Ultrastructural Immunocytochemical Localization of Chondroitin Sulfate Proteoglycan in Bruch’s Membrane of the Rat

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Two monoclonal antibodies (Mab 4D5 and 2D6) raised against the core protein of a basement membrane chondroitin sulfate proteoglycan from Reichert’s membrane of the rat, were used for ultrastructural immunoperoxidase localization of this protein in Bruch’s membrane of the rat. Immunoreactivity for both antibodies was found in the basal lamina (basement membrane) of the choriocapillary endothelium and retinal pigment epithelium, in collagen fibers in the collagenous zones, and surrounding the elastic layer. Invest Ophthalmol Vis Sci 33:2072–2075, 1992

Bruch’s membrane is a multi-layered structure located at the interface of the retina and choroid. The layers include the basal lamina (basement membrane) of the retinal pigment epithelium (RPE) and the choriocapillary endothelium (CCE), the inner and outer collagenous zones, and a central elastic layer separating the two collagenous zones.¹ These morphologic features suggest that Bruch’s membrane has the composition of an extracellular matrix (ECM). Indeed, a number of ECM molecules, including laminin, fibronectin, collagens, and heparan sulfate proteoglycan have been identified biochemically in Bruch’s membrane, and their localization has been confirmed by immunocytochemistry.² Members of a family of heparan sulfate proteoglycan (HSPG) were thought to be the sole proteoglycan component of basement membranes. Recent studies, however, have demonstrated the presence of a chondroitin sulfate proteoglycan (BM-CSPG) in basement membranes.³⁻⁴ This proteoglycan has a core protein that is immunologically distinct from that of HSPG and, unlike HSPG, has been shown to be heavily substituted with CS glycosaminoglycan chains in some basement membranes.⁵ Using core protein-specific monoclonal antibodies, we have shown this proteoglycan to have an almost ubiquitous tissue distribution.⁶ Only a few studies have dealt with the localization of CSPG in Bruch’s membrane, despite several biochemical studies in which CSPG has been isolated from primate Bruch’s membrane⁷⁻⁸ and cultured chick RPE cells.⁹

Until the advent of monoclonal antibodies that recognize the core protein of BM-CSPG, studies of its distribution were performed with the use of antibodies that recognize carbohydrate epitopes on the molecule¹⁰ or by the use of histochemical reagents. Pino and coworkers were the first to use cytochemical methods to study proteoglycans in Bruch’s membrane of the rat.¹¹ Using cationic dyes to stain anionic groups of glycosaminoglycans with enzymes specific for degrading glycosaminoglycans, they showed that the basal lamina of the RPE contains HSPG, whereas that of the CCE contains primarily CSPG. Recently, Call and Hollyfield,¹² using cupromeronic blue in conjunction with enzyme digestion and nitrous acid treatment, reported that in human Bruch’s membrane, HSPG is associated with the basal lamina of the RPE and CCE, whereas CSPG is associated with collagen fibrils but not with the basal laminae. In the present report, we examine the distribution of BM-CSPG in Bruch’s membrane of the rat using two monoclonal antibodies to different epitopes of the core protein of CSPG and the ultrastructural immunoperoxidase method.

Materials and Methods. Female Lewis rats (200 gm, Harlan Sprague Dawley, Inc., Indianapolis, IN) were used according to the ARVO Resolution on the Use of Animals in Research. Animals were perfused retrogradely via the abdominal aorta with 4% paraformaldehyde (freshly depolymerized) and 0.1% glutaraldehyde (EM grade) in 0.1 mol/l Sörensen’s phosphate buffer (pH 7.4) containing 7.5% sucrose. The eyes were removed, slit at the limbus, and immersed in the same fixative for 1 hr at 4°C. During fixation, the posterior eyecup, including the retina, choroid, and sclera was separated from the anterior segments. After an overnight wash in cold buffer containing sucrose, the mid-peripheral region of the eyecup was sliced into 40 μm-thick, nonfrozen sections using a Sorvall TC-2 tissue sectioner (Sorvall, Newton, CT). The sections were blocked with phosphate-buffered...
saline (PBS) containing 1% bovine serum albumin for 30 min and then exposed to monoclonal antibodies 4D5 or 2D6 in moistened, multi-well culture plates for >20 hr at 4°C. The antibodies were prepared and characterized as described previously\(^3\) and used without dilution. After incubation in the primary antibodies, the sections were rinsed in PBS and then exposed to rabbit antimouse IgG horseradish peroxidase (0.1 mg/ml; E-Y Laboratories, San Mateo, CA) for 1 hr at room temperature in the dark.

For control experiments, the monoclonal antibodies were omitted or nonimmune mouse IgG was used in place of the antibodies. After a thorough rinse in PBS, the sections were incubated for peroxidase activity in medium containing 3,3'-diaminobenzidine (1 mg/ml) and hydrogen peroxide (0.01% final concentration) in 0.1 mol/l Tris-HCl, pH 7, for 15 min in the dark at room temperature. After the cytochemical reaction, the sections were fixed in 2.5% glutaraldehyde, dehydrated in a series of ethanols and propylene oxide, and embedded in Epon 812 (Polysciences, Inc., Warrington, PA). Thin sections unstained or stained with lead citrate were examined with a Philips (Mahwah, NJ) 301 electron microscope.

**Results.** The ultrastructure of Bruch's membrane appeared to be well preserved despite overnight exposure to the antibodies. After exposure to either monoclonal antibody, peroxidase immunostaining was found in the basal lamina of the CCE and RPE (Figs. 1 and 2). Immunostaining of the RPE basal lamina with Mab 2D6 (Fig. 2) tended to be weaker and less uniform than with Mab 4D5 (Fig. 1). The interchorioretinal stroma, collagen fibers in both collagenous zones, and the cortical region of the elastic layer also were stained by the antibodies. Controls showed virtually no staining in any region of Bruch's membrane (Fig. 3).

**Discussion.** Two monoclonal antibodies directed to different epitopes of the core protein of a basement membrane CSPG\(^3,4\) were used to localize this proteoglycan in Bruch's membrane of the rat. The results obtained indicate that CSPG is present in the basal lamina of the CCE and RPE, as well as in association with collagen fibers in the collagenous zones of the membrane. Recently, immunofluorescence studies using the same monoclonal antibodies have demonstrated the presence of this protein in the basement membrane of a wide variety of adult rat tissues.\(^4\)

The localization of CSPG to the basal lamina of the RPE is consistent with biochemical and cytochemical studies that showed that CSPG synthesized by cultured chick RPE cells was located in the basal lamina.\(^9\) However, our findings do not agree with previous cytochemical studies in which CSPG was not detected in the RPE basal lamina using ruthenium red\(^11\) or in the RPE and CCE basal lamina using cupromeronic blue.\(^12\)

Because these agents are cationic dyes that react with the sulfate groups but not the core protein of proteoglycans, their failure to stain the RPE basal lamina could be due to lack of or reduced sulfation of the glycosaminoglycans of CSPG. Couchman and his coworkers have demonstrated that CSPG with sulfated or unsulfated glycosaminoglycans have distinct and restricted distributions in different tissues, including basal laminae.\(^10,13\) Further studies using antibodies against unsulfated or sulfated side chains of CSPG\(^13\) may help clarify this problem in the RPE basal lamina. Another possibility is that the amount of CSPG in the RPE basal lamina is relatively low compared to that of the CCE basal lamina or is masked by other components. Such conditions may make the sulfate groups of CSPG less accessible to the dyes. The relatively weak, variable immunostaining seen in the RPE basal lamina with Mab 2D6 (Fig. 2) could indicate that the epitope recognized by the antibody is present in lesser amounts or is masked. A third possibility may be related to the molecular orientation of CSPG in the basal lamina. It has been demonstrated that the core protein of cartilage CSPG contains three regions in the following order: a chondroitin sulfate-rich region, a keratan sulfate-rich region, and the terminal region with almost no sulfated chains.\(^14\) The CSPG molecule in the RPE basal lamina could be oriented with its unsulfated terminal region in the basal lamina and the sulfated region extended into the collagenous zone. Such a configuration could explain our immunostaining of the core protein in the basal lamina and collagenous zone. It also could explain the lack of staining by anionic dyes in the basal lamina but the presence of staining in the collagenous zone. A similar model has been proposed for the large heparan sulfate proteoglycan in the basal lamina.\(^15\)

The localization of CSPG to the basal lamina of the CCE agrees with previous cytochemical studies that demonstrated chondroitinase-sensitive, ruthenium red-positive anionic sites in this structure.\(^11\) Taken together, these findings suggest that sulfated and unsulfated regions of CSPG are located within the basal lamina. It should be noted, however, that such sites were not detected in this structure of the human eye with cupromeronic blue.\(^12\) Whether this discrepancy is a result of species variations or technical differences is not clear.

It may be argued that the immunostaining observed in the basal laminae in this study is a result of HSPG, which is known to be present in these struc-
Figs. 1, 2. Exposure to monoclonal antibody 4D5 (Fig. 1) and 2D6 (Fig. 2). Peroxidase reaction product is localized to the basal lamina (BL) of the choriocapillary endothelium (CCE) and retinal pigment epithelium (RPE). Note, in Figure 2, the variable intensity of reaction product in the basal lamina of the RPE. Reaction product is also found in the intercapillary stroma (IS, Fig. 1), in association with collagen fibers in the inner and outer collagenous zones (ICZ, OCZ) and in the cortical region (arrows, Fig. 2) of the elastic layer (EL). L, choriocapillary lumen. Bars = 1 μm.

However, this is unlikely because it has been shown that the CSPG core protein used to generate monoclonal antibodies 4D5 and 2D6 is not related immunohistochemically to HSPG.

Monoclonal antibodies showed immunoperoxidase staining in association with collagen fibers in the cortex of the central elastic layer. Using ruthenium red, Pino et al. demonstrated the presence of chondroitinase-sensitive anionic sites in these same locations. More recently, Call and Hollyfield showed that these sites react with cupromeronic blue, suggesting that they contain sulfated polyanions. However, the reaction in the elastic layer was insensitive to chondroitinase treatment. The relationship between the immunostaining we observed in the elastic layer and that seen with cationic dyes remains to be clarified.
that in addition to the lamina densa region of basal lamina, staining of the immediately subjacent collagen fibers was evident.

**Key words:** chondroitin sulfate proteoglycan, immunocytochemistry, basal lamina, retinal pigment epithelium, choriocapillary endothelium

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**References**