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GLYCOSAMINOGLYCANS ARE INTEGRAL CONSTITUENTS OF RENAL GLOMERULAR BASEMENT MEMBRANE

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SUMMARY

Basement membranes from canine renal glomeruli were isolated following osmotic lysis and sequential detergent treatment. Substantial amounts of uronic acid in unfractionated membranes were demonstrated with the carbazole and orcinol reactions. About 10-15% of basement membrane uronic acid was solubilized with neutral salt solutions. Denaturation in 8M urea solubilized ≈70% of the uronic acid but only ≈10% of basement membrane hydroxyproline; the latter was solubilized after reduction and alkylation. Uronic acid containing glycoprotein isolated by denaturation did not bind to carboxymethylcellulose and migrated as a high molecular weight band on SDS-gel electrophoresis. The ability of isolated rat glomeruli to incorporate radioactive sulfate in vitro was demonstrated. These findings indicate that sulfated glycosaminoglycans are integral components of glomerular basement membrane.

Despite some continued controversy, the majority of current evidence indicates that the collagen components of basement membrane are high molecular weight chains which resemble procollagen in size (1-5), in the presence of α-chain like segments associated with non-collagenous sequences and disulfide linkages (1,2, 6-8) and in their partial susceptibility to limited pepsin digestion, at least some of which can be explained on the basis of conversion of procollagen-like molecules to collagen-like chains (2,9-11). Recent reports suggest that several tissues contain more than one genetically distinct type of collagen chain (11-14), although the molecular organization of these chains and their interaction with pro collagen sequences is not well-defined. The nature of the non-collagen moieties of basement membrane is even less clear, and glycoproteins other than procollagen extension sequences may constitute an integral part of these extracellular structures. That the latter may include glycosaminoglycans is suggested by the charge-selective glomerular capillary barrier which retards passage of acidic molecules (15), the ability of glomerular basement membrane to absorb ruthenium red in anionic sites (16), and the elimination of these anionic sites after treatment with heparitinase (17). The present communication describes the intimate association

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of glycosaminoglycans with purified glomerular basement membrane and reports the incorporation by isolated glomeruli of labeled sulfate into the basement membrane matrix.

MATERIALS AND METHODS

Glomeruli were isolated from adult male white rats or mongrel dogs as previously described (18) and either used directly (canine) or first incubated (rat) with [^{35}S]-sulfate before purification of the basement membranes. Incubations were performed with shaking for 90 and 180 minutes at 37°C in 2.5 ml of modified Krebs solution containing 10% fetal calf serum, 10mM glucose, and 20 µCi/ml of [^{35}S]-sulfate (New England Nuclear, 880 mCi/mM) in an atmosphere of 95% O_2 with 5% CO_2. Glomerular concentration was adjusted with Krebs solution so that timed incubations contained comparable numbers of glomeruli.

Basement membranes were purified with a novel technique of osmotic lysis followed by selective solubilization with detergents (19); the architectural and molecular integrity of glomerular basement membrane isolated with this method has been described (20). Basement membranes were extracted overnite at 4°C in 0.5M or 2.0M NaCl, .05M Tris HCl, pH 7.4 containing inhibitors of proteolysis (25mM EDTA, 10mM N-ethylmaleimide, 1mM benzamidine HCl and 1mM phenylmethylsulfonylflouride), were denatured at 50°C in 8M urea, .05M Tris pH 8.6 or 8M urea, .05M sodium acetate, pH 4.8, and were reduced in .05M dithiothreitol or reduced and alkylated with .05M dithiothreitol and .11M iodoacetic acid (21).

Total protein was measured by the method of Lowry (22) using albumin as standard; uronic acid was determined with the carbazole (23) and orcinol (24) reactions using glucuronolactone as standard. Hydroxyproline was measured by the method of Rojkind and Gonzalez (25). Carboxymethylcellulose chromatography was performed on a 1.6 x 12 cm column equilibrated with 0.05M sodium acetate, pH 4.8, 1M urea and eluted with a superimposed linear salt gradient from 0 to 0.22M NaCl over a total volume of 400 ml at 42°C. SDS-gel electrophoresis was performed according to the method of Goldberg et al (26) in gels 5% and 7.5% in acrylamide. Samples were denatured or reduced and denatured prior to application to the gels.

RESULTS AND DISCUSSION

The technique of glomerular basement membrane isolation used in these experiments yields preparations with retention of architectural integrity and hydroxyproline : proline ratios of 0.8 (canine) to 0.9 (rat). These preparations of unfractionated basement membrane contained substantial (2-400 µg/mg protein) amounts of uronic acid with a carbazole : orcinol ratio of 1.8. Extraction with neutral salt solutions solubilized limited amounts of uronic acid (see Table 1). Most of the uronic acid, but <10% of the hydroxyproline, was solubilized with denaturation in 8M urea; virtually all of the hydroxyproline was solubilized after reduction (Table 1).

Differential solubilization of collagen and uronic acid-containing glycoproteins was also demonstrated with SDS-gel electrophoresis of material solubilized

TABLE 1 - URONIC ACID IN CANINE GLOMERULAR BASEMENT MEMBRANE

Solubilization	% Total Uronic Acid Solubilized	% Total OH-Proline Solubilized
(a) 0.5M NaCl	11.6	-
(b) 2.0M NaCl	14.3	-
(c) Residue from (a) or (b); Denatured in 8M urea	75	10
(d) Residue from (c); Reduced and Denatured	10	85

Uronic acid determined by the carbazole method (23) after dialysis against distilled water; hydroxyproline determined by the method of Rojkind and Gonzalez (25) after desalting and acid hydrolysis.

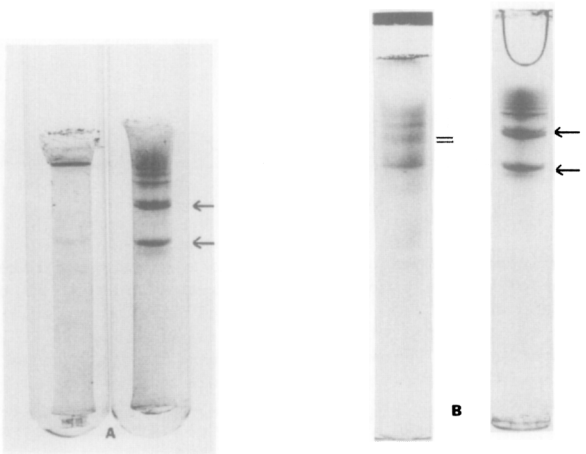

FIGURE 1
Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of canine glomerular basement membrane purified with Triton X-100 and sodium deoxycholate treatment of osmotically lysed whole glomeruli in the presence of protease inhibitors. A) ≈100 µg (protein) denatured; solubilized material applied to the gel; B) 100 µg (protein) reduced and denatured and applied to gel; positions of collagenase-sensitive bands migrating between α and β components of Type I collagen indicated by fine lines. The electrophoretic pattern of calf skin collagen, 75 µg reduced and denatured, shown on right in A and B; migration positions of α-chains and β-components indicated by arrows. Gels 5% in acrylamide in A and 7.5% in B.

with denaturation alone (Figure 1A) versus that solubilized after reduction (Figure 1B). The latter migrated as several bands with the most prominent components having electrophoretic mobilities similar to collagen α chains and between the α and β components of Type I collagen (see arrows and fine lines, Figure 1). The former migrated as a high molecular weight band just penetrating the gel (Figure

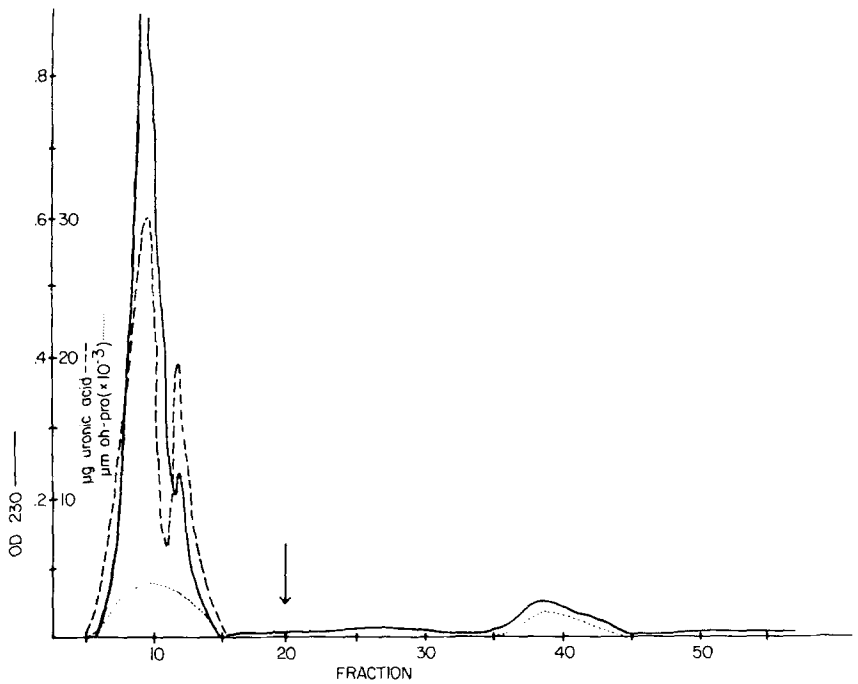

FIGURE 2
Carboxymethyl-cellulose chromatography of canine glomerular basement membrane. ≈1.8 mg (protein) of basement membrane denatured at 50°C in 8M urea/.05M sodium acetate, pH 4.8 and centrifuged to remove unsolubilized material. The column 1.6 x 12 cm) was equilibrated with 0.05M sodium acetate, pH 4.8, 1M urea and eluted with a superimposed linear salt gradient from 0-0.22M NaCl over a total volume of 400 ml at 42°C. Fractions (5 ml) were monitored for absorbance at 230nm (——), for hydroxyproline after acid hydrolysis (.....) and for uronic acid by the carbazole reaction (- - -) after desalting by dialysis.

1A). This material did not bind to carboxymethylcellulose and was almost all recovered in the fraction eluting before the start of the salt gradient (Figure 2). The small amount of material solubilized by denaturation in 8M urea which adsorbed to CM-cellulose contained hydroxyproline but no uronic acid (Figure 2).

Isolated rat renal glomeruli incubated in vitro incorporated [^{35}S]-sulfate into glomerular basement membrane. About 70% of the label and uronic acid was recovered in glycoprotein solubilized by denaturation in 8M urea. Incorporation both as cpm/mg uronic acid and cpm/mg protein increased with time (Table 2).

Almost all previous biochemical analyses of glomerular basement membrane, in which uronic acid or sulfate were not detected, were performed on material obtained after sonic disruption of whole glomeruli (27-29). This procedure may disrupt or remove anionic sites, as suggested by Kanwar and Farquhar (17). These investigators

TABLE 2 - [^{35}S]-SULFATE INCORPORATION INTO RAT GLOMERULAR BASEMENT MEMBRANE

Solubilization	Time	Cpm/mg Uronic Acid	Cpm/mg Protein
(a) Denatured in 8M urea	90 mins.	13,700	1,043
	180 mins.	34,700	2,000
(b) Residue from (a) 0.1N NaOH	90 mins.	13,300	1,260
	180 mins.	33,200	4,000

≈1 x 10^5 glomeruli freshly isolated from renal cortex of adult male white rats incubated for 90 and 180 minutes in modified Krebs buffer containing 10mM glucose, 10% fetal calf serum, and 20 μCi/ml [^{35}S]-sulfate. Basement membranes, purified as described in text, repeatedly washed with distilled water containing protease inhibitors until wash was free of radioactivity. Uronic acid determined by the carbazole reaction (23) and protein by the method of Lowry et al (22). Samples in (b) contained 20-25% of the total uronic acid.

found that anionic sites were preserved in glomerular basement membrane isolated by detergent treatment and, furthermore, that these sites contained heparan sulfate since they disappeared after treatment with purified heparitinase. In the present report, the lower than expected carbozole : orcinol ratio is also compatible with the presence of heparan sulfate (30) although the latter was not specifically isolated or identified by enzymatic digestion. Recent identification of a macromolecular proteoglycan associated with extracts obtained from a mouse tumor that produces basement membrane provides further evidence that the basement membrane matrix contains glycosaminoglycans (31).

The method for glomerular basement membrane isolation employed in these studies offers several advantages for identification and characterization of basement membrane components. It preserves mesangial as well as peripheral basement membrane and allows recovery of ultrastructurally and chemically intact membrane. It also provides relatively pure material that can be prepared without resorting to limited pepsin digestion for liberation from whole tissues and can be solubilized without the use of pepsin. These considerations pertain to the present results, which demonstrate that glycosaminoglycans are integral constituents of renal glomerular basement membrane.

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REFERENCES

1. Daniels, J.R. and Chu, G.H. (1975) J. Biol. Chem. 250:3531-3537
2. Dehm, P. and Kefalides, N.A. (1978) J. Biol. Chem. 253:6680-6686
3. Timpl, R., Martin, G.R., Bruckner, P., Wick, G. and Wiedemann, H. (1978) Eur. J. Biochem. 84:43-52
4. Minor, R.R., Clark, C.C., Strause, E.L., Koszalka, T.R., Brent, R.L. and Kefalides, N.A. (1976) J. Biol. Chem. 251:1780-1794
5. Heathcote, J.G., Sear, C.H.J. and Grant, M.E. (1978) Biochem. J. 176:283-294
6. Kefalides, N.A. (1971) Biochem. Biophys. Res. Comm. 45:226-234
7. Kefalides, N.A. (1972) Biochem. Biophys. Res. Comm. 47:1151-1158
8. Olsen, B.R., Alper, R. and Kefalides, N.A. (1973) Eur. J. Biochem. 38:220-228
9. Orkin, R.W., Gehron, P., McGoodwin, E.B., Martin, G.R., Valentine, T. and Swarm, R. (1977) J. Exp. Med. 145:204-220
10. Tryggvason, K. and Kivirikko, K.I. (1978) Nephron 21:230-235
11. Timpl, R., Bruckner, P. and Fietzek, P. (1979) Eur. J. Biochem. 95:255-263
12. Glanville, R.W., Rauter, A. and Fietzek, P. (1979) Eur. J. Biochem. 95:383-389
13. Kresina, T.F. and Miller, E.J. (1979) Biochem. 18:3089-3097
14. Sage, H. and Bornstein, P. (1979) Biochem. 18:3815-3822
15. Caulfield, J.P. and Farquhar, M.G. (1976) Proc. Natl. Acad. Sci. 73:1646-1650
16. Kanwar, Y.S. and Farquhar, M.G. (1978) J. Cell Biol. 79:150a
17. Kanwar, Y.S. and Farquhar, M.G. (1979) Proc. Natl. Acad. Sci. 76:1303-1307
18. Cohen, M.P. and Vogt, C.A. (1975) Biochem. Biophys. Acta 393:78-87
19. Carlson, E.C., Brendel, K., Hjelle, J.T. and Meezan, E. (1978) J. Ultrastruc. Res. 62:26-53
20. Cohen, M.P. and Surma, M. (1979) J. Biol. Chem. (in press)
21. Monson, J.M., Click, E.M. and Bornstein, P. (1975) Biochem. 14:4088-4092
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J.R. (1951) J. Biol. Chem. 194:265-275
23. Bitter, T. and Muir, H.M. (1962) Analyt. Biochem. 4:330-334
24. Svennerholm, L. (1956) J. Neurochem. 1:42-53
25. Rojkind, M. and Gonzalez, E. (1974) Analyt. Biochem. 57:1-7
26. Goldberg, B., Epstein, E.H. and Sherr, C.J. (1972) Proc. Nat. Acad. Sci. USA 69:3655-3659
27. Westberg, N.G. and Michael, A.F. (1970) Biochem. 9:3837-3846
28. Kefalides, N.A. (1973) Int. Rev. Conn. Tiss. Res. 6:63-104
29. Hudson, B.G. and Spiro, R.G. (1972) J. Biol. Chem. 247:4229-4247
30. Barker, S.A. (1965) Carbohydrate Res. 1:52-61
31. Hassell, J.R., Robey, P.G., Barrach, H.J., Wilczek, J., Rennard, S.I. and Martin, G.R. (October 1979) 5th Int. Symp. on Glycoconjugates.