The Extracellular Matrix Composition of the Monkey Optic Nerve Head

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The presence and distribution of laminin, heparan sulfate proteoglycan and collagen types I, III and IV were immunohistologically determined in cynomolgus monkey optic nerve heads using the avidin-biotin-peroxidase complex technique. Collagen types I and III were detected within the collagenous plates of the scleral lamina cribrosa, in the septa and pia mater of the postlaminar optic nerve and in blood vessel walls in all regions of the optic nerve head. Collagen type IV, laminin and heparan sulfate proteoglycans were all localized to the margins of the collagenous laminar plates of the scleral lamina cribrosa and along the margins of the optic nerve septa and the pia mater. All three components also appeared beneath the blood vessel endothelium throughout the optic nerve head. Within the lamina cribrosa, collagen types I and III occupy the core of the scleral laminar plates and may provide structural support for optic nerve bundles exiting the eye. The distribution of collagen type IV, laminin and heparan sulfate proteoglycan corresponds to basement membranes from two sources: vascular endothelial cells and glial cells lining the axonal bundles. Abnormalities of these substances may influence optic nerve function and susceptibility to elevated intraocular pressure by altering their mechanical support functions within the nerve head, by interfering with axonal nutrition, or both.

Defining the extracellular elements of the optic nerve head is important to our understanding of the development of glaucomatous optic nerve damage. The lamina cribrosa consists of several collagenous sheets that span the scleral opening and possess perforations for the exit of nerve fiber bundles from the eye. Eyes with advanced glaucomatous cupping exhibit collapse and prominent posterior bowing of the scleral lamina cribrosa. Both light and scanning electron microscopy have demonstrated that the collagenous laminar beams in the superior and inferior regions of the optic nerve head appear less dense than in the horizontal regions. Hence, the lamina cribrosa in these regions may provide less mechanical support for their corresponding nerve fiber bundles, rendering them more susceptible to increased intraocular pressure.

However, the extracellular matrix of the optic nerve head may provide more than simple mechanical support for ganglion cell axons leaving the eye. In addition to collagen, the lamina cribrosa contains basement membrane, both within the laminar beams surrounding capillary endothelial cells and along the margins of the beams themselves. Vascular basement membranes modulate ultrafiltration in addition to providing physical support. Therefore, subtle alterations in the biochemical composition of this structure may affect ganglion cell nutrition and function at the level of the optic nerve head.

We have studied the presence and distribution of five extracellular matrix components within the cynomolgus monkey optic nerve head using indirect immunoperoxidase labelling. By defining the normal biochemical composition of the optic nerve head in this laboratory animal, these examinations provide initial information to suggest how the connective tissue of the optic nerve head functions to support ganglion cell axons under normal conditions and in disease states.

Materials and Methods

Tissue Preparation

These experiments were performed in compliance with the ARVO Resolution on the Use of Animals in
Research. Adult, normal cynomolgus monkeys anesthetized with 9 mg/kg intramuscular ketamine (Bristol, Syracuse, NY) and 11 mg/kg intravenous pentobarbital (Harvey Laboratories, Philadelphia, PA) were exsanguinated and perfused with normal saline. Next, the eyes were enucleated and the optic nerve heads washed in 0.1 M phosphate buffer (pH 7.2) and bisected into temporal and nasal halves. These were then embedded in O.C.T. (Miles, Naperville, IL) and frozen in liquid hexanes cooled with liquid nitrogen.

Horizontal, 8 to 10 μm sagittal sections of the optic nerve head were cut on a Reichert-Jung cryostat and collected on albumin-coated slides for immunohistochemistry and plastic embedding. The avidin-biotin-peroxidase complex (ABC) technique. In addition, 20 μm sections were transferred into 0.1 M phosphate buffer for immunohistochemistry and plastic embedding.

Immunohistochemistry

Sections on slides from vertical and horizontal optic nerve head regions were fixed in absolute methanol at −20°C, air-dried and then treated in 3% hydrogen peroxide to “quench” endogenous tissue peroxidase. After several washes in Dulbecco’s phosphate buffer, sections were incubated for 20 min at room temperature in blocking serum to decrease nonspecific binding. The blocking serum was then drained off and primary antibodies applied to the sections, all diluted in phosphate buffer with 1% bovine serum albumin.

Sections were incubated for 1 hr at room temperature in one of the following polyclonal antibodies: rabbit anti-mouse EHS tumor laminin (1:200 and 1:400) and collagen IV (1:200), rabbit anti-bovine collagen type III (1:500 and 1:1000), affinity-purified rabbit antibodies to heparan sulfate proteoglycan (1:50) and affinity-purified sheep anti-human collagen type I (1:500 and 1:1000). Antibodies to laminin and type IV collagen were purified by immunoprecipitation and evaluated by Western blot analysis on purified laminin and type IV collagen. With the exception of antibodies to type III collagen and heparan sulfate proteoglycans, all antibodies were the gift of Drs. Kleinman, Chandrasekhar, and Grotendorst of the National Institute of Dental Research, National Institutes of Health (Bethesda, MD). The rabbit antibodies against collagen type III were the gift of Dr. L. Paglia, Pfizer Hospital Products (Groton, CT). Antibodies against heparan sulfate proteoglycans were the gift of Dr. John Hassell, of the National Institute of Dental Research, National Institutes of Health.

Control sections were incubated with appropriate dilutions of nonimmune rabbit and sheep IgG. Antibody specificity was evaluated by incubating other control sections with antibodies to collagen types I, III, IV and laminin which were preabsorbed with excess quantities of rat tail collagen type I, fetal calf skin collagen III, and murine EHS tumor collagen IV and laminin, respectively. These antigens were the gift of Dr. Kleinman and their purity established using SDS-polyacrylamide gel electrophoresis. In addition, all collagen antibodies were checked for nonspecific binding to human serum fibronectin (a gift of Dr. Kleinman, purity judged by SDS-polyacrylamide gel electrophoresis) by dot-blot analysis (Biorad Laboratories, Richmond, CA) and no cross-reactivity was noted.

Additional sections were tested using affinity-purified goat anti-human collagen type I, III and IV (1:400) (Southern Biotechnologies, Inc., Birmingham, AL), each of which had been cross-adsorbed against the remaining three purified human collagen subtypes and their specificity determined by ELISA.

Sections were next washed in phosphate buffer and then overlayed for 30 min at room temperature with biotinylated secondary antibodies (goat anti-rabbit or rabbit anti-goat IgG) (Vector Laboratories, Burlingame, CA) diluted 1:250 with phosphate buffer and 1% BSA. The sections were then washed in phosphate buffer and incubated for 45 min in avidin-biotin-peroxidase complex (ABC) (Vector) diluted 1:125 in phosphate buffer with 1% BSA.

Following a wash in phosphate buffer, the sections were incubated for 15 min in a 0.017% solution of 3-amino-9-ethylcarbazole with 0.02% hydrogen peroxide in 0.1 M sodium acetate buffer (pH 5.3). A final wash in distilled water was followed by counterstaining with Harris’s hematoxylin and mounting a coverslip with Gelvatol (Monsanto, Springfield, MA) mounting media.

Because the 8–10 μm cryosections had limited resolution, further ABC immunolabelling experiments were performed on 20 μm frozen sections for plastic embedding and semithin sectioning. After a 30 min wash in 0.1 M phosphate buffer, these sections were transferred using a wire loop into droplets of blocking serum. Primary antibodies were as described previously, except for the substitution of affinity-purified, cross-adsorbed goat anti-human collagen types I (1:200 and 1:400) and IV (1:100 and 1:200) antibodies purchased from Southern Biotechnologies. The remaining antibodies were diluted approximately one-half as much as in the previously described experiments. Controls in these experiments consisted of appropriate dilutions of nonimmune goat and rabbit IgG.

After a 1 hr incubation with primary antibodies at room temperature, sections were washed in 0.1 M phosphate buffer for 30 min on a rotating wheel.
Fig. 1. Immunolabelling of optic nerve head frozen sections with antibodies to collagen type I (A) and III (C) demonstrates prominent dark reaction product over the laminar beams (arrows), optic nerve septa (arrowheads) and adjacent sclera (S). Reaction product anterior to the lamina cribrosa corresponds to blood vessels of the choroidal lamina and nerve fiber layer regions of the optic nerve head. Labelling is virtually eliminated when the antibodies are preincubated in an excess of their corresponding antigens, collagen type I (B) and type III (D) (hematoxylin counterstain, ×125).

They were then transferred into the secondary antibodies, washed and treated with the ABC as previously described. After a final wash, sections were fixed for 30 min in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed in 0.05 M Tris buffer and then treated for 5 min in 0.05% 3,3‘diaminobenzidine (DAB) with 0.02% hydrogen peroxide in Tris buffer.

The sections were then fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded ethanol series and then transferred via propylene oxide into LX-112 (Ladd Research Industries, Inc., Burlington, VT). Final embedding was accomplished by flattening the sections against a teflon block with the flat end of prehardened blocks of resin according to the method of Laurie et al.14 After polymerization at 60°C for 48 hr, 1 μm sections were then cut with...
Fig. 2. (A) One micron plastic section of the lamina cribrosa labeled with affinity-purified, cross-adsorbed antibodies to type I collagen. Reaction product appears localized to the core of the laminar beams (arrow) and to the septa of the anterior optic nerve (arrowhead), standing out against the unlabelled nerve fiber bundles. This is in contrast to the control section (Fig. 2B), prepared with substitution of nonimmune goat IgG for the primary antibody (phase contrast, uncounterstained, ×800).

glass knives on a Reichert (Vienna, Austria) Ultracut microtome and mounted on glass slides. Some sections were stained lightly with toluidine blue while others were not counterstained. Sections prepared by either method were examined with a Zeiss light microscope, using both bright field and phase contrast techniques under low and high power magnification.

Fig. 3. One micron plastic sections of a monkey optic nerve head labeled with affinity-purified, cross-adsorbed antibodies to collagen type I. Anterior to the lamina cribrosa (A), reaction product is limited to the walls of blood vessels (arrows) in the nerve fiber layer and glial columns. Posterior to the lamina (B), dense labelling appears within the optic nerve septa and walls of their contained blood vessels (C) (phase contrast, uncounterstained, ×800).
Fig. 4. A frozen section incubated with antibodies to laminin (A) demonstrates reaction product in the prelaminar, laminar (*) and postlaminar regions of the nerve head, as compared with a control section (B) exposed to antibodies preincubated with excess laminin. Note the absence of reaction product over the peripapillary sclera (S) in both experimental and control sections (hematoxylin counterstain, ×125).

Fig. 5. One micron plastic section from tissue incubated with antibodies to collagen type IV demonstrates distinct, linear labelling of blood vessels, laminar beams and optic nerve septa (toluidine blue counterstain, ×340).
Results

Collagen Types I and III

Collagen types I and III were found in the scleral lamina cribrosa with a labelling intensity that was equal to that of the adjacent sclera and the pia mater (Fig. 1A, C). These patterns were present regardless of the source of the antibody used. Incubation of sections with antibodies to collagen types I and III that had been preincubated with an excess of their purified corresponding antigens resulted in elimination of the label to both types of collagen (Fig. 1B, D). The label for both antibodies appeared to be confined to the collagenous portions of the lamina cribrosa and was absent over the neural bundles. This distribution was confirmed by examination of 1 μm plastic sections (Fig. 2).

Anterior to the scleral lamina, label was associated primarily with the walls of blood vessels and capillaries of the glial columns and nerve fiber layer regions of the optic nerve head (Fig. 3A). Posterior to the lamina cribrosa, incubation with antibodies to collagen types I and III resulted in heavy labelling of the core of the optic nerve septa and the walls of the blood vessels contained within them (Fig. 3B). In these preparations, labelling of optic nerve septa was consistently heavier than that over the laminar beams (Figs. 2A, 3B).

Collagen Type IV, Laminin and Heparan Sulfate Proteoglycan

Frozen sections incubated with antibodies against collagen type IV, laminin and heparan sulfate proteoglycan all demonstrated labelling of structures in the prelaminar, laminar and postlaminar regions of the optic nerve head (Fig. 4A). This label was not present in sections exposed to antibodies that had been preincubated with their corresponding antigen (Fig. 4B).

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In 1 μm plastic sections, all three components had predominantly linear distributions along the blood vessels and connective tissue elements of the optic nerve head (Fig. 5). At the level of the scleral lamina cribrosa, they lined the interface between the peripapillary sclera and the most peripheral axon bundle (Fig. 6). The majority of label within the peripapillary sclera was associated with the numerous blood vessels that occupy this region. In addition, small amounts of reaction product were found unassociated with blood vessels, lying between the peripapillary collagen bundles.

Within the lamina cribrosa, labelling with collagen IV, laminin and heparan sulfate proteoglycan was linearly distributed along the margins of the collagenous laminar beams, appearing as parallel stacks of reaction product (Fig. 7). Label was also associated with the blood vessels lying within laminar beams, indicating adequate antibody penetration. Similar labelling was noted when the 20 μm sections were cut in cross-section (data not shown), demonstrating adequate reagent penetration throughout the thickness of the section. Control sections, both with and without counterstain, were entirely negative (data not shown).

Just anterior to the lamina cribrosa, the linear stacks of label gradually tapered into a less orderly deposition of reaction product within the glial columns (Fig. 8). In more anterior regions of the nerve head, the reaction product was scattered anterior to the glial columns, between the nerve bundles, often accompanying them in their 90 degree turn into the nerve fiber layer (Fig. 9). The only linear deposits of label seen in this region were associated with blood vessels.

Posterior to the lamina cribrosa, all three antibodies produced linear deposition of label along the margins of the optic nerve septa and beneath the endothelium of their associated blood vessels (Fig. 10A). The label along the septa was continuous with that lining the pia mater (Fig. 10B).

**Discussion**

Collagens I, III, IV, laminin and heparan sulfate proteoglycan all contribute to the extracellular matrix...
of the human and nonhuman primate optic nerve head. Their distribution falls into two patterns, both of which are in agreement with the known ultrastructure of the optic nerve head as well as with the accepted functions of these macromolecules. Collagens I and III were consistently localized to blood vessel walls in all regions of the optic nerve head. They were also found in the peripapillary sclera, lamina cribrosa, pia mater and optic nerve septa.

We found strong labeling of both collagen types I and III within the lamina cribrosa of the monkey optic nerve head. These results were obtained using two sets of antibodies that satisfied several criteria for specificity. These include controls that used prior adsorption of one set of antibodies with an excess of purified antigens (Fig. 1) and affinity purification and cross-adsorption of another set followed by ELISA. We therefore propose that collagen types I and III are significant components of the primate lamina cribrosa; they represent the densely-packed, cross-banded collagen fibrils that comprise the core of the laminar beams and ultrastructurally resemble those found in sclera.

Our results differ significantly from prior studies using monoclonal antibodies in human eyes that have found "relatively little" type I and III collagen within the lamina cribrosa and concluded that these macromolecules are not a major component of the lamina cribrosa. This discrepancy may reflect methodological differences, since monoclonal antibodies are by definition directed against a single epitope and may therefore be less sensitive, or possibly more easily masked from their antigen by other macromolecules, than are the affinity-purified and cross-adsorbed polyclonal antibodies used here. A species difference in laminar composition is unlikely, since other laboratories have demonstrated prominence of collagens I and III as well as type VI within the beams of the human, rat and bovine lamina cribrosa. Hernandez et al have recently proposed that the poor staining of collagens I and III observed by them may be due to the young age of the specimens they have studied, less than 25 years old.
However, while the thickness of laminar sheets increases throughout life, electron microscopic analysis indicates that the density of collagen fibrils within these sheets is well established during the first decade of life. Animals used in our study were all adults; although precise ages were not obtainable, they would therefore be analogous to the human patient population in which primary open angle glaucoma is most prevalent.

Collagen type I is widely distributed throughout the body and provides tensile strength to numerous structures, including skin, tendon and cornea. Its presence, along with collagen III, comprises a major structure of the scleral lamina and makes it unique from the surrounding neural bundles and anterior regions of the optic nerve head. Anchored to the peripapillary sclera, the lamina may present an unyielding structure in the face of elevated intraocular pressure which then would impinge on shifting axons. This may underlie previous observations that axonal transport blockade in human and experimental glaucoma is most evident at the level of the lamina cribrosa.

Collagen IV, laminin and heparan sulfate proteoglycan exhibited identical patterns of reaction product throughout the optic nerve head that was distinct from that of collagen I and III. All are basement membrane components and their coincident distributions suggest the presence of basement membranes from two sources: vascular endothelium and astrocytes. The linear deposition of reaction product within the laminar beams and optic nerve septa corresponds to basement membrane deposited by vascular endothelial cells. That deposited on the margins of these structures and the pia mater most likely indicates the presence of basement membrane associated with glial cells lining the axon bundles. In general, these results agree with similar studies in human tissue and previous ultrastructural findings, thus supporting the utility of this experimental animal as a model for glaucomatous optic nerve damage.

Collagen type IV is thought to contribute tensile strength to basement membranes for a structural role in tissue organization. Its presence within basement membranes lining the laminar beams and optic nerve septa suggests that these structures may contribute to maintaining optic nerve head strength and structure. Likewise, the large amount of basement membrane components observed within the glial columns (Fig. 9) may contribute mechanical support to axons in their 90 degree turn into the nerve head, although electron microscopic study is required to determine precisely its anterior extent.

In glomerular capillary basement membranes, the high anionic charge of heparan sulfate proteoglycan is thought to modulate filtration across the vessel wall. Although not confirmed in the vasculature of the central nervous system, it is possible that an altered biochemical composition of capillary basement membranes may alter their filtration function and affect axonal nutrition within the optic nerve head. Thus, elevated intraocular pressure could still affect axonal function on a vascular basis even though both blood flow studies and pathologic evaluations have failed to demonstrate vascular abnormalities at the level of the lamina cribrosa. However, our results indicate that, under normal conditions, the basement membrane composition of capillaries in the lamina cribrosa is not unique from those in other regions of the optic nerve head and sclera.

Key words: extracellular matrix, optic nerve head, immunohistochemistry, basement membranes, lamina cribrosa

References

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