Serum Autoantibodies to Optic Nerve Head Glycosaminoglycans in Patients With Glaucoma

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Background: Serum autoantibodies that cross-react with glycosaminoglycans have been proposed to play a significant role in specific tissue injury in patients with systemic autoimmune diseases.

Objective: To investigate whether serum immunoreactivity to glycosaminoglycans is present in patients with glaucoma who have aberrant serum autoantibodies to DNA, RNA, nuclear proteins, or retinal proteins, as proteoglycans and their glycosaminoglycan side chains are important components of the optic nerve head and its vasculature.

Methods: We performed Western blotting using patient serum samples and human optic nerve head homogenates that were treated with or without specific glycosaminoglycan degrading enzymes. Monoclonal antibodies that recognize different determinants of glycosaminoglycans were used to identify specific substrate antigenicity. We compared the serum immunoreactivity to glycosaminoglycans in 60 age-matched patients with normal-pressure glaucoma, 36 patients with primary open-angle glaucoma, and 20 control subjects by enzyme-linked immunosorbent assay. In addition, immunohistochemistry was performed to compare the distribution patterns of glycosaminoglycans in the optic nerve head of postmortem eyes of age-matched patients with normal-pressure glaucoma, primary open-angle glaucoma, and control subjects.

Results: Western blotting demonstrated that serum samples from patients with glaucoma who have circulating autoantibodies can recognize optic nerve head proteoglycans, including chondroitin sulfate and heparan sulfate. The level of serum autoantibodies binding purified chondroitin sulfate and heparan sulfate glycosaminoglycans in an enzyme-linked immunosorbent assay was approximately 100% higher in patients with normal-pressure glaucoma than that in control subjects and approximately 50% higher than that in patients with primary open-angle glaucoma. We also observed increased immunostaining of glycosaminoglycans in the optic nerve head of eyes with glaucoma, particularly those with normal intraocular pressure, compared with control eyes.

Conclusion: There are increased levels of autoantibodies recognizing glycosaminoglycans of the optic nerve head in the serum samples of some patients with glaucoma.

Clinical Relevance: These autoantibodies may increase the susceptibility of the optic nerve head to damage in these patients by changing the functional properties of the lamina cribrosa, its vasculature, or both.

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PATIENTS AND METHODS

PATIENT SELECTION

Blood samples were obtained after detailed consent was obtained from each patient according to the recommendations of the World Medical Association Declaration of Helsinki. Sixty age-matched patients with normal-pressure glaucoma, 36 patients with primary open-angle glaucoma, and a control group of 20 healthy subjects were included. Twelve (20%) of the patients with normal-pressure glaucoma had antinuclear antibodies or serum antibodies to extractable nuclear antigens, including Ro/SS-A, La/SS-B, U1 ribonuclear protein, and Smith antigen. The inclusion and exclusion criteria for these groups were described previously. Briefly, normal-pressure glaucoma consisted of the presence of open iridocorneal angles, no evidence of intraocular pressure higher than 23 mm Hg, glaucomatous changes in visual fields and optic nerve cupping, and the absence of alternative causes of optic neuropathy. The diagnostic criteria for patients with primary open-angle glaucoma were similar to those of patients with normal-pressure glaucoma, except that their intraocular pressure levels were higher than 23 mm Hg. The visual field loss of patients was evaluated (Humphrey Field Analyzer, 30-2 program; Humphrey, San Leandro, Calif). Our criteria for visual field abnormalities included a corrected-pattern SD with P<.05 or a glaucoma hemifield test outside normal limits obtained with at least 2 reliable and reproducible visual field examinations. The subjects in the control group had no evidence of ocular or systemic disease, including that related to autoimmune serum abnormalities.

The qualitative and quantitative presence of antinuclear antibodies was assessed by indirect immunofluorescence using a substrate (HEp-2; Sanofi, Chaska, Minn). Antibodies to the extractable nuclear antigens (Ro/SS-A, La/SS-B, U1 ribonuclear protein, and Smith antigen) were assessed semiquantitatively by enzyme-linked immunosorbent assay kits (Gull, Salt Lake City, Utah) in the Barnes Hospital (St Louis, Mo) laboratory. Patient serum samples were further examined in a masked manner by Western blotting and enzyme-linked immunosorbent assay.

PROTEIN SOLUBILIZATION

The human optic nerve heads were homogenized in ice-cold lysis buffer containing HEPES, 2 mmol/L; EDTA, 2 mmol/L; pH 7.4; and protease inhibitors (phenylmethylsulfonyl fluoride, 50 mmol/L; and apro tinin, antipain, bacitracin, bestatin, chymostatin, leupeptin, and pepstatin A, 1 mg/mL each) for 5 minutes at 4°C. After centrifugation of homogenates at 10000g for 10 minutes, the pellet (consisting of nuclei and unbroken cells) was discarded. Membrane fractions were incubated in a buffer containing Tris, 20 mmol/L; sodium chloride, 150 mmol/L; potassium chloride, 1 mmol/L; calcium chloride, 1 mmol/L; magnesium chloride, 1 mmol/L; pH 7.4; and apro tinin, antipain, bacitracin, bestatin, chymostatin, leupeptin, pepstatin A, and 1% Triton X-100, 1 mg/mL each, for 1 hour at 4°C. After centrifugation for 10 minutes at 100 000g at 4°C, supernatant was removed. Fractions were stored at −80°C until use. The protein concentrations in the solubilized fractions were determined using the bicinchoninic acid method. All of the reagents were purchased from Sigma Aldrich Corp (St Louis, Mo).

ANTIBODIES

We used monoclonal antibody to heparan sulfate proteoglycan (Chemicon, Temecula, Calif) and specific monoclonal antibodies raised against different determinants of chondroitin sulfate proteoglycans. These monoclonal antibodies recognize determinants present on chondroitin sulfate oligosaccharide stubs attached to the proteoglycan core protein after chondroitinase digestion and partial removal of dermatan and chondroitin sulfate side chains of the proteoglycan (ie, A-unsaturated 4- and 6-sulfated chondroitin and unsulfated chondroitin on the proteoglycan core) (ICN, Costa Mesa, Calif) as well as similar structures on the native glycosaminoglycans.19,20

ENZYMATIC TREATMENT

Specific enzymes were used to expose the proteoglycan macromolecules via sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For digestion of the chondroitin sulfate proteoglycans, the solubilized fractions of the human optic nerve heads were incubated in Tris–hydrochloric acid, 0.1 mol/L; pH 8.0, containing sodium acetate, 0.03 mol/L, with chondroitinase ABC, 0.02 U/mL (Seikagaku, Tokyo, Japan), at 37°C for 40 minutes. That enzyme catalyzes the partial removal of chondroitin sulfate, dermatan sulfate, and hyaluronic acid side chains of proteoglycans. Remaining proteoglycan protein core contains short oligosaccharide stubs consisting of the chondroitin sulfate linkage region sugars covalently bound to protein and one or more chondroitin sulfate disaccharide units with either 4-sulfated, 6-sulfated, or unsulfated N-acetylglactosamine residues. Enzymatic treatment with heparinase III (Sigma Aldrich Corp) to lyse heparan sulfate and heparin side chains was similarly performed.

sition of sulfate substitution (chondroitin sulfate and dermatan sulfate; keratan sulfate; heparan sulfate and heparin; and hyaluronic acid). These macromolecular components are believed to have an important role in the strength and elasticity of the optic nerve head because of their hydration properties, which contribute to the overall rigidity of the extracellular matrix, whose elements include collagen, elastin, laminin, and fibronectin.8,9

Serum autoantibodies, such as those directed against nuclear DNA that occur in patients with autoimmune disease, can cross-react with glycosaminoglycans and thereby play a major role in tissue injury.10-12 Considerable evidence13-17 suggests that in some patients with glaucoma, particularly those with normal intraocular pressure, an autoimmune mechanism may contribute to their optic neuropathy. In particular, increased serum antibodies to DNA, RNA, and nuclear proteins have been found in some patients with glaucoma.14 We, therefore, sought to determine whether serum immunoreactivity to glycosaminoglycans of the optic nerve head is present in patients with glaucoma who also have aberrant serum autoantibodies. We reasoned that the presence of such an immunoreactivity might have pathogenic importance by contributing to the optic nerve injury.
Before performing immunohistochemistry on optic nerve head sections to examine the presence of different chondroitin sulfate side chains, deparaffinized sections were treated with the same buffer previously described containing chondroitinase ABC, 0.5 U/mL, at 37°C for 2 hours.

WESTERN BLOTTING

Human optic nerve head homogenates were separated by electrophoresis in 12.5% sodium dodecyl sulfate–polyacrylamide gels at 160 V for 1 hour and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Marlboro, Mass) at 40 V for 2 hours using a transfer apparatus (BioRad, Hercules, Calif). After transfer, membranes were incubated in a buffer (a combination of Tris–hydrochloric acid, 50 mmol/L; sodium chloride, 154 mmol/L; and 0.1% polysorbate 20, pH 7.5) containing 5% nonfat dry milk for 1 hour to block nonspecific binding sites, then overnight in the same buffer containing a dilution of primary antibody and sodium azide. Primary antibodies consisted of serum samples from patients with glaucoma and controls and monoclonal antibodies against Δ-unsaturated 4- and 6-sulfated and unsulfated chondroitin on the proteoglycan core (ICN) or monoclonal antibody to heparan sulfate proteoglycan (Chemicon). After several washes and a second blocking incubation for 20 minutes, the membranes were incubated with secondary antibodies (goat antihuman or goat antimouse IgG) conjugated with horseradish peroxidase (Fisher, Pittsburgh, Pa) (1:2000) for 1 hour. Immunoreactive bands were visualized by enhanced chemiluminescence using commercial reagents (Amersham, Arlington Heights, Ill).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Ninety-six-well microtiter plates (Packard, Meriden, Conn) coated with purified chondroitin sulfate or heparan sulfate (Seikagaku) (2.5 µg per well in sodium carbonate buffer, pH 8.8) were incubated overnight at 4°C. After washing the plates, nonspecific binding sites were blocked using 1% normal goat serum and 0.1% sodium azide at room temperature for 2 hours. Serum samples from patients or controls diluted 1:500 in phosphate-buffered saline containing polysorbate 20, plus 1% normal goat serum and sodium azide, were added to duplicate wells of antigen-coated plates and incubated overnight at 4°C. The serum was removed by washing with phosphate-buffered saline, then secondary antibody conjugated with horseradish peroxidase (goat antihuman IgG) (Fisher) was added (1:2000). After a 2-hour incubation at room temperature, the secondary antibody was washed with phosphate-buffered saline and color was developed by adding substrate buffer, including hydrogen peroxide and ABTS (2,2′-azino-di-[3-ethyl-benzthiazolin-sulfonat] (6) diamonium salt) (Boehringer Mannheim, Mannheim, Germany) for 40 minutes. The plates were read at 410 nmol/L in a plate reader (model MR700; Dynatech, Chantilly, Va). Negative control wells prepared without antigen or primary antibody, and positive control wells in which increased concentrations of monoclonal antibodies to chondroitin sulfate or heparan sulfate were used as primary antibody, were simultaneously processed.

IMMUNOHISTOCHEMISTRY

Four postmortem human eyes with a diagnosis of normal-pressure glaucoma, 4 eyes with a diagnosis of primary open-angle glaucoma, and 4 eyes from age-matched normal donors were obtained. Clinical findings of the patients with glaucoma, including intraocular pressure readings, optic disc changes, and visual field changes, were well documented during 5 to 13 years of follow-up. Information about the laboratory findings could also be obtained from one of the patients with normal-pressure glaucoma, which included the presence of a monoclonal gammapathy and aberrant serum antibodies to extractable nuclear proteins and retinal proteins. The level of optic nerve damage was similar in glaucomatous eyes as identified by previous clinical records and microscopic findings of the optic nerve heads. All eyes were enucleated within 4 hours of death and processed within 12 hours. All eyes were fixed in 10% formalin solution, processed, and embedded in paraffin. Serial 4-mm-thick longitudinal and cross-sections of the optic nerve heads were prepared and mounted on glass slides.

For immunostaining, deparaffinized and enzymatically treated sections with chondroitinase ABC as previously described were incubated with 3% bovine serum albumin at 37°C for 30 minutes to block nonspecific binding sites. At the end of the incubation time, sections were washed and incubated at 37°C for 2 hours with monoclonal antibodies against Δ-unsaturated 4-, 6-sulfated, or unsulfated chondroitin on the proteoglycan core (1:50) or monoclonal antibody to heparan sulfate (1:25). After washing the sections twice, they were incubated for 1 hour with Cy3-conjugated secondary antibodies (1:200). The sections were then examined using a fluorescence microscope (Olympus, Tokyo, Japan). Negative control specimens consisting of sections without primary antibody incubation were processed simultaneously.

head changes that accompany glaucomatous optic neuropathy in these patients.

RESULTS

WESTERN BLOTTING

We examined patient serum samples from 20 age-matched patients with normal-pressure glaucoma, 20 patients with primary open-angle glaucoma, and 20 control subjects against human optic nerve head homogenates using Western blotting. Western blots demonstrated marked immunoreactivity of glaucomatous patient serum samples against optic nerve head proteoglycans after enzymatic digestion of optic nerve head homog- enates using either chondroitinase ABC or heparinase III. Treatment with specific enzymes to expose the proteoglycan macromolecules on sodium dodecyl sulfate–polyacrylamide gel electrophoresis either increased the immunoreactivity or revealed new immunoreactive bands between approximately 40 and 90 kd, which suggests that the immunoreactivity is indeed to chondroitin sulfate or heparan sulfate proteoglycan (Figure 1). Serum immu- noreactivity against optic nerve head proteoglycans was
most strongly present in patients with glaucoma who have other evidence of aberrant systemic humoral autoimmunity, such as autoantibodies to nuclear proteins or retinal proteins, as shown by representative patients in Figure 1. No immunoreactivity was observed using serum samples from normal age-matched controls.

Western blots using specific monoclonal antibodies or the serum samples of patients with glaucoma as the primary antibody source revealed similarly sized immunoreactive bands against solubilized and enzymatically digested fractions of the human optic nerve head tissue. Although the specific monoclonal antibodies to different determinants of chondroitin sulfate glycosaminoglycans used can recognize carbohydrate structures on proteoglycans, but not protein core, similar immunoreactive bands seen on Western blots cannot differentiate whether the patient serum samples recognize an antigenic site on glycosaminoglycan or a different structure on protein core of proteoglycan exposed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis. We, therefore, performed an enzyme-linked immunosorbent assay using purified glycosaminoglycans as an antigen source to examine and quantify the serum immunoreactivities against glycosaminoglycans of proteoglycans.

ENZYME-LINKED IMMUNOSORBENT ASSAY

We tested patient serum samples from a cohort of 60 age-matched patients with normal-pressure glaucoma, 36 patients with primary open-angle glaucoma, and 20 healthy controls against purified glycosaminoglycans using enzyme-linked immunosorbent assay. The sex distribution of the groups was similar (female-male ratio, normal-pressure glaucoma, 36:24; primary open-angle glaucoma, 21:15; and controls, 12:8), and the titers of serum antibodies recognizing glycosaminoglycans were not different between the female and male groups (Mann-Whitney U test, P > .05). However, serum immunoreactivities to chondroitin sulfate and heparan sulfate glycosaminoglycans in the normal-pressure glaucoma group were approximately 100% higher than those found in the control group (Mann-Whitney U test, P < .001 and P = .003, respectively) and 50% higher than those found in the primary open-angle glaucoma group (Mann-Whitney U test, P = .009 and P = .04, respectively). Differences in the serum immunoreactivities to glycosaminoglycans between the primary open-angle glaucoma and control groups (approximately 20%) were not statistically significant (Mann-Whitney U test, P > .05) (Table).

IMMUNOHISTOCHEMISTRY

Hematoxylin-eosin–stained longitudinal sections and cross-sections of the lamina cribrosa are provided in

Figure 1. Western blot findings. Human optic nerve head homogenates were used (15 µg per lane) as antigen with or without treatment with specific enzymes. Primary antibody was either patient serum (1:1000) or monoclonal antibodies to unsulfated chondroitin (lane 11), 4-sulfated chondroitin (lane 12), 6-sulfated chondroitin (lane 13), or heparan sulfate (lane 26) (1:100-500). Lanes show before (1, 3, 5, 7, and 14) and after (2, 4, 6, 8, 10, 12, 13, and 15) treatment with chondroitinase ABC or before (16, 18, 20, 22, 24, and 27) and after (17, 19, 21, 23, 25, 26, and 28) treatment with heparinase III. The serum samples in lanes 1 and 2, 3 and 4, 16 and 17, and 18 and 19 are from representative patients with primary open-angle glaucoma; lanes 5 and 6, 7 and 8, 9 and 10, 20 and 21, 22 and 23, and 24 and 25, from patients with normal-pressure glaucoma; and lanes 14 and 15 and 27 and 28, from control subjects. Immunoreactive bands detected using serum samples from patients with glaucoma against enzymatically treated human optic nerve head homogenates were in the same region as those seen using monoclonal antibodies to glycosaminoglycans. Secondary antibody (goat antihuman IgG or goat antimouse IgG) dilution, 1:2000.

Enzyme-Linked Immunosorbent Assay Results for Immunoreactivities to Glycosaminoglycans

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chondroitin Sulfate</th>
<th>Heparan Sulfate</th>
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<tbody>
<tr>
<td>Group*</td>
<td></td>
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<tr>
<td>NPG (n = 60)</td>
<td>1.61 ± 0.48 (1.87)</td>
<td>1.26 ± 0.64 (1.10)</td>
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<tr>
<td>POAG (n = 36)</td>
<td>1.05 ± 0.65 (0.95)</td>
<td>0.81 ± 0.51 (0.61)</td>
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<tr>
<td>Control (n = 20)</td>
<td>0.87 ± 0.64 (0.81)</td>
<td>0.58 ± 0.33 (0.64)</td>
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<tr>
<td>P†</td>
<td></td>
<td></td>
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<tr>
<td>NPG vs POAG</td>
<td>.009</td>
<td>.04</td>
</tr>
<tr>
<td>NPG vs control</td>
<td>&lt;.001</td>
<td>.003</td>
</tr>
<tr>
<td>POAG vs control</td>
<td>.49</td>
<td>.46</td>
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*Data are expressed as mean ± SD (median) optical density. NPG indicates normal-pressure glaucoma; POAG, primary open-angle glaucoma. †Mann-Whitney U test.
The density of the immunostaining for fate was also increased in eyes with glaucoma compared with control eyes. The immunostaining for chondroitin 6-sulfate and heparan sulfate was more intense and confluent in eyes with glaucoma. The immunostaining patterns of glycosaminoglycans in the lamina cribrosa of postmortem glaucomatous eyes, particularly in the eyes of patients with normal-pressure glaucoma. We propose that, in some patients, these findings may explain in part why optic nerve head cupping accompanies glaucomatous optic neuropathy in either the absence or the presence of elevated intraocular pressure.

Glycosaminoglycans have an important role in the construction of the tissues because of their organizational and space-filling functions. In addition, they are essential for various cell functions and in the interaction between cells and their environment. They take part in various biological processes, such as adhesion, migration, proliferation, differentiation, and intercellular transport of the cells. Glycosaminoglycan macromolecules are characterized by a strong polyanionic charge and hydrophilia that is due to multiple carboxyl residues of uronic acids (except for keratan sulfate, where the uronic acid molecule is replaced by galactose) as well as an ester-bound monosulfation or polysulfation, with the exception of hyaluronic acid that is unsulfated. These biopolymers have been identified in the human optic nerve head, except keratan sulfate and hyaluronic acid, the latter being observed only in the cavernous spaces of Schwalbe optic atrophy.

The biological properties of glycosaminoglycans make them well suited for the specialized requirements of the lamina cribrosa. Chondroitin sulfate proteoglycans help assemble and hold together the components of the extracellular matrix with the correct spacing that is essential for tensile strength in certain tissues such as cartilage and tendon. Chondroitin sulfate and dermatan sulfate proteoglycans may similarly contribute to the biomechanical properties of the lamina cribrosa in which they fill the loose space in the laminar beams to serve as a compression absorber, allowing reversible deformation in the tissue. Furthermore, since the water-holding capacity of chondroitin 6-sulfate is higher than that of chondroitin 4-sulfate, the proportions of these glycosaminoglycans within a tissue determine the degree of hydration and consequently the rigidity of the tissue. Since they also inhibit neurite outgrowth in vitro and in vivo, an increase in these extracellular matrix proteins within the optic nerve head may affect the ability of injured retinal ganglion cells to repair axolemmal dam-
Heparan sulfate proteoglycans are associated with the basal laminae of glial cells and blood vessels and play an important role in physiological characteristics of filtration, such as in the renal glomerular basement membrane. In addition, they also have diverse functions in the nervous system and have been shown to bind various growth factors and take part in cell-cell and cell-matrix adhesions.

It has been suggested that the ability of the optic nerve head to withstand pressure decreases with increasing age due to age-related differences in the proportion of various components of glycosaminoglycans. Indeed, alterations in proteoglycan content and distribution have been observed in the optic nerve heads of glaucomatous eyes with elevated intraocular pressure. These changes of the optic nerve head may be due to a direct effect of elevated intraocular pressure. Alternatively, they may reflect secondary changes that accompany the destruction of collagen bundles and thus represent cellular healing responses to intraocular pressure elevation, mechanical forces, or neural damage. Although we observed increased immunostaining for glycosaminoglycans in eyes with either primary open-angle or normal-pressure glaucoma, it remains unanswered whether these findings signify an accumulation of glycosaminoglycans in the glaucomatous optic nerve heads we examined. For example, increased immunostaining may represent a response of the activated glial cells of the lamina cribrosa by different stress factors that increase the release or exposure of glycosaminoglycans. These would subsequently act as an antigen in a secondary immune reaction to induce the production of serum antibodies that recognize glycosaminoglycans.

Whether viewed as increased autoantibodies to optic nerve head glycosaminoglycans, or simply a cross-reaction of the elevated serum antibodies, it seems quite feasible that the antibodies that bind to glycosaminoglycans may change the organization and physical characteristics of the optic nerve head tissue by some modifications in the shape, size, length, level of sulfation, anionic sites, or water content of glycosaminoglycans, thereby increasing the susceptibility of the optic nerve head to damage. By changing the microenvironment surrounding injured axons, alterations in glycosaminoglycan composition in the lamina cribrosa of some patients may account for the increased vulnerability of remaining axons to sustain further damage regardless of intraocular pressure. Alternatively, since glycosaminoglycan molecules...
are in close contact with the other components of the extracellular matrix, including collagen and elastin networks, involved in their formation, and hold them with correct spacing. Alterations in the optic nerve head glycosaminoglycans may also affect the compliance of the optic nerve head. For example, curling of the elastin fibers as seen in the glaucomatous optic nerve head might be secondary to their disconnection from altered glycosaminoglycans within the connective tissue matrix, which may account for the cupping even in the absence of elevated intraocular pressure. We, therefore, suggest that serum antibodies against optic nerve head glycosaminoglycans, in part, may underlie the glaucomatous damage that occurs in patients in whom such antibodies are present, most often those with normal-pressure glaucoma.

This hypothesis is supported by findings in which alterations in the distribution patterns, composition, and functional properties of the glycosaminoglycans secondary to cross-reactivity of serum autoantibodies with glycosaminoglycans mediate specific tissue injury in several autoimmune diseases. Negatively charged sulfate and carboxyl groups of glycosaminoglycans are thought to play a role in the immunodominant site. For example, in systemic lupus erythematosus, the neutralization of negatively charged heparan sulfate in the glomerular basement membrane by cross-reactive anti-
DNA antibodies alters the charge and size selective barrier function in the renal glomerulus and leads to proteinuria. Glycosaminoglycans not only appear to play a major role in the pathogenesis of autoimmune diseases but have been considered as an activity marker of the disease. In several autoimmune-mediated diseases, including systemic lupus erythematosus, diabetes mellitus, thyroid disease, and scleroderma, elevated production and local accumulation of collagen and glycosaminoglycan in the affected tissues has been reported. A similar cross-reactivity to optic nerve head glycosaminoglycans may occur in persons who have abnormal serum antibodies and may render an increased susceptibility for glaucomatous damage regardless of the intraocular pressure level. Therefore, our findings may not have only pathogenic significance but also may indicate an important serum marker for the increased susceptibility to glaucomatous optic neuropathy. 

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