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Distribution of Proteoglycans Antigenically Related to Corneal Keratan Sulfate Proteoglycan*

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Three antibodies reacting with corneal keratan sulfate proteoglycan were used to detect antigenically related molecules in 11 bovine and 13 embryonic chick tissues. Two monoclonal antibodies recognized sulfated epitopes on the keratan sulfate chain and a polyclonal antibody bound antigenic sites on the core protein of corneal keratan sulfate proteoglycan. Competitive immunoassay detected core protein and keratan sulfate antigens in guanidine HCl extracts of most tissues. Keratan sulfate antigens of most bovine tissues were only partially extracted with guanidine HCl, but the remainder could be solubilized by CNBr treatment of the guanidine-extracted residue. Keratan sulfate and core protein antigens co-eluted with purified corneal keratan sulfate proteoglycan on ion exchange high-performance liquid chromatography (HPLC). Endo-β-galactosidase digestion of the HPLC-purified keratan sulfate antigens eliminated the binding of monoclonal anti-keratan sulfate antibodies in enzyme-linked immunosorbent assay. Extracts of all 11 bovine tissues, except those from brain and cartilage, could bind both anti-keratan sulfate monoclonal antibodies and anti-core protein polyclonal antibody simultaneously. Binding was sensitive to competition with keratan sulfate and to digestion with endo-β-galactosidase. These results suggest widespread occurrence of a proteoglycan or sulfated glycoprotein bearing keratan sulfate-like carbohydrate and a core protein resembling that of corneal keratan sulfate proteoglycan.

Keratan sulfate is a linear polymer of *N*-acetyllactosamine (Galβ1-4GlcNAc) dissacharides joined by β1-3 linkage (lactosaminoglycan). Keratan sulfate is sulfated at the C-6 positions of both glucosamine and galactose moieties. Proteoglycans bearing keratan sulfate chains have been characterized in three different tissues: cornea, cartilage, and brain (1, 2). In each of the three tissues, the core proteins of the proteoglycans are distinct, with keratan sulfate attached to the core by different linkage oligosaccharides. Corneal keratan sulfate is attached to core protein through an N-linked branched mannose-containing oligosaccharide that is identical to that found in complex-type oligosaccharides of neutral glycoproteins. Keratan sulfate from cartilage is linked to serine by an *O*-glycosidic bond through galactosamine (1) and brain keratan sulfate is O-linked to serine through mannose (2). The term keratan sulfate, therefore, describes a diverse group of polysaccharides which share the same sulfated poly-*N*-acetyllactosamine structure.

The presence of keratan sulfate-containing proteoglycans as major components of the extracellular matrix of the cornea and of cartilage has been known for some time (1). More recently keratan sulfate has been identified in brain (2), in the skin of several marine fish (3, 4), in bone (5), in mastocytoma tumor (6), and in teratocarcinoma cells (7). Smaller sulfated oligosaccharides structurally related to keratan sulfate have been found in lymphocytes and virus-infected chick embryos (8, 9). Lactosaminoglycan, a nonsulfated analogue of keratan sulfate, has been reported in cell membrane glycoproteins of several cell types (10–12), in mucins from the gastrointestinal tract and ovarian cysts (13, 14), and in extracellular matrix covalently linked to fibronectin (15). These reports suggest that distribution of keratan sulfate-containing proteoglycans and glycoproteins may be more widespread than originally thought.

During the last 5 years several antibodies have been isolated to keratan sulfate and to core proteins of keratan sulfate-containing proteoglycans (16–23). These antibodies appear highly specific (24, 25) and have provided sensitive detection of keratan sulfate by immunohistochemistry in cultured chondrocytes (19, 26, 27) and in developing cornea and skeletal tissues (21, 28–30). Immunoassay using anti-keratan sulfate antibodies has been used to quantitate keratan sulfate antigens in human serum (31) and in extracts of developing chick cornea (30).

In this study we used three previously characterized antibodies with reported activities against keratan sulfate proteoglycans to survey bovine and embryonic chick tissues for the presence of antigens related to keratan sulfate and to corneal KSPG[1] core protein. We find that compounds antigenically related to corneal KSPG can be detected in all tissues.

EXPERIMENTAL PROCEDURES

Materials

Purification of monoclonal antibodies I22 against rabbit corneal keratan sulfate (16), and 5-D-4 against bovine cartilage keratan sulfate (17) was described previously. Each of these antibodies recognizes sulfated epitopes on the keratan sulfate chains from both cartilage and corneal keratan sulfate but not epitopes in the protein or linkage carbohydrate (16, 17). Polyclonal rabbit antiserum against bovine KSPG (18) was further purified by affinity for immobilized bovine corneal KSPG (30). Purified bovine corneal keratan sulfate was obtained from Miles Laboratories. The preparation of bovine KSPG, desulfated bovine corneal keratan sulfate, whale cartilage

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[1] The abbreviations used are: KSPG, corneal keratan sulfate proteoglycan; ELISA, enzyme-linked immunosorbent assay; PBS, 10 mM sodium phosphate, 0.15 M NaCl, 0.01% (w/v) thimerosal, pH 7.4; HPLC, high-performance liquid chromatography.

keratan sulfate (18), and of rabbit corneal keratan sulfate (16), were described previously, as was purification of endo-β-galactosidase from *Escherichia freundii* (32). Bovine corneal KSPG was deglycosylated 3 h at 0 °C, with trifluoromethane-sulfonic acid as described (33). The free core protein was recovered by ion exchange HPLC as described below. The deglycosylated core did not adhere to the column and was detected by protein assay and by ELISA with the polyclonal antibody.

Tissue Extraction

Tissues (fresh or frozen at −70 °C) were freed of connective tissue and fat, minced, and extracted twice, 24 h each, at 4 °C, in 10 volumes (w/v) 4 M guanidine HCl, containing protease inhibitors (0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 mM *N*-ethylmaleimide), 0.05 M acetate buffer, pH 6.0, and 1% Triton X-100. Solutions were cleared by centrifugation at 10,000 × *g* for 10 min. Pooled guanidine HCl extracts were dialyzed at 4 °C against 6 M deionized urea in 20 mM Tris-HCl, pH 6.8 with the same protease inhibitors as above, then filtered through a glass fiber filter and stored at 4 °C. Protein content of the extract was determined with the Bio-Rad protein assay (34). Tissue remaining after guanidine HCl extraction was rinsed free of guanidine HCl in distilled water (4–6 changes), at 4 °C then dried in a vacuum centrifuge. Residue from 0.5 g of wet weight tissue was resuspended in 5 ml of 70% formic acid, containing 100 mg of CNBr and incubated capped at 25 °C overnight, vortexing occasionally. Addition of 100 mg of CNBr and a second overnight incubation was usually performed. CBNr was removed by repeated evaporation to dryness from 70% formic acid at reduced pressure. For quantitative immunoassay the dry residue was resuspended in 70% formic acid and dialyzed against 17 mM acetic acid in the cold. For ion exchange chromatography the dried residue was resuspended directly in 6 M urea with 20 mM Tris sulfate, pH 6.8. Insoluble residue was removed by centrifugation at 10,000 × *g*, 10 min.

Immunoassays

All ELISA procedures were carried out at 25 °C, with 2–3 PBS rinses between incubations, and dilutions were made in 1% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20 detergent in PBS (blocking buffer), except as noted.

Competitive ELISA—Competitive ELISA using a biotin-labeled purified corneal KSPG standard was described previously (30). Competitive assays with polyclonal antibody used biotin-labeled corneal KSPG, affinity-purified on antibody I22-agarose as described previously for ^{125}I-KSPG (16).

Direct ELISA—Direct ELISA was carried out by coating a polystyrene ELISA plate (Nunc 439454, Vanguard International, Neptune, NJ) with 50 μl of 0.07% glutaraldehyde freshly made in 0.1 M phosphate, pH 5.0, for 30 min. Samples diluted to 50 μl in 6 M urea were incubated in the wells overnight, and additional binding was blocked by incubation with blocking buffer for 1 h. Anti-KSPG antibody in 50 μl was added for 2 h (overnight for antibody I22 assay of column fractions) followed by incubation with peroxidase-labeled goat anti-mouse or anti-rabbit IgG F(ab)$_2$ (TAGO, Burlingame, CA) for 1 h. Color was developed with 3,3′,5,5′-tetramethylbenzidine as described (35) for 10 min.

Double Antibody ELISA—Double antibody ELISA was carried out by adsorption of monoclonal I22 or 5-D-4 to ELISA plates overnight at 4 °C in 0.1 M $NaHCO_3$ buffer, pH 9. After blocking, primary incubation was carried out for 2 h with tissue extracts diluted in blocking buffer. Inhibition by keratan sulfate was assessed by addition of 1 mg/ml bovine corneal keratan sulfate during the primary incubation. Sensitivity of the bound antigen to endo-β-galactosidase was assessed after primary incubation, by incubation with endo-β-galactosidase for 30 min at 37 °C as described below, omitting urea in the buffer. Binding of polyclonal antibody to the antigen-monoclonal antibody complex was assessed by a 2-h incubation with the diluted antibody followed by reaction with peroxidase-labeled goat anti-rabbit IgG F(ab)$_2$ and color development as described above.

Ion Exchange HPLC

One-half-ml aliquots of the filtered tissue extracts in 6 M urea were injected on a 1-ml Pharmacia Mono Q anion exchange HPLC column. Elution was carried out in 6 M urea, 20 mM Tris sulfate, pH 6.8, using a gradient of increasing slope from 0–1.5 M Na_2SO_4 at a flow rate of 1 ml per min at room temperature. Fractions of 0.3 ml were collected and assayed for protein and for KSPG antigens by direct ELISA as described above.

Endo-β-galactosidase Treatment

Pooled peak fractions from ion exchange columns (fractions 60–80) were dialyzed against 6 M urea, 20 mM Tris-sulfate, pH 6.8, and KSPG standards were dissolved in the same solution. *E. freundii* endo-β-galactosidase was added to a final concentration of 0.025 units/ml, sodium acetate, pH 5.8, was added to 0.2 M, and four protease inhibitors (16) were added. Digestion was conducted for 2 h at room temperature. Dilutions of digests were bound onto a glutaraldehyde-treated ELISA plate and assayed with 5-D-4, I22, or polyclonal antibodies in direct ELISA as described above.

RESULTS

Comparison of Anti-keratan Sulfate Antibodies—Three previously described antibodies made to different keratan sulfate-containing proteoglycans (16–18) were compared using whole and partially degraded bovine corneal KSPG. In direct ELISA, the two monoclonal antibodies bound to whole KSPG, but only weakly to endo-β-galactosidase-treated KSPG. No binding was detected to chemically deglycosylated KSPG protein core (Fig. 1, *A* and *B*). This activity is consistent with the previously determined specificity of the monoclonal antibodies for epitopes localized in keratan sulfate glycosaminoglycan chains and not for epitopes in core protein or linkage

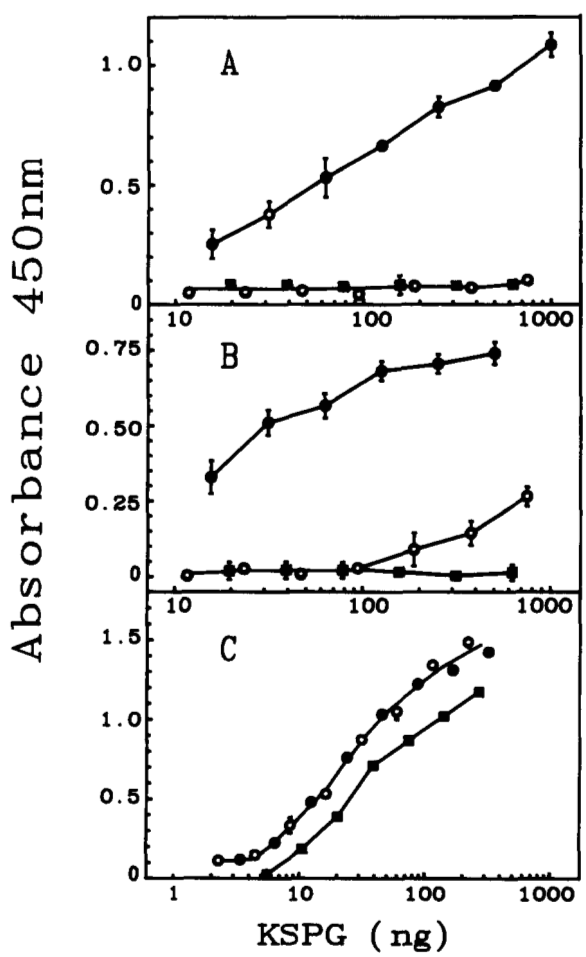

FIG. 1. **Direct ELISA of corneal KSPG components.** Bovine KSPG (*solid circles*), endo-β-galactosidase-treated KSPG (*open circles*), and deglycosylated KSPG core protein (*squares* and *broken line*) were assayed for their ability to bind each of three antibodies by ELISA as described under "Experimental Procedures." Error bars show ±1 S.D. and are omitted if the standard deviation was less than the diameter of the symbol. *A*, antibody I22; *B*, antibody 5-D-4; *C*, polyclonal antibody.

region of the keratan sulfate (16, 17). In contrast, a polyclonal antibody to purified bovine corneal KSPG (18), affinity-purified on KSPG-agarose (30), bound KSPG and endo-β-galactosidase-treated KSPG equally well (Fig. 1*C*). Binding to deglycosylated KSPG core was somewhat reduced compared to whole proteoglycan (Fig. 1*C*). The affinity-purified polyclonal antibody, therefore, appears to recognize antigens on the core protein of KSPG.

In the competitive assay, keratan sulfate preparations from several sources varied in ability to bind the monoclonals. As shown in Table I, purified keratan sulfate glycosaminoglycan preparations were weaker competitors than KSPG and also differed in their competitive abilities according to the source of the keratan sulfate. The weakest binding to both antibodies was from chemically desulfated keratan sulfate, confirming the requirement for sulfation of the binding site recognized by the monoclonal antibodies. The monoclonal antibodies also differed from each other in relative response to each keratan sulfate preparation, suggesting that these two antibodies may recognize slightly different sulfated epitopes in the keratan sulfate chain. Monoclonal antibodies had no response to heparin or other glycosaminoglycans, nor did the polyclonal antibody bind purified keratan sulfate or other glycosaminoglycans in this assay (not shown).

Keratan Sulfate Antigens in Tissue Extracts—KSPG-related antigens recognized by the three antibodies were measured in extracts of several bovine and embryonic chick tissues. As shown in Table II, antigens competing with corneal KSPG were detected in most of the bovine tissues examined. Antigens competing for binding of the affinity-purified polyclonal antibody were most widespread and abundant, whereas monoclonal 5-D-4 was the most restrictive in identifying KSPG-related antigens. The amount of antigen varied widely among tissues: cornea > cartilage ≫ tissues containing muscle (skeletal and cardiac muscle, lung, and intestine). Fibrous tissue (skin and bovine sclera) were very low in keratan sulfate antigens but rich in core protein antigens recognized by the polyclonal antibody. Liver consistently contained the least KSPG antigens. In a similar survey of 13 tissues from 20-day embryonic chick, keratan sulfate antigens were found in all except liver and skin, with abundance distribution similar to that of bovine tissues (not shown). The tissues surveyed were: aorta, bone, brain, cartilage, cornea, gizzard, intestine, liver, lung, skeletal muscle, cardiac muscle, sclera, and skin.

In many bovine tissues, part of the KSPG antigens were not extracted by guanidine HCl, but could be solubilized by treatment of the previously guanidine HCl-extracted tissue with CNBr. The guanidine HCl-resistant antigen was most abundant in striated muscle tissues, where it comprised the majority of the total keratan sulfate antigen (99% in bovine cardiac muscle (Table II)). Significant amounts of guanidine HCl-resistant KSPG antigens were also found in bovine and chick cornea and in bovine lung, kidney, and skin. The CNBr-solubilized antigens were typically more reactive with antibody 5-D-4 than with I22, indicating possible differences in the structure of keratan sulfate antigens in this fraction (Table II).

TABLE I

Comparison of keratan sulfate antigens with anti-keratan sulfate monoclonal antibodies by competitive ELISA

Values show the amount of each type of keratan sulfate (μg of galactose) required to reduce the binding of a biotin-labeled bovine corneal KSPG standard to 50% of uninhibited levels. Amounts were normalized to KSPG = 1 for comparison of the two antibodies.

Keratan sulfate type	Relative competitive abilities	
	I22	5-D-4
KSPG, bovine cornea	1	1
Keratan sulfate, bovine cornea	5.3	11.4
Keratan sulfate, rabbit cornea	1.4	5.5
Keratan sulfate, whale cartilage	252	51
Desulfated keratan sulfate, bovine cornea	>1000	930

Double Antibody Binding—The data in Table II show wide distribution of antigens from both carbohydrate and protein components of corneal KSPG. Table III demonstrates the physical association of these two antigens in the tissue extracts by assessing the ability of the antigens to bind both anti-keratan sulfate monoclonal and polyclonal antibodies simultaneously. As seen in Table III, color development was dependent on incubation with both monoclonal and polyclonal antibodies. Competition with keratan sulfate glycosaminoglycan blocked >90% of the color development resulting from incubation with guanidine HCl extracts and blocked 40–50% of the color developed by CNBr-extracted antigens from muscle and skin. Treatment of the keratan sulfate antigen-antibody complex with endo-β-galactosidase, resulted in a 20–50% reduction of color development. These controls demonstrate specificity of the monoclonal antibody for legitimate keratan sulfate antigens in this assay. Of bovine tissues listed in Table II, all but cartilage (shown) and brain (not shown) had antigens responding in this assay. Poor response from antigens in these two tissues would be expected because in these two tissues keratan sulfate is linked to core proteins different from corneal KSPG core protein. Monoclonal antibody 5-D-4 also participated in double antibody binding in a similar manner (not shown).

Ion Exchange HPLC of the Tissue Extracts—Fig. 2 compares elution patterns of guanidine HCl extracts of bovine cornea and kidney tissue on anion exchange HPLC. In each case, more than 95% of the protein in the sample was eluted by 0.1 M sulfate. Keratan sulfate antigens, detected by ELISA, eluted between 0.35–1.5 M sulfate in a single broad peak (Fig. 2, *A* and *B*). Similar elution profiles were obtained from purified bovine corneal KSPG, as well as from guanidine HCl extracts of all the bovine tissues listed in Table II (except from liver, which had no detectable activity). Assay with the affinity-purified polyclonal antibody found KSPG core protein-related antigen in fractions of the gradient overlapping the peak of keratan sulfate antigen in extracts of cornea (Fig. 2*C*), kidney (Fig. 2*D*), and all other tissues examined in Table II (data not shown). For several tissues (brain, liver, cardiac and skeletal muscle, intestine, and kidney) core protein antigen was found only in the high ionic strength elutate (*e.g.* Fig. 2*D*), but some tissues (cornea, lung, skin, cartilage, and sclera) also contained related antigens eluting earlier in the gradient (as in Fig. 2*C*).

Chromatography of CNBr-solubilized antigens of bovine cornea and cardiac muscle is shown in Fig. 3. Keratan sulfate antigens reacting with antibody I22, from these two samples (Fig. 3, *A* and *B*), eluted in a similar manner to keratan sulfate antigens from the guanidine HCl extract shown in Fig. 2. Antigenic components reacting with the polyclonal antibody (Fig. 3, *C* and *D*) were found in low ionic strength eluate and also in a typically broad peak co-eluting with the keratan sulfate antigens. CNBr digests of bovine skeletal muscle, skin, kidney, and lung were also chromatographed with similar results (not shown).

Sensitivity of Keratan Sulfate Antigen to Endo-β-galactosidase—Fig. 4 shows endo-β-galactosidase sensitivity of antigens in pooled HPLC peak fractions of corneal guanidine HCl extracts (Fig. 2*A*) and cardiac muscle CNBr extracts (Fig.

TABLE II

Quantitative estimate of KSPG antigens in guanidine HCl extracts and CNBr digests of bovine tissues

KSPG antigens were measured in extracts and digests of tissues using the competitive ELISA, as described under "Experimental Procedures." Values shown indicate the weight of bovine corneal KSPG containing the same amount of antigen as the experimental sample. Repeatability of the assay was typically from 50 to 200% of the values shown. Sensitivity of the assay was equivalent to ~1 μg of KSPG/g of tissue (I22, polyclonal) and ~0.1 μg of KSPG/g of tissue (5-D-4).

Tissue	Antibody	Amount of antigen			Guanidine HCl	CNBr
		Guanidine HCl	CNBr	Total		
		μg KSPG/g tissue			*% Total*	
Brain	I22	10	0	10	100	0
	5-D-4	0	0	0		
	Polyclonal	43	0	43	100	0
Cartilage	I22	1195	0	1195	100	0
	5-D-4	196	7	203	97	3
	Polyclonal	1653	5	1658	100	0
Cornea	I22	3019	943	3962	76	24
	5-D-4	3722	1124	4846	77	23
	Polyclonal	8154	122	8276	99	1
Muscle (skeletal)	I22	9	144	153	6	94
	5-D-4	0	22	22	0	100
	Polyclonal	1386	144	1530	91	9
Muscle (cardiac)	I22	1	91	92	1	99
	5-D-4	0	9	9	0	100
	Polyclonal	249	57	306	81	19
Intestine	I22	10	0	10	100	0
	5-D-4	0	0	0		
	Polyclonal	2000	6	2006	100	0
Kidney cortex	I22	28	6	34	82	18
	5-D-4	0.1	0	0.1		
	Polyclonal	55	15	70	78	22
Kidney medulla	I22	28	5	33	85	15
	5-D-4	0	0	0		
	Polyclonal	333	11	344	97	3
Liver	I22	0	0	0		
	5-D-4	0	0	0		
	Polyclonal	0	8	8	0	100
Lung	I22	22	3	25	88	12
	5-D-4	0.1	0.2	0.3	33	67
	Polyclonal	41	23	64	64	36
Sclera	I22	3	0	3	100	0
	5-D-4	1	0	1	100	0
	Polyclonal	734	27	761	96	4
Skin	I22	3	3	6	50	50
	5-D-4	0	0	0		
	Polyclonal	1541	25	1566	98	2

3*B*). Reactivity in direct ELISA with both monoclonal antibodies was eliminated by endo-β-galactosidase treatment of the keratan sulfate antigens from both corneal and cardiac muscle extracts (Fig. 4, *A–D*). Similar endo-β-galactosidase sensitivity was found for chromatographically purified keratan sulfate antigen from all other tissues with sufficient antigen for assay (guanidine HCl extracts from cartilage, kidney, lung, and skin; CNBr extracts from skeletal muscle).

Binding of the polyclonal antibody to purified KSPG antigens in guanidine HCl extracts from cornea (Fig. 4*E*), as well as other tissues (not shown), was not affected by endo-β-galactosidase treatment. Endo-β-galactosidase treatment of pooled peak fractions of CNBr digests from muscle (Fig. 4*F*), and other CNBr digests, reduced but did not eliminate polyclonal antibody binding. This pattern of sensitivity demonstrates that endo-β-galactosidase digests the keratan sulfate antigen without blocking reactivity of protein antigens in the same fractions.

DISCUSSION

The data presented here demonstrate that proteoglycans with antigenic and physical properties similar to corneal KSPG can be extracted from many body tissues. The abundance and extractability of these proteoglycans vary with respect to the tissue. We hypothesize that these antigens may be a family of proteoglycans related to corneal KSPG.

All of the tissues listed in Table II reacted with the anti-keratan sulfate monoclonal antibodies in the competitive assay (Table II) or in one of the direct ELISA tests (Table

TABLE III

Double antibody binding by tissue extracts

Tissue extracts and digests were assayed for simultaneous binding of polyclonal and monoclonal antibody I22 by double antibody ELISA as described under "Experimental Procedures." Values are averages of four replicate data points. Values for the "complete" assay are significantly ($p < 0.025$) greater than all other conditions for each assay (except for cartilage). Abbreviations: Muscle (S), skeletal muscle. Kidney (C), kidney cortex.

Tissue	Conditions of assay				
	Complete	Plus keratan sulfate	Endo-β-galactosidase	Minus monoclonal	Minus polyclonal
	absorbance ×450 nm				
Guanidine HCl extracts					
Cornea	0.894	0.060	0.692	−0.018	−0.015
Muscle (S)	0.872	−0.016	0.559	−0.001	0.095
Kidney (C)	0.195	0.019	0.107	0.002	−0.026
Liver	0.186	0.058	0.112	−0.003	−0.030
Cartilage	0.007	0.079	0.063	0.020	0.105
CNBr digests					
Cornea	0.149	−0.027	0.042	−0.023	0.002
Muscle (S)	0.176	0.108	0.084	−0.008	0.016
Skin	0.342	0.168	0.261	−0.011	−0.017

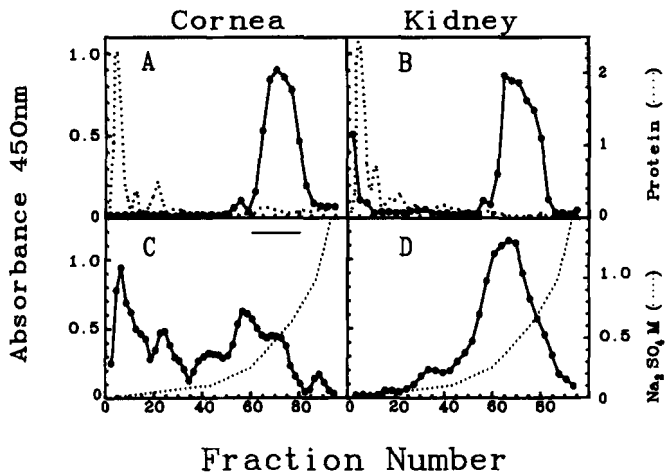

FIG. 2. **Ion exchange HPLC of guanidine HCl tissue extracts.** HPLC of the unfractionated extracts of bovine cornea (*A* and *C*) and kidney (*B* and *D*) was carried out in 6 M urea, 20 mM Tris-SO_4, with a 0–1.5 M gradient of Na_2SO_4 as described under "Experimental Procedures." *Solid circles* show direct ELISA with monoclonal antibody I22 (*A* and *B*) and with polyclonal antibody (*C* and *D*). *Dotted line* (*C* and *D*) shows concentration of eluting Na_2SO_4. *Dashed lines* (*A* and *B*) show protein concentration in mg/ml. *Bar* in *C* shows fractions pooled for endo-β-galactosidase digestion in Fig. 4.

III, Figs. 2 and 3). The recent literature contains an increasing number of reports of compounds related to keratan sulfate in tissues other than cartilage and cornea. However, this study appears to be the first to suggest that some keratan sulfate may be present in virtually all tissues. This discovery was made possible by the monoclonal antibodies against keratan sulfate. Previous characterization of these antibodies showed that they bind sulfated regions of keratan sulfate, but not other glycosaminoglycans, or related sulfated glycoproteins such as mucins (17, 18, 24). Recent studies with monoclonal 5-D-4 found that this antibody does not recognize fully sulfated keratan sulfate fragments shorter than hexasaccharides, nor are nonsulfated keratan sulfate fragments bound (24, 25). This requirement for more than two repeated sulfated lactosamine disaccharides insures that the antigens detected by this antibody are sulfated large oligo- or polysaccharides

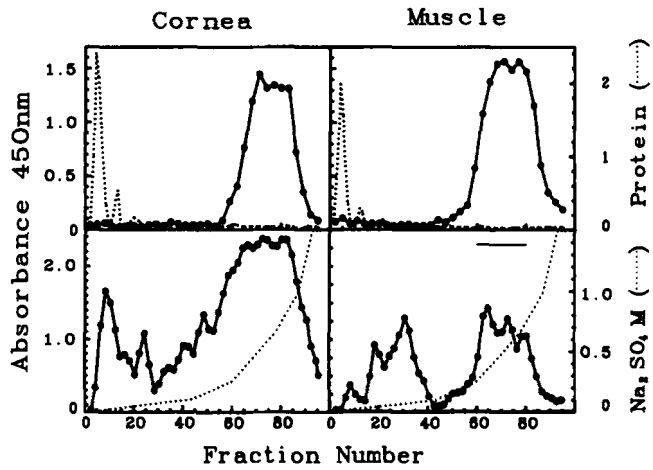

FIG. 3. **Ion exchange HPLC of CNBr extracts.** CNBr digests of bovine cornea (*A* and *C*) and of cardiac muscle (*B* and *D*) were fractionated by HPLC as described under "Experimental Procedures." Data are displayed as in Fig. 2. The absorbance scale in *D* is 0.4 × scale values of *C*. *Bar* in *D* shows fractions pooled for endo-β-galactosidase digestion in Fig. 4.

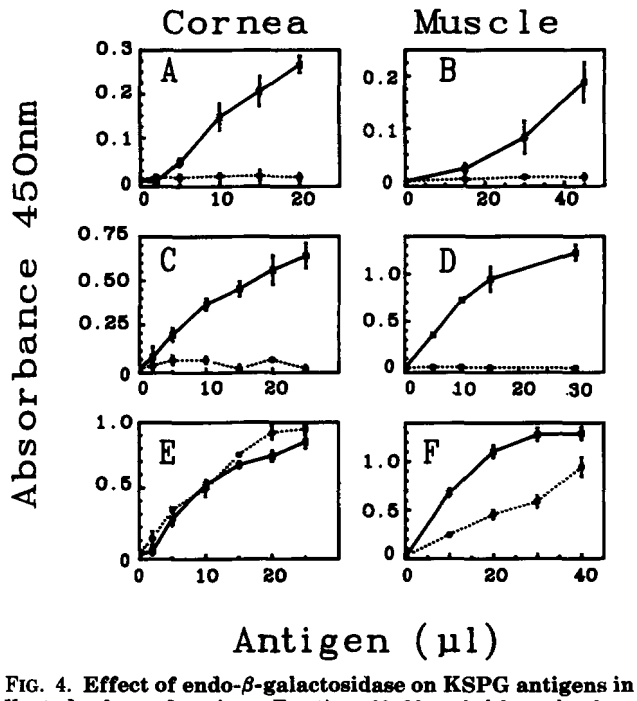

FIG. 4. **Effect of endo-β-galactosidase on KSPG antigens in collected column fractions.** Fractions 60–80 pooled from the chromatograms shown in Figs. 2*C* and 3*D* were treated with endo-β-galactosidase in the presence of protease inhibitors as described under "Experimental Procedures." Direct ELISA of the treated (*broken lines*) and untreated (*solid lines*) antigens was compared for corneal guanidine HCl extract (*A*, *C*, and *E*) and for cardiac muscle CNBr digest (*B*, *D*, and *F*) with three antibodies: I22 (*A* and *B*); 5-D-4 (*C* and *D*); polyclonal (*E* and *F*). Error bars are shown as Fig. 1.

rather than nonsulfated lactosaminoglycans or short sulfated lactosamine-containing oligosaccharides, such as those of lymphocytes and virus-infected chicken embryos (8, 9).

Even though the monoclonal antibodies react with a well-defined component of keratan sulfate, the possibility exists that the structures recognized in tissue extracts are unknown keratan sulfate-like carbohydrates different from the keratan sulfate of cornea and cartilage. Sensitivity of the tissue antigens to endo-β-galactosidase, therefore, provides independent confirmation of the poly-*N*-acetyllactosamine carbohydrate composition of these antigens. Endo-β-galactosidase is a well-

characterized and highly specific enzyme requiring a minimum structure of glcNAcβ(1-3)galβ(1-4)glcNAC (32). Degradation of the antigenic material with endo-β-galactosidase demonstrates the presence of legitimate keratan sulfate which was inferred from the antibody reactivity. Together, monoclonal antibody reactivity and endo-β-galactosidase sensitivity leave little question that the tissue antigens described here contain oligosaccharide regions identical to the polymeric keratan sulfate of cornea and cartilage. On the basis of the current data, no conclusions can be drawn about the possible presence of branched chains, the chain length, or the nature of the protein-keratan sulfate linkage regions. These analyses must await detailed structural studies on purified tissue KSPG antigens.

The polyclonal antibody provided specificity to a completely different portion of the KSPG molecule than that recognized by the monoclonal antibodies. Although the unfractionated antiserum does bind keratan sulfate (18), it is clear from Fig. 1, that after affinity purification, no reactivity with free keratan sulfate remains. Reaction with deglycosylated KSPG core (Fig. 1*C*) suggests that the polyclonal primarily recognizes the polypeptide sequence, and not the neutral oligosaccharides, of the KSPG core protein. Further characterization of this antiserum[2] found >90% of the reactivity of purified KSPG was in endo-β-galactosidase-sensitive material. Thus, antigens recognized by this antibody are keratan sulfate-linked proteins, not unrelated proteins which may have contaminated the KSPG originally used as immunogen. Competitive assay with the polyclonal antibody was carried out against a labeled KSPG standard which had been purified by adsorption to immobilized antibody I22. This procedure further insured that the antigens detected in tissue extracts were related to keratan sulfate-linked protein of corneal KSPG and not to contaminating proteins.

Antigens from two separate components of corneal KSPG (keratan sulfate and core protein) were found widely distributed, with a generally similar abundance (Table II). A portion of the two antigens co-chromatographs on HPLC ion exchange (Figs. 2 and 3). This circumstantial evidence for presence of these antigens in the same molecule was corroborated by the demonstration (Table III) of a physical association of the two antigens. Specificity of this double antibody binding for keratan sulfate was shown by sensitivity to competition with purified keratan sulfate and by sensitivity to endo-β-galactosidase treatment of the antibody-antigen complex. An important control regarding specificity of the polyclonal antibody in this experiment was the nonreactivity of brain and cartilage extracts, both of which contain keratan sulfate linked to a protein core differing from that of cornea (1, 2). These data suggest that the keratan sulfate in many non-corneal tissues is part of a proteoglycan containing protein and keratan sulfate antigenic sites related to those of corneal KSPG. The existence of families of proteoglycans ranging over many tissues, containing similar or identical core proteins, has recently been reported by Heinegard and coworkers (36). The antigens detected in our study may represent a previously undetected family of proteoglycans related to corneal KSPG.

It is apparent from Table II that the proteins recognized by the polyclonal antibody were relatively more abundant than the keratan sulfate antigens in tissues other than cornea and cartilage. This was most evident in fibrous tissues, such as sclera and skin. These data suggest the presence of core protein antigens with little or no sulfated keratan sulfate. HPLC profiles of cornea (Fig. 2*C*), as well as those of skin and sclera (not shown), indeed contain much material reacting with polyclonal antibody that elutes at lower ionic strength than does keratan sulfate. This material might be unrelated protein containing antigenic sites in common with KSPG core protein. On the other hand, these data could be explained by the presence, in skin, sclera, and other tissues, of a KSPG-like proteoglycan that has relatively low sulfation. Its low sulfation would result in little or no reaction with anti-keratan sulfate monoclonal antibodies and in elution at a low ionic strength from the HPLC. A low ionic charge would confer physical properties different from those of highly sulfated proteoglycans, thus explaining why such a compound has not been previously described.

An interesting observation of the current work is that from some tissues KSPG antigens were marginally soluble in 4 M guanidine HCl. Insolubility of a portion of corneal KSPG (37), cartilage proteoglycan (38), and basement membrane proteoglycans (39) in guanidine HCl has been reported previously. The resistance of much collagen to extraction with guanidine HCl and its ready solubilization with CNBr suggests an association of guanidine HCl-resistant KSPG with collagen. Recent ultrastructural studies found corneal KSPG associated with regions of corneal collagen fibrils corresponding to the ends of collagen molecules (40). This association of KSPG with a specific portion of the corneal collagen molecules and its resistance to extraction with guanidine HCl suggest a possible covalent collagen-KSPG link. Recent findings of covalent linkages between chondroitin sulfate and collagen (41) and between heparan sulfate and fibronectin (15) lend credibility to this possibility.

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[2] J. L. Funderburgh, B. Caterson, and G. W. Conrad, unpublished observations.

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