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THE PRESENCE OF A KERATAN SULFATE-LIKE GLYCOCONJUGATE IN MICROSOMES OF THE CEREBRAL CORTEX

Hyman G. Weinstein,[a] Lawrence J. Blacik,[a] Moira Breen,[a,b,*] and Paul A. Knepper

[a]Research-in-Aging Laboratory, Veterans Administration Medical Center, North Chicago, Illinois 60064;

[b]Department of Pathology, University of Health Sciences, Chicago Medical School, North Chicago, Illinois 60064

[c]Laboratory for Oculo-Cerebrospinal Investigation, Children's Memorial Hospital, Chicago, Illinois 60614

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SUMMARY: Density gradient centrifugation of a 4 M guanidine hydrochloride preparation of the cerebral cortex showed that most of the keratan sulfate-like glycoconjugate was bound to the microsomes; the latter also contained heparan sulfate and one-third of the chondroitin sulfate proteoglycans. A hyaluronate-containing chondroitin sulfate proteoglycan was present in the soluble fraction.

INTRODUCTION: Keratan sulfate-like oligosaccharides have been found in the ovary (1) and erythrocyte membranes (2). Sialyl-transferase enzymes involved in the synthesis of such oligosaccharides have been detected in the microsomes of fetal liver, embryonic brain, placenta, testis, spleen and kidney (3).

Recently, our laboratory has reported the presence of a keratan sulfate-like glycoconjugate in the cerebral cortex and cerebellum, the concentration of which varied with growth and senescence (4-7).

The subcellular location of the keratan sulfate-like glycoconjugate in the brain has yet to be determined. Since half of the glycosaminoglycans (GAG) in the brain are present in the microsomes and at least 40% of the total brain GAG can be solubilized with a phosphate buffered isotonic sucrose solution

* Address reprint requests to: Moira Breen, Research-in-Aging Laboratory, Veterans Administration Medical Center, North Chicago, Illinois 60064.

Abbreviations used are: GAG, glycosaminoglycans; GuHCl, quanidine hydrochloride; CS, chondroitin sulfate; HA, hyaluronic acid; HS, heparan sulfate in Fig. 1d(5); also represents (KS) keratan sulfate plus heparan sulfate in Figs. 1b(4), 1d(1) and 1f(6); UA, uronic acid; HexN, heoxsamine; GluN, glucosamine; GalN, galactosamine; DNase, deoxyribonuclease; RNase, ribonuclease.

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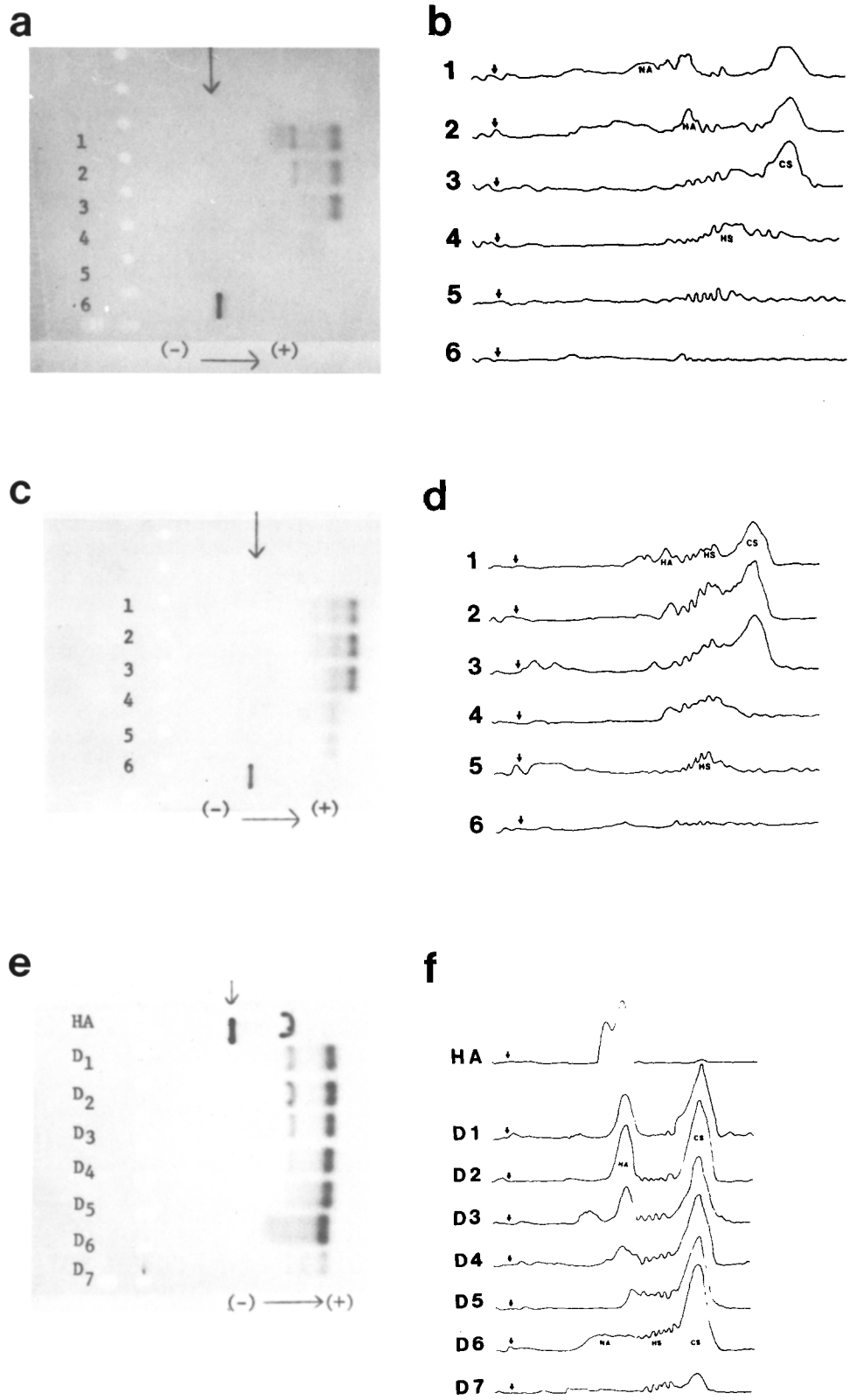
(8,9), our aim in this preliminary study was to determine whether the keratan sulfate-like glycoconjugate in the brain was also associated with microsomes. The methods used by Hascall and Sajdera for the isolation of cartilage proteoglycans (10) and of Margolis et al. (11) and Branford White and Hudson (12) for brain proteoglycans were adapted for the separation of the microsomes and their bound proteoglycans from the soluble proteoglycans and other subcellular particulates of brain.

MATERIALS AND METHODS: The cerebral cortices from twenty-eight three-month-old rats were homogenized with 4 M guanidine hydrochloride (GuHCl) in the presence of protease inhibitors as described by Oegema et al. (13). The homogenate was subsequently centrifuged at 10,400 x g to remove nuclei, mitochondria and synaptosomes. The crude supernatant containing the soluble macromolecules, microsomes and non-synaptic plasma membranes of the cerebral cortex (14) was centrifuged at 96,000 x g at 4°C for 71 hours in a cesium chloride (CsCl) gradient. After centrifugation, 6 equivolume fractions were obtained by displacement with a CsCl solution and labelled D_1 to D_6 from the bottom up. Lipid-containing material floating on the top was labelled fraction D_7.

Aliquots of the cerebral cortex, the whole 4 M GuHCl extract and the seven density gradient fractions were delipidated, dried and analyzed for the GAG as previously described by Breen et al. (15-17). Total protein was measured by the method of Lowry et al. (18).

RESULTS AND DISCUSSION: At least 80% of the GAG substituents (glucosamine, galactosamine and uronic acid) in cerebral cortex appeared in the 4 M GuHCl extract. Recovery of the GAG substituents in the D_1 to D_7 fractions from the 4 M GuHCl extract was at least 83%; protein recovery was 95%.

Four GAG polymers present in the cerebral cortex were also found in the 4 M GuHCl extract and in the light fractions D_6 and D_7; these were: hyaluronic acid, heparan sulfate, chondroitin 4(6)-sulfate and a keratan sulfate-like glycoconjugate. In the heavier fractions, D_1 to D_5, only hyaluronic acid and chondroitin 4(6)-sulfate were present. Hyaluronic acid was most concentrated in fraction D_2, less concentrated in fractions D_1 and D_3 and still less in fractions D_4 and D_5. The light fractions D_6 and D_7 contained very little hyaluronic acid. Chondroitin sulfate was found in all density gradient fractions but was much less evident in D_7. Heparan sulfate was not present in the heavy fractions D_1 to D_5 but appeared in the light fractions D_6 and D_7 (Fig. 1).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In each of the fractions D_1 to D_5, the uronic acid concentration was stoichiometrically equivalent to that of the amino sugar, indicating that almost all of the amino sugar was derived from hyaluronic acid and chondroitin sulfate. In contrast, the amino sugar in the fraction D_6 and D_7, was very much in excess of uronic acid indicating a high concentration of a hexosamine containing glycoconjugate. Our previous studies of the GAG-containing fraction from the cerebral cortex showed that the excess amino sugar content was primarily due to glucosamine. The latter varied directly

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Fig. 1a. Cellulose acetate electrophoresis of the glycosaminoglycans (GAG) of the 4 M guanidine HCl (GuHCl) extract of the cerebral cortex, before and after sequential enzymatic and chemical degradation. Stained with Alcian Blue at pH 5.8.
- Lane 1: GAG of the 4 M GuHCl extract.
- Lane 2: GAG from 4 M GuHCl extract after treatment with deoxyribonuclease (DNase) and ribonuclease (RNase).
- Lane 3: GAG from 4 M GuHCl extract after treatment with DNase, RNase and hyaluronidase.
- Lane 4: GAG from 4 M GuHCl extract after treatment with DNase, RNase, hyaluronidase and chondroitinase ABC.
- Lane 5: GAG from 4 M GuHCl extract after treatment with DNase, RNase, hyaluronidase, chondroitinase ABC and endo-B-D-galactosidase (keratanase).
- Lane 6: GAG from 4 M GuHCl extract after treatment with DNase, RNase, hyaluronidase, chondroitinase ABC, endo-B-D-galactosidase (keratanase) and nitrous acid.

Fig. 1b: Densitometric tracings of Fig. 1a.

Fig. 1c: Cellulose acetate electrophoresis of the GAG from fraction D_7 of the 4 M GuHCl extract of the cerebral cortex before and after sequential enzymatic and chemical degradation.
- Lane 1: GAG in fraction D_7.
- Lane 2: GAG in fraction D_7 after treatment with DNase and RNase.
- Lane 3: GAG in fraction D_7 after treatment with DNase, RNase and hyaluronidase.
- Lane 4: GAG in fraction D_7 after treatment with DNase, RNase, hyaluronidase and chondroitinase ABC.
- Lane 5: GAG in fraction D_7 after treatment with DNase, RNase, hyaluronidase, chondroitinase ABC and endo-B-D-galactosidase (keratanase).
- Lane 6: GAG in fraction D_7 after treatment with DNase, RNase, hyaluronidase, chondroitinase ABC, endo-B-D-galactosidase and nitrous acid.

Fig. 1d: Densitometric tracing of Fig. 1c.

Fig. 1e: Cellulose acetate electrophoresis of the GAG from the buoyant density fractions D_1 to D_7 of the 4 M GuHCl extract of the rat cerebral cortex.

Fig. 1f: Densitometric tracing of Fig. 1e. D_1 to D_7 are buoyant density fractions of the 4 M GuHCl extract obtained under dissociative conditions as described in the text and Table 1.

Arrow: origin
Direction of mobility: from left to right.

with the galactose, sulfate and sialic acid content of the fraction. Specific enzymatic degradation followed by electrophoresis revealed the presence of keratan sulfate (4,17). A critical comparison of the electrophoresis pattern in this study (Fig. 1c and its corresponding densitometer tracings in Fig. 1d) revealed a loss in optical density in the position of the keratan sulfate standard when degraded by endo-B-D-galactosidase. This suggests that a keratan sulfate-like glycoconjugate is present in fraction D_7. In contrast, as shown in Figs. 1e and 1f, a keratan sulfate-like glycoconjugate was not visible in the electrophoresis patterns of the GAG in fraction D_1 to D_5. In terms of our chemical analysis for hexosamine and uronic acid, the excess hexosamine was found primarily in the light fractions D_6 and D_7 (Table 1).

TABLE 1

DISTRIBUTION OF PROTEOGLYCANS IN RAT CEREBRAL CORTEX, 4M GUANIDINIUM HCl EXTRACT AND ITS BUOYANT DENSITY SUBFRACTIONS

			←Glycosaminoglycan Substituents→			
	Density g/ml	Protein mg/g	GluN	GalN	HexN	UA
			μmol/g lipid-free dry wt. cerebral cortex			
Cerebral Cortex		786 ± 34	7.10	3.63	10.73	5.94
4M GuHCl extract		758	5.86	2.91	8.77	4.75
Buoyant Density Fractions of 4M GuHCl extract Starting Density = 1.28						
D_7	<1.224	355	3.230	0.957	4.190	1.13
D_6	1.224	192	0.114	0.131	0.245	0.072
D_5	1.230	109.2	0.121	0.217	0.338	0.328
D_4	1.292	44.4	0.160	0.305	0.465	0.492
D_3	1.402	13.5	0.436	0.530	0.966	0.829
D_2	1.547	3.6	0.576	0.435	1.011	0.930
D_1	1.679	2.2	0.202	0.180	0.382	0.328
Total (D_1 - D_7)		719.9	4.839	2.755	7.597	4.109
% extracted with 4M GuHCl		96.4	82.5	80.2	81.2	80.0
% recovered of 4M GuHCl extract from buoyant density fractions		95.0	82.6	94.6	86.6	86.5

Glycoconjugates other than keratan sulfate may be present in the low density fractions, however, unless such compounds are charged, they will not move electrophoretically or take the Alcian Blue stain at pH 5.8. Although our study indicates that a keratan sulfate-like substance is present in the GAG-enriched fraction, other glycoconjugates may also be present.

Our analysis always included delipidation prior to proteolysis, therefore, although glycolipids may be present initially in the low density fractions, they do not contribute to the data shown in Table 1.

Prior to density gradient centrifugation of the 4 M GuHCl supernatant, all subcellular particulates, except microsomes were removed (8,9,11). The microsomes having buoyant densities less than 1.20 g/ml (9,14) were located at the top of the gradient, in fractions D_6 and D_7. Seventy-two percent of the total amino sugar in the GAG-enriched fraction obtained from these low density regions represented keratan sulfate-like and other glycoconjugates, compared to only 45% in the original cerebral cortex. Therefore, the microsomal fraction was significantly enriched with the keratan sulfate-like moiety. Although earlier reports showed that 90% of the brain glycoproteins and GAG occurred in the subcellular fractions, particularly the synaptic and microsomal membranes (8,9), a keratan sulfate-like substance was not identified.

At least 75% of the whole brain heparan sulfate is bound to microsomal membranes (9). Hook and co-workers have shown that heparan sulfate proteoglycans in hepatocytes exists in two forms: non-covalently bound to carbohydrate receptors on the cell surface or covalently bound to a protein core, which in turn, is intercalated in the lipid bilayer of the membrane (19,20). It is likely that the GAG in our fractions D_6 and D_7, (heparan sulfate, keratan sulfate-like glycoconjugate and chondroitin sulfate) are strongly bound to microsomes. If these sulfate-containing GAG had been free, they would have been found at higher densities; it is the presence of the heavy sulfate radical that contributes markedly to the density of the proteoglycan molecular (21,22).

The bulk of the soluble proteoglycans, D_1 to D_3, contained equimolar concentrations of hyaluronic acid and chondroitin sulfate. The buoyant density of free hyaluronic acid is 1.46 (23,24). In this study, most hyaluronic acid was found at higher buoyant densities of 1.547 (D_2) and 1.679 (D_1) indicating that it may be bound to a soluble chondroitin sulfate proteoglycan. This contrasts with other studies in which hyaluronic acid was reported at levels of 5% or less of the total GAG in the soluble proteoglycans (12,25). We attribute the substantially higher proportion of hyaluronic acid in our soluble proteoglycans to the use of protease inhibitors in the 4 M GuHCl extract.

In conclusion, the soluble proteoglycans of the cerebral cortex were separated from those bound to the microsomes; the latter contained most of the keratan sulfate-like glycoconjugate. Heparan sulfate and at least one-third of the chondroitin sulfate glycoconjugates of the cerebral cortex are also associated with the microsomes. Hyaluronic acid was associated with a high density soluble chondroitin sulfate proteoglycan.

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