Dermatan sulphate proteoglycans of human articular cartilage

The properties of dermatan sulphate proteoglycans I and II

Peter J. ROUGHLEY* and Robert J. WHITE

Joint Diseases Laboratory, Shriners Hospital for Crippled Children, and Division of Surgical Research, McGill University, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada.

Dermatan sulphate proteoglycans were purified from juvenile human articular cartilage, with a yield of about 2 mg/g wet wt. of cartilage. Both dermatan sulphate proteoglycan I (DS-PGI) and dermatan sulphate proteoglycan II (DS-PGII) were identified and the former was present in greater abundance. The two proteoglycans could not be resolved by agarose/polyacrylamide-gel electrophoresis, but could be resolved by SDS/polyacrylamide-gel electrophoresis, which indicated average M_r values of 200 000 and 98 000 for DS-PGI and DS-PGII respectively. After digestion with chondroitin ABC lyase the M_r values of the core proteins were 44 000 for DS-PGI and 43 000 and 47 000 for DS-PGII, with the smaller core protein being predominant in DS-PGII. Sequence analysis of the *N*-terminal 20 amino acid residues reveals the presence of a single site for the potential substitution of dermatan sulphate at residue 4 of DS-PGII and two such sites at residues 5 and 10 for DS-PGI.

INTRODUCTION

Hyaline cartilage contains both aggregating and non-aggregating proteoglycans (Heinegård *et al.*, 1982). The aggregating proteoglycans are characterized by their large size, numerous chondroitin sulphate and keratan sulphate chains and the ability to interact with hyaluronic acid. The non-aggregating proteoglycans are smaller in size, and possess only the chondroitin sulphate class of glycosaminoglycan. The non-aggregating proteoglycans can be further divided into large moieties containing multiple chondroitin sulphate chains, and small molecules containing only one or two chains. The chondroitin sulphate in the small non-aggregating proteoglycans may be in the form of dermatan sulphate, with some glucuronate residues having been epimerized to iduronate. This has led to the term dermatan sulphate proteoglycan (DS-PG) being applied to the small non-aggregating molecules. Two distinct proteoglycans are generally considered in this class, and they have been referred to as DS-PGI and DS-PGII (Rosenberg *et al.*, 1985). In recent years type IX collagen (van der Rest *et al.*, 1985) has been shown to bear a chondroitin sulphate chain (Huber *et al.*, 1986) and to be equivalent to a proteoglycan previously termed PG-L₁ (Vaughan *et al.*, 1985). This, therefore, could also be considered as another type of small non-aggregating proteoglycan.

Bovine articular cartilage contains both DS-PGI and DS-PGII (Rosenberg *et al.*, 1985). The two molecules are of similar size, but can be distinguished both immunologically and by the ability of DS-PGI to self-associate. Both molecules possess core proteins with M_r values of about 45 000, and nearly half of their uronic acid is in the form of iduronate. Similar dermatan sulphate proteoglycans have also been described in human fetal membranes (Brennan *et al.*, 1984), bovine

sclera (Cöster & Fransson, 1981), human cervix (Uldbjerg *et al.*, 1983), bovine and human bone (Franzén & Heinegård, 1984; Fisher *et al.*, 1987), bovine tendon (Vogel & Evanko, 1987) and rat, calf and pig skin (Miyamoto & Nagase, 1980; Fujii & Nagai, 1981; Damle *et al.*, 1982). Indeed, it is likely that such DS-PGs are ubiquitous to all connective tissues, though the abundance of DS-PGI relative to DS-PGII, the degree of epimerization of uronic acid and the size of the glycosaminoglycan chains may vary (Heinegård *et al.*, 1985a; Vogel & Fisher, 1986; Cöster *et al.*, 1987). The *N*-terminal amino acid sequences for DS-PGI and DS-PGII from human bone have been established, confirming that the two classes of proteoglycan are distinct entities (Fisher *et al.*, 1987), and the total protein sequence for DS-PGII has been deduced from cDNA analysis (Krusius & Ruoslahti, 1986; Day *et al.*, 1986). Although several potential sites for glycosaminoglycan substitution are present on the core protein of DS-PGII, it appears that dermatan sulphate is only located at the *N*-terminal site (Chopra *et al.*, 1985).

The aim of the present work was to extend these observations to human articular cartilage, where the presence of the non-aggregating DS-PGs has recently been reported in young and mature individuals (Stanescu *et al.*, 1988; Sampaio *et al.*, 1988). At present, however, it has not conclusively been established that both DS-PGI and DS-PGII are present, and how their properties may differ.

EXPERIMENTAL

Purification of non-aggregating proteoglycan

Human articular cartilage was obtained from the epiphyses of the proximal tibia and distal femur of

Abbreviation used: DS-PG, dermatan sulphate proteoglycan.

* To whom correspondence should be addressed, at: Joint Diseases Laboratory, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada.

juveniles (3–15 months old) at the time of autopsy. Tissue was collected within 20 h of death, chopped into fine pieces, then extracted with 10 vol. of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 6.0, containing proteinase inhibitors (Roughley & White, 1980) for 48 h at 4 °C. Solid CsCl was then added to the extract at a concentration of 0.48 g/ml to give a density of 1.4 g/ml, and this solution was subjected to density-gradient centrifugation at 100 000 *g* for 48 h at 10 °C. Non-aggregating proteoglycan was recovered from the centre of the gradient, and then purified by ion-exchange and subsequent gel-filtration chromatography as described by Rosenberg *et al.* (1985).

Electrophoretic techniques

Analysis of proteoglycan by agarose/polyacrylamide-gel electrophoresis was performed in slab gels as described by Heinegård *et al.* (1985b). Interaction with hyaluronic acid was assayed by adding 20% (w/w) hyaluronic acid to the sample before electrophoresis. Authentic samples of DS-PGI and DS-PGII from bovine articular cartilage were used as reference markers, and had been kindly supplied by Dr. L. C. Rosenberg (Montefiore Hospital, New York, NY, U.S.A.). For analysis of fractions from chromatography or density gradients, samples were first dialysed into water. Gels were stained with Toluidine Blue.

SDS/polyacrylamide-gel electrophoresis was performed in 4–20%-gradient slab gels as described by Rosenberg *et al.* (1985). Gels were stained successively with Coomassie Brilliant Blue R-250 then Alcian Blue as described by Fisher *et al.* (1983). For analysis of samples from chromatography or density gradients, samples were first dialysed into 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS. Estimation of core protein size was performed after digestion of samples with chondroitin ABC lyase, with 0.05 unit of enzyme/mg of proteoglycan in 0.1 M-sodium acetate/0.1 M-Tris/HCl buffer, pH 7.3, for 20 h at 40 °C. The chondroitin ABC lyase preparation used in this work appeared to be free of any proteinase contamination capable of degrading the core protein of the DS-PGs, as prolonged incubation in the presence of the enzyme did not alter the electrophoretic mobility of the core protein.

Chromatographic techniques

The sizes of the intact proteoglycans and their component dermatan sulphate chains were estimated by chromatography on a Sepharose CL-4B column (115 cm × 1 cm) under dissociative conditions (4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5), with a flow rate of 6 ml/h and collection of 1 ml fractions. Dermatan sulphate chains were analysed following digestion of the proteoglycan with 50 mM-NaOH/1 M-NaBH₄ for 48 h at 45 °C (Carlson, 1968). The ability of the DS-PGs to self-associate was determined by chromatography on a Sepharose CL-4B column (115 cm × 1 cm) under associative conditions (200 mM-sodium acetate buffer, pH 5.5). Fractions from the columns were analysed for either absorbance at 280 nm or uronic acid content (Bitter & Muir, 1962).

The position of sulphation along the dermatan sulphate chains was estimated by h.p.l.c. (Hjerpe *et al.*, 1979) following release of component disaccharides by digestion of the proteoglycan with chondroitin ABC lyase (Lee & Tieckelmann, 1979). The same chromato-

graphic technique was used to estimate the proportion of glucuronic acid and iduronic acid in the dermatan sulphate chains following digestion with chondroitin AC lyase. Disaccharides were analysed by their absorbance at 232 nm.

Protein sequencing

The *N*-terminal amino acid sequences for the DS-PGs were determined by using an Applied Biosystems 470A gas-phase protein sequencer. Resulting amino acid phenylthiohydantoin derivatives were determined by using an on-line Applied Biosystems 120A analyser after separation by reverse-phase h.p.l.c. on a C₁₈ column. For analysis, 100 µg of proteoglycan in water was applied to a glass-fibre filter coated with Biobrene.

RESULTS

The yield of small proteoglycan obtained by the preparative procedure averaged about 2 mg/g wet wt. of cartilage, whereas the yield of aggregating proteoglycan obtained from the same cartilage was about 40 mg/g wet wt. of cartilage. Isolation of glycosaminoglycan chains from the small proteoglycan preparation by alkali/

Fig. 1. Sepharose CL-4B chromatography and agarose/polyacrylamide-gel electrophoresis of DS-PGs

Chromatography was performed in the presence of 4 M-guanidinium chloride, and fractions were analysed by absorbance at 280 nm (a) and agarose/polyacrylamide-gel electrophoresis (b). The electrophoretic migration of bovine articular-cartilage DS-PG and the chromatographic elution position of cartilage aggregating proteoglycan (Agg.-PG) are indicated, as are the void volume (*V*₀) and total volume (*V*_t) of the column.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of DS-PGs and their core proteins

Fractions from dissociative Sepharose CL-4B chromatography (Fig. 1) were analysed by electrophoresis under reducing conditions and the gels were stained consecutively with Coomassie Brilliant Blue and Alcian Blue. The position of electrophoretic migration of bovine articular-cartilage DS-PGI and DS-PGII and human neonatal cartilage aggregating proteoglycan (Agg.-PG) are indicated, together with the positions of M_r markers. Column fractions were analysed directly (a) or were pooled (1, 2 and 3) and treated with chondroitin ABC lyase before analysis (b).

borohydride treatment yielded a single population of chains, with a K_{av} of 0.54 on Sepharose CL-4B under dissociative conditions. This is larger than the size of chondroitin sulphate chains from the aggregating proteoglycan obtained from the same cartilage, which, under identical chromatographic conditions, were eluted with a K_{av} of 0.67. The intact DS-PGs migrated with a K_{av} of 0.44 on the same column (Fig. 1a), and on analysis of the column fractions by agarose/polyacrylamide-gel electrophoresis only a single component was observed (Fig. 1b). Analysis of the column fractions by SDS/polyacrylamide-gel electrophoresis indicated that two components were present in the DS-PG preparation, corresponding to DS-PGI and DS-PGII (Fig. 2a). The average M_r values of the two populations were estimated to be 200000 and 98000 respectively by this technique, and the greater size of DS-PGI was supported by its concentration in fractions that were less retarded. DS-PGI also appears to be present in greater amounts than DS-PGII.

On Sepharose CL-4B chromatography under associative conditions, two distinct components, with K_{av} values of 0.21 and 0.40, were observed, indicating partial self-association of the preparation. In bovine cartilage such self-association is characteristic of DS-PGI but not DS-PGII (Rosenberg *et al.*, 1985). Analysis of the two components by SDS/polyacrylamide-gel electrophoresis indicated that in the human system DS-PGI also shows a marked tendency to self-associate under non-dissociative conditions, whereas DS-PGII remains mainly in the monomeric form.

The partial separation of DS-PGI and DS-PGII on the dissociative Sepharose CL-4B column enabled fractionation into three pools: one enriched in DS-PGI, one containing similar amounts of DS-PGI and DS-PGII, and one enriched in DS-PGII (Fig. 2a). The molecular

size of the core proteins from the proteoglycans in these pools was determined following incubation with chondroitin ABC lyase. The pool enriched in DS-PGI showed a single core protein of M_r 44000, whereas that enriched in DS-PGII showed two core proteins of M_r 43000 and 47000, with the smaller species predominating (Fig. 2b). Analysis of disaccharides obtained from the three pools by digestion with chondroitin ABC lyase and chondroitin AC lyase revealed that both DS-PGI and DS-PGII possessed dermatan sulphate chains of similar structure, with both appearing to contain at least 80% of their uronic acid as glucuronate. Only 20–25% of the *N*-acetylgalactosamine residues bore sulphate at the 6-position, and this was all associated with the glucuronate-rich blocks within the dermatan sulphate chains. The less prevalent iduronate-rich blocks appeared to be exclusively associated with 4-sulphated *N*-acetylgalactosamine residues.

The pooled fractions of the small proteoglycans enriched in DS-PGI and DS-PGII were also used for investigating the *N*-terminal amino acid sequences of the two proteoglycan populations. Analysis of the pool enriched in DS-PGI gave a single sequence, whereas that enriched in DS-PGII gave a double sequence, and the sequence of DS-PGII was deduced by eliminating the residues present in the sequence of DS-PGI (Table 1). Apart from the first two amino acid residues, the sequences show no additional similarity within the 20 amino acid residues sequenced. Blank cycles were obtained at residue 4 for DS-PGII and residues 5 and 10 for DS-PGI, and all three sites are compatible with the Asp/Glu-Xaa-Ser-Gly sequence postulated as the recognition sequence for chondroitin sulphate addition in the aggregating proteoglycans (Oldberg *et al.*, 1987). At present, however, it is not known which of these sites may bear dermatan sulphate chains in the human.

Table 1. *N*-Terminal amino acid sequence analysis of DS-PGI and DS-PGII from human articular cartilage

Sequences for DS-PGI and DS-PGII were obtained by analysis of pools 1 and 3 (Fig. 2) respectively. —, Blank cycle, presumably due to glycosylation.

Residue no.	Amino acid residue	
	DS-PGI	DS-PGII
1	Asp	Asp
2	Glu	Glu
3	Glu	Ala
4	Ala	—
5	—	Gly
6	Gly	Ile
7	Ala	Gly
8	Asp	Pro
9	Thr	Glu
10	—	Val
11	Gly	Pro
12	Val	Asp
13	Leu	Asp
14	Asp	Arg
15	Pro	Asp
16	Asp	Phe
17	Ser	Glu
18	Val	Pro
19	Thr	Ser
20	Pro	Leu

DISCUSSION

Juvenile human articular cartilage was shown to contain the two classes of DS-PG previously characterized in bovine articular cartilage and other connective tissues (Rosenberg *et al.*, 1985). The larger of these proteoglycans (DS-PGI) predominates over the smaller (DS-PGII) in about a 3:2 molar ratio. The dermatan sulphate chains contain no more than 20–25% of the uronic acid as iduronate, which is lower than that previously reported for DS-PGs from human fetal membranes (Brennan *et al.*, 1984), bovine fetal tendon (Vogel & Evanko, 1987; Honda *et al.*, 1987) and bovine fetal cartilage (Rosenberg *et al.*, 1982), where iduronate contents greater than 40% were observed. The core protein of the DS-PGI appeared to be a single species of M_r 44000, whereas DS-PGII had two core proteins of M_r 43000 and 47000. The existence of such a pair of core protein sizes has been ascribed to differing degrees of oligosaccharide substitution (Glössl *et al.*, 1984). A single core protein for DS-PGI and two core proteins for DS-PGII have been previously reported in other tissues (Brennan *et al.*, 1984; Fisher *et al.*, 1987; Vogel & Fisher, 1986). However, unlike these tissues, the smaller core protein for DS-PGII appears to predominate in the human cartilage.

The homology of the DS-PGI and DS-PGII from the human cartilage with the DS-PG from other species and tissues was shown by *N*-terminal amino acid sequence analysis. The only previous report of *N*-terminal analysis for DS-PGI was from human bone (Fisher *et al.*, 1987), and the cartilage sequence proved to be identical for the first 20 amino acid residues. In common with the bone sequence, blank cycles were obtained at residues 5 and 10, compatible with the substitution at these positions by

dermatan sulphate or *O*-linked oligosaccharides. The possibility for substitution by two dermatan sulphate chains in this region exists. The *N*-terminal sequence for DS-PGII from human cartilage differed from that reported for human bone by the presence of glycine rather than alanine at residue 7 and of aspartic acid rather than proline at residue 15. It was, however, identical with the first 20 amino acid residues reported for the DS-PG from human fetal membranes (Brennan *et al.*, 1984) and those derived from the cDNA corresponding to the small proteoglycan expressed by human fibroblasts (Krusius & Ruoslahti, 1986). In comparison with bovine cartilage, skin and sclera, which show identical sequences for DS-PGII for the first 20 amino acid residues (Cöster *et al.*, 1987), the human cartilage sequence is identical for the first nine residues, but then shows considerable divergence. It thus appears that the core proteins for DS-PGI and DS-PGII from human cartilage are analogous to those utilized by other human tissues, though the structure of the dermatan sulphate chains and the amount of oligosaccharide substitution vary.

The yield of extracted DS-PG from human juvenile cartilage was about 2 mg/g wet wt. of cartilage. This is about 20 times less than that obtained for the large aggregating proteoglycans. However, when one takes into account the different molecular sizes of the two proteoglycan classes, they could both be present in similar molar amounts, and be equally important in maintaining cartilage function. The DS-PGs have been postulated as having a role in collagen fibril organization (Scott & Orford, 1981; Scott, 1984), and it has also been shown that DS-PGII will inhibit fibrillogenesis (Vogel *et al.*, 1984; Scott *et al.*, 1986). It is, however, still unclear how the role of DS-PGI may differ from that of DS-PGII, and how the function of these two proteoglycans relates to that of type IX collagen, which has also been shown to interact with the collagen fibrils (Müller-Glauser *et al.*, 1986). Furthermore, it is not clear whether the structure of the dermatan sulphate chains has any effect on the function of the proteoglycans.

We thank Dr. J. Michaud of Hôpital Ste. Justine for access to autopsy specimens, Dr. M. van der Rest for help with the amino acid sequencing, Ms. M. Burman Turner for typing the manuscript, and Mr. M. Lepik for preparing the Figures. This work was supported by research grants from the Medical Research Council of Canada and the Shriners of North America.

REFERENCES

- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
- Brennan, M. J., Oldberg, Å., Pierschbacher, M. D. & Ruoslahti, E. (1984) *J. Biol. Chem.* **259**, 13742–13750
- Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
- Chopra, R. K., Pearson, C. H., Pringle, G. A., Fackre, D. S. & Scott, P. G. (1985) *Biochem. J.* **232**, 277–279
- Cöster, L. & Fransson, L.-Å. (1981) *Biochem. J.* **193**, 143–153
- Cöster, L., Rosenberg, L. C., van der Rest, M. & Poole, A. R. (1987) *J. Biol. Chem.* **262**, 3809–3812
- Damle, S. P., Cöster, L. & Gregory, J. D. (1982) *J. Biol. Chem.* **257**, 5523–5527
- Day, A. A., Ramis, C. I., Fisher, L. W., Gehron-Robey, P., Termine, J. D. & Young, M. F. (1986) *Nucleic Acids Res.* **14**, 9861–9876

- Fisher, L. W., Termine, J. D., DeJter, S. W., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Kleinman, H. K., Hassell, J. R. & Nilsson, B. (1983) *J. Biol. Chem.* **258**, 6588–6594
- Fisher, L. W., Hawkins, G. R., Tuross, N. & Termine, J. D. (1987) *J. Biol. Chem.* **262**, 9702–9708
- Franzén, A. & Heinegård, D. (1984) *Biochem. J.* **224**, 59–66
- Fujii, N. & Nagai, Y. (1981) *J. Biochem. (Tokyo)* **90**, 1249–1258
- Glössl, J., Beck, M. & Kresse, H. (1984) *J. Biol. Chem.* **259**, 14144–14150
- Heinegård, D., Paulsson, M. & Sommarin, Y. (1982) in *Limb Development and Regeneration* (Kelley, R. O., Goetinck, P. F. & MacCabe, J. A., eds.), part B, pp. 35–43, Alan R. Liss, New York
- Heinegård, D., Björne-Person, A., Cöster, L., Franzén, A., Gardell, S., Malmström, A., Paulsson, M., Sandfalk, R. & Vogel, K. (1985a) *Biochem. J.* **230**, 181–194
- Heinegård, D., Sommarin, Y., Hedbom, E., Wieslander, J. & Larsson, B. (1985b) *Anal. Biochem.* **151**, 41–48
- Hjerpe, A., Antonopoulos, C. A. & Engfeldt, B. (1979) *J. Chromatogr.* **171**, 339–344
- Honda, T., Katagiri, K., Kuroda, A., Matsunaga, E. & Shinkai, H. (1987) *Collagen Relat. Res.* **7**, 171–184
- Huber, S., van der Rest, M., Bruckner, P., Rodriguez, E., Winterhalter, K. H. & Vaughan, L. (1986) *J. Biol. Chem.* **261**, 5965–5968
- Krusius, T. & Ruoslahti, E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7683–7687
- Lee, G. J. & Tieckelmann, H. (1979) *Anal. Biochem.* **94**, 231–236
- Miyamoto, I. & Nagase, S. (1980) *J. Biochem. (Tokyo)* **88**, 1793–1803
- Müller-Glauser, W., Humbel, B., Glatt, M., Sträuli, P., Winterhalter, K. H. & Bruckner, P. (1986) *J. Cell Biol.* **102**, 1931–1939
- Oldberg, A., Antonsson, P. & Heinegård, D. (1987) *Biochem. J.* **243**, 255–259
- Rosenberg, L., Tang, L., Choi, H., Pal, S., Johnson, T., Poole, A. R., Roughley, P., Reiner, A. & Pidoux, I. (1982) in *Limb Development and Regeneration* (Kelley, R. O., Goetinck, P. F. & MacCabe, J. A., eds.), part B, pp. 67–84, Alan R. Liss, New York
- Rosenberg, L. C., Choi, H. U., Tang, L.-H., Johnson, T. L., Pal, S., Webber, C., Reiner, A. & Poole, A. R. (1985) *J. Biol. Chem.* **260**, 6304–6313
- Roughley, P. J. & White, R. J. (1980) *J. Biol. Chem.* **255**, 217–224
- Sampaio, L. de O., Bayliss, M. T., Hardingham, T. E. & Muir, H. (1988) *Biochem. J.* **254**, 757–764
- Scott, J. E. (1984) *Biochem. J.* **218**, 229–233
- Scott, J. E. & Orford, C. R. (1981) *Biochem. J.* **197**, 213–216
- Scott, P. G., Winterbottom, N., Dodd, C. M., Edwards, E. & Pearson, C. H. (1986) *Biochem. Biophys. Res. Commun.* **138**, 1348–1354
- Stanescu, V., Chaminade, F. & Muriel, M.-P. (1988) *Connect. Tissue Res.* **17**, 239–252
- Uldbjerg, N., Malmström, A., Ekman, G., Sheehan, J., Ulmsten, U. & Wingerup, L. (1983) *Biochem. J.* **209**, 497–503
- van der Rest, M., Mayne, R., Ninomiya, Y., Seidah, N. G., Chretien, M. & Olsen, B. R. (1985) *J. Biol. Chem.* **260**, 220–225
- Vaughan, L., Winterhalter, K. H. & Bruckner, P. (1985) *J. Biol. Chem.* **260**, 4758–4763
- Vogel, K. G. & Evanko, S. P. (1987) *J. Biol. Chem.* **262**, 13607–13613
- Vogel, K. G. & Fisher, L. W. (1986) *J. Biol. Chem.* **261**, 11334–11340
- Vogel, K., Paulsson, M. & Heinegård, D. (1984) *Biochem. J.* **223**, 587–597

Received 25 January 1989/29 March 1989; accepted 12 April 1989