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Analysis of glycosaminoglycans in bovine retinal microvessel basement membrane

Van-Yu Wu [b], Bryan Wilson [a], Henry Klepser [b] and Margo P. Cohen [b]

[a] *University of Medicine and Dentistry of New Jersey, NJ and* [b] *University City Science Center, Philadelphia, PA (U.S.A.)*

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Glycosaminoglycans (GAG) were isolated from bovine retinal microvessel basement membrane (RMV-BM) and quantitatively analyzed using a recently described competitive binding assay that is specific for and sensitive to nanogram amounts of heparan and chondroitin sulfates. Treatment of osmotically lysed retinal microvessels with the ionic detergent deoxycholate (DOC), required for liberation of the extracellular matrix for plasma membrane lipoproteins and purification of the insoluble matrix, solubilized less than 5% of the GAG in the water-insoluble material. Total GAG content in the DOC-insoluble basement membranes was approx. 0.52 μg/mg dry weight; about 70% of the measurable GAG was resistant to both chondroitinase ABC and chondroitinase AC digestion and was sensitive to nitrous acid treatment, indicating its heparan sulfate nature. Cellulose acetate electrophoresis revealed two bands, one of which had an electrophoretic mobility similar to heparan sulfate standard and was sensitive to nitrous acid; the other migrated in the same position as chondroitin sulfate standard and was sensitive to chondroitinase ABC and chondroitinase AC digestion. These results provide evidence that RMV-BM contains chondroitin sulfate(s) as well as heparan sulfate, and offer the first quantitative analysis of GAG in this extracellular matrix.

Introduction

The retinal microvasculature contains endothelial cells, pericytes, and extracellular matrix. The latter underlies endothelial cells which line the vessel lumen, and envelops intramural pericytes which lie outside the basement membrane surrounding the endothelial cells. As in the intracerebral vasculature, to which it is anatomically similar, the retinal microvasculature partitions the blood retinal interface and restricts the passage of substances from the blood [1]. Although it is generally believed that this barrier to the diffusion of molecules resides in the tight junctions of the endothelial cells [1–3], the identification of glycosaminoglycans (GAG) as integral constituents of basement membranes [4–6] suggests that other mechanisms may be operative. Basement membrane GAG are negatively charged components, present in covalent linkage with protein to form proteoglycans, that constitute anionic sites which impart charge-selective properties to the capillary filtration barrier [4,5,7–9]. The evidence for this largely stems from studies of the renal glomerular basement membrane in which heparan sulfate is the principal GAG species. For example, removal of heparan sulfate in glomerular basement membrane by in situ enzymatic digestion leads to a dramatic increase in the permeability of ferritin [10] and ^{125}I-albumin [11]. Nevertheless, the presence of both anionic sites and GAG appears to be a feature common to basement membranes in diverse anatomic loci [12–14], although the anionic

Correspondence: M.P. Cohen, University City Science Center, 3508 Market Street, Suite 420, Philadelphia, PA 19104, U.S.A.

sites of different basement membranes may have different electrostatic properties [14].

Because of the limited amounts of this matrix that can be recovered from most tissues, analyses of GAG in basement membranes other than the renal glomerulus are scant. Only two previous studies have analyzed GAG of the retinal microvessel basement membrane (RMV-BM); both identified heparan sulfate on the basis of mobility on cellulose acetate electrophoresis, resistance to digestion with chondroitinase, and sensitivity to treatment with heparitinase or nitrous acid, and suggested that this was the major, if not the only GAG species in this matrix [12,15]. In the present study, we examined the GAG in bovine RMV-BM using a sensitive and specific competitive binding assay that allows accurate measurement of nanogram amounts of heparan and chondroitin sulfates. The results provide the first quantitative analysis of GAG in this basement membrane, and indicate that both heparan sulfate and chondroitin sulfate are present in this extracellular matrix.

Materials and Methods

Retinal vessels were prepared from bovine eyes obtained from a local abbatoir within 2 h of slaughter. The microvessels were isolated according to previously described techniques, which entail differential sieving, homogenization, and centrifugation [16,17]. This procedure yields preparations that are free of nonvascular contaminants and contain endothelial cells surrounded by basement membrane in which intramural pericytes are embedded [12]. Microvessels from 50–100 eyes were osmotically lysed by stirring, in the cold, in 50 ml distilled water containing proteinase inhibitors (20 mM EDTA, 1 mM benzamidine hydrochloride, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM *N*-ethylmaleimide). The preparations were monitored microscopically at the end of the stirring period to ensure that lysis was complete. The water-insoluble material, representing basement membrane and cell membranes, was collected by centrifugation for 30 min at 20000 rpm and either directly extracted with 5 ml of deoxycholate (DOC) or first treated overnight at 37 °C with 1000 units of DNAase in 5 ml of 5 mM Tris buffer, pH 7.4 containing 5 mM $MgCl_2$, and then treated with 5 ml of the detergent. DOC extraction was performed twice with a 4% solution containing 1 mM PMSF, collecting the membranes by centrifugation for 15 min at 20000 rpm, followed by washing three times with distilled water to remove adherent extraction fluid. We have previously shown that osmotic lysis followed by detergent treatment yields acellular preparations that have the requisite amino acid composition generally associated with basement membranes [12]. Treatment with DOC is a traditional step in basement membrane purification that removes cells and cell debris and that separates plasma membrane lipoproteins from insoluble matrix [18,19]. It is also required for dissociation of basement membrane GAG from cell membrane lipids and for their subsequent measurement in the assay described below. However, since we have found that DOC solubilized a small fraction of the total extracellular GAG associated with glomerular basement membrane [20], both the DOC-soluble and -insoluble fractions were further processed for GAG isolation. The DOC-soluble fraction was brought to dryness and extracted with absolute ethanol to remove the detergent.

For purification of GAG, lyophilized samples of the above preparations (approx. 30 mg dry wt.) were digested with 2 ml of 2% papain (w/v) overnight at 60 °C in 0.5 M sodium acetate buffer, pH 5.5, containing 5 mM EDTA and 1 mM cysteine. Digested proteins and residual nucleic acids were removed by precipitation with 10% trichloroacetic acid and centrifugation. The supernatants were lyophilized, extracted with several volumes of absolute alcohol to remove trichloroacetic acid, dissolved in water, and then made 1% in cetylpyridinium chloride to precipitate the GAG. The cetylpyridinium chloride-precipitated material was dissolved in 0.2 ml of 2 M NaCl, reprecipitated with 0.4 ml of absolute ethanol, and washed with chloroform/methanol (1 : 2, v/v) and then three times with 95% ethanol. The final GAG precipitate was dried and reconstituted in a small volume of distilled water. Appropriate analysis confirmed that these GAG isolates were devoid of DNA (diphenylamine reaction) even when DNAase treatment was not included earlier in the preparative scheme (see above).

Aliquots of the GAG isolates were subjected to electrophoresis on cellulose acetate strips with 0.05 M sodium acetate buffer, pH 5.5. Authentic standards of chondroitin or dermatan sulfate (Miles Chemical Co.) and bovine kidney heparan sulfate (Sigma Chemical Co.) were run simultaneously. Samples or standards were applied in a 2 μl volume and run for 15 min at 0.8 mV/cm. The strips were stained with alcian blue in 3% acetic acid, and destained with 3% acetic acid in water.

Quantitative analysis of GAG was performed by a previously described competitive binding assay, modified as described below to increase sensitivity, in which unlabeled GAG competes with radiolabeled heparan sulfate for complexation with serum lipoproteins in a dose-dependent and saturable manner [20,21]. Tritiated heparan sulfate (500 ng), prepared by reaction with [^{3}H]acetic anhydride [22], with a specific activity of $1.3 \cdot 10^7$ cpm/mg was incubated in the presence of zinc acetate (2 mg/ml), normal human serum (1 : 10000 final dilution in water, pH 6.2), and varying amounts of unlabeled standard or basement membrane samples in a total volume of 1 ml. The precipitated complexes were removed by centrifugation at 3000 rpm for 60 min, and radioactivity in the supernatants, which is proportional to the amount of added (unlabeled) heparan sulfate, was measured in a liquid scintillation counter after the addition of Bray's solution. Since equivalent amounts of chondroitin sulfate, but not hyaluronate, compete in the assay, aliquots of the samples (approx. 1/10 of sample) were first measured without prior treatment to determine total sulfated GAG, followed by digestion with chondroitinase ABC or chondroitinase AC (Sigma Chemical Co.) to specifically determine heparan sulfate. Chondroitinase digestion was performed with 2 units of enzyme in 0.2 ml of 0.01 M Tris buffer, pH 7.4, overnight at 37 °C. Preliminary assessment of the GAG content in each of the membrane preparations and soluble fractions was performed to ensure that subsequent aliquots taken for quantitative measurement were appropriate for the limits of the assay system. Since maximum displacement using the described conditions was observed with 0–1 μg of unlabeled standard, all aliquots contained amounts of GAG within this range, yielding results falling within the linear portion of the displacement curve.

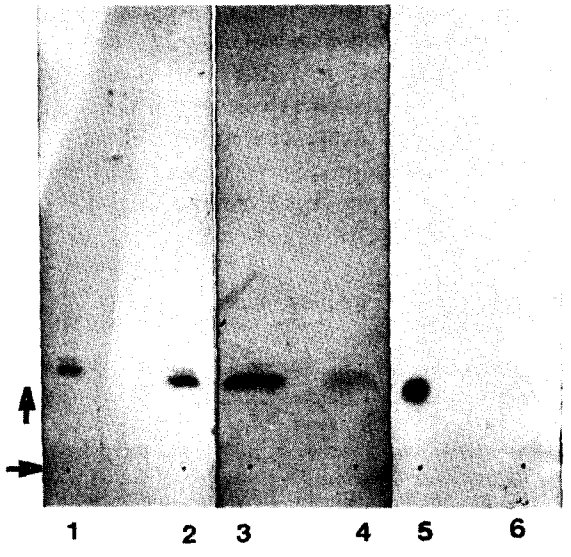

Fig. 1. Cellulose acetate electrophoresis of GAG purified from RMV-BM in Experiment 2. Arrows denote origin and direction of electrophoretic migration. Spots from left to right represent: 1, RMV-BM, untreated; 2, RMV-BM, untreated; 3, RMV-BM, chondroitinase ABC-digested; 4, RMV-BM, chondroitinase AC-digested; 5, heparan sulfate standard; 6, RMV-BM, chondrotinase ABC-digested, followed by nitrous acid treatment. (AC-digested material yielded a lighter band due to lower recovery compared to ABC digestion; see legend to Table II).

Results

Cellulose acetate electrophoresis of GAG purified from bovine RMV-BM revealed two bands with electrophoretic mobilities similar to those of chondroitin sulfate B and heparan sulfate standard. The fastest moving of these bands disappeared after digestion with chondroitinase ABC or AC, compatible with chondroitin sulfates A and/or C (Fig. 1). The other more densely staining band exhibited an electrophoretic mobility similar to that of heparan sulfate standard, disappeared after treatment with nitrous acid, but was unaffected by digestion with either chondroitinase preparation (Fig. 1).

As originally reported, the competitive binding assay was sensitive to about 5–25 μg of GAG. Preliminary experiments indicated that enhanced sensitivity was needed for measurement of GAG

in RMV-BM, since the amount of tissue (and, hence, GAG isolate) is limiting. This was accomplished by radiolabeling of purified heparan sulfate to high specific activity, achieved by reaction with [^{3}H]acetic anhydride [22], coupled with maximal serum dilution in the presence of minimal but saturating amounts of radiotracer. For example, using [^{3}H]heparan sulfate of specific activity $1.3 \cdot 10^7$ cpm/mg and serum at 1 : 10000 dilution, a linear relationship was observed between radioactivity displaced into the supernatant and the amount of added unlabeled heparan sulfate between 0 and 1.0 μg, thus allowing reproducible and accurate detection of as little as 50 ng of heparan sulfate (Fig. 2).

It is worth noting that the above experiments demonstrating enhanced sensitivity with [^{3}H]acetic anhydride-labeled heparan sulfate of high specific activity document the validity of the assay by showing that binding of radiolabeled heparan sulfate to serum lipoproteins occurs regardless of the manner in which radiolabel is introduced. Since GAG derived after proteinase digestion presumably contain no reducible groups, it has not been clear where the radiolabel is incorporated with borotritide reduction, which was the method used in the original assay. This was also recognized by the investigators who described the borotritide-radiolabeling procedure [23]. Attempts to clarify this issue by using borotritide-reduced GAG prepared by β-elimination, which generates reducible termini in the released polysaccharide, were unsuccessful largely because specific activity was low and the radiolabeled material so prepared did not interact well in the assay. This was interpreted as reflecting a need for remnant peptide, as is present in GAG released by proteinase digestion, for binding to lipoprotein [24]. In contrast, reaction with *N*-[^{3}H]acetyl introduces the radiolabel into a defined locus of the heparan sulfate chain, and this preparation exhibited properties identical to those of the borotritide-treated material in the assay. These results provide unequivocal evidence that radiolabeled heparan sulfate indeed binds to lipoprotein and is competitively displaced by pure preparations of the unlabeled GAG.

Total GAG in DOC-insoluble bovine RMV-BM was about 0.52 μg/mg dry weight, where dry weight refers to lyophilized crude membrane after osmotic lysis (Table I). This value for total GAG content was determined without chondroitinase digestion in duplicate aliquots of four separate preparations of material, each derived from about 50 bovine eyes. Total GAG content was similar whether or not DNAase treatment was included in the preparative scheme, as long as residual nucleic acids had been adquately removed with trichloroacetic acid precipitation steps (see Materials and

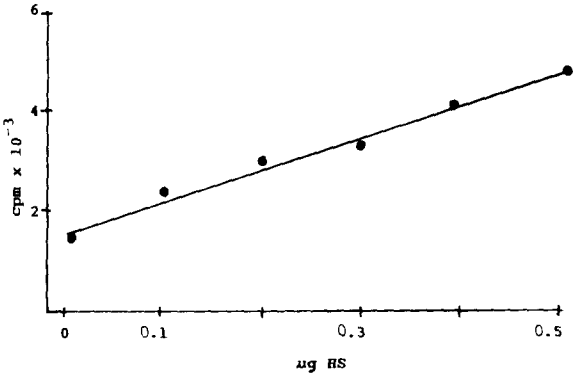

Fig. 2. Competitive displacement of [^{3}H]heparan sulfate (HS) with unlabeled heparan sulfate. Radiolabeling achieved by reaction with [^{3}H]acetic anhydride. Incubations performed with varying amounts of unlabeled heparan sulfate and with zinc acetate (2 mg), in the presence of 0.5 μg [^{3}H]heparan sulfate and serum at 1 : 10000 dilution. Radioactivity was determined in the supernatants.

TABLE I

EFFECT OF DETERGENT TREATMENT ON GAG IN RMV-BM

Results are expressed as μg/mg dry wt. of the starting material, defined as lyophilized membrane prepared after osmotic lysis of isolated retinal microvessels obtained from about 50 eyes. Dry weight of starting material in a typical preparation was about 70 mg; dry weight of DOC-insoluble fraction was about 35 mg. Crude membranes were treated with DNAase before DOC extraction in Experiments 1, 3 and 4, and were directly extracted with DOC in Experiment 2.

Preparation	DOC-insoluble	DOC-soluble
Experiment 1	0.454	0.012
Experiment 2	0.369	0.009
Experiment 3	0.785	0.016
Experiment 4	0.472	0.026
Mean ± S.E.	0.520 ± 0.091	0.016 ± 0.004
Percent of total	95–98	2–5

TABLE II

GAG COMPOSITION OF RMV-BM

Aliquots of DOC-insoluble membranes (see Table I and text) were taken for chondroitinase ABC or AC digestion, followed by GAG measurement in duplicate in the competitive binding assay. Results after chondroitinase digestion have been corrected for losses as a result of the digestion procedure, established by performing the assay before and after subjecting known amounts of authentic heparan or chondroitin sulfate to identical procedures. After ABC digestion, recovery = 80%; after AC digestion = 65%. n.d., not determined.

Preparation	GAG (μg)				
	Initial	After ABC	Percent of initial	After AC	Percent of initial
Experiment 1	52.8	40.9	77.4	n.d.	–
Experiment 2	56.3	44.5	79.2	48.0	85.3
Experiment 3	58.1	35.0	60.2	43.0	74.0
Experiment 4	44.4	27.0	61.1	24.0	54.3
Mean ± S.E.			69.5 ± 5.0		71.2 ± 9.0

Methods). Less than 5% of the GAG in the water-insoluble material recovered after osmotic lysis of isolated microvessels was solubilized with DOC, suggesting that a very small amount of matrix-associated GAG derives from material that abuts on the surface of adjacent plasma membrane.

About 70% of the GAG in RMV-BM was resistant to digestion with chondroitinase ABC or chondroitinase AC (Table II). GAG remaining after chondroitinase digestion became unmeasurable after treatment with nitrous acid. These results indicate that, although heparan sulfate is the predominant GAG in RMV-BM, this extracellular matrix also contains a relatively substantial amount of condroitin sulfate(s). However, in view of the relative intensity of the chondroitin sulfate band on electrophoresis (Fig. 1), and the fact that the relative binding strengths of chondroitin 6-sulfate, dermatan sulfate and chondroitin 4-sulfate (Miles Chemical Co.) in the assay are respectively 1.8, 0.95 and 0.80 compared to heparan sulfate (Sigma Chemical Co.), it is possible that the assay results for the chondroitin sulfate species of RMV-BM are not completely quantitative. Although little effect on the results with the binding assay would be expected if the proportion of the three chondroitin sulfate species is similar or if chondroitin 4-sulfate and dermatan sulfate predominate, the assay would overestimate the amount of chondroitin sulfate(s) by 15–30% if chondroitin 6-sulfate predominates.

Discussion

The experiments reported herein provide the first quantitative analysis of GAG in RMV-BM. The study utilized a recently developed competitive binding assay for sulfated GAG that is specific for heparan sulfate in chondroitinase-treated samples, and can detect as little as 50 ng of heparan sulfate. In view of the relatively small amounts of GAG which this tissue contains, it is clear that such analysis would not have been possible without this assay, unless hundreds of eyes were employed as starting material. The results definitvely establish that RMV-BM contains chondroitin sulfate as well as heparan sulfate. This is partially consistent with the findings of a previous study of this basement membrane, in which some diminution in the intensity of the single band that migrated in the same location as heparan sulfate standard was noted following chondroitinase ABC or chondroitinase AC digestion [15], although photographic evidence of the decreased intensity with chondrotinase ABC or AC digestion was not provided in that report. The present experiments not only demonstrated the existence of two GAG species on cellulose acetate electrophoresis, but also that chondroitinase digestion resulted in a quantitative decrease in the measureable GAG content of purified RMV-BM. Chondroitin sulfate also has been identified in the extracellular matrix of the renal glomerulus [25].

Consideration of the anatomic orientation of the extracellular matrix in the retinal microvasculature and the biosynthetic properties of adjacent cells offers some explanation for the presence of both chondroitin and heparan sulfate, and for the solubility properties observed. The basement membrane lies between two cell layers (the endothelium and the mural cells) and also surrounds one of these (mural pericytes). Cultured retinal endothelial cells produce a mixture of GAG consisting of about 60% heparan sulfate and 40% chondroitin sulfate, whereas pericytes in culture have been shown to synthesize primarily chondroitin sulfate [15]. Thus, constituents of the RMV-BM probably derive from the two cell types with which the matrix is anatomically associated. Chondroitin sulfate has been found to be present exclusively in the mesangial matrix of the renal glomerulus, which contains cells analogous to retinal pericytes [26]. However, it is possible that the RMV-BM contains a hybrid proteoglycan which bears both heparan sulfate and chondroitin sulfate, as has been reported for cell surface and basement membrane proteoglycans of other tissue [27,28].

The GAG of RMV-BM appear to be similar to those in glomerular basement membrane in at least one other respect in that a small amount of the GAG in the water-insoluble material recovered after osmotic lysis of either tissue is solubilized by treatment with the ionic detergent DOC. This suggests the presence of two populations of heparan sulfate, analogous to those associated with cell surfaces of other tissues, in these basement membranes; one that is liberated and solubilized by ionic detergents and has been designated intercalated heparan sulfate, and another that may be bound to cell surface receptors, is not solubilized by detergents, and has been called peripheral heparan sulfate [29–31]. Although the biologic significance of these two populations of heparan sulfate in the retinal microvascular matrix is presently speculative, it is worth noting that treatment with DOC, which is a traditional step in basement membrane purification, apparently removes a small amount of the material of interest. Nevertheless, the present findings offer insight into quantitative and qualitative aspects of the GAG in the RMV-BM, and provide a basis for comparative studies of these components in disorders affecting the retinal microvasculature.

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