristics from those in skin and tendon (DSPGs), the core proteins have been shown to be very similar [35–37]. Taken together, modification of the core proteins of proteoglycans is probably important for interaction between proteoglycans and collagen fibrils. For example, partial degradation of proteoglycans by the removal of the hydrophobic domain in core proteins may lead to the dissociation of proteoglycans from the bands of collagen fibrils in bone, thus creating initiation sites for mineralization.

Synthesis of proteoglycans by MC3T3-E1 cells at two different culture phases with or without mineralization showed distinct features. The content of the large CSPG in the G extract at early phase was more than that at the mineralization phase. A similar observation has been reported by Reddi *et al.* [38] in the process of osteogenesis induced by the implantation of demineralized bone powder in rats. In endochondral ossification a decrease of large cartilagenous proteoglycans has been demonstrated to accompany the mineralization process [5]. In addition, we have observed that 1,25-dihydroxyvitamin D_3, which stimulated mineralization by MC3T3-E1 cells *in vitro* (T. Matsumoto, Y. Takeuchi & E. Ogata, unpublished work), caused a decrease in the content of the large CSPG [39]. Thus it is possible that the large CSPG may have an inhibitory role in mineralization. It has been reported that a large CSPG was actively synthesized at the undifferentiated stage and was decreased through a differentiation process in some systems [40,41]. MC3T3-E1 cells were proliferative after confluency at the undifferentiated early phase (phase 1), whereas the cell number did not increase at mineralization phase (phase 2) [12]. Thus the content of the large CSPG may reflect a stage of differentiation or may be involved in cell proliferation activity. Even if this were the case, it is still possible that the differences in proteoglycan synthesis contribute to mineralization because the change in proteoglycan synthesis due to loss of potency to proliferate may be one of the triggers for mineralization.

The MC3T3-E1 cell line provides a good model for studying mineralization *in vitro*, and further investigation on precise roles of bone proteoglycans in the mineralization process with the use of this system will be important.

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