Biosynthesis of Proteoglycans Present in Primate Bruch's Membrane

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The proteoglycan components of Bruch's membrane have not been well characterized to date. In this study, the glycosaminoglycans present in Bruch's membrane were identified and found to be heparan sulfate with small amounts of chondroitin and/or dermatan sulfate and hyaluronic acid. The biosynthesis of glycosaminoglycans and their incorporation into proteoglycans was investigated using an eye organ culture in which the cornea, iris, and sclera had been removed. The newly synthesized proteoglycan(s) extracted with 4 M guanidine hydrochloride bound to a DEAE-cellulose column and eluted with approximately 0.44 M NaCl. The proteoglycan(s) had a molecular weight ranging from 100,000 to 150,000 daltons. After papain treatment, the glycosaminoglycan side chains had a molecular weight of approximately 44,000 daltons. The newly synthesized proteoglycan(s) contained 65% chondroitin and/or dermatan sulfate and 35% heparan sulfate. This organ culture system should be useful in studying disease states of Bruch's membrane. Invest Ophthalmol Vis Sci 24:898-905, 1983

Bruch's membrane is a composite extracellular matrix lying between the pigmented epithelium of the retina and the choriocapillaris. It contains two basement membranes: one associated with the retinal pigmented epithelium and one with the choriocapillaris. The inner and outer collagenous layers are separated from each other by a thin elastin-bearing lamina (Fig. 1A).1 Bruch's membrane plays several important roles such as providing structural support and an attachment site for the pigmented epithelium. Since recent studies in the kidney show that basement membranes are responsible for selective filtration of blood components,2 the basement membrane layers of Bruch's membrane may also provide a selective filtration barrier through which nutrients gain entrance to the interior portions of the eye from the choriocapillaris.

The biochemical components of Bruch's membrane, including the proteoglycans, have not been well characterized. These sulfated glycoconjugates are especially important as discrete macromolecular structural elements and in the selective filtration properties of the basement membrane.3-5 A preliminary report indicated that bovine Bruch's membrane contains the sulfated glycosaminoglycan, chondroitin sulfate.6 In order to learn more about the types of these important macromolecules in a Bruch's membrane, we isolated and identified the glycosaminoglycans of Bruch's membranes from monkey eyes. In addition, we used a novel organ culture system to radiolabel the chorioretinal complex. Bruch's membranes were prepared from the radiolabeled complexes and the newly synthesized proteoglycans were also isolated and characterized.

Materials and Methods

Preparation of Glycosaminoglycans from Bruch's Membranes

Chorioretinal complexes were dissected from 80 fresh cynomolgus (Macaca mulatta) eyes by first removing the cornea, sclera, iris, and lens. An incision was made through the full thickness of the eye from the ciliary body back to the optic nerve, and the optic nerve was removed. Separation of the neural retina from the chorioretinal complex was facilitated by placing the preparation in phosphate-buffered saline (0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4) and gently pulling on the ciliary body and the neural retina. Subsequently, the ciliary body was removed from the chorioretinal complex, which was washed extensively in cold distilled water. Pigmented epithelial and some choroidal cells were removed easily by gently shaking the membrane in cold water with fine forceps. After washing in cold water, the remaining choriocapillaries became fluffy and were removed by gentle teasing with jeweler's forceps under a microscope. A semitransparent white membrane was obtained. Portions of these preparations were fixed in...
10% formalin and embedded in paraffin. Seven micron sections were cut and stained with periodic acid-Schiff reagent for light microscopy.

The cleaned membranes were homogenized in 4 M guanidine hydrochloride containing 0.05 M sodium acetate, pH 5.8, 0.01 M EDTA, and 0.005 M benzamidine (Sigma), and extracted overnight at 4°C with stirring. Solubilized proteoglycans were separated from other proteins by centrifugation with cesium chloride (0.35 g/g of extract) at 37,000 rpm for 72 hrs in a SW 50.1 rotor. The resulting gradients were separated into thirds. Each fraction was dialyzed against water to remove salt and lyophilized. All fractions were dissolved in 1 M sodium acetate, pH 5.0, 0.05 M EDTA, 1 mM cysteine and 3.0 units of papain (Sigma), and incubated at 55°C for 7 hrs to generate glycosaminoglycans. Glycosaminoglycans from the fractions were identified by cellulose acetate electrophoresis before and after chondroitinase ABC as described by Saito et al (Miles), and nitrous acid treatment, as described by Hovingh and Linker. Glycosaminoglycans used as standards were obtained from Drs. M. B. Matthews and J. A. Cifonelli, University of Chicago (NIAMDD contract No. N01-AM-52205).

Labeling of Proteoglycans by Organ Culture

Cynomolgus eyes were obtained immediately upon terminal exsanguination and were placed into sterile Coon’s modified Ham’s F12 medium. The cornea, iris, and sclera were removed with care not to rupture the chorioretinal complex or vitreous humor. In cynomolgus eyes, the vitreous humor is tightly adherent to the retina and its removal causes disruption of retinal layers. Consequently, the vitreous was not removed in order to maintain normal relationships between the cell layers of the retina and choroid. Six eyes were then placed into Coon’s modified Ham’s F12 medium containing 20% dialyzed fetal calf serum (GIBCO) with freshly added ascorbic acid (50 μg/ml), and the antibiotic lincomycin (500 μg/ml). The amount of glucose was reduced to 50 mg/100 ml and MgSO4 to 0.2 mM with an increase by 0.2 mM of MgCl2. The eyes were preincubated in individual conical tubes with 2.5 ml of this medium for 30 min to reduce intracellular pools of glucose and sulfate. Subsequently the preincubation media was replaced with 2.5 ml of fresh medium containing 0.2 mCi of 3H-glucosamine and 1.0 mCi of 35S-sodium sulfate (Amersham). The eyes were incubated in a humidified atmosphere of 95% air, 5% CO2 at 37°C for 5 hrs, and gently agitated at hourly intervals during the incubation. The chorioretinal complexes were then dissected free of the neural retina, optic nerve, ciliary muscle, and vitreous humor. Radiolabeled chorioretinal complexes were used initially to assess incorporation of isotope at various intervals up to 6 hrs. Subsequently the labeled chorioretinal complexes were treated as described above to obtain isolated, radiolabeled Bruch’s membranes.

Extraction and Characterization of Newly Synthesized Radiolabeled Proteoglycans

Proteoglycans were extracted from either the chorioretinal complexes or Bruch’s membranes in 4 M guanidine hydrochloride with protease inhibitors and dialyzed extensively against 8 M urea, 0.05 M Tris-HCl, pH 6.8. Extracts were passed through a DEAE-cellulose column (2 X 10 cm) (Whatman DE52) equilibrated in the same buffer. Bound material was eluted from the column with a linear salt gradient ranging from 0 to 1 M NaCl. Fractions of 2.5 ml were collected and 0.2 ml aliquots from every other tube were assayed for radioactivity by liquid scintillation spectroscopy using ACS aqueous counting scintillant (Amersham). Those fractions containing 35S were concentrated by Amicon filtration using a PM10 filter, and passed through a Sepharose CL-4B (Pharmacia) column (1.5 X 160 cm) equilibrated with 4 M guanidine hydrochloride, 0.01 M Tris-HCl, pH 7.0, 2.5 fractions were collected and 0.2 ml aliquots were assayed for radioactivity from every other tube. 35S-containing material was again concentrated and passed through a Sepharose CL-6B column (1.5 X 80 cm) (Pharmacia) before and after papain treatment as described above. The glycosaminoglycans liberated following papain digestion were characterized by chromatography on Sepharose G-50 (1 X 50 cm column) (Pharmacia) before and after treatment with chondroitinase ABC (Miles) and nitrous acid. Fractions of 2.0 ml were collected and 0.5 ml aliquots assayed for radioactivity.

Results

Histologic Appearance of Bruch’s Membranes

After washing and mechanical cleaning of obvious material, the Bruch’s membrane preparations had a “live” or somewhat rubbery consistency. By light microscopy, the ribbon-like membrane had a small amount of fine amorphous material on its chorio-capillaris aspect (Fig. 1B). The membrane retained PAS positivity and gross structural integrity as determined by comparison of histologic sections to those prepared from undissected posterior eye walls.

Identification of Glycosaminoglycans Isolated from Bruch’s Membranes

Papain treatment of the cesium chloride gradient fractions (top, middle, and bottom thirds) from na-
Figs. 1A-B. A, Schematic representation of layers of Bruch's membrane. RPE—retinal pigmented epithelium; LL—lamina lucida; LD—lamina densa; BM—basement membrane; ICL—inner collagenous layer, EL—elastic layer, OCL—outer collagenous layer; CC—choriocapillaris. B, Histologic section of acellular Bruch's membrane. Cells were removed from Bruch's membrane as described in Materials and Methods. A small portion was fixed with 10% formalin, embedded in paraffin and 7 micron sections were stained with the PAS reaction. (X450).

tive, unlabeled Bruch's membranes yielded glycosaminoglycans identifiable by alcian blue staining of cellulose acetate strips following electrophoresis (Fig. 2). The top and middle fractions did not contain enough glycosaminoglycans to be visualized by alcian blue (data not shown). Without any enzymatic or chemical treatment, the bottom third of the cesium chloride gradient migrated as a fairly diffuse band between the migration positions of standard heparan sulfate and hyaluronic acid. After nitrous acid treatment, these glycosaminoglycans disappeared. Small amounts of material migrating in positions similar to those of authentic hyaluronic acid and dermatan sulfate were still visible, but not in sufficient quantities to be seen in the photograph of the dry cellulose acetate strip. Authentic heparan sulfate in the standard mixture was destroyed completely. When the Bruch's membrane glycosaminoglycans were treated with chondroitinase ABC, their electrophoretic appearance was largely unaffected, while authentic chondroitin sulfate, dermatan sulfate, and hyaluronic acid in the standard mixture disappeared. These results indicate that the most plentiful glycosaminoglycan present in Bruch's membrane is heparan sulfate, with very small amounts of other glycosaminoglycans, possibly chondroitin and/or dermatan sulfate and hyaluronic acid.

Identification of Newly Synthesized Components in Chorioretinal Complexes

Incorporation of radioisotope into the chorioretinal complex was found to be linear up to 6 hrs. A 5-hr incubation was chosen since the neural retina visibly began to detach from the pigmented epithelium after approximately 6 hrs in culture.
Guanidine extracts of chorioretinal complexes contained newly synthesized $^3$H-labeled glycoproteins and $^{35}$S-labeled materials that were separable from each other by DEAE-cellulose column chromatography (Fig. 3). The $^{35}$S-containing material eluted from the column as a heterogeneous peak with approximately 0.44 M NaCl. The fractions from this peak were pooled and further characterized and found to be proteoglycans as judged by (1) their change in molecular weight following papain treatment to remove the protein core, and (2) sensitivity to chondroitinase ABC and nitrous acid (data not shown). These experiments indicated that the chorioretinal complex isolated from our stripped whole eye preparation was capable of incorporating radiolabeled precursors into the chorioretinal complex in organ culture. Subsequent experiments used this tissue to prepare radiolabeled Bruch’s membranes.

Characterization of Newly Synthesized Proteoglycans from Bruch’s Membranes

Bruch’s membranes prepared from radiolabeled chorioretinal complexes contained a guanidine extractable fraction that could be bound and eluted from DEAE-cellulose (Fig. 4). Unlike guanidine extracts of whole, labeled chorioretinal complexes, Bruch’s membrane extracts contained small amounts of labeled glycoproteins, in comparison with the total amounts of $^{35}$S-labeled components. In addition, the $^{35}$S-containing material eluted as a less heterogeneous peak than that from the whole chorioretinal complex. This sulfated peak was used for further characterization. Fractions containing $^{35}$S were pooled and passed through a Sepharose CL-4B column (Fig. 5). The $^{35}$S material eluted as a somewhat heterogeneous peak with a $K_v$ of approximately 0.44. Compared to the elution positions of other proteoglycans such as that from chick sterna (chondroitin sulfate proteoglycan), cornea (keratan sulfate proteoglycan), and a basement membrane tumor (heparan sulfate proteoglycan), this material contains molecules with molecular weights ranging between 100,000 and 150,000 daltons.

The latter part of this peak was pooled (tubes 80–120), and the proteoglycan nature of this material was investigated by passing it through Sepharose CL-6B before and after papain treatment to remove the pro-
protein core (Fig. 6). Before papain treatment, the $^{35}$S-labeled material eluted close to the void volume of the column (Fig. 6A). Following papain treatment, the resulting glycosaminoglycan fraction shifted to a lower molecular weight with a Kav of 0.39 (Fig. 6B), indicating the presence of a protein core. The glycosaminoglycan(s) have an approximate molecular weight of 44,000 daltons as determined by comparison with other glycosaminoglycans of known molecular weight.

In some experiments, the heterogeneous sulfated peak eluting from Sepharose CL-4B (Fig. 5) was divided into two fractions, the first containing the early fractions (tubes 55–79) and the second containing the...
later fractions (tubes 80–120). Both of these fractions were treated with papain and chromatographed on Sepharose CL-6B. The resulting glycosaminoglycans from both fractions eluted identically with a peak at tube 30. These results suggest that the two fractions contain similar material and that the material eluting earlier from Sepharose CL-4B may be a biosynthetic precursor of later-eluting material. This is also supported by the fact that variable amounts of the early eluting fractions were present, and in some preparations only very small amounts were present. However, the identity of the early eluting material was not investigated further.

The glycosaminoglycan fraction was further characterized by chromatography on Sepharose G-50 before and after treatment with chondroitinase ABC and nitrous acid (Fig. 7). Without treatment, the glycosaminoglycan fraction eluted in the void exclusion volume of the column. After digestion with chondroitinase ABC, approximately 65% of the 35S eluted in the included volume, indicating the presence of chondroitin and/or dermatan sulfate. After nitrous acid treatment, approximately 35% of the 35S eluted in the included volume, indicative of heparan sulfate.

**Discussion**

The proteoglycan constituents of primate Bruch's membranes have previously been uncharacterized. From our preparations of unlabeled Bruch's membrane, we have identified heparan sulfate as the primary glycosaminoglycan liberated by papain digestion, with small amounts of other glycosaminoglycans, possibly chondroitin and/or dermatan sulfate and hyaluronic acid. Although the localization of
these glycosaminoglycans to one layer of Bruch's or another cannot be assigned by the methods used here, it is possible that the heparan sulfate is located in the basement membranes of the retinal pigmented epithelium and the choriocapillaris, while the other glycosaminoglycans may reside in the inner and outer collagenous layers.

Since glycosaminoglycans usually occur attached to a protein core to form proteoglycans, an organ culture system was developed to study the biosynthesis of the glycosaminoglycans and their incorporation into proteoglycans present in the chorioretinal complex, and more specifically in Bruch's membranes. Proteoglycans isolated from Bruch's membranes have molecular weights ranging from 100,000 to 150,000 daltons. These proteoglycans are considerably smaller than the complex units of cartilage, but are similar in molecular weight to other characterized proteoglycans, particularly those of cornea. The presence of a protein core was verified by the reduction in molecular weight following papain treatment. Papain digested the protein core and liberated glycosaminoglycan side chains with a molecular weight of approximately 44,000 daltons. The glycosaminoglycan fraction was found to contain \( \frac{1}{3} \) chondroitin and/or dermatan sulfate and \( \frac{1}{3} \) heparan sulfate. Further characterization of the proteoglycans will require larger amounts of labeled material.

The relative amounts of glycosaminoglycans liberated from Bruch's membranes with or without radiolabeled precursors are different. Heparan sulfate was liberated in higher concentration from uncultured preparations. The reason for these differences is unknown. Some possible reasons include (1) different rates of biosynthesis, (2) variations in dissection, and (3) organ culture conditions. Unlabeled chorioretinal preparations were exposed to longer periods of distilled water wash than the radiolabeled material. It is possible that some components were leached out, although glycoconjugates are not particularly soluble in distilled water. Our culture medium has allowed human and primate corneas to synthesize stromal proteoglycans of normal type and proportion. With the present preparation, the medium may not be ideally supportive. This possibility bears further investigation. It is thought that basement membrane components have longer half lives than components of other connective tissues, and it is possible that in the organ culture system, heparan sulfate proteoglycans were turning over more slowly than chondroitin, and/or dermatan sulfate proteoglycans. Thus, different rates of turnover seem to be a good possible explanation for the differences we observed.

Interestingly, the radiolabeled extracts of chorioretinal complex contained both radiolabeled glycoproteins and proteoglycans, while radiolabeled Bruch's membrane extracts contained mainly proteoglycans. Thus, it is likely that the proteoglycans were turning over more rapidly than the other membrane components. Such a greater turnover has been seen in other tissues, such as in corneal stroma (Dr. J. R. Hassell, personal communication). This observation may indicate that proteoglycans play a more dynamic role in the maintenance of normal function.

While it is useful to know the static composition of proteoglycans present in Bruch's membrane, the use of the biosynthetic radiolabeling system may be more helpful in detecting changes in proteoglycan metabolism as a result of disease and/or injury. Deformations in the ratios of Bruch's membrane proteoglycans may not be detected by analyzing unla-
beled Bruch’s membranes since small changes could be masked by pre-existing material. Therefore, the organ culture system may be quite useful in detecting abnormalities in proteoglycan metabolism in disease states such as senile macular degeneration.

**Key words:** Bruch’s membrane, proteoglycans, glycosaminoglycans, biosynthesis, organ culture, basement membranes

**References**


