Identity of the core proteins of the large chondroitin sulphate proteoglycans synthesized by skeletal muscle and prechondrogenic mesenchyme

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Large, chondroitin sulphate-containing proteoglycans are synthesized by three prominent tissues in the embryonic chick limb. One of these proteoglycans is aggrecan, the phenotype-specific proteoglycan of cartilage. Another, PG-M, is produced by prechondrogenic mesenchymal cells. The third, M-CSPG, is made by developing skeletal muscle cells. While the carbohydrate components of PG-M and M-CSPG share some similarities, both of these proteoglycans clearly have different carbohydrate moieties from those of aggrecan. To compare these three proteoglycans at another level, their core protein structures were analysed in three ways: by the presence or absence of monoclonal antibody epitopes, by one-dimensional peptide display of the cyanogen bromide-cleaved core proteins and by electron microscopic imaging of the molecules. Monoclonal antibodies whose epitopes are present in aggrecan core protein were tested with core protein preparations from M-CSPG and PG-M. One of these, 7D1, recognizes both PG-M and M-CSPG, while another, 1C6, shows no reactivity for the non-cartilage proteoglycans. The absence of 1C6 reactivity is of interest, as its epitope is in a region of the aggrecan core protein known to have a functional homologue in the core proteins of PG-M and M-CSPG. The

cyanogen bromide-fragmented peptide pattern of M-CSPG is the same as that of PG-M, and both are different from that of aggrecan. The aggrecan pattern has one prominent large band (molecular mass 130 kDa), some less prominent large bands (molecular mass 70–100 kDa) and several smaller bands. In contrast, the PG-M and M-CSPG patterns show no bands with molecular masses > 73 kDa, and the smaller bands (molecular mass < 40 kDa) have a different pattern to that of the smaller bands from aggrecan. The electron microscopic images of aggrecan show a core protein with one end having two globular regions separated by a short linear segment; adjacent to this is a long linear segment, which sometimes contains a third globular region at the end of the core protein opposite the end with the double-globe structure. M-CSPG and PG-M core proteins never show images with the double-globe structure. Instead, one end of the molecule has a single globular domain, and a second globular region is variably present at the opposite end of the core protein. Thus, by all three methods, the core proteins of PG-M and M-CSPG appear to be the same and both differ from the core protein of aggrecan.

INTRODUCTION

Proteoglycans, covalent adducts of protein and glycosaminoglycans, are involved in many cellular processes necessary for embryonic development, such as migration, adhesion and binding of cytokines and growth factors [1,2]. Large, galactosaminoglycan-containing proteoglycans are synthesized by many different cell types. Large proteoglycans containing chondroitin sulphate are produced by brain [3–5], retina [6], embryonic skeletal muscle [7], aorta [8], arterial smooth muscle [9], developing kidney [10,11], tendon [12], fetal and newborn skin [13], prechondrogenic mesenchyme [14,15] and periodontal tissue [16]. Large dermatan sulphate proteoglycans are synthesized by skin fibroblasts [17] and scleral tissue [18]. In addition, chondrocytes produce large proteoglycans which contain both chondroitin sulphate and keratan sulphate [19,20]. Because the polysaccharide components are added to the molecules post-translationally, it is necessary to analyse the core proteins in order to understand the relationships between the various proteoglycans better.

Three large, chondroitin sulphate-containing proteoglycans are synthesized by different cells of the embryonic chick limb. One of these is aggrecan, the chondroitin sulphate/keratan sulphate proteoglycan produced by chondrocytes [21–23]. An-

other, PG-M, is synthesized by the prechondrogenic mesenchymal cells which give rise to chondrocytes [14]. The third, (abbreviated M-CSPG) is synthesized by developing skeletal muscle cells, both as myoblasts and myotubes [7,24]. PG-M and M-CSPG are similar to each other in size and contain chondroitin sulphate as the only glycosaminoglycan [7,14,25]; the embryonic chick limb aggrecan is smaller and contains both chondroitin sulphate and keratan sulphate [21–23]. Consistent with the larger size of the intact molecules, the chondroitin sulphates of PG-M and M-CSPG are larger than that of aggrecan (molecular masses ≥ 60 kDa compared with 20 kDa) [7,14,21–23]. However, the sulphation patterns of the chondroitin sulphates of PG-M and M-CSPG differ [7,14], with M-CSPG having a higher percentage of 6-sulphated chondroitin sulphate (90 % versus 69 %) and PG-M having a higher percentage of unsulphated chondroitin (24 % versus 8 %). All three of these proteoglycans contain small O-linked and N-linked oligosaccharides and are able to aggregate with hyaluronic acid [14,21–23,25]. Because of the structural similarities between the carbohydrate constituents of PG-M and M-CSPG, their core proteins were compared with each other as well as with the core protein of aggrecan. Several methods were used to assess the degree of similarity between the three core proteins, including the presence or absence of various monoclonal antibody epitopes, electrophoretic mobility of core proteins,

Abbreviations used: M-CSPG and PG-M, chondroitin sulphate proteoglycans of skeletal muscle and prechondrogenic mesenchyme respectively; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulphoxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; PPO, 2,5-diphenyloxazole; NBT, Nitrotetrazolium Blue; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

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analysis of fragment patterns of the core proteins after cyanogen bromide cleavage and electron microscopic visualization after rotary shadowing. By all of these criteria, the core proteins of PG-M and M-CSPG appear to be the same, while the aggrecan core protein appears to be different, although some structural similarities exist between all three core proteins. These results indicate therefore that the same core protein is used by myogenic cells and prechondrogenic mesenchymal cells for synthesis of their large chondroitin sulphate proteoglycans.

MATERIALS AND METHODS

Materials

Urea, guanidinium chloride, CsCl, chondroitinase ABC, chondroitinase AC II, keratanase and carrier-free [35 S]sulphate (sodium salt) were obtained from ICN. Phenylmethane sulphonyl fluoride (PMSF), EDTA, *N*-ethylmaleimide (NEM), pepstatin and cyanogen bromide were purchased from Sigma and 6-aminohexanoic acid and benzamidine from Aldrich. Acrylamide, bisacrylamide, glycine, SDS, molecular-mass standards and 2-mercaptoethanol were from Bio-Rad. Formic acid and dimethyl sulphoxide (DMSO) were from Fisher, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) was from Boehringer-Mannheim and 2,5-diphenyloxazole (PPO) was from New England Nuclear. Molecular-mass standards labelled with 14 C, L-[4,5- 3 H]leucine and D-[6- 3 H]glucosamine were from Amersham and X-Omat film from Kodak. Tissue-culture reagents were from Grand Island Biological Company (GIBCO) and plasticware from Falcon. Secondary antibody for dot-blots and immunoblots [alkaline phosphatase-conjugated goat anti-mouse IgG] antibody and alkaline phosphatase substrates, Nitroterazolum Blue (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Promega and were purchased from Fisher. Sepharose CL-2B and CL-6B, Sephadex G-25 and G-50 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals. All other reagents were the purest commercially available.

Preparation of cell cultures of chick limb-bud cartilage

Chick limb buds were isolated from White Leghorn stage-24 embryos and cell suspensions obtained by trypsin digestion as described previously [26,27]. The single-cell suspensions were inoculated into 100-mm-diam. tissue-culture dishes at a density of 5×10^7 cells in 5 to 10 ml of medium, which consisted of 84% (v/v) Eagle's Minimal Essential Medium, 7% (v/v) horse serum, 3% (v/v) fetal-calf serum, 5% (v/v) chick embryo extract and 1% (v/v) GIBCO antibiotic-antimycotic solution and was replaced daily. At this high inoculation density, the majority of the cells eventually differentiate into chondrocytes [21,26,27]. On the day after cell seeding (day 1), most of the cells are undifferentiated mesenchymal cells [21,26,27].

Preparation of skeletal muscle cultures

Chick leg-muscle cultures were prepared by a vortex procedure as described previously [7,28]. Briefly, muscle tissue from the lower limbs of day 11 chick embryos was dissected free of fascia, skin and other tissue, minced and vortexed vigorously to liberate single cells. After removal of clumps by filtration through sterile cheesecloth, the cells were plated at 2.5×10^6 cells per 100-mm-diam. gelatin-coated culture dish. Medium consisted of 84% (v/v) Eagle's Minimal Essential Medium with Earle's salts, 10% (v/v) horse serum, 5% (v/v) chick embryo extract and 1%

(v/v) GIBCO antibiotic-antimycotic solution and was changed every day.

Radioisotopic labelling

For preparation of aggrecan, chick limb-bud cartilage cultures were labelled on day 8, the time of maximal synthesis [21]. Cultures were labelled for 6 h in 4–5 ml of medium, which contained 200 μ Ci/ml [3 H]leucine. The cell layers and associated matrix were rinsed twice with Tyrode's balanced salt solution, scraped with a polyethylene scraper into plastic tubes, frozen in a bath of solid CO₂-ethanol and stored at -70°C .

For preparation of PG-M for core protein analysis, day 1 limb-bud cell cultures were labelled for 19 h with 100 μ Ci/ml [3 H]leucine in 4–5 ml of medium per 100-mm-diam. dish. The cell layers were collected as for the day 8 cultures. Because the medium of day 1 cultures contains a significant proportion of the proteoglycans, the medium was saved at -20°C for proteoglycan isolation. For some experiments, the PG-M material was prepared from organ-cultured stage-22–33 chick limb buds [14]; this material was a gift from Dr. Koji Kimata (Aichi Medical University, Japan). The PG-M molecules isolated from day 1 limb-bud cell cultures do not differ from those isolated from organ-cultured limb buds with regard to monomer size, chondroitin sulphate size, the ability to aggregate with hyaluronic acid and the presence of O-linked and N-linked oligosaccharides.

Muscle cultures were labelled on day 3 for 6 h in 4–5 ml of medium per culture. Samples for proteoglycan analysis were labelled with 100 μ Ci/ml [35 S]sulphate and 500 μ Ci/ml [3 H]glucosamine. Samples for core protein analysis were labelled with 100 μ Ci/ml [3 H]leucine in leucine-free medium. At the end of the labelling period, the medium from the muscle cultures was collected and stored at -20°C until it was processed, and the cell layers were collected as for the other cultures. The medium from the cartilage cultures was discarded, as it contains less than 5% of the incorporated [35 S]sulphate.

Extraction and purification of limb-bud-cartilage proteoglycans

Cell layers were extracted in 4 M guanidinium chloride/0.05 M sodium acetate, pH 5.8, plus protease inhibitors (EDTA, PMSF, NEM, 6-aminohexanoic acid and benzamidine) and isolated by CsCl equilibrium-density-gradient centrifugation as described previously [22,25]. The high buoyant density or D1 fraction was reconstituted in 4 M guanidinium chloride/0.5% CHAPS/0.05 M sodium acetate, pH 6.0, and chromatographed on a column of Sepharose CL-2B to purify the proteoglycan monomer further.

Extraction and isolation of non-cartilage proteoglycans

Proteoglycans synthesized in cultures of chick embryonic leg muscle cells were extracted and isolated by a minor modification of an anion-exchange isolation procedure [9,29,30]. Cell layers were extracted in a solution of 4 M guanidinium chloride/0.5% CHAPS/0.05 M sodium acetate, pH 5.8, containing protease inhibitors. Tissues were extracted by stirring overnight (15–18 h) at 4°C . Extracts were clarified by centrifugation at 4°C and the residues digested with papain to facilitate determination of unextracted radioisotope, which accounted for 5% or less of the incorporated [35 S]sulphate.

Proteoglycans and other macromolecules were separated from unincorporated radioisotope and transferred out of the extraction solution by chromatography of the clarified extracts over dis-

possible columns of Sephadex G-50 in a buffer consisting of 0.05 M sodium acetate, pH 7.0, containing 8 M urea, 0.15 M NaCl and 0.5% CHAPS. Macromolecular material in the medium was likewise separated from unincorporated radioisotope by Sephadex G-50 chromatography after addition of solid guanidinium chloride to the medium to a final concentration of 4 M. The pooled void volume material, which contains macromolecules transferred into DEAE starting buffer, was applied to a 2 ml (5.2 cm × 0.7 cm) DEAE-Sepacel anion-exchange column. Samples were applied to the DEAE-Sepacel column by gravity at a flow rate of 5–10 ml/h, and the unbound material was collected in bulk. The column was then washed with 10 ml of the same buffer, and a linear gradient of NaCl was established from 0.15 M to 0.70 M in 8 M urea/0.5% CHAPS/0.05 M sodium acetate, pH 7.0. The total volume of the gradient was 46 ml and it was applied with a pump at a flow rate of 2.5 ml/h. The column was then rinsed with 8 M guanidinium chloride to desorb any remaining material, which accounted for 5–10% of the total ^{35}S radioactivity. In all cases, recovery of ^{35}S from the ion-exchange columns ranged from 80–90%. The conductivity of each fraction was measured, and aliquots were removed from each fraction for determination of radioactivity by scintillation spectrometry. Appropriate fractions were pooled, dialysed against distilled water at 4 °C and lyophilized to dryness. Samples were reconstituted in 4 M guanidinium chloride/0.5% CHAPS/0.05 M sodium acetate, pH 6.0, and chromatographed over a column of Sepharose CL-2B to separate the large chondroitin sulphate proteoglycans (M-CSPG) from smaller proteoglycans.

After establishment of the optimal conditions for gradient elution of the proteoglycans from muscle cultures, which was done with the samples labelled with [^3H]glucosamine and [^{35}S]sulphate, the samples for core protein analysis were simply eluted in a stepwise manner with solutions containing 0.25 M and 1 M NaCl in DEAE eluent. Previous work with several tissues from chick embryos, including skeletal muscle, showed that virtually all of the incorporated [^3S]sulphate is recovered in the 1 M NaCl eluate [31]. Stepwise elution from DEAE-Sepacel, followed by Sepharose CL-2B chromatography of the 1 M eluate, was used to isolate the [^3H]leucine-labelled M-CSPG for core protein analysis. This procedure was also used for isolation of [^3H]leucine-labelled PG-M from day 1 limb-bud cell cultures, as the conditions for gradient elution of PG-M have already been reported for organ-cultured limb buds [14].

Analysis of proteoglycan core protein

To generate peptide displays of the core proteins, isolated proteoglycans were treated with cyanogen bromide to cleave the core proteins and then the glycosaminoglycans were removed from aliquots of each sample by glycosidase treatment. Cyanogen bromide incubation conditions were a modification of those described elsewhere [32,33]. Lyophilized proteoglycans were reconstituted in a solution of 4% cyanogen bromide in 70% formic acid. Samples were incubated with constant stirring for 90 min, after which they were diluted approx. 10-fold with distilled water and dialysed exhaustively against distilled water at 4 °C with frequent changes of the dialysate. Samples were then lyophilized and stored at –20 °C until required for further analysis.

Glycosaminoglycans were removed from uncleaved and cyanogen bromide-cleaved proteoglycans by digestion with chondroitinase AC II (and keratanase for aggrecan) in the presence of protease inhibitors (EDTA, NEM, PMSF and pepstatin) as described elsewhere [33,34]. Samples were analysed

by electrophoresis on SDS/polyacrylamide slab gels prepared and run as reported previously [31,35]. Samples of uncleaved core proteins were applied to 5% (w/v) polyacrylamide analytical gels with 3% (w/v) polyacrylamide stacking gels; cyanogen bromide-cleaved core proteins were applied to 5–17.5% polyacrylamide gradient analytical gels with 5% polyacrylamide stacking gels. A Hoefer Mighty Small II slab-gel unit was used for electrophoresis. Immediately after electrophoresis, the gels were prepared for fluorography [36]. Gels were fixed for 1 h in 45% (v/v) methanol/10% (v/v) acetic acid, rinsed for 45 min with DMSO and then rinsed for 45 min in a solution of 22% (w/v) PPO in DMSO, all procedures being performed on a shaker. After impregnation with PPO, gels were rinsed briefly with water to precipitate the PPO, dried and used to expose Kodak X-Omat film.

Immunoblotting and dot-blotting

Samples for immunoblotting were electrophoresed as described above and then transferred to Immobilon [poly(vinylidene fluoride)] membranes by a slight modification of the procedure used previously [31]. Electrotransfer was performed with a Hoefer Transphor apparatus at 0.6 A for 1 h. The transfer buffer consisted of 25 mM Tris/192 mM glycine, pH 8.3, containing 20% (v/v) methanol [37] and 0.1% SDS. Inclusion of SDS in the transfer buffer enhances the rate of transfer of the high-molecular-mass core proteins [38,39], but requires the use of lower current and shorter transfer times than those used previously [31] as the detergent interferes with binding of proteins to the membranes [40]. After transfer, the membranes were blocked with 5% (w/v) powdered non-fat dry milk in TBS-T (10 mM Tris, pH 7.5/140 mM NaCl/0.05% Tween-20), incubated in primary antibody, rinsed with TBS-T, incubated in secondary antibody, rinsed again with TBS-T and then incubated in alkaline phosphatase substrates, NBT and BCIP [31]. After allowing sufficient time for colour development, the membranes were rinsed extensively with distilled water and allowed to dry. Dot-blots were performed as described previously [31] with slight modification. Samples for dot-blotting were diluted into TBS (TBS-T without Tween-20) so that the aliquots contained 10, 1 and 0.1 μg per 100 μl , based on the dry weight of lyophilized proteoglycan. Samples were applied to 0.45 μm -pore-diam. nitrocellulose with a Bio-Rad dot-blot apparatus at 100 μl per well. After the samples were drawn on to the nitrocellulose by vacuum, the wells were rinsed once with 100 μl of TBS and then the membranes were blocked and processed as for the immunoblots. The antibodies used for immunoblotting or dot-blotting are MY-174, which recognizes an epitope on the core protein of PG-M [41] S103L, which recognizes an epitope in the chondroitin sulphate-attachment region of aggrecan [42,43] and 2A5, 7D1 and 1C6, which recognize reduced and alkylated aggrecan core protein [44]. MY-174 was a gift from Dr. Koji Kimata (Aichi Medical University, Japan); S103L was generously provided by Dr. Nancy Schwartz (University of Chicago, Chicago, IL, U.S.A.); and 2A5, 7D1 and 1C6 were kindly provided by Dr. Bruce Caterson (University of North Carolina, Chapel Hill, NC, U.S.A.).

Analytical procedures

Columns (110 cm × 0.6 cm) of Sepharose CL-2B and CL-6B were poured and eluted in 4 M guanidinium chloride/0.5% CHAPS/0.05 M sodium acetate, pH 6.0, and 0.5 M sodium acetate, pH 7.0, respectively, as described previously [7]. Glycosaminoglycan chains were released from proteoglycans by in-

cubation with alkaline sodium borohydride [45]. The molecular masses of the glycosaminoglycans were estimated by comparison with previously published standard curves [46], with the assumptions that the column resin used has sieving properties similar to that employed for the previous report and that other glycosaminoglycans have elution properties like those of chondroitin sulphate. Disaccharide analysis of chondroitinase digests of glycosaminoglycans was accomplished by t.l.c. separation of the digestion products [47,48]. Nitrous acid treatment of glycosaminoglycans was performed as reported previously [49]; resistant and degraded material were separated on a column of Sephadex G-25.

Electron microscopic visualization of proteoglycans

Rotary shadowing of proteoglycans was performed in two ways: the glycerol spray technique [50] and the mica sandwich technique [51]. For the glycerol spray technique, lyophilized samples of proteoglycans were dissolved in 0.15 M NaCl/0.01 M Tris, pH 7.5, at a concentration of 100 µg/ml. A 15-µl aliquot of each sample was mixed with 15 µl of 0.12 M ammonium acetate, pH 5.0, in 60% (v/v) glycerol, and then 25 µl of this mixture were sprayed on to freshly cleaved mica squares with a nitrogen-driven airbrush operated at 193.2 kPa. For the mica sandwich technique, samples were reconstituted at a concentration of 1 or 5 µg/ml in 0.2 M ammonium bicarbonate containing 55% (v/v) glycerol. Aliquots of 5 µl of each sample were placed separately on to 1-cm² pieces of freshly cleaved mica and allowed to adsorb for 5 to 15 min. The samples were then placed into a Denton DV-502 vacuum evaporator on a rotating stage. At pressures less than 5×10^{-6} mmHg, the samples were shadowed at a 6° angle with 10–15 mm of 0.008-in 100% platinum wrapped around 0.05-in tungsten. The samples were floated off on to distilled water containing 5–10% formic acid and picked up on to 400-mesh copper grids. The samples were viewed in a JEOL JEM-100S transmission electron microscope operated at 60 keV. The magnification was calibrated at low magnification with a diffraction grating containing 2160 lines/mm (Ted Pella, Tustin, CA, U.S.A.) and at high magnification with negatively stained catalase crystals [52]. Images were recorded on Kodak Electron Microscope Film-4489. Video images were captured for computer-assisted measurements of molecules with Java video analysis software (Jandel Scientific).

RESULTS

Ion-exchange chromatography of skeletal muscle proteoglycans

Previously, the proteoglycans of skeletal muscle (including M-CSPG) have been isolated by CsCl equilibrium-density-gradient centrifugation [7,24,25]. For the studies reported here, ion-exchange chromatography was used to isolate M-CSPG, as this procedure better facilitates isolation of the lower-buoyant-density proteoglycans of non-cartilaginous tissues and to allow a direct comparison of M-CSPG with PG-M, for which ion-exchange chromatography was used for isolation [14]. The majority of the macromolecular ³⁵S extracted from the muscle-culture cell layer binds to the DEAE-Sephacel and was eluted during the NaCl gradient. In contrast with the [³⁵S]sulphate, of which 90–95% binds to the resin, only 50–60% of the incorporated [³H]glucosamine and 5–10% of the [³H]leucine do so, which is consistent with their incorporation into a wider spectrum of macromolecules than the [³⁵S]sulphate. Three major peaks of radioactivity are eluted from the DEAE-Sephacel column during the NaCl gradient (Figure 1a). Some radioactivity was also eluted with the 8 M guanidinium chloride, which is detected in

Figure 1 Column chromatographic isolation and analysis of the proteoglycans synthesized in day 3 chick leg-muscle cell cultures and extracted from the cell layer (—, ³⁵S c.p.m.; ---, ³H c.p.m.; ····, conductivity)

(a) DEAE-Sephacel column elution profile of the extracted material. Labelled material extracted from the cell layer was applied to a DEAE-Sephacel column and eluted with a linear gradient of 0.15–0.70 M NaCl. The radioactivity and conductivity of each fraction were measured, and the fractions were pooled as indicated, dialysed against distilled water at 4 °C, lyophilized and used for further analysis. (b) and (c) Sepharose CL-2B column elution profiles of DEAE-Sephacel pools 2 and 3 respectively. The lyophilized DEAE-Sephacel pools were reconstituted in Sepharose CL-2B column eluent and chromatographed. The radioactivity of each fraction was measured, and fractions were pooled as indicated, dialysed, lyophilized and treated with alkaline sodium borohydride to liberate the glycosaminoglycans. (d)–(h) Sepharose CL-6B column elution profiles of the glycosaminoglycans released from the various proteoglycans recovered from Sepharose CL-2B. To facilitate comparison, the vertical lines indicate the elution position of the glycosaminoglycans of pool 3a (i.e. the chondroitin sulphate of the large chondroitin sulphate proteoglycans). Fractions were pooled as indicated, dialysed, lyophilized and subjected to compositional analysis (Table 1).

Table 1 Glycosaminoglycan composition of the proteoglycans isolated from leg muscle cultures

Samples were generated by pooling fractions from the Sepharose CL-6B column as indicated in Figure 1. Values were calculated from the amounts of incorporated [^{35}S]sulphate which behaved as indicated (% resistant, the percentage at the origin of the thin layer plate for the chondroitinase assay and in the void volume of the Sephadex G-25 column for the nitrous acid assay; % C6S and % C4S, the percentages migrating as the disaccharide digestion products from chondroitin 6-sulphate and chondroitin 4-sulphate respectively; ABC, AC and HNO_2 , separate aliquots treated with chondroitinase ABC, chondroitinase AC II and nitrous acid respectively; the last line of the table is the percentage of 6-sulphated chondroitin sulphate calculated from the amount of [^{35}S]sulphate recovered as the digestion products of the two isomers of chondroitin sulphate.

	Pool 2a		Pool 2b		Pool 3a		Pool 3b		Pool 3c	
	ABC	HNO_2	ABC	AC	ABC	AC	ABC	AC	ABC	AC
% resistant	77	25	28	63	10	9	11	12	9	20
% C6S	—	—	36	29	82	85	78	81	70	69
% C4S	—	—	36	8	8	7	11	8	21	11
$\left(\frac{\text{C6S}}{\text{C6S} + \text{C4S}}\right) \times 100$	—	—	50	78	91	93	88	91	77	87

the elution profile as a rapid increase in conductivity; this material was not further analysed.

The material in Pool 1 (Figure 1a) elutes at the lowest conductivity of the three peaks and contains ^3H but essentially no ^{35}S . Pool 1 displays a prominent peak at the void volume of a Sepharose CL-2B column and a plateau of included material (not shown). This void volume material and almost all of the included material is hyaluronic acid, as assessed by digestion with chondroitinase ABC and t.l.c. analysis of the digestion products (results not shown). Thus, Pool 1 represents hyaluronic acid, of which a significant proportion is of high molecular mass.

Pools 2 and 3 are labelled by both [^3H]glucosamine and [^{35}S]sulphate (Figure 1a) and can also be labelled with [^3H]leucine (not shown). The prominent peak of Pool 3 elutes at a conductivity which indicates that the NaCl concentration is approx. 0.5 M, similar to that at which other large chondroitin sulphate proteoglycans, such as PG-M, were eluted [9,14]. Pool 3 contains the large skeletal muscle chondroitin sulphate proteoglycans, as indicated by the major peak at $K_{av} = 0.10$ in its Sepharose CL-2B elution profile (Figure 1c). The glycosaminoglycans of these proteoglycans elute from Sepharose CL-6B with $K_{av} = 0.25$ (Figure 1f). Most of the ^{35}S in this material is degraded by either chondroitinase ABC or AC II and approx. 90% of the chondroitin sulphate is chondroitin 6-sulphate (Table 1). These structural parameters indicate that the proteoglycans in Pool 3a are the large chondroitin sulphate proteoglycans of skeletal muscle. Other proteoglycans recovered in Pool 3 (i.e. Pool 3b, Figure 1c) are smaller in size and have somewhat smaller chondroitin sulphate chains (Sepharose CL-6B $K_{av} = 0.33$, Figure 1g) which likewise possess 90% 6-sulphated isomer (Table 1); these may represent molecules recovered at lower buoyant density on the CsCl gradients [7,24,25]. The smallest proteoglycans recovered in Pool 3 (i.e. Pool 3c, Figure 1c) have still smaller glycosaminoglycans (Sepharose CL-6B $K_{av} = 0.38$, Figure 1h) and appear to contain dermatan sulphate, as indicated by the increased percentage of material resistant to digestion with chondroitinase AC II compared with chondroitinase ABC (Table 1). The presence of this material in Pool 3 may result from the incomplete separation of Pools 2 and 3 (see below).

The proteoglycans of Pool 2 are relatively small in size and

were eluted from Sepharose CL-2B as a main peak of $K_{av} = 0.62$ and a trailing shoulder of $K_{av} = 0.76$. The larger of these two proteoglycans (Pool 2a) has glycosaminoglycans which were eluted from Sepharose CL-6B with $K_{av} = 0.35$ (Figure 1d) and are largely resistant to chondroitinase but sensitive to nitrous acid (Table 1). This indicates that its glycosaminoglycans are composed of heparan sulphate. The other proteoglycan (Pool 2b) has smaller glycosaminoglycans (Sepharose CL-6B $K_{av} = 0.47$, Figure 1e) which contain a significant proportion of dermatan sulphate (Table 1) and are sulphated equally at the 4 and 6 position (Table 1). Based on previous work, this proteoglycan appears to be decorin [31].

Therefore, in the day 3 skeletal muscle cultures, three major proteoglycan species are synthesized: the large chondroitin sulphate proteoglycan of skeletal muscle, which has chondroitin sulphate chains of 60–70 kDa and 90% 6-sulphated isomer; a small heparan sulphate proteoglycan, similar to that previously reported in chick pectoral muscle cultures [53], with glycosaminoglycans of 40–50 kDa; and a small dermatan sulphate proteoglycan, which appears to be decorin and has glycosaminoglycans of 20–30 kDa with as much as 30–40% dermatan sulphate. This analysis is for leg-muscle-culture cell layers labelled on day 3, at which time the major cell type is freshly fused myotubes [7,28]. The same proteoglycan species are observed in the medium, which contains approx. half of the incorporated [^{35}S]sulphate, except that the relative amount of the heparan sulphate proteoglycans is less, consistent with previous results [54]. With cultures labelled on day 1, at which time the cells consist primarily of unfused, proliferating myoblasts [7,28], the same proteoglycans are observed, but the proportion of heparan sulphate proteoglycans is less than on day 3, which correlates with previous results [53,54] and is consistent with the developmental progression of proteoglycan synthesis in culture and *in vivo* [7,24,30].

Monoclonal antibody analysis

Several monoclonal antibodies were used to compare the core proteins of aggrecan, PG-M and M-CSPG. Core protein preparations, generated by treatment of isolated proteoglycans with chondroitinase AC II (plus keratanase for aggrecan), were tested by dot-blot assay for reactivity with the monoclonal antibodies. S103L, which recognizes an epitope in the chondroitin sulphate-attachment region of chick aggrecan core protein [42,43], does not react with either M-CSPG or PG-M (results not shown). Previously, it was found that MY-174, which recognizes an epitope in the core protein of PG-M, cross-reacts with the core protein of M-CSPG [55]. The other monoclonal antibodies which were tested, 2A5, 7D1 and 1C6, react with the core proteins of mammalian aggrecan after reduction and alkylation [44]. Of these, 2A5 fails to recognize any of the chick proteoglycan core proteins, including that of aggrecan, while bovine and rat aggrecan core proteins are clearly recognized (Figures 2 and 3). This suggests that the 2A5 epitope is not present in avian proteoglycans. In contrast, 7D1 recognizes the core proteins of all three of the chick large chondroitin sulphate proteoglycans (Figures 2 and 3). However, while 1C6 recognizes the core protein of chick aggrecan, this antibody shows no reactivity with the core proteins of M-CSPG and PG-M (Figures 2 and 3).

To compare the core proteins of aggrecan, PG-M and M-CSPG further, they were analysed by Western immunoblot with MY-174. The core protein preparation from chick aggrecan shows a single band of high molecular mass (Figure 4); this pattern is also observed with S103L (results not shown). PG-M and M-CSPG show two bands, both of which have less mobility

Figure 2 Dot-blot analysis of core protein preparations from chondroitin sulphate proteoglycans

Core proteins were prepared from various proteoglycans by treatment with glycosidase(s) (either chondroitinase AC II or chondroitinase AC I and keratanase, as appropriate) in the presence of protease inhibitors and then subjected to dot-blot analysis with monoclonal antibodies 1C6, 2A5 and 7D1. The core protein preparations analysed are from aggrecan isolated from chick limb-bud chondrocyte day 8 cultures (C-CSPG), the large chondroitin sulphate proteoglycans isolated from chick embryo leg-muscle day 3 cell cultures (M-CSPG), aggrecan isolated from bovine nasal septum (BNS) and aggrecan isolated from rat chondrosarcoma (RCS). Separate aliquots were assayed without or after reduction and alkylation; as expected, control samples which were not reduced gave minimal, if any, reactivity.

than the aggrecan core protein; the more slowly migrating of the two bands is more prominent (Figure 4). Another sample of muscle proteoglycan which was tested by immunoblot is from day 14 chick embryonic leg muscle. (This sample is designated 'M-PG' in Figure 4.) The M-PG sample shows the same core protein bands as those recognized by MY-174 for M-CSPG and PG-M. The M-PG sample also contains two other prominent bands, one migrating slightly slower and one somewhat faster than the aggrecan core protein (Figure 4). These bands are faintly present in the M-CSPG and PG-M samples and, as discussed below, may represent catabolic products. There are other faint bands in the three non-cartilage samples, and these may likewise result from catabolism. These bands become progressively much more prominent in samples prepared from older leg muscle (day 20 embryos, hatchling chick and two-year-old chicken; data not shown). This is also consistent with these bands resulting from catabolism, as new synthesis of large chondroitin sulphate proteoglycans is much reduced or absent in older skeletal muscle [24,30]. None of the bands seen in Figure 4 is observed if the samples are not treated with chondroitinase before electrophoresis or if the immunoblot is not exposed to MY-174 (results not shown).

Cyanogen bromide cleavage fragment patterns

Aggrecan (from day 8 limb-bud cell cultures), PG-M (from day

Figure 3 Dot-blot analysis of core protein preparations from chondroitin sulphate proteoglycans

Core proteins were prepared from various proteoglycans by treatment with chondroitinase AC II in the presence of protease inhibitors and then subjected to dot-blot analysis with monoclonal antibodies 1C6, 2A5 and 7D1. One core protein preparation analysed is from the large chondroitin sulphate proteoglycans isolated from stage 22–23 chick limb buds (PG-M); M-CSPG and RCS are as defined in the legend for Figure 2.

Figure 4 Western immunoblot of core protein preparations from chondroitin sulphate proteoglycans

Core proteins were prepared from various proteoglycans by treatment with glycosidase(s) in the presence of protease inhibitors. These preparations were electrophoresed and transferred to Immobilon. The immunoblot was probed with monoclonal antibody MY-174. M-CSPG, PG-M and C-CSPG are as described in the legends for Figures 2 and 3. M-PG is the core protein preparation from the proteoglycans isolated from day 14 chick embryo leg muscle.

1 limb-bud cell cultures) and M-CSPG (from day 3 leg-muscle cell cultures) were isolated after metabolic labelling with [3 H]leucine. Core protein preparations were generated from each proteoglycan and compared by electrophoresis and fluorography (Figure 5). Aggrecan core protein consists of a single band with an estimated molecular mass of 440 kDa. The M-CSPG core protein preparation shows two bands with estimated molecular masses of 570 and 490 kDa. These are similar to the values previously reported for PG-M [14]. Moreover, the results for 3 H-labelled M-CSPG core protein (Figure 5) are similar to those

Figure 5 Fluorograph of ^3H -labelled core protein preparations from chondroitin sulphate proteoglycans

Appropriate cell cultures were labelled with ^3H leucine and the proteoglycans isolated. Core proteins were prepared by treatment with glycosidase(s) in the presence of protease inhibitors and electrophoresed, and the gel was subjected to fluorography. C-CSPG, M-CSPG and PG-M are as defined in the legends for Figures 2 and 3, except that PG-M was isolated from the medium of chick limb-bud day 1 cell cultures.

Figure 6 Cyanogen bromide cleavage patterns of chondroitin sulphate proteoglycan core proteins

Proteoglycans labelled with ^3H leucine were treated with cyanogen bromide. Aliquots of each sample were electrophoresed either without (CNBr only) or after (CNBr + CSase) removal of the glycosaminoglycans by treatment with chondroitinase AC II (plus keratanase for aggrecan) in the presence of protease inhibitors. After electrophoresis, the gel was subjected to fluorography. C-CSPG, M-CSPG and PG-M are as defined in the legend for Figure 5. Not all of the previously identified aggrecan bands [33] are visible. Prolonged exposure of the fluorograph (not shown) allows detection of all of the previously observed aggrecan bands [33] and reveals no other M-CSPG or PG-M bands.

Table 2 Cyanogen bromide peptide fragments from large chondroitin sulphate proteoglycan core proteins

Cyanogen bromide peptide patterns were generated for ^3H leucine-labelled core proteins of aggrecan, M-CSPG and PG-M (Figure 6). The relative molecular mass of each peptide was estimated from its mobility relative to protein standards. The presence (+) or absence (–) of glycosaminoglycan was determined by comparison of samples electrophoresed without or after glycosaminoglycan removal. Not all of the previously reported aggrecan peptides [33] are indicated; only those peptides observable in Figure 6 are noted. However, prolonged exposure of the fluorograph allows detection of all of the aggrecan peptides previously described.

Core protein	Peptide	Molecular mass (kDa)	Glycosaminoglycan
Aggrecan	1	130	+
	2	100	+
	3	87	+
	6	65	+
	8A	54	+
	8B	48	+
	9	43	+
	10A	35	–
	10B	29	–
	11A, B	21	+
	12	17	–
	13	13	–
M-CSPG/PG-M	1	73	–
	2	58	–
	3	52	+
	4	47	–
	5	40	–
	6	35–39	–
	7	28–31	+
	8	20–23	+
	9	13	–

seen with the MY-174 immunoblot (Figure 4); in both cases, there is a more prominent, slower-migrating band and a less prominent, faster-migrating band. In addition to PG-M and M-CSPG, a doublet pattern has been reported for the core proteins of large galactosaminoglycan-containing proteoglycans from other tissues, such as newborn skin [13], arterial smooth muscle [56] and periodontal fibroblasts [16].

The ^3H -labelled core protein preparation for PG-M has a more complex pattern, with four prominent bands and several fainter bands (Figure 5). The largest two of the prominent bands have the same mobilities as the bands from M-CSPG. The smallest two of the prominent bands have mobilities similar to the smallest two prominent bands observed in the MY-174 immunoblot of the core protein preparation from day 14 leg-muscle proteoglycans (M-PG of Figure 4). Thus, the four most intense bands in the MY-174 immunoblot of M-PG and in the ^3H -labelled PG-M have the same mobilities. In addition, the fainter ^3H -labelled PG-M bands appear to have mobilities similar to the fainter, MY-174-stained M-PG bands. The more complicated pattern observed with the ^3H -labelled PG-M may be due to increased catabolism, since a longer labelling period was required to allow incorporation of sufficient ^3H leucine for the cyanogen bromide analysis.

Other aliquots of the ^3H -labelled proteoglycans were treated with cyanogen bromide and then electrophoresed either after or without removal of the glycosaminoglycans by glycosidase treatment in the presence of protease inhibitors (Figure 6). If its

Figure 7 Electron microscopic images of rotary-shadowed molecules of M-CSPG (a–d) and PG-M (e–h)

M-CSPG isolated from day 3 muscle cultures and PG-M isolated from organ-cultured stage-22–23 limb buds were processed for rotary-shadowed electron microscopy. Black arrows indicate globular regions and white arrows indicate glycosaminoglycan (chondroitin sulphate) side chains; bar = 100 nm. M-CSPG molecules contain core proteins with variable thickness and few clearly discernible globular domains. PG-M molecules have clearly defined globular regions, a consistently thin core protein, and barely detectable side chains.

glycosaminoglycans are removed, aggrecan gives rise to a pattern of 17 resolvable peptides which has been reported previously ([33]; Figure 6). These peptides range in molecular mass from 130 to 11 kDa (Table 2). Comparison with the pattern observed for the aggrecan sample from which the glycosaminoglycans were not removed shows that several of the smaller peptides do not have attached glycosaminoglycans. This is indicated by the similarity in mobility of the peptides in samples electrophoresed without or after prior glycosidase treatment and is seen for aggrecan peptides 10A, 10B and 12 ([33]; Figure 6). Many of the aggrecan peptides (e.g. peptides 1–9) appear to contain attached glycosaminoglycan, as shown by their absence from the pattern obtained without previous glycosidase treatment ([33]; Figure 6); instead, an intense band is observed at the top of the stacking gel and a fainter, diffuse band is seen above the position to which aggrecan peptide 1 migrates (Figure 6).

The patterns for M-CSPG and PG-M are the same as each other and different from the aggrecan pattern. If their chondroitin sulphate is removed, both M-CSPG and PG-M give rise to nine discernible bands, which range in molecular mass from 73 to 13 kDa (Figure 6 and Table 2). Peptides 1–5 and 9 resolve as sharp bands, while bands 6–8 are diffuse. Peptides 3, 7 and 8 are absent, or much reduced, if the glycosaminoglycans are not removed before electrophoresis, which indicates that these peptides have chondroitin sulphate attached to them. The pattern for the PG-M and M-CSPG samples which were not glycosidase-treated before electrophoresis contains a diffuse band with a molecular mass of 60–100 kDa (Figure 6); this, presumably, represents the chondroitin sulphate peptides. These M-CSPG/PG-M chondroitin sulphate peptides are much smaller than those of aggrecan (which barely enter the gel), even though the chondroitin sulphate chains of PG-M and M-CSPG are larger

[7,14]. This suggests that there are fewer chondroitin sulphate chains per cyanogen bromide fragment for PG-M and M-CSPG. Some of the peptides in the M-CSPG/PG-M pattern have mobilities similar to bands in the aggrecan pattern, such as peptides 3, 4, 7, 8 and 9 of M-CSPG/PG-M and peptides 8A, 8B, 10B, 11A, 11B and 13 of aggrecan respectively. However, some of the similarly migrating peptides differ with respect to the presence or absence of glycosaminoglycan attachments (Figure 6 and Table 2). Control samples of the proteoglycans were electrophoresed on gradient gels either without or after removal of the glycosaminoglycans by glycosidase treatment, but without cyanogen bromide treatment. These samples showed none of the bands seen in Figure 6 (results not shown). This indicates that cyanogen bromide is required to generate the peptides and that spurious cleavage is not responsible for the patterns observed for each sample.

Electron microscopic visualization

The ultrastructure of PG-M and M-CSPG was examined by electron microscopic observation of high-resolution rotary-shadowed specimens. The images of PG-M and M-CSPG both show elongated linear molecules with globular domains located at their ends (Figure 7). The number of globular domains is variable between 0 and 2, but these domains are always located at the terminus of the linear segment (arrows in Figure 7). The variability in occurrence of the globular domains may be a result of variations inherent in the rotary-shadowing methodology. For example, folds in the core protein or differences in spreading of the glycosaminoglycan side chains can obscure details within individual molecules to a varying degree. The structure of PG-M and M-CSPG is different from that of bovine, rat [57,58] and

Figure 8 Histogram of core protein length measurements from rotary-shadowing electron micrographs of proteoglycan monomers of PG-M (a) and M-CSPG (b)

The core protein of PG-M has a mean length of 383 nm (S.D. = 80 nm; $n = 36$) and the core protein of M-CSPG has a mean length of 298 nm (S.D. = 88 nm; $n = 141$).

chicken [43] aggrecan. Aggrecan molecules have a structure with three globular (G1–G3) and two linear domains (L1 and L2). The G1 and G2 structures reside at one end of the molecule and are connected by a short L1 segment. G3 is variably present at the end of the molecule opposite that of the G1–G2 double-globe structure and is separated from G2 by the long linear L2 segment. M-CSPG and PG-M do not show a double-globe structure and have, at most, a single globular region at each end of the molecule (Figure 7). In addition to the differences in globular domain structure between aggrecan on the one hand and M-CSPG and PG-M on the other, PG-M and M-CSPG contain longer but fewer glycosaminoglycan side chains, which is consistent with the biochemical analysis of these proteoglycans [7,14].

The M-CSPG molecules have clearly visible side chains and a linear core protein with regions of varying thickness randomly spaced along its length. The cause of these thicker regions is unknown, although collapsed side chains seem likely. From these images, it is difficult to distinguish a true globular region from a core protein thickening. The PG-M molecules have clearly defined globular domains which tend to be highly electron dense. The PG-M side chains are not as apparent as those in the M-CSPG preparations and often appear as a diaphanous formation rather than clearly traceable linear structures. The PG-M core protein does not contain alternately thickened regions along its length as does M-CSPG core protein. Whether these ultrastructural differences reflect a true biochemical difference in these molecules or preparative differences cannot be ascertained from these data.

Measurements of core protein lengths (Figure 8) show a total length (mean $\pm$ S.D.) of 382 ± 80 nm ($n = 36$) for PG-M, while

that of M-CSPG is 298 ± 88 nm ($n = 141$). We previously reported that chick aggrecan has a core protein length of 145 ± 31 nm [43], which is less than half that of either PG-M or M-CSPG. This correlates with the immunoblot and fluorographic data, which show that aggrecan has a smaller core protein than PG-M or M-CSPG (Figures 4 and 5). To determine whether glycosaminoglycans attached to the proteoglycan core protein influence the core protein length, perhaps by repulsive forces between negatively charged sulphate and carboxyl groups of the chondroitin sulphate, measurements were made of PG-M molecules which had been digested with chondroitinase AC II and which clearly contained two terminal globular regions. Measurements on 25 molecules show a length of 388 ± 56 nm (data not shown), which is nearly identical with the length for the core protein measured in PG-M monomers. Hence, the chondroitin sulphate chains do not significantly affect the length of the core protein measurements.

DISCUSSION

The results presented above suggest that the large chondroitin sulphate proteoglycans of skeletal muscle (M-CSPG) and prechondrogenic mesenchyme (PG-M) contain the same core protein. One caveat to these findings is the observation that the sources of PG-M (stage-23 limb buds and day 1 limb-bud mesenchymal cell cultures) contain myogenic cells [59,60], and the source of M-CSPG (day 3 cultures of leg-muscle cell cultures) contains mononucleated cells of indeterminate origin [7], some of which may be mesenchymal cells. Thus, the possibility exists that only a single cell type is responsible for the synthesis of both PG-M and M-CSPG. Immunohistochemistry with MY-174 shows staining in the prechondrogenic core region of stage-23–25 limb buds [41] and pericellular staining around myotubes of stage-36 leg muscle [55]. This suggests that both myogenic cells and prechondrogenic mesenchymal cells produce MY-174-reactive molecules. When cDNA probes are available for the PG-M/M-CSPG core protein, *in situ* hybridization analysis should clarify the cellular source(s) of these molecules.

If both cell types do, in fact, utilize the same core protein for synthesis of their large chondroitin sulphate proteoglycans, then this is of interest for several reasons. First, the myogenic cells synthesize M-CSPG well after differentiation of the cells has begun. M-CSPG synthesis in myogenic cell cultures is observed even after fusion into myotubes [7]. In contrast, PG-M is made by the mesenchymal cells of the limb-bud core region before their differentiation into chondrocytes [14,41]. In addition, the myogenic and prechondrogenic cells of the limb arise from different lineages. While the prechondrogenic cells arise from the lateral plate mesoderm which initially forms the limb bud, the myogenic cells of the limb migrate into the limb bud from the somites [59].

Several findings about the PG-M/M-CSPG core protein are also of interest. The lack of reactivity with 1C6 and the absence of a G2 domain may be correlated, as one of the 1C6 epitopes in aggrecan is located in G2 [61]. It has also been shown that lack of reactivity of 1C6 with the G2 epitope in pig cartilage aggrecan is due to the presence of keratan sulphate [62]. However, neither PG-M nor M-CSPG contains keratan sulphate [14,25], and the G1 1C6 epitope in pig cartilage aggrecan is not masked [62]. Unless some other masking occurs for 1C6 in PG-M and M-CSPG, the 1C6 epitope appears to be absent from this core protein. It should be noted that the sequence for the 1C6 epitope [61] is altered by one amino acid in the core protein of the non-cartilage proteoglycan, versican [63]. Also, the structure predicted from the amino acid sequence of versican includes only a single globular region at each end of the core protein [63]. In addition,

while the distribution of methionine residues in the amino acid sequence of rat aggrecan predicts one large cyanogen bromide-cleaved peptide from the chondroitin sulphate-attachment region, as well as several smaller peptides [64], the methionine distribution in versican would not lead to any peptides as large as the largest one from rat aggrecan [63].

Thus, the PG-M/M-CSPG core protein may be of a versican type. The large proteoglycans of aorta have been found to possess a pattern of globular domains like that of PG-M and M-CSPG [65]. The absence of a double-globe (G1-G2) structure from the large proteoglycans of aorta correlates with evidence that arterial smooth muscle cells synthesize versican [56], because, as mentioned above, the amino acid sequence of versican predicts only a single globular region at each end of the molecule [63]. However, not all large, non-cartilage galactosaminoglycan-containing proteoglycans appear to be of the versican type. It has been shown by electron microscopic visualization that the large proteoglycans of sclera and tendon have a pattern of globular domains like that of aggrecan [65]. As the core proteins of the large proteoglycans of sclera and tendon show some differences from the aggrecan core protein [66], there appear to be at least three classes of large, hyaluronic acid-binding, galactosaminoglycan-containing proteoglycans: aggrecan, sclera/tendon and versican/PG-M/M-CSPG/aorta.

We are grateful to Amy Johnson and to Carol Ingle for technical assistance, to Don Lennon for establishing and maintaining the cultures, and to Christine Kehoe for typing the manuscript. This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

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