Fluorescence-Labeled Lectins, Glycoconjugates, and the Development of the Mouse AOP

Terry L. Vanden Hoek, William Goossens, and Paul A. Knepper

The development of the aqueous outflow pathway (AOP) in early postnatal mouse eyes was examined for the presence of a variety of lectin receptors using fluorescein isothiocyanate (FITC) conjugated lectins, 1 μm araldite plastic sections, and computer-aided fluorescence photography. The trabecular meshwork anlage (days 1–4) was characterized by the presence of loosely arranged cells and an extracellular matrix that exhibited intense areas of Con A- and RCA-lectin staining, and absence of WGA- and LPA-lectin staining. By day 6, trabecular meshwork LPA- and WGA-positive materials were observed as focal areas of staining. By day 10, LPA- and WGA-positive materials were present as diffuse areas of staining, as the AOP differentiated into an organized and functional biological filter. The age-dependent pattern of LPA- and WGA-positive materials indicated that there were time-dependent points in the synthesis of glycoconjugates in the developing AOP. The results suggest: (1) The composition and/or conformation of the glycoconjugates on cells and extracellular matrix changed as the AOP differentiated into a functional tissue. (2) The use of FITC lectins as biological markers for studies of the AOP provided information on the potential role of glycoconjugates in the development of the normal AOP. (3) Modification in the type, amount, and distribution of glycoconjugates may provide a basis for understanding the cellular mechanisms of abnormal development of the AOP, eg, congenital glaucoma.


The morphogenesis of the structures of the aqueous outflow pathway (AOP), ie, the trabecular meshwork (TM) and the aqueous plexus, requires a series of complex cellular events that depend on precise timing and interaction between the cells and their extracellular matrix.1 Complex carbohydrates, ie, glycoconjugates, on the cell surface provide some of the required signals for cells to migrate, develop, and mature into an organized tissue.2–4 Carbohydrate residues of glycoconjugates in tissues can be localized by specific techniques using lectin-sugar interactions. The use of lectins is often problematic, and careful controls (type and length of fixation, embedding medium for tissue sections, and preservation of lectin staining) are required for analysis of the lectin staining patterns.5 In addition, under customary fluorescent excitation conditions, the fluorescence of fluorescein isothiocyanate (FITC) conjugated lectins fades quickly.5

This study presents a comparison of lectin staining patterns in the development of the mouse AOP using newer methods: FITC-lectin staining of 1 μm pre-treated araldite sections, p-phenylenediamine to prevent fluorescence fading, and image processing to evaluate the distribution of FITC-lectin staining of the cells and extracellular matrix. The development periods of the mouse AOP are: (1) anlage formation (prenatal to day 6 postnatal), (2) differentiation into definite and functional structures (days 6–14), and (3) maturation of structures (days 12–life). The results suggest that the composition and/or conformation of the carbohydrate components change as the AOP differentiates into a functional tissue.

Materials and Methods

The following FITC lectins were purchased from E·Y Laboratories, Inc. (San Mateo, CA): Concanavalia ensiformis (Con A), Triticum vulgaris agglutinin (WGA), Ricinus communis agglutinin (RCA-120), and Limulus polyphemus agglutinin (LPA). The manufacturer's specifications for the FITC concentration relative to protein concentration were: Con A, 1.48; WGA, 1.69; RCA-120, 1.26; and LPA, 1.69. Haptene sugar inhibitors used were D-glucose, α-D-methyl manno-pyranoside, D-galactose, mucin, and N,N',N"-triacetyltchiotriose (Sigma Chemical Company, St. Louis, MO). Para-phenylenediamine (PPD) was purchased from Fisher Scientific Company (Fair Lawn, NJ).
Tissue Preparation

Normal C57BL/6J mouse eyes were fixed on postnatal days 1, 4, 6, 8, 10, and 14 for a total of 3 hr by 15 min vascular perfusion with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M cacodylate buffer, pH 7.2, and immersion of the anterior globe, including the lens. After fixation, the tissue was rinsed in the same buffer for 12 hr and dehydrated in a series of ethanol. Embedding in araldite was done according to routine protocols. One μm sections were cut with glass knives and placed on ethanol-cleaned glass slides. Control sections were stained with Richardson's stain. All procedures used in this study conformed to the ARVO Resolution on the Use of Animals in Research.

Lectin Staining Protocol

To enhance the FITC-lectin staining, 1 μm araldite sections were pretreated with freshly prepared 5% sodium hydroxide in ethanol:propylene oxide (vol/vol). The sections were preincubated in a 14 mM phosphate buffered saline (PBS) for 5 min and then digested for 5 min in 0.05% trypsin-14 mM PBS solution at room temperature. Sections were then washed in 14 mM PBS, water, and allowed to air dry. Slides were incubated with 20 μl of FITC lectins for 10 hr at room temperature in a moist chamber. Con A was diluted in 15 mM sodium chloride containing 5 mM Tris, 1 mM calcium chloride, and 1 mM manganese chloride, pH 7.0, to give a staining concentration of 0.1 mg/ml. The other FITC lectins, WGA, RCA-120, and LPA, were diluted in 10 mM PBS, pH 7.45, to give a staining concentration of 0.2 mg/ml. The specificity of FITC-lectin staining was determined by a comparison of the concentration range of 10^-8 M to 10^-3 M. All measurements were made with a planapochromatic 40X/1.0 oil immersion objective.

The rate and amount of fluorescent fading was evaluated by the use of a computer-controlled spectrophotometer (Zonax®, Carl Zeiss, Inc., Thornwood, NY). The fluorescence fading rate^10 per time is expressed by:

$$FR_t = \frac{1 - (Fl - I_0)}{(Fl - I_0)} \times 100$$

where $FR_t =$ percent of loss of fluorescence fading during time t, $Fl - I_0 =$ fluorescence intensity after time t, and $Fl - I_0 =$ initial fluorescence intensity.

The optimum pH and concentration of PPD^11 were determined by measuring the fluorescence fading of FITC-Con A staining of the lens capsules during a 3 min exposure to exciting light with a wide-open diaphragm. The loss of fluorescence was 0.3% (pH 9.0, 0.20%); 42.0% (pH 9.1, 0.01%); and 87.6% (pH 7.2, 0%). In addition, the effects of storage and temperature conditions were determined by measuring the fluorescence fading during a 1 min exposure to exciting light with a semi-closed diaphragm, which was used for the analyses of all tissue sections. The loss of fluorescence was 0.5% (10 days, -10°C), 14.4% (30 days, -10°C), 20.4% (10 days, 20°C), and 46.4% (30 days, 20°C). Consequently, all measurements of fluorescence intensity and image processing were made with the lowest excitation light level, and FITC-stained sections were analyzed within 10 days of staining in a mounting medium of 90% glycerol-10% Tris containing 0.2% PPD, pH 9.0.

Evaluation of Lectin Staining

After incubation with the FITC lectins, the sections were washed with the same buffer which was used to dissolve the lectin; mounted with 90% glycerol-10% Tris containing 0.2% PPD, pH 9.0, to prevent fading of the FITC lectins; and coverslips were applied. The sections were sealed with nail polish and stored at -10°C. The slides were examined on a Zeiss Photomicroscope II (Carl Zeiss, Inc., Thornwood, NY) equipped for epi-illumination (excitation filter BP 550-590 and emission filter 520), and an Osram HBO 100-W (Osram GmbH., Berlin-Munich, West Germany) high-pressure mercury lamp was used for fluorescence microscopy.

Results

The primitive AOP of a day 1 eye of a postnatal mouse was primarily composed of a triangular mass of loosely arranged cells (Fig. 1A). The angle was covered by a continuous endothelium from the periphery of the corneal endothelium to the anterior border of the iris. By day 6, the AOP was organized into a lamellar pattern, although the base of the angle consisted of a block of three to five cells that was covered by a persistent endothelial lining (Fig. 1B). By day 10, the morphological appearance of the AOP had changed. Spaces were present between the trabecular lamellae, collagenous material was present within the AOP, and
there was a well-defined collecting channel, the aqueous plexus (Fig. 1C).

FITC-Con A, which reacts with α-D-mannosyl and α-D-glucosyl residues, stained the TM at all time points. The anlage of the TM contained patches of intense Con A fluorescence (Figs. 2A, 2B). At days 6 and 8, the patches were fewer and less intense, displaying weak to moderate binding (Fig. 2C), whereas by day 10, the TM was diffusely stained, having a laminated appearance that included localized spots of intense staining. FITC-Con A binding to the corneal and scleral stroma, Descemet's membrane, and the lens capsule was strong. The staining of the basement membrane of the ciliary epithelium was weak to moderate on days 1 through 10 (see Figs. 2A–2D), whereas the staining of the Bowman's membrane was moderate at day 10 (Fig. 2D).

The intensity of fluorescence obtained with FITC-RCA-120, specific for β-D-galactosyl residues, showed a diffuse staining of the AOP. The staining intensity of the TM was strong at all time points (Figs. 3A–3D). In contrast to the staining pattern observed with Con A, RCA-120 labeled the cell membranes of the endothelium of the TM (Figs. 3A–3C). By day 10, the TM was diffusely stained, having a laminated staining pattern. There was weaker RCA-120 binding to the cell membranes (Fig. 3D). Similarly, the ciliary epithelium (both the cell membranes and the organelles) was moderately stained during days 1 and 4, and more weakly stained by day 10 (Fig. 3D). RCA-120 staining of the corneal and scleral stroma, Descemet's membrane, and the lens capsule was moderate to strong at all time points. The staining of the basement membrane of the ciliary epithelium was strong on days 1 and 4, and moderate on days 6 and 10 (Figs. 2A–2D).

FITC-WGA, specific for sialyl and β-N-acetylglucosaminosyl residues, stained the TM in an age-dependent pattern. The intensity of the fluorescence was...
absent to weak at the early time points (days 1, 4, and 6) (Fig. 4A), but the TM at day 8 stained weakly to moderately, including a number of bright spots. By day 10, the TM was diffusely stained, having a laminated appearance (Fig. 4B). This pattern of fluorescent staining continued on day 14, with the laminated staining pattern assuming a more corded appearance. WGA staining of the corneal and scleral stroma and the lens capsule bound WGA moderately to strongly on days 1, 10, and 14, but was weak to moderate on days 4, 6, and 8. Staining of the corneal and ciliary body epithelium was moderate at all time points, while binding of WGA by Descemet’s and Bowman’s membranes and the ciliary body was weak or absent.

The staining intensity of fluorescence obtained with FITC-LPA, specific for sialyl residues, also stained the TM in an age-dependent pattern. LPA staining of the TM was absent to weak on days 1, 4 (Fig. 5A), 6 and 8, and was strong on days 10 and 14. By day 10, the TM was diffusely stained with focal areas of intense staining. In addition, LPA staining was present on the cell membranes of the TM (Fig. 5B). At all time points studied, LPA binding to Descemet’s and Bowman’s membranes, the lens capsule, the ciliary body epithelium, and the corneal epithelium was weak. The corneal and scleral stroma binding of LPA was moderate to strong on day 1, moderate on days 4, 6, and 8, and strong on days 10 and 14.

The staining intensity of a day 10 aqueous plexus, the collecting channel, is shown in Figures 6A-6D. All the FITC lectins strongly stained the sclera and the adjacent TM, and moderately stained the endothelium...
Fig. 3. Computer-aided fluorescence micrographs of the development of the AOP after incubation with FITC-RCA-120: (A) day 1; (B) day 4; (C) day 6; (D) day 10. The trabecular meshwork cells and matrix are variably stained on days 1 through 6 and are uniformly stained on day 10. ac = anterior chamber; b = basement membrane of ciliary epithelium; * = aqueous plexus (×650).

of the cells that bulged into the lumen (Fig. 6A). FITC-WGA (Fig. 6B) and FITC-LPA (Fig. 6D) appeared to stain the endothelium more than FITC-Con A or FITC-RCA-120.

The subjective impression of the differences in the staining intensity during differentiation was substantiated by a comparison of the intensities obtained from a digital image of the cells and matrix elements of the AOP and lens capsule (Table 1). A quantitative estimate of the amount of lectin staining was obtained by measuring the percent transmission of fluorescence (0% representing no fluorescence and 100% representing maximum fluorescence) over background (ie, autofluorescence) and control values (ie, competing sugar plus FITC lectin). In all Con-A-, RCA-, and WGA-lectin stained sections, the presence of the appropriate competing sugar inhibited fluorescence; however, LPA-lectin modestly stained the TM, day 10 through day 14, despite the presence of mucin, its specific inhibitor.

Discussion

These findings indicate that receptors for a variety of FITC lectins were present on the cells and extracellular matrix components of the developing mouse AOP. Although the intensity and the distribution of FITC-lectin fluorescence changed as the AOP differentiated into a functional tissue, two considerations must be noted. First, the intensity of fluorescence is dependent on the number of FITC residues attached...
Fig. 4. Computer-aided fluorescence micrographs of the development of the AOP after incubation with FITC-WGA: (A) day 6; (B) day 10. The trabecular meshwork cells and matrix show patchy fluorescence on day 6 and intense fluorescence on day 10. ac = anterior chamber; * = aqueous plexus (X650).

To each lectin molecule, according to the commercial source of the FITC lectins (E. Y Laboratories, Inc.), FITC concentration was similar for each lectin, and thus it was likely that the intensity of fluorescence was independent of the number of FITC residues attached to each lectin. Second, the binding sites for lectins can be masked by the presence of more distal carbohydrate moieties. At this time, there is no information regarding the structure of sugar chains of TM glycoconjugates. Nonetheless, the results of this study suggest that FITC lectins bind sufficiently to 1 μm pretreated araldite sections, and the presence of 0.2% PPD, pH 9.0, allowed for detection of fluorescence without fading by computer-aided image processing and photography.

The anlage of the AOP, which was characterized by the presence of loosely arranged cells and extracellular matrix, exhibited intense areas of Con A- and RCA-120-positive materials that were most likely extracellular matrix elements. For example, collagen and basement membranes contain short carbohydrate chains (glucosyl-galactosyl and/or galactosyl residues) which

Fig. 5. Computer-aided fluorescence micrographs of the development of the AOP after incubation with FITC-LPA: (A) day 4; (B) day 10. Note the intense fluorescence on day 10. ac = anterior chamber (X650).
are Con A and RCA-120 receptors. In the early stage of differentiation of the AOP, no LPA- and minimal WGA-positive materials were observed.

The differentiation of the AOP of the mouse eye into a definite and functional tissue occurred during the time period of days 6 to 10. The AOP was organized into a lamellar pattern, and a collecting channel, the aqueous plexus, was observed. By day 6, LPA- and WGA-positive materials in the TM were observed as focal areas of staining. LPA- and WGA-positive materials were characterized by diffuse areas of staining, having a laminated appearance by day 10. The age-dependent pattern of LPA- and WGA-positive materials in the TM and aqueous plexus suggested that there were time-dependent points in the appearance of glycoconjugates, which have also been described in other differentiating tissues.24,14

RCA-120 staining of cell membranes was observed to decrease in the transition from the anlage stage to the period of differentiation into a functional structure. RCA-120 receptors of endothelium of the TM cells and the cell membranes and cell organelles of the ciliary epithelium were considerably decreased, whereas WGA and LPA receptors of the TM cells were observed to increase.

The decrease in RCA-120 staining of the AOP and ciliary epithelium may indicate further biochemical modifications of cell surface glycoconjugates. Certain

Table 1. Estimate of the amount of lectin staining of TM* and lens capsule of the developing mouse eye

<table>
<thead>
<tr>
<th>FITC-Lectin</th>
<th>Tissue</th>
<th>Age (Day)</th>
<th>1</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>TM</td>
<td></td>
<td>21.6†</td>
<td>23.5</td>
<td>13.0</td>
<td>19.5</td>
<td>20.7</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td>Lens Capsule</td>
<td></td>
<td>44.7</td>
<td>36.8</td>
<td>36.1</td>
<td>36.6</td>
<td>51.8</td>
<td>41.4</td>
</tr>
<tr>
<td>RCA-120</td>
<td>TM</td>
<td></td>
<td>6.0</td>
<td>5.0</td>
<td>3.8</td>
<td>4.0</td>
<td>5.2</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Lens Capsule</td>
<td></td>
<td>4.0</td>
<td>3.9</td>
<td>4.2</td>
<td>3.7</td>
<td>5.2</td>
<td>3.6</td>
</tr>
<tr>
<td>WGA</td>
<td>TM</td>
<td></td>
<td>3.9</td>
<td>1.8</td>
<td>0</td>
<td>1.8</td>
<td>5.2</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Lens Capsule</td>
<td></td>
<td>6.1</td>
<td>6.2</td>
<td>4.8</td>
<td>3.7</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td>LPA</td>
<td>TM</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.4</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Lens Capsule</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Readings were made in duplicate by outlining with an interactive cursor the TM that was stained with the FITC-lectin, with and without the competing sugar.

†Data are expressed as the percent transmission value (0–100) over background plus control values.
differentiating cells are characterized by the induction of specific glycosidases that act to remove and remodel the microenvironment of the tissue. For example, β-galactosidase may be actively removing galactosyl-containing glycoconjugates. Alternatively, the sequence of events leading to cellular differentiation may involve glycosyltransferases, by which sugars are added to the glycoconjugates. The induction of a specific enzyme(s) may represent an important step(s) in the differentiation process of the AOP. Since changes in the cell surface have been implicated in both the type and the timing of the development process, studies of enzymes that alter the cell surface properties, such as β-galactosidase or glycosyltransferases, have taken on increasing importance.

Presently, theories regarding the normal development of the TM (for review, see reference 18) are described anatomically; cleavage within the tissue, perforation and/or removal of a membrane, or tissue atrophy and cell death, all of which are evidence of a remodeling process. The end product of differentiation in the AOP is the generation of a functional biological filter for aqueous humor. In this study, FITC lectins have indicated that development of the AOP of the mouse eye involves a series of specific morphogenetic and biochemical events. Our results suggested: (1) Glycoconjugates on cells and in the extracellular matrix were useful as biological markers of AOP differentiation; and (2) modifications in the type, amount, and distribution of glycoconjugates may provide a basis for understanding the cellular mechanisms of certain defects in the differentiation of the AOP, namely congenital glaucoma.

Key words: trabecular meshwork, glycoconjugates, lectins, fluorescence, p-phenylenediamine, differentiation, video microscopy

Acknowledgments

The authors thank Mr. James W. Sullivan of Eberhardt Instrument Company, Downers Grove, Illinois, for assistance with the video microscopy; and Carol Fabian for preparation of the manuscript.

References