Identification of Glycosaminoglycans in Age-Related Macular Deposits

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We investigated the presence and localization of glycosaminoglycans in basal laminar deposit and drusen in age-related maculopathy. Conventional histological staining techniques and monoclonal antibodies specific for several glycosaminoglycans were used on paraffin-embedded human maculae. Furthermore, macular homogenates were analyzed with two-dimensional electrophoresis. Quantitative analysis of glycosaminoglycans was done spectrophotometrically using dimethylmethylene blue. Immunohistochemically, all basal laminar deposit stained positive for chondroitin 4-sulfate and focally positive for heparan sulfate proteoglycan. Drusen were not stained with any of the monoclonal antibodies. With two-dimensional electrophoresis, it was demonstrated that macular extracts with and without age-related maculopathy contained chondroitin sulfate. Heparan sulfate was only expressed in maculae with age-related maculopathy. The total amount of glycosaminoglycans was significantly higher in maculae with basal laminar deposit than in maculae without basal laminar deposit ($P=.001$). There were significant differences in the amount and composition of glycosaminoglycans between maculae with and without age-related maculopathy. *Arch Ophthalmol.* 1996;114:1009-1014

The late stages of age-related maculopathy (ARM) can severely affect central vision and are the main cause of blindness in the elderly in the Western World.¹⁻³ Information on the pathogenesis of this disease is still incomplete.⁴⁻⁸ Recently, a classification system for ARM has been proposed for use in epidemiologic studies.⁹ However, this classification does not cover the many histopathologic changes in the aging human macula.^{8,10-13}

Drusen and basal laminar deposit (BLD) are both extracellular deposits that accumulate in ARM. Hard, nodular drusen are located external to the basement membrane of the retinal pigment epithelium (RPE). Soft drusen and confluent drusen are located between the basement membrane of the RPE and the remainder of Bruch's membrane or external to a diffuse thickening of the inner aspect of

Bruch's membrane. Basal laminar deposit is located between the cell membrane of the RPE and its basement membrane.^{8,10} These macular deposits are assumed to be important determinants in the pathogenesis of the late stages of ARM, but their origin and chemical composition are mostly unknown.^{6,14-18} In previous studies, we have demonstrated that BLD consists partly of basement membrane material adjacent to the RPE¹⁹ and contains carbohydrate structures, most likely as components of glycoproteins or complex carbohydrate molecules.^{18,20} Glycosaminoglycans (GAGs) are large carbohydrate molecules of repeating disaccharides, mostly linked to proteins to form proteoglycans. Proteoglycans occur in the extracellular matrix and on cell surfaces. On cell surfaces, they function in cell adhesion. In the extracellular matrix, an important function of the hydrophilic proteoglycans is to retain water molecules and thus to provide tissue turgor. Proteoglycans function in that capacity in the maintenance of the vitreous.²¹ Proteoglycans also pro-

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vide an environment favorable for cell migration, as in angiogenesis.^{22,23} Matrix growth factors, such as fibroblast growth factor, platelet-derived growth factor, and transforming growth factor, are bound to proteoglycans, especially heparan sulfate proteoglycan. Therefore, GAGs may play a role in the development of neovascular growth in the late stages of ARM.

The purpose of this study was to investigate the presence and localization of GAGs in human maculae with and without early stages of ARM, especially in eyes with drusen and BLD.

MATERIALS AND METHODS

PREPARATION OF HUMAN MACULAR TISSUE

We used 25 fresh human eye-bank eyes from 17 subjects between 56 and 94 years of age (mean age, 82 years), with postmortem intervals between 5 and 14 hours (mean, 9 hours). Each macular specimen (1 cm²) was divided into two equal parts by cutting along its horizontal diameter. In one half, the choroid and retina together were separated from the sclera and vitreous, frozen, and stored in liquid nitrogen until further use. The other half was fixed in phosphate-buffered formaldehyde solution (4% vol/vol, pH 7.4) and embedded in paraffin for histopathological classification and immunohistochemical testing.

HISTOPATHOLOGICAL CLASSIFICATION OF HUMAN MACULAR TISSUE

Paraffin sections (5 μ m) were stained with hematoxylin-eosin, the periodic acid-Schiff reaction, and Mallory staining. The maculae were histologically classified according to their gradation of BLD and drusen as follows: no BLD, class 0; small, solitary patches of BLD, class 1; a thin, continuous layer of BLD, class 2; and a thick layer of BLD of at least half the height of the RPE cells, class 3; no drusen, class 0; one to three drusen, class 1; four to 10 drusen, class 2; and many or confluent drusen, class 3.¹³ Since limited histologic changes are present in maculae with BLD class 0 or class 1 and

in maculae with drusen class 0 or class 1, these eyes were categorized as control samples. All other maculae were classified as samples with ARM. No eyes with histologic geographic atrophy or neovascularization were included.

HISTOCHEMICAL EXAMINATION

Alcian blue staining at different acid concentrations (pH 0.2, 1.0, 2.5, and 3.2) and Hale's colloidal iron staining as modified according to Müller were used to identify GAGs. To obtain more specific staining of the distinct GAGs with their different critical electrolyte concentrations,²⁴ alcian blue staining was performed at pH 5.8 with increasing magnesium chloride concentrations (0.06, 0.1, 0.3, 0.7, and 1.0 mol/L).

IMMUNOHISTOCHEMICAL EXAMINATION

Paraffin sections (5 μ m) of all maculae were mounted on glass slides coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co, St Louis, Mo). After deparaffinization and rehydration, the slides were rinsed with water and phosphate-buffered saline. The slides were then placed in a Sequenza Immunostaining Workstation (Life Sciences International, Velthoven, the Netherlands) and incubated for 30 minutes with a mouse monoclonal antibody directed against chondroitin 4-sulfate, basement membrane heparan sulfate proteoglycan, or keratan sulfate disaccharides. These antibodies were obtained commercially (Chemicon International, Temecula, Calif) and were used at dilutions of 1:400, 1:10, and 1:3200, respectively. After the slides were washed with phosphate-buffered saline, they were incubated for 30 minutes with biotinylated secondary antibodies (multilink, 1:100 dilution, BioGenex Laboratories, San Ramon, Calif). The slides were washed again and incubated for 30 minutes with alkaline phosphatase-conjugated streptavidin (1:50 dilution, BioGenex Laboratories). Finally, the slides were rinsed with 0.2-mol/L TRIS hydrochloride, pH 8.0, and stained for 30 minutes with

0.3% new fuchsin/TRIS hydrochloride (Sigma Chemical Co). The sections were counterstained with Mayer's hematoxylin. Prior to incubation with anti-chondroitin 4-sulfate and with anti-heparan sulfate proteoglycan antibodies, the sections were treated with 0.1-U/mL chondroitinase ABC (Sigma Chemical Co) for 2 hours at 37°C and with 5.5-mU/mL pronase E (Sigma Chemical Co) for 10 minutes at 37°C, respectively.

In negative controls, the primary antibodies were replaced by normal mouse serum (1:10 dilution, Dako Corp, Glostrup, Denmark). As a second negative control for anti-chondroitin 4-sulfate antibodies, chondroitinase ABC digestion was omitted prior to the incubation with the primary antibody. Photoreceptors and perineurium were used as internal positive controls for chondroitin 4-sulfate and keratan sulfate, respectively. Basement membranes in human skin tissue were used as a positive control for heparan sulfate proteoglycan monoclonal antibodies.

TWO-DIMENSIONAL ELECTROPHORESIS AND QUANTITATIVE ANALYSIS OF GAGS

The choroid/retina preparations frozen in liquid nitrogen were defrosted and homogenized on ice in 0.5 mL of Milli-Q water (Millipore Corp, Etten-Leur, the Netherlands). The main part (450 μ L) of these homogenates was acidified with one drop of 1N hydrochloric acid and subsequently added to 40 mL of 0.05% alcian blue in 0.05-mol/L sodium acetate buffer (pH 5.8). Subsequently, GAGs were isolated according to the method of Mossman and Patrick (1982).²⁵ These isolated GAGs were used for two-dimensional electrophoresis on cellulose acetate membranes according to the method of Abeling et al (1974).²⁶ Quantitative analysis of GAGs was done on the homogenates, using dimethylmethylene blue as described previously for measuring GAGs in urine.²⁷ Furthermore, a protein assay (bicinchoninic acid, Pierce, Rockford, Ill) was performed.

Figure 1. Section of a human macula with age-related maculopathy (age 86 years), stained with alcian blue. There is slight blue staining in the basal laminar deposit (large arrows) (original magnification $\times 400$). rpe indicates retinal pigment epithelium; bm, Bruch's membrane.

Figure 2. Sections of human maculae with age-related maculopathy (age 81 years), stained with Hale's colloidal iron staining (original magnification $\times 400$). Top, The basal laminar deposit (arrows) is stained blue. Bottom, Both the small nodular drusen and the larger confluent drusen are stained blue (arrows).

Student's *t* test was used to compare the amounts of GAGs per milligram of protein between ARM maculae and control maculae. To avoid bias, only one eye of a single donor was used in this analysis.

RESULTS

HISTOPATHOLOGICAL CLASSIFICATION OF HUMAN MACULAR TISSUE

Twenty maculae were classified as having ARM. Five maculae had no ARM and were used as controls.

HISTOCHEMICAL FINDINGS

Alcian blue at pH 0.2 stained BLD blue (**Figure 1**). As the pH increased to 1.0 and 2.0, the staining decreased gradually, and there was no staining at pH 3.2. With increasing magnesium chloride concentrations, alcian blue staining of BLD showed reduced intensity at a critical electrolyte concentration of 0.7 mol/L; however, weak staining persisted at a concentration of 1.0

mol/L. Hale's colloidal iron stained BLD blue to purple (**Figure 2**, top). Hyaluronidase treatment markedly reduced the staining intensity of BLD, although some staining persisted. In contrast with BLD, drusen were not stained by alcian blue, but they were stained by Hale's colloidal iron (**Figure 2**, bottom). There was no staining of drusen after treatment with hyaluronidase.

IMMUNOHISTOCHEMICAL FINDINGS

Specific staining with a monoclonal antibody against chondroitin 4-sulfate was found diffusely in BLD (**Figure 3**, top left). The choroid, the outer collagenous zone of Bruch's membrane (focally), the apical side of the RPE, and the photoreceptor outer segments were also stained with this antibody. With heparan sulfate proteoglycan immunostaining, BLD was stained focally positive adjacent to the RPE in two of four maculae with BLD class 3 (**Figure 3**, top right). Since the BLD in these two maculae was stained

only at places overlying a soft drusen, there might be basal linear deposit (diffuse thickening of the inner aspect of Bruch's membrane) at these places as well, because it is difficult to discriminate between BLD and basal linear deposit.¹⁰ The monoclonal antibody against keratan sulfate revealed specific staining in the outer collagenous zone of Bruch's membrane but not in BLD (**Figure 3**, bottom left). Drusen were not stained with these immunohistochemical techniques, except for a large, confluent drusen in one macula that reacted with the antibody directed against keratan sulfate (**Figure 3**, bottom right). These immunohistochemical staining patterns are summarized in **Table 1**. The several subtypes of drusen are not specified in this table because only one confluent drusen showed positive staining.

Negative controls showed no staining except for weak to moderate staining in the choroid after incubation with the monoclonal antibody directed against chondroitin 4-sulfate without prior treatment with chondroitinase ABC. The internal and external positive controls stained positive.

TWO-DIMENSIONAL ELECTROPHORESIS AND QUANTITATIVE ANALYSIS OF GAGS

Seven human maculae with ARM (mean age, 83 years) and four controls (mean age, 71 years) were used for two-dimensional electrophoresis and quantitative analysis of GAGs (**Table 2**). Chondroitin sulfate was demonstrated in all these maculae (**Figure 4**). In five of the seven maculae with ARM, heparan sulfate was also detected (**Figure 4**, right). In two controls without BLD or drusen, no heparan sulfate was detected. In the two controls with BLD class 1 (cases 10 and 11, **Table 2**), relatively small amounts of heparan sulfate were detected along with chondroitin sulfate. On the other hand, one of the two maculae with ARM that showed no heparan sulfate had drusen class 3 (case 1, **Table 2**) but no BLD. Other GAGs (hyaluronic acid, dermatan sulfate, and keratan sulfate) could not be de-

tected in the macular extracts with two-dimensional electrophoresis. Discrimination between chondroitin 4-sulfate and chondroitin 6-sulfate is not possible with this technique.

The results of quantitative analysis of the human macular homogenates are summarized in Table 2. The average amount was 15.1 μg of GAG per milligram of protein in macular extracts with ARM, compared with 12.4 μg of GAG per milligram of protein in extracts of control maculae. This difference was not statistically significant ($P=.06$). However, the difference in the average amount of GAG per milligram of protein between macular extracts with BLD class 2 and class 3 (16.2 $\mu\text{g}/\text{mg}$, $n=5$) and macular extracts with BLD class 0 and class 1 (12.4

$\mu\text{g}/\text{mg}$, $n=6$) was highly statistically significant ($P=.001$; SEM, 0.41) (**Figure 5**). There was no association between the total amount of GAG per milligram of protein and drusen.

COMMENT

IMMUNOHISTOCHEMICAL FINDINGS

Chondroitin 4-sulfate was immunohistochemically demonstrated in BLD; alcian blue staining disappeared at a critical electrolyte concentration of 0.7-mol/L magnesium chloride. It is possible that deposition of chondroitin 4-sulfate in BLD and in the outer collagenous zone of Bruch's membrane is one reason for the reduced hydrau-

lic conductivity of Bruch's membrane reported in aging eyes,^{12,14,28} given the hydrophilic nature and negative charges of chondroitin sulfates. On the other hand, chondroitin 4-sulfate deposition at these sites could have been caused by the reduced hydraulic conductivity in Bruch's membrane. Since there is no BLD in normal eyes and since chondroitin sulfates are not broken down in the extracellular matrix,²⁹ it is possible that these GAGs are normally transported through Bruch's membrane to the choriocapillaris.

Nodular drusen, confluent drusen (but not soft drusen), and the intercapillary pillars of thickened Bruch's membranes were stained strongly with Hale's colloidal iron staining, which means that they contained GAGs. This staining totally disappeared after treatment with hyaluronidase. However, there was no staining with any of the monoclonal antibodies or with alcian blue (except for one confluent drusen that was stained with antibodies directed to keratan sulfate). These data suggest that nodular drusen and confluent drusen as well as thickened Bruch's membrane contain hyaluronic acid. Hyaluronic acid is also a GAG and is normally present in the extracellular matrix.

The purple color of BLD with Hale's colloidal iron staining and the persisting weak staining of BLD after alcian blue staining with 1.0-mol/L magnesium chloride could be caused by the presence of a collagenous component²⁴ in BLD, eg, long-spacing collagen. This would also explain the remaining weak staining of

Figure 3. Sections of human maculae with age-related maculopathy, stained with monoclonal antibodies (new fuchsin, original magnification $\times 400$). Top left, The monoclonal antibody was directed against chondroitin 4-sulfate; the basal laminar deposit (arrows) is stained positive (red). The patient was 94 years old. Top right, The monoclonal antibody was directed against heparan sulfate; the basal laminar deposit (arrows) is stained positive (red). The patient was 81 years old. Bottom left, The monoclonal antibody was directed against keratan sulfate; the outer collagenous zone of Bruch's membrane is stained positive (red). The basal laminar deposit (arrows) is not stained. The patient was 83 years old. Bottom right, The monoclonal antibody was directed against keratan sulfate; a large, confluent drusen (arrows) is stained positive (red). The patient was 88 years old.

Table 1. Immunohistochemical Staining Patterns of Human Maculae*

Glycosaminoglycans	Macula Type	Basal Laminar Deposit†	Drusen‡	Bruch's Membrane		Retinal Pigment Epithelium	Choroid
				Outer Collagenous Zone	Inner Collagenous Zone		
Chondroitin 4-sulfate	ARM	+ (19/19)	—	—	—	+ (20/20)	+ (20/20)
	Control	+ (3/3)	—	—	—	+ (3/5)	+ (5/5)
Keratan sulfate	ARM	—	+ (1/8)§	+ (20/20)	+ (2/20)	+ (3/20)	+ (16/20)
	Control	—	—	+ (5/5)	—	+ (2/5)	+ (4/5)
Heparan sulfate	ARM	+ (2/19)	—	—	—	—	—
	Control	—	—	—	—	—	—

*ARM indicates age-related maculopathy; plus sign, moderate to strong staining; and minus sign, no staining.

†Nineteen of 20 maculae with ARM had basal laminar deposit, and three of five controls had class 1 basal laminar deposit.

‡Eight of 20 maculae with ARM had drusen, and one of five controls had class 1 drusen.

§One confluent drusen was stained.

Table 2. Histopathological Classification of Human Maculae and Results of Quantitative Analysis of GAGs*

Case No./ Age, y	Drusen Class†	BLD Class†	Macula Type	GAGs, μg/mg of Protein
1/79	3	0	ARM	12.5
2/75	3	1	ARM	12.2
3/86	0	2	ARM	15.0
4/81	0	2	ARM	17.2
5/81	2	2	ARM	16.3
6/94	0	3	ARM	14.1
7/86	2	3	ARM	18.2
8/71	0	0	Control	10.5
9/56	0	0	Control	12.4
10/70	0	1	Control	13.2
11/86	1	1	Control	13.6

*GAGs indicates glycosaminoglycans; BLD, basal laminar deposit; and ARM, age-related maculopathy. All 11 cases were used for two-dimensional electrophoresis.

†See the "Materials and Methods" section for explanations of the histopathological classifications.

Figure 4. Cellulose acetate plates after two-dimensional electrophoresis with (left) a control human macular homogenate (age 71 years), and (right) a human macular homogenate with age-related maculopathy (age 81 years). On the plate that has homogenate with age-related maculopathy, there is a second spot (heparan sulfate [hs]) under the large spot of chondroitin sulfate (cs). Plus sign indicates positive electrode; minus sign, negative electrode.

BLD after hyaluronidase digestion. Except for a small rim of collagen type IV adjacent to the RPE, no special collagen types have been demonstrated in BLD.^{19,30}

TWO-DIMENSIONAL ELECTROPHORESIS AND QUANTITATIVE ANALYSIS OF GAGS

With two-dimensional electrophoresis, we found chondroitin sulfate and heparan sulfate in extracts of human maculae with BLD. The significant quantitative increase in total GAGs in these macular extracts could be attributed to the presence of chondroitin sulfate and heparan sulfate in BLD. However, GAGs (in particular, chondroitin sulfate) are also abundantly present in the normal extracellular matrix and in the

interphotoreceptor matrix.²¹ Since the amount of BLD in the macular extracts was small in comparison with the total amount of tissue, it is possible that these GAGs in extracts of maculae with BLD are also derived from another part of the macula related to BLD. It has been demonstrated that synthesis of heparan sulfate and chondroitin sulfate in the inner retinal cells is increased following photoreceptor degeneration,³¹ a pathologic feature also occurring in ARM. Furthermore, an increased proportion of heparan sulfate proteoglycan has also been shown in Bruch's membrane in several retinal diseases, including ARM.³² In contrast to others,³³ we could demonstrate heparan sulfate proteoglycan neither in Bruch's membrane nor in drusen. This can be attributed to methodological dif-

Figure 5. Relationship between the quantitatively measured glycosaminoglycans (GAGs) and the class of basal laminar deposit (see the "Materials and Methods" section for definitions) ($n=11$). The increase in mean GAGs (horizontal lines) between maculae with basal laminar deposit class 0 and class 1 and maculae with basal laminar deposit class 2 and class 3 is statistically significant ($P=0.001$).

ferences. Newsome et al³³ used a different antibody and used cryostat sections instead of paraffin sections. Although formaldehyde fixation alters the molecules, the antibodies we used in this study were specifically made for these fixed molecules. However, the long fixation time of our tissue samples (up to 2 weeks) could have caused loss of the heparan sulfate antigen, which could also explain the absence of reaction in the RPE basement membrane.

It has been demonstrated that chondroitin sulfates induce epiretinal membranes.³⁴ They may also be involved in the pathogenesis of intravitreal membranes in proliferative vitreoretinopathy.³⁵ In the same way, GAGs could play a role in the development of subretinal neovascular membranes occurring in the late stages of ARM, as GAGs are very suitable for the growth of new blood vessels, and GAGs enhance the biological activity of growth factors.^{22,23,35} Higher levels of fibroblast growth factor and transforming growth factor have been reported in choroidal neovascular membranes³⁶; therefore, the accumulation of GAGs in BLD might play a role in the development of subretinal neovascularization. This could also explain the clinical observation that subretinal neovascular growth in ARM is mostly directed toward the macula, where BLD is most prominent.¹⁰

In conclusion, we have demonstrated that chondroitin 4-sul-

fate and heparan sulfate proteoglycan are present in BLD and that human macular extracts with BLD contain a significantly higher amount of GAGs than macular extracts without BLD. The deposition of these GAGs in BLD could play a role in the pathogenesis of subretinal neovascularization in the late stages of ARM.

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