Chondroitin Sulfate Proteoglycan Distribution in the Primate Optic Nerve Head

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**Purpose.** To evaluate the presence and distribution of chondroitin and dermatan sulfate-containing proteoglycans in normal human and monkey optic nerve heads by light microscopic immunohistochemistry.

**Methods.** Monoclonal antibodies specific for glycosaminoglycan attachment sites remaining after incubation of tissues with chondroitinase ABC and ACII were used to detect proteoglycans containing unsulfated chondroitin (OS), chondroitin-4 and/or dermatan sulfate (4S), and chondroitin-6 sulfate (6S) glycosaminoglycans.

**Results.** 4S antibody labeling after chondroitinase ABC was heavily and evenly distributed within the peripapillary sclera and in the core of laminar beams and optic nerve septa. Preincubation with chondroitinase AC, which exposes only chondroitin sulfate attachment sites, diminished labeling intensity in the lamina cribrosa and sclera and almost completely eliminated it in the retrolaminal optic nerve septa. In contrast, 6S antibodies demonstrated a more intermittent linear distribution throughout the laminar beams and optic nerve septa. No qualitative differences were seen between human and monkey optic nerve heads.

**Conclusion.** Chondroitin and dermatan sulfate-containing proteoglycans exist throughout the support tissues of the optic nerve head. The specific distribution patterns demonstrated by these monoclonal antibodies, and, in particular, the unique confinement of one of them to the lamina, indicate the presence of different core proteins or different functional glycosaminoglycan side chains that may influence the behavior of the lamina cribrosa. Invest Ophthalmol Vis Sci. 1994;35:838-845.

The structural components of the optic nerve head may influence the susceptibility of optic nerve fibers to elevated intraocular pressure as they exit the eye. It has been previously shown that the monkey and human lamina cribrosa are composed of several collagen subtypes, basement membrane components, and elastin. These components provide an array of structural properties to the optic nerve head, ranging from tensile strength (the interstitial collagens) and resiliency (elastin and interstitial collagen type III), to cell attachment, organization, and barrier functions (collagen type IV, laminin, and other basement membrane components). The relative amounts and organization of these constituents have been shown to vary with age and with optic nerve diseases, including glaucomatous and neurogenic optic atrophy. Because of their diverse functions, changes in the extracellular matrix components of the optic nerve head may alter its function and thus the susceptibility of optic nerve fibers to intraocular pressure.

Determining the concentration and location of the major structural proteins alone will not provide a complete understanding of how these proteins support optic nerve fibers, particularly if their interrelationships are not completely understood. In this regard, little is known of proteoglycans in the optic nerve head. Proteoglycans, major components of the extracellular matrix, are a diverse group of proteins with attached carbohydrate side chains, called glycosaminoglycans (GAGs). GAGs adhere to many
other macromolecules through their charge interactions and thus contribute greatly to macromolecular and cell adhesion phenomena. Because of their diverse functions, understanding the presence and distribution of proteoglycans within the lamina cribrosa may enhance our understanding of the optic nerve head extracellular matrix and how it functions in glaucoma and other optic neuropathies.

We have used a battery of monoclonal antibodies directed against the carbohydrate stubs of unsulfated chondroitin (OS), chondroitin-4 sulfate (4S), and chondroitin-6 sulfate (6S) remaining after exposure to chondroitinase enzymes to demonstrate the presence of chondroitin and dermatan sulfate-containing proteoglycans in various locations within the normal human and monkey optic nerve head.

MATERIALS AND METHODS

Normal, adult human and rhesus monkey optic nerve heads were dissected from freshly obtained, enucleated eyes immersed in OCT mounting media and frozen in 2-methyl butane chilled in liquid nitrogen. Tissues from seven white human donors (six men and one woman), ranging from 54 to 83 years of age, were obtained from the Oregon Eye Bank and frozen within 24 hours of death. All eyes were judged normal by a history of no ocular disease and direct, magnified inspection of the globes, retina, and optic nerve heads to rule out ocular disease at the time of dissection. Normal monkey eyes (four male and three female), aged 10 to 25 years, were obtained from the Oregon Regional Primate Research Center and frozen within 3 hours of sacrifice. All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Longitudinal optic nerve sections 3 μm thick were cut on a cryostat, collected on silane-coated glass slides, and stored at −80°C until use. Sections were first fixed in methanol for 15 minutes at 4°C and then washed in phosphate-buffered saline (PBS). Experimental sections were then incubated for 90 minutes at 37°C in enzyme buffer (pH 7.2) consisting of 20 mM Tris, 50 mM sodium acetate, 100 mM sodium chloride, 0.01% bovine serum albumin (fraction V, Sigma, St. Louis, MO), and 0.1 U/ml chondroitinase ABC or chondroitinase ACII (Sigma). Chondroitinase ABC cleaves both chondroitin and dermatan sulfate glycosaminoglycan side chains from the core protein, whereas the latter is specific for chondroitin sulfates. All slides were then rinsed in PBS and processed for light microscopic immunohistochemistry.

After digestion, sections were incubated for 30 minutes in PBS with 1% bovine serum albumin (PBS/BSA) containing 20% normal horse serum to block nonspecific binding. Excess blocking serum was then removed, and the sections were overlaid with primary antibodies diluted 1:3000 to 1:5000 in PBS/BSA overnight at 4°C. Primary antibodies were mouse monoclonal IgG antibodies to unsulfated chondroitin (OS) and chondroitin-4 sulfate (4S) and mouse monoclonal IgM

FIGURE 1. Overviews of human (A) and monkey (B) tissues exposed to 4S antibodies after chondroitinase ABC preincubation demonstrate heavy labeling of all connective tissue components. S, peripapillary sclera. *Lamina cribrosa (original magnification, ×110).
antibodies to chondroitin-6 sulfate (6S), all purchased from ICN Immunobiologicals (Costa Mesa, CA). These antibodies are well characterized, specifically recognize the carbohydrate stubs that remain attached to the proteoglycan core protein after enzymatic removal of dermatan and chondroitin sulfate side chains, and have provided important information on proteoglycan distribution in several organ systems, including the eye.21-28

Sections were then washed with PBS and overlaid for 30 minutes with biotinylated secondary antibodies (Vector laboratories, Burlingame, California) diluted 1:200 in PBS/BSA. After a second wash, the slides were exposed to avidin biotin peroxidase complex (Vector laboratories) mixed 1:200 in PBS/BSA for an additional 45 minutes. Sections were developed in 0.05% 3,3-diaminobenzidine with 0.02% hydrogen peroxide in 20 mM Tris buffer, pH 7.2, for 3 minutes,
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washed, counterstained lightly with hematoxylin, dehydrated, and coverslipped for viewing with a Zeiss Axiofot light microscope. Findings were documented by black-and-white photography using a green filter.

Control experiments consisted of substituting the corresponding dilutions of appropriate mouse purified immunoglobulins for primary antibodies on enzyme-treated tissues.

RESULTS

We found evidence that proteoglycans containing chondroitin-4 and chondroitin-6 sulfate exist in all regions of the optic nerve head. Antibodies to 4S and 6S proteoglycans produced distinctive labeling of the connective tissues of both the lamina cribrosa and the retrolaminar optic nerve, without fundamental differences between human and monkey tissues.

Overview studies illustrated that label with 4S antibodies after chondroitinase ABC was heavy over the sclera, lamina cribrosa, and optic nerve septa and pia in both human and monkey optic nerve heads (Fig. 1). Antibodies to unsulfated chondroitin (OS) labeled only the posterior sclera (Fig. 2A). Purified immunoglobulin controls showed no consistent staining over any region of the optic nerve head (Fig. 2B). Control tissues incubated in buffer without enzyme before antibody exposure were similarly negative.

Substitution of chondroitinase ACII for chondroitinase ABC dramatically altered labeling at all levels of the optic nerve head (Fig. 3). Label in these experiments was limited to the lamina cribrosa and anterior half of the peripapillary sclera but was almost

FIGURE 5. High-power view of human lamina cribrosa demonstrates less dense but still diffuse label of laminar beams (asterisks) with 4S antibodies after chondroitinase ACII predigestion. (original magnification, ×1100).

FIGURE 6. Chondroitin-6 sulfate antibodies exposed to human optic nerve heads after chondroitinase ABC predigestion produce less intense label of lamina cribrosa (asterisk) and retrolaminar optic nerve septa compared to 4S antibodies, and minimal label of peripapillary sclera (S) (original magnification, ×110).

FIGURE 4. High-power view of human lamina cribrosa exposed to 4S antibodies after chondroitinase ABC confirms heavy, confluent labeling of laminar beams (asterisks) (original magnification, ×1100).
FIGURE 7. High magnification of human lamina cribrosa indicates filamentary 6S labeling throughout the laminar beams (asterisks). Intermittent labeling also appears along the beam margins (arrows) (original magnification, $\times 1100$).

totally eliminated in the optic nerve septa and pia, particularly in the monkey.

High-power examination of the lamina cribrosa confirmed that label with 4S antibodies after chondroitinase ABC predigestion was heavy and confluent over the collagenous laminar beams (Fig. 4). Label of beams with these same antibodies after predigestion with chondroitinase ACII was less intense (Fig. 5) but not qualitatively different from that seen after digestion with chondroitinase ABC.

Overviews of tissues labeled with 6S antibodies after chondroitinase ABC predigestion showed generally less intense label of the lamina cribrosa than that produced by 4S antibodies, with minimal staining of the peripapillary sclera (Fig. 6). At high magnification, 6S label appeared intermittent and linear throughout the laminar beams with filamentary deposits often along the beam margins (Fig. 7). This was a marked contrast to the confluent labeling seen with 4S antibodies after either chondroitinase ABC or chondroitinase ACII predigestion.

Posterior to the lamina cribrosa, 4S antibodies produced heavy, confluent labeling over the optic nerve septa and the pia (Fig. 8). This label was completely eliminated by preincubation with chondroitinase ACII. In contrast, 6S antibodies resulted in linear, filamentary labeling throughout the optic nerve septa, similar to the pattern seen in the beams of the lamina cribrosa.

DISCUSSION

Proteoglycans are a diverse group of core proteins with carbohydrate side chains called glycosaminoglycans (GAGs). GAGs bind to many other macromolecules through their charge interactions and thus pro-
mote participation in macromolecular and cell adhesion phenomena within the extracellular matrix.\textsuperscript{15–18} Each GAG is a polymer of a disaccharide, and can vary in its length and degree of sulfation, both of which affect the proteoglycan’s space-occupying properties and affinity for other macromolecules. The classes of GAG chains are heparan sulfate, keratan sulfate, chondroitin sulfate, and dermatan sulfate. The latter two can exist either as homogeneous or mixed polymers of one or more of these disaccharides with varying degrees of sulfation. Heparan sulfate-containing proteoglycans specific for basement membranes have previously been identified within the lamina cribrosa.\textsuperscript{8,24} Although analytic histochemical studies suggest that keratan sulfate exists within the lamina,\textsuperscript{25} keratan sulfate proteoglycans have not been found using specific core protein antibodies.\textsuperscript{24}

Our data suggest that chondroitin sulfate and dermatan sulfate proteoglycans are heavily represented throughout the primate optic nerve head, including the lamina cribrosa and peripapillary sclera, and, posteriorly, the optic nerve septa and pia. These proteoglycans appear to have distinct distributions within the nerve head, depending on their content of dermatan sulfate, chondroitin-4 sulfate, and chondroitin-6 sulfate.

One of our most striking findings was the heavy, diffuse 4S labeling of laminar beams, the sclera, and the retrolaminar optic nerve septa and pia mater after chondroitinase ABC digestion, indicating the presence of chondroitin-4 sulfate, dermatan sulfate, or both. Preincubation of tissues with chondroitinase ACII, which does not reveal the dermatan sulfate epitope, reduced the labeling intensity in the lamina cribrosa. This confirms that at least part of the heavy 4S staining after chondroitinase ABC was due to proteoglycans that contain dermatan sulfate. The presence of both chondroitin and dermatan sulfate in the optic nerve head has been suggested by recent electron microscopic\textsuperscript{26} and immunohistochemical studies.\textsuperscript{24}

Although specific chondroitin and dermatan sulfate proteoglycan core proteins have not yet been localized in the optic nerve head, several are known to be present in other eye tissues.\textsuperscript{27–29} In addition, their molecular characteristics and presumed functional roles make them likely candidates for some of the core proteins of the proteoglycan GAGs demonstrated here.

The majority of heavy labeling with 4S antibodies after chondroitinase ABC appears to be localized to regions known to contain large amounts of interstitial collagen.\textsuperscript{7–14} This pattern may reflect, in part, GAG chains attached to the small proteoglycan decorin, whose core protein demonstrates strong and specific interactions with interstitial collagen and which is known to modify both collagen fibril alignment and the rate of fibrillogenesis.\textsuperscript{17,30–32} It may also reflect the presence of biglycan, which is a structurally related proteoglycan associated with pressure-responsive tissues such as cartilage and aorta.\textsuperscript{27,33} Both of these proteoglycans may contain either chondroitin or dermatan sulfate, or both. Both have been localized in sclera,\textsuperscript{27} as has aggrecan, the large cartilage chondroitin sulfate proteoglycan.\textsuperscript{28} Additional possible core proteins include versican, syndecan, serglycin, and others, the characteristics of which are beyond the scope of this discussion but are summarized in recent reviews.\textsuperscript{17,34,35}

Preincubation of tissues with chondroitinase ACII not only diminished the intensity of 4S antibody labeling in the lamina cribrosa, it virtually eliminated this label posterior to the lamina, over both the optic nerve septa and pia. Limitation of chondroitin-4 sulfate labeling to the lamina and inner sclera is unique among extracellular matrix components studied to date. This pattern suggests possible localized differences in optic nerve head intracellular GAG iduronization, proteoglycan core protein identity, or both. Although these differences may reflect the unique developmental history of these regions of the eye,\textsuperscript{36} they may also have functional implications important to glaucomatous optic nerve damage because the lamina and sclera are load-bearing structures in the normal eye constantly exposed to intraocular pressure.

Antibodies to chondroitin-6 sulfate demonstrated a labeling pattern that was qualitatively different from that seen with 4S antibodies. The 6S labeling had a linear distribution throughout the laminar beams and optic nerve septa often extending to the margins of these structures. Labeling of laminar and septal margins resembles that previously described for heparan sulfate proteoglycans, collagen type IV, and laminin, all basement membrane components deposited by vascular endothelium astrocytes.\textsuperscript{6–9} Two basement membrane-associated heparan sulfate proteoglycans have been isolated, one of which may contain a chondroitin sulfate chain.\textsuperscript{37,38} In addition, a distinct chondroitin-6 sulfate-containing proteoglycan associated with basement membranes has also been described.\textsuperscript{39}

The 6S antibodies also produced linear labeling within the laminar beams and nerve septa. This pattern may represent one or more cell surface-associated proteoglycans, such as versican, a large chondroitin sulfate-containing proteoglycan derived from fibroblasts,\textsuperscript{40–42} or may possibly indicate proteoglycans associated with other matrix components known to exist in these regions, such as elastin.\textsuperscript{11} Further light and ultrastructural immunohistochemical studies will be required to identify the precise proteoglycan core proteins involved, determine their association with other extracellular components, and improve our un-
derstanding of the cell-matrix interactions that govern the physical response of the lamina cribrosa to normal and elevated intraocular pressure.

**Key Words**

lamina cribrosa, extracellular matrix, proteoglycans, chondroitin sulfate, dermatan sulfate

**References**


