Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 95-101

ISOLATION AND CHARACTERIZATION OF CARTILAGE PROTEOGLYCANS IMMUNOREACTIVE WITH AN ANTIBODY TO SKIN PROTEODERMATAN SULFATE CORE PROTEIN

Satoshi Onodera and Yutaka Nagai

Department of Tissue Physiology, Medical Research Institute,
Tokyo Medical and Dental University, Tokyo 101, Japan

Received March 13, 1985

SUMMARY : Low density proteoglycans showing cross-reaction with an antibody to skin proteodermatan sulfate (PDS) core protein were isolated from the bovine articular cartilage, by CsCl density gradient centrifugation followed by repeated DEAE-cellulose chromatography. The size and amino acid composition of the core proteins of the immunoreactive proteoglycans, eluted at 0.25M and 0.5M NaCl on DEAE-cellulose column, were quite similar to that of PDS. The glycosaminoglycan components of both proteoglycans were shown to be composed of a hybrid structure of chondroitin sulfate and dermatan sulfate, based on chondroitinase treatments followed by two-dimensional electrophoresis.

It is well known that the proteodermatan sulfate plays important roles in the promotion of collagen fibrillogenesis and deposition of the resulting fibrils into the interstitial space of the connective tissue (1-3). Our previous studies on glycosaminoglycan synthesis during cell growth using embryonic chick tendon cell cultures showed that synthesis of hyaluronic acid, which is the major component at the early phase of culture, remained constant during cell proliferation, while chondroitin sulfate synthesis increased rapidly during an actively growing phase and dermatan sulfate and heparan sulfate syntheses increased gradually and became the major components at the stationary phase of the cell culture (4). Furthermore, during the course of studies on the tissue distribution of PDS using an antibody against the core protein of the proteoglycan, we found that hyaline cartilage such as articular and bronchial cartilage was well stained with the antibody, when pretreated with testicular hyaluronidase (5).

These observations prompted us to elucidate whether or not proteoglycan(s) from cartilage shares a common core protein with skin PDS.

0006-291X/85 $1.50

This paper describes the isolation and characterization of articular cartilage proteoglycans immunoreactive with antibody to PDS core protein.

MATERIALS AND METHODS

Isolation of proteodermatan sulfate

PDS was isolated from fresh calf skin as described previously (6) with some modifications. Three molar $MgCl_2$ extract of the skin in the presence of protease inhibitors (10mM EDTA, 10mM N-ethylmaleimide, 1mM phenylmethanesulfonyl fluoride and 0.35mM pepstatin), was exhaustively dialyzed against distilled water to precipitate PDS and collagen which was then dissolved in 7M urea, 0.05M Tris-HCl buffer, pH 7.0 (buffer A). The resulting solution was directly applied to a DEAE-cellulose (Whatman DE32) column (2.6 x 24 cm) equilibrated with buffer A. After washing the column with 0.15M NaCl in buffer A, PDS was eluted with 2.0M NaCl in buffer A. For further purification, the 2.0M NaCl eluate was dissolved in 4M urea, 0.15M NaCl, 0.05M Tris-HCl buffer, pH 7.0 (buffer B), rechromatographed on a DEAE-cellulose column equilibrated with buffer B, and then eluted with stepwise increases of NaCl concentration at 0.15M, 0.25M and 1.0M. PDS was recovered at 0.25M NaCl fraction in the pure form.

Preparation and purification of antibody to proteodermatan sulfate core protein

Purified PDS (10mg) was digested with 0.5 units of chondroitinase (Chase) ABC (Seikagaku Kogyo Co. Tokyo) at pH 7.6 and 37°C for 16hr in the presence of a cocktail of four protease inhibitors (6) and its core protein was obtained from the reaction mixture by passing through an anti-bovine serum albumin antibody-Sepharose 4B column (1.5 x 5 cm) to remove albumin contained in the enzyme preparation. Antisera to the core protein were obtained by sensitizing Sprague-Dawley (SD) rats with antigen (0.4mg/body) emulsified with an equal volume of Freund's complete adjuvant followed by booster injections twice. Antibody to PDS core protein was prepared by passing over a Chase ABC-Sepharose 4B column (3ml gel) and then by adsorbing to and eluting from an PDS-Sepharose 4B column (3ml gel).

Immunofluorescent staining

Fresh normal bovine tissues were quickly flozen with n-hexane cooled with dry ice acetone. Six-eight μm sectioned slices were mounted on glass slides coated with egg albumin and immunoreacted with antibodies to PDS, as described previously (7).

Isolation of cartilage proteoglycans immunoreactive with antibody to PDS

Sliced bovine articular cartilage (wet weight 36g) was extracted with 10 volumes of 4M guanidinium chloride (GuHCl), 0.15M NaCl, 0.05M acetate buffer, pH 5.8 containing four protease inhibitors at 4°C for 48hr (8). The extract was dialyzed against 9 volumes of 0.05M acetate buffer, pH 5.8, containing protease inhibitors and concentrated with an Amicon YM-5 membranes. After adding solid CsCl to the resulting solution to give an initial density of 1.61g/ml, the mixture was ultracentrifuged at 100,000 xg and 15°C for 68hr, then collected from the bottom (A1) with the aid of peristaltic pump. A fraction immunoreactive with antibody to PDS (top fraction A4) was dissolved in 6M urea, 0.05M Tris-HCl buffer, pH 7.6, dialyzed against the buffer, applied on a DEAE-cellulose column (1 x 12 cm) equilibrated with the same buffer and eluted by stepwise increases of NaCl concentration. Fractions immunoreactive with antibody to PDS were eluted at 0.25M and 0.5M NaCl concentrations.

Passive hemagglutination assay (PHA) and immunodiffusion technique

The titer, specificity and cross reactivity of isolated cartilage proteoglycans with antibody to PDS were determined by the PHA (9) and immunodiffusion methods (10).

Electrophoresis

SDS-polyacrylamide gel electrophoresis (11) and two-dimensional electrophoresis on cellulose acetate membranes (12) were performed, as reported previously.

Chemical analysis

Amino acid analysis was performed with a JEOL-6AS autoanalyzer after hydrolysis of sample proteins with 6M HCl in an evacuated sealed tube at 110°C for 24hr. Uronic acid was measured by the carbazol (13) and orcinol (14) methods.

RESULTS AND DISCUSSION

Proteoglycans, extracted from bovine articular cartilage with 4M GuHCl, were fractionated into A1 (bottom) to A4 (top) fractions by CsCl density gradient centrifugation under associative conditions (Fig. 1) and subjected to immunological analysis by the Ouchterlony method using a polyclonal antibody against PDS. The antibody used was specific to the core protein of PDS and showed cross-reactions neither with dermatan sulfate component and its Chase digests nor with type I, II, III or IV collagen as well as bovine plasma fibronectin. Only fraction A4 ($<\rho=1.50$), which consisted of low density proteoglycans, collagen and some other proteins, showed an immunoreaction with the antibody. No immunoreaction was observed between the antibody and fractions A1 and A2 ($>\rho=1.60$) consisting of high density proteoglycan aggregates either by PHA or by enzyme-linked immunosorbent assay (data not shown), suggesting that low density proteoglycan(s) in cartilage is immunologically in close

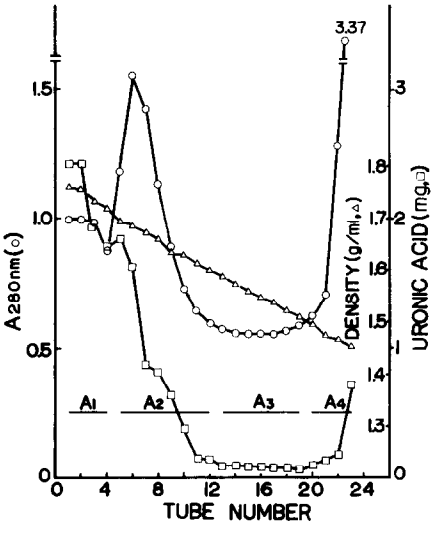

Fig. 1 A typical pattern of CsCl density gradient centrifugation of 4M GuHCl extract from bovine articular cartilage under associative conditions. 1.5ml each of effluent fractions was collected. Initial density : 1.61g/ml.

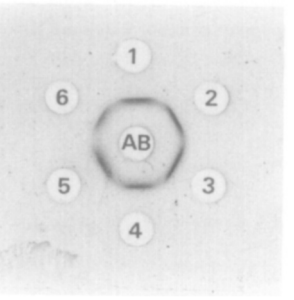

Fig. 2 Double immuno-diffusion patterns of core proteins of PGO.25 and PGO.5 with antibody to PDS core protein. The plate was stained with coomassie brilliant blue after exhaustive washing with 0.02M phosphate buffer. 1 and 4, PGO.25; 2 and 5, PDS; 3 and 6, PGO.5. AB: antibody to PDS.

association with skin PDS in terms of their core proteins (5). Therefore, further purification of the immunoreactive proteoglycans was undertaken using fraction A4. Two proteoglycan subfractions eluted at 0.25M NaCl (PGO.25) and 0.5M NaCl (PGO.5) concentrations on a DEAE-cellulose column were shown to immunoreact with antibody to PDS by the Ouchterlony method, giving a single precipitin line fused with both core proteins of PGO.25 and PGO.5 (Fig. 2), indicating that these cartilage proteoglycans possess a common antigenic determinant(s) in each molecule.

SDS-polyacrylamide gel electrophoresis of the isolated proteoglycans before and after Chase ABC digestion showed that estimated molecular weights of PGO.25 and PGO.5 were quite different from each other, corresponding to 112 kilo daltons and more than 250 kilo daltons, respectively (Fig.3-1 and Fig.3-3). However, after Chase digestion, the core proteins of both proteoglycans migrated down to the same distance as that of PDS, which corresponded to 57 kilo daltons (6). The amino acid compositions of both cartilage proteoglycans were also quite similar to that of PDS, showing high contents of aspartic acid, glutamic acid and leucine, but different from that of A1-D1 monomer, which was prepared from the high density proteoglycan aggregates by CsCl density gradient centrifugation under dissociative conditions (Table I). From the results described above, one can conclude that both PGO.25 and PGO.5 may have a common core

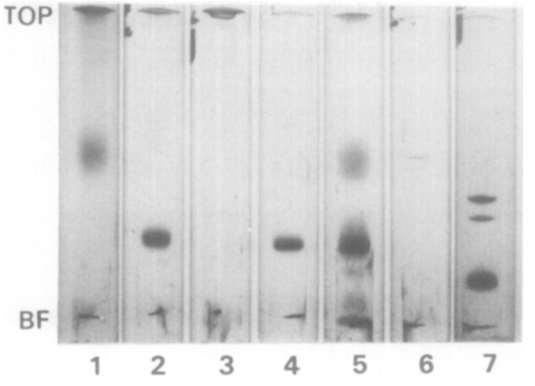

Fig. 3 SDS-PAGE patterns of proteoglycans and their core proteins cross-reacted with anti-PDS core protein antibody. 1, PGO.25; 2, Chase ABC digest of PGO.25; 3, PGO.5; 4, Chase ABC digest of PGO.5; 5, a mixture of PDS and its core protein as references; 6, Chase ABC enzyme alone and 7, a mixture of bovine serum albumin and ovalbumin. BF: buffer front.

protein with skin PDS. Similar low density proteoglycans in terms of amino acid composition were also isolated from bovine nasal (16) and articular (17) cartilages, bone (18) and sclera (19) and chick embryo cartilage (20).

Two-dimensional electrophoresis of glycosaminoglycan components of PGO.25 and PGO.5 revealed that they migrated to a position between

Table I. The amino acid composition of bovine articular cartilage proteoglycan subfractions (residues/1000 residues)

Amino acid	Articular cartilage proteoglycan			Calf skin proteodermatan sulfate
	A1 - D1 fraction	0.25M NaCl fraction	0.5M NaCl fraction	
Hyp	0	0	0	0
Asp	63	125	120	127
Thr	66	50	47	46
Ser	128	70	63	58
Glu	146	103	107	105
Pro	97	69	70	77
Gly	129	82	81	94
Ala	65	50	46	51
½Cys	3	17	17	12
Val	69	59	58	58
Met	4	12	12	13
Ile	35	51	52	59
Leu	75	116	127	115
Tyr	17	33	29	21
Phe	36	34	36	29
His	17	25	31	24
Lys	17	60	57	79
Arg	33	44	47	32

reference dermatan sulfate and chondroitin sulfate as a broad spot. However, after Chase AC digestion, the migrating position of both components shifted to that of reference dermatan sulfate, giving a clear spot, which was sensitive to the action of Chase ABC. Relative contents of Chase AC resistant portion in PG0.25 and PG0.5 subfractions were 21.5% and 15.7% respectively, based on alcian blue staining and the carbazole to orcinol ratio. These results strongly suggest that both low density proteoglycans from cartilage are composed of a hybrid structure of chondroitin sulfate and dermatan sulfate. A similar hybrid structure of glycosaminoglycans was also found in the meniscus of human knee joint containing skin type collagen (21).

The biological function of these low density proteoglycans in cartilage tissue remains obscure at present. However, it seems most likely that the proteoglycans may serve as a promoter of collagen fibrillogenesis in the tissue, as PDS does in the skin. Although precise mechanisms of the formation of proteoglycan copolymers are not fully understood yet at present, levels of synthetic and / or modification enzymes for glycosaminoglycan synthesis such as an enzyme responsible for C-5 epimerization of D-glucuronate residues in polysaccharide precursors (22) may determine the degree of hybridization of chondroitin sulfate and dermatan sulfate in a given tissue.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid for Co-operative Research (56380018 and 59380015) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Nakamura, T., and Nagai, Y. (1980) J. Biochem. 87, 629-637.
2. Toole, B.P., and Lowther, D.A. (1968) Arch.Biochem.Biophys. 128, 567-578.
3. Parry, D.A.D., Flint, M.H., Gillard, G.C., and Craig, A.S. (1982) FEBS Lett. 149, 1-7.
4. Ninomiya, Y., and Nagai, Y. (1979) J. Biochem. 86, 111-119.
5. Nagai, Y., Onodera, S., and Konomi, H. (1983) Proceedings of the 7th International Symposium on Glycoconjugates. Lund-Ronneby, Sweden. pp.114-115.
6. Fujii, N., and Nagai, Y. (1981) J. Biochem. 90, 1249-1258.

7. Konomi, H., Hori, H., Sano, J., Sunada, H., Hata, R., Fujiwara, S., and Nagai, Y. (1981) Acta Pathol. Jpn. 31(4), 601-610.
8. Sajdera, S.W., and Hascall, V.C. (1969) J. Biol. Chem. 244, 77-87.
9. Herbert, W.J. (1978) in Handbook of Experimental Immunology (Weir, D.M. ed.), Vol. 1, 3rd Edition, Chap.20, Blackwell Scientific Publications, Oxford.
10. Ouchterlony, Ö., and Nilsson, L.Å. (1978) in Handbook of Experimental Immunology (Weir, D.M. ed.), Vol. 1, 3rd Edition, Chap. 19, Blackwell Scientific Publications, Oxford.
11. Weber, K., and Osborn, M. (1975) in The Protein (Neurath, H., Hill, R.L. eds.), Vol. 1, 3rd Edition, pp. 179-223, Academic Press, New York.
12. Hata, R., and Nagai, Y. (1972) Anal. Biochem. 45, 462-468.
13. Dische, Z. (1947) J. Biol. Chem. 167, 189-198.
14. Brown, A.H. (1946) Arch. Biochem. 11, 269-278.
15. Onodera, S., Konomi, H., and Nagai, Y. (1984) Connective Tissue, 15(3,4), 136-137. in Japanese.
16. Heinegård, D., Paulsson, M., Inerot, S., and Carlström, C. (1981) Biochem. J. 197, 355-366.
17. Swann, D.A., Garg, H.G., Sotman, S.L., and Hermann, H. (1983) J. Biol. Chem. 258, 2683-2688.
18. 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., and Nilsson, B. (1983) J. Biol. Chem. 258, 6588-6594.
19. Cöster, L., and Fransson, L.-Å. (1981) Biochem. J. 193, 143-153.
20. Shinomura, T., Kimata, K., Oike, Y., Noro, A., Hirose, N., Tanabe, K., and Suzuki, S. (1983) J. Biol. Chem. 258, 9314-9322.
21. Habuchi, H., Yamagata, T., Iwata, H., and Suzuki, S. (1973) J. Biol. Chem. 248, 6019-6028.
22. Malmström, A., and Åberg, L. (1982) Biochem. J. 201, 489-493.