Localisation of $\alpha(2,3)$ and $\alpha(2,6)$ linked terminal sialic acid groups in human trabecular meshwork

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Abstract

Sialic acid specific lectins were used to localise isomers of sialyl glycosides in human trabecular meshwork (TM) at the ultrastructural level. A lectin immunogold method demonstrated that sialic groups were concentrated on the endothelial surface of Schlemm's canal (SC) and in the adjacent juxtacanalicular tissue (JCT). One sialyl glycoside, $\alpha(2,6)$ linked *N*-acetyl neuraminic acid, was present mainly on the luminal aspect of the SC endothelium and in the cytoplasm of the JCT cells. Another, $\alpha(2,3)$ linked *N*-acetyl neuraminic acid, was localised predominantly to the extracellular fibrillar material of the JCT. The existence of a topographical segregation of these two sialyl glycosides within the TM supports the view that highly charged anionic molecules may be of significance in regulating aqueous outflow.

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The trabecular meshwork (TM), Schlemm's canal (SC), and collector channels are involved in the regulation of aqueous outflow and the control of intraocular pressure (IOP). Primary open angle glaucoma (POAG) is considered to arise from a 'functional disturbance' of the outflow system, no pathognomonic morphological features having been identified.¹ Resistance to aqueous outflow within the TM is thought, on the basis of physiological and ultrastructural studies, to occur mainly at the level of the juxtacanalicular tissue (JCT) (also known as the endothelial meshwork or cribriform layer), located beneath the inner wall of the SC and consisting of several layers of endothelial cells surrounded by extracellular matrix.¹² Recently, interest has been revived in the role of hyaluronic acid in the regulation of anterior chamber outflow.³

The cells lining the TM, including those of the JCT, perform a variety of functions including elaboration of extracellular matrix components,^{4,5} and may be important in maintenance of the integrity of the outflow pathway.⁶ The passage of aqueous from the JCT into the canal of Schlemm is generally thought to occur by an intracellular route through 'giant vacuoles' found in the inner wall endothelium.⁷⁻⁹ A 'paracellular' route, with channels bound by negatively charged membranes, has also been suggested.¹⁰

Sialic acids are highly charged anions and are the terminal constituent of many cell surface glycoconjugates, gangliosides, and extracellular glycoproteins, including fibronectin and laminin. Previous studies have demonstrated the presence within the TM of sialic acid, which has thus been implicated in the maintenance of the

normal IOP and in the pathogenesis of POAG. At the ultrastructural level, investigations using polycationic ferritin (PCF) and colloidal iron (CI) concluded that sialyl residues are present on the endothelial surfaces of the canal of Schlemm and the trabecular beams, and also within the cores of beams.¹¹ Using the same techniques, a comparative study of normal TM and TM from cases of POAG revealed an increase in the level of sialic acids on the abluminal surface of the SC endothelium in glaucomatous eyes.¹²

The term 'sialic acid' is generic for a group of natural derivatives of neuraminic acid, an acid amino sugar in pyranose form where the amino group is substituted by an acetyl or glycolyl group; in humans *N*-acetyl neuraminic acid (Neu5NAc) is the most abundant. Sialic acids are α -glycosidically linked from C2 of the pyranose ring usually to C3 or C6 of the subterminal monosaccharide group. The most common sialyl groupings in human tissue are Neu5NAc linked $\alpha(2,3)$ to galactose (Gal) and Neu5NAc linked $\alpha(2,6)$ to galactose (Gal) or *N*-acetyl galactosamine (GalNAc).¹³ The type of linkage is important in determining the properties of the grouping – for example, $\alpha(2,3)$ linkages are hydrolysed much faster than $\alpha(2,6)$ bonds.¹³ The high electronegative charge of sialic acids influences the conformation of glycoproteins and is thought to be important in the stabilising of cell membranes^{14,15} and other functions such as resistance to proteolytic degradation.¹³

It is now possible to differentiate between some of the sialic acids via their glycosidic linkages using lectins with different specific binding requirements. The lectin from the bark of elderberry, *Sambucus nigra* (SNA) binds with highest affinity to the terminal glycoconjugate sequence Neu5NAc $\alpha(2,6)$ Gal/GalNAc.¹⁶ The leucoagglutinin from *Maackia amurensis* (MAA) binds specifically to Neu5NAc $\alpha(2,3)$ Gal $\beta(1,4)$ (Glc)/GlcNAc, but not to Neu5NAc $\alpha(2,6)$ glycoconjugates.¹⁷ A more general sialic acid specificity is claimed for the lectin from the yellow slug, *Limax flavus* (LFA).¹⁸ We have applied biotinylated SNA, MAA, and LFA to normal human TM and have visualised lectin binding at the ultrastructural level by an immunogold technique. We find a distinct, although not absolute, topographical specificity for the two sialic acid isomers. By differentiating between the $\alpha(2,3)$ and $\alpha(2,6)$ linkages and providing information on their ultrastructural localisation within the human TM, further

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Abbreviations: Neu5NAc = *N*-acetyl neuraminic acid; Gal = galactosamine; Glc = glucosamine; 3' sialyllactose = Neu5Ac $\alpha(2,3)$ Gal $\beta(1,4)$ Glc; 6' sialyllactose = Neu5Ac $\alpha(2,6)$ Gal $\beta(1,4)$ Glc.

insight has been gained into the topographical relations of these biochemical components of the human outflow pathway.

Materials and methods

TISSUE

TM was taken from five human eyes at post mortem within a maximum of 11 hours from death. The ages of the patients ranged from 32 to 82 years and there was no history of glaucoma. The eyes were macroscopically and histologically normal. Four of the globes were initially taken into 10% (v/v) buffered formalin and subsequently part of the iridocorneal angle was removed and cut into 1 mm thick pieces for further fixation in 2.5% (v/v) glutaraldehyde in 0.1 mol/litre sodium cacodylate buffer containing 3 mmol/litre calcium chloride for 2–4 hours. One globe was fixed immediately in 2.5% (v/v) glutaraldehyde. The tissue was then processed into London resin white (LRW) for transmission electron microscopy (TEM).

REAGENTS

Biotinylated LFA was obtained from EY Laboratories Inc (suppliers in the UK, Bradsure Biologicals Ltd, Market Harborough, Leics); biotinylated SNA and MAA were obtained from Boehringer Mannheim (Lewes, East Sussex,

UK); bovine serum albumin (BSA, A7906) and Neu5Ac were obtained from Sigma Chemical Co (Poole, Dorset, UK); gold labelled anti-biotin antiserum, raised in goat, was supplied by Biocell Research Laboratories (Cardiff, UK); 3'sialyllactose, and 6'sialyllactose were obtained from Biocarb Chemicals (suppliers in the UK, Russell Fine Chemicals, Chester); Tween 20 was supplied by BDH (Poole, Dorset); London resin white was obtained from Taab Laboratories (Reading, Berks, UK).

LECTIN IMMUNOGOLD LABELLING

A method was developed using biotinylated lectin and gold labelled antibiotin. The anti-biotin gold technique was adapted from that of Slot and Geuze.¹⁹ Ultrathin sections (90 nm approx) on nickel grids were incubated successively as follows: 0.5 mol/litre Tris buffered saline, pH 7.6 (TBS) with 1% (w/v) BSA for 5 minutes; TBS with 1% (w/v) gelatine for 10 minutes followed by TBS with 0.2 mol/litre glycine for 3 minutes. The grids were then washed in TBS containing 1 mmol/litre CaCl_2 and 0.5% (v/v) Tween 20 before incubating with the biotinylated lectins (concentration 10, 50, or 100 $\mu\text{g/ml}$) for 1 hour at room temperature. After washing as before, the grids were finally incubated in antibiotin gold (10 nm or 15 nm) diluted 1:30 with TBS containing 1% (w/v) BSA. The grids were finally washed in distilled water before counterstaining with 2% (w/v) uranyl acetate and Reynold's lead citrate before examination on a Philips 301 transmission electron microscope at 80 kV.

CONTROLS

A negative control with 1% (w/v) BSA substituted for the lectin was included with every experiment.

The specificity of staining was confirmed for each of the lectins with inhibitory sugars as follows.

SNA

SNA was mixed with 4 mmol/litre 3'sialyllactose. A second aliquot of SNA was mixed with 6 mmol/litre 6'sialyllactose. After 30 minutes grids were then incubated separately with each mixture and the normal labelling procedure was then followed. Labelling with SNA was reduced very slightly by 3'sialyllactose and was reduced considerably by 6'sialyllactose. The principal specificity is therefore for the sialyl group in $\alpha(2,6)$ linkage, with much lower affinity for the $\alpha(2,3)$ linkage. This is in agreement with the work of Shibuya *et al.*¹⁶ Also, it has been reported that SNA has some affinity for the subterminal group GalNAc²⁰ which explains the residual labelling after inhibition with 6'sialyllactose. SNA was used at a concentration of 10 $\mu\text{g/ml}$ to keep low affinity binding to a minimum.

MAA

An aliquot of MAA was likewise mixed with 3'sialyllactose and a second aliquot with

Figure 1 Typical low power view of the trabecular meshwork adjacent to the Schlemm's canal (SC). (Magnification 2900; scale bar = 5 μm approx). JCT, juxtacanalicular tissue; CM, corneoscleral meshwork; e, endothelial cell, inner wall of canal of Schlemm; v, giant vacuole; tb, trabecular beam.

Figure 2 *Sambucus nigra* agglutinin (SNA) binding to the Schlemm's canal (SC) endothelium. (a) Immunogold label is somewhat heavier on the luminal aspect of the SC endothelial cells compared with the abluminal surface. It is also present within a cytoplasmic vesicle (arrowhead). Very little label is seen on the granular material (G) of the extracellular matrix. Juxtacanalicular tissue cells (JC) are not labelled. (Magnification 38 750; scale bar=0.25 μ m approx). (b) Immunogold label is visible on the luminal and abluminal aspect of the canal endothelium and, to some extent, within the cytoplasm of the endothelial cell shown. In this view, as opposed to (a), the cytoplasm of a juxtacanalicular tissue cell (JC) is labelled. (Magnification 54 250; scale bar=0.25 μ m approx).

6'sialyllactose and the same procedure was followed. MAA labelling was reduced almost to zero with 3'sialyllactose and was unaffected by 6'sialyllactose confirming that MAA was labelling specifically the $\alpha(2,3)$ linked sialic acid group. MAA was used at 50 and 100 μ g/ml in labelling experiments.

LFA

LFA was mixed with Neu5NAc (30 mmol/litre) and after 30 minutes the usual labelling procedure was followed. LFA labelling was reduced to zero by the inhibiting sugar confirming the specificity of the lectin in this procedure. LFA was used at a concentration of 10 μ g/ml.

Results

The preservation and resulting ultrastructure was variable because of the different time periods before fixation. This did not appear to affect the labelling which was consistent in all the cases examined. Figure 1 shows at low magnification a typical area of JCT and adjacent structures from one of the cases studied. Labelling of serial sections with the three lectins was possible with one case where a large filled vacuole on the SC endothelium was identified.

SNA

Labelling was observed mainly on the endothelial cell membrane of the inner and outer walls of SC, with a greater concentration on the luminal rather than the abluminal aspect of the cells (Fig 2a, 2b). Label was also present within cytoplasmic vesicles of the SC endothelium and, to a lesser extent, within the cytoplasm of endothelial cells. Label was seen within the cytoplasm of a few JCT cells (Fig 2b). Very occasionally label was seen on fine fibrillar material of the extracellular matrix (ECM) within the JCT. The filled vacuole which was serially sectioned, shown at low magnification in Fig 3a, clearly showed SNA label predominantly on the luminal surface of the endothelium, with occasional label within the cytoplasm of endothelial cells and sparsely decorating the fibrillar material of the ECM (Fig 3b). In the corneoscleral meshwork, generally there was negligible labelling of the external surfaces of trabecular endothelial cells and within their cytoplasm, but very occasional areas of moderately dense label were observed. The cores of trabecular beams showed sparse labelling.

MAA

Occasionally there was label on the cell membrane of the SC endothelium and the extracellular granular material of the JCT, but labelling was concentrated on the fine fibrillar material beneath the endothelial cell layer of the inner and outer walls of SC. There was considerable variation in the amount of fibrillar material observed in the subendothelial matrix and labelled fibrils sometimes took the form of a narrow band adjacent to abluminal surface of the SC endothelial cell layer (Fig 4a) or larger tangles in the JCT extending away from the endothelial cell layer (Fig 4b). Label also decorated basal lamina which was present focally beneath the SC endothelium (Fig 4a). In the serial sections (Fig 3), the vacuole which bound SNA predominantly on its luminal surface (Fig 3a) was observed to bind MAA mainly to the fibrils of the subendothelial space (Fig 3b). Very occasional grains of label were seen decorating the cytoplasm of the SC endothelial cell lining the vacuole. Trabecular beams of the corneoscleral meshwork showed sparse labelling in the basement membrane of the trabecular endothelium.

LFA

Moderately dense label was seen on the endothelium of the inner and outer walls of SC (Fig 5a). It was concentrated in the glycocalyx of the luminal aspect of the endothelial cells, with less label on the abluminal side. Label was also observed in cytoplasmic vesicles and occasionally in the cytoplasm of the SC endothelium. The density of label on the SC endothelium was greater than that observed with SNA. In the JCT, label was seen on the plasmalemmal surface of cells of the JCT (Fig 5a) and on the fibrillar material beneath the SC endothelium (Fig 5b). The granular material and elastic material were not convincingly labelled. The vacuole which clearly showed the differential localisation of

Figure 3 *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) binding to the same filled vacuole on the Schlemm's canal (SC) endothelium. (a) Low power view of the vacuole and adjacent structures. Serial sections from areas adjacent to that delineated by the box are shown at higher magnification in (b) and (c). (Magnification 4650; scale bar = 2.5 μ m approx). (b) SNA: label is heaviest on the luminal aspect of the canal endothelium (arrowheads). There is only slight binding to the subendothelial fibrils. Some label appears to be present within the endothelial cell cytoplasm at the top left hand corner. This may reflect tangential cutting of the cell at this point. (Magnification 38 750; scale bar = 0.25 μ m approx). (c) MAA: label is concentrated on the subendothelial fibrils of the extracellular matrix with very occasional label on the SC endothelium (arrows and box). (Magnification 38 750; scale bar = 0.25 μ m approx).

$\alpha(2,6)$ and $\alpha(2,3)$ isomers, showed no such differentiation with LFA. In the corneoscleral meshwork, sparse label was observed on trabecular endothelial cells, in the basal lamina and on the collagen of the trabecular beams (Fig 5c).

Discussion

The results show that, by applying biotinylated lectin probes followed by gold labelled antibiotin

antiserum, it is possible to localise residues of different isomeric sialyl glycosides in human TM at the ultrastructural level. $\alpha(2,6)$ Sialic acid, demonstrated by SNA, was concentrated mainly in the glycocalyx of the luminal aspect of endothelial cells of the CS; it was present to a much lesser extent on the endothelium of trabecular cells of the corneoscleral mesh and within the cores of trabecular beams. Conversely, sialic acid in the $\alpha(2,3)$ linkage, as shown by MAA, was observed mainly in the fine fibrillar material beneath the endothelium of the CS and to a much lesser extent in the basement membrane of trabecular beams. LFA confirmed the findings with SNA and MAA, binding to both the CS endothelium and the fine fibrils of the subendothelial extracellular matrix as well as to the endothelium of trabecular cells. The pattern of LFA binding, with its broad specificity for sialyl glycosides, is in broad agreement with studies using polycationic ferritin (PCF) and colloidal iron (CI) where it was found that, in normal human TM there is a greater concentration of neuraminidase sensitive label on the luminal than on the abluminal aspect of SC endothelium, with lesser amounts present within the extracellular matrix.¹²

With respect to this and other similar studies it should be noted that both PCF and CI are general markers for polyacids which include polyuronides, sialyl glycoconjugates, polysulphated and polyphosphorylated molecules. Staining at pH 1.8–1.9 suggests sulphate, not sialic acid and sialyl glycosides would, in any case, undergo an appreciable degree of hydrolysis at this low pH. Binding of PCF and CI above pH 3.3 suggests non-sialyl carboxyl. Only reduction of staining with neuraminidase pretreatment provides good evidence for sialic acid, but does not prove that the label has bound directly to it. The label may have bound to a non-sialyl acidic molecule which was attached to the sialic acid, hence showing reduction in staining with neuraminidase treatment. Our reagents detect sialyl glycosides directly and do not cross react with, for example, hyaluronic acid which shows a broadly similar distribution in other studies using PCF and CI.^{21–23} Despite these chemical and methodological considerations in interpreting PCF and CI data, it is nevertheless of interest that there appears to be a concentration of a variety of highly anionic species in the distal regions of the TM. A recent study employing cationic dyes and an anti-hyaluronic acid monoclonal antibody suggests that glycosaminoglycans including hyaluronic acid may have an 'osmotic' function in controlling water flow at the level of the chamber angle in addition to a 'structural' function within the TM itself.³

The binding patterns we observed with SNA and MAA indicate that there is molecular and spatial heterogeneity in the expression of sialyl glycosides within the human TM. It is likely that they are constituents of different sialoglycoconjugates with different roles in aqueous outflow. $\alpha(2,6)$ Linked sialic acid appears to be associated predominantly with the cell membrane and may thus be a component of membrane-bound glycoprotein or glycolipid. It is known from nuclear magnetic resonance spectroscopy to form a more

Figure 4 *Maackia amurensis* agglutinin (MAA) binding to subendothelial fibrils of the juxtacanalicular tissue (JCT). (Magnification 50 000; scale bar=0.5 μ m approx). (a) Label can be seen on fibrillar tangles of the JCT as well as focally on the basal lamina of the Schlemm's canal (SC) endothelium (arrows). Very occasional label is present on the luminal surface of the endothelium (arrowheads). (b) Label is visible mainly on fibrils closely juxtaposed to the basal lamina of the SC endothelium. No label is visible on the luminal surface.

Figure 5 *Limax flavus* agglutinin (LFA) binding to trabecular meshwork. (a) Heaviest label is on the luminal aspect of the Schlemm's canal (SC) endothelium, but it can also be seen in the cytoplasm and on the abluminal surface. Label is also present on the plasmalemmal surface of juxtacanalicular cells. (Magnification 30 875; scale bar=0.5 μ m approx). (b) Label is visible on the SC endothelium and on fibrils of the JCT. (Magnification 40 625; scale bar=0.5 μ m approx). (c) A trabecular beam of the corneoscleral mesh shows sparse labelling on the trabecular cell surface and in the cytoplasm. There is some binding to the basal lamina (BL) with very occasional label in the core. (Magnification 40 625; scale bar=0.5 μ m approx).

flexible structure than the $\alpha(2,3)$ form¹⁴ and, because of the exposed terminal position and high electronegative charge, it is likely to be involved in functions such as stabilisation of membranes¹⁵ and maintenance of cellular attachment to basement membrane.²⁴ A decrease in sialyl residues at the cell surface has been shown to be associated with an increase in cell membrane permeability and endocytosis in myeloid sinusoidal endothelium²⁵ and renal glomeruli.²⁶ Further, a sialoprotein, identified on rat glomerular epithelial membrane, shows a decreased sialic acid content in the rat model of human renal 'minimal change disease'.²⁷ Glucocorticoids, which increase the expression of sialyltransferase activity,^{28,29} are used therapeutically to reverse minimal change disease in the kidney and may affect aqueous outflow through a similar mechanism ('steroid glaucoma'). The $\alpha(2,3)$ linked sialic acid residues were localised mainly in the fine fibrillar material in the subendothelial region of the JCT, the so-called 'type I plaque',³⁰ and were also noted to be associated with the small amount of basal laminar material seen focally here. Other highly negatively charged molecules such as heparan sulphate, chondroitin sulphate, and dermatan sulphate are also found in this location. It has been suggested that these proteoglycans, which are associated with a mixture of fine fibrils and basal lamina-like material, may have a role in aqueous outflow resistance.³¹ The co-localisation of MAA binding to this fibrillar material in our study implies an ECM based role of sialic acids in aqueous outflow.

Laminin, a multidomain sialoglycoprotein of the extracellular matrix, has been identified (together with type I, III, and IV collagen) in the subendothelial layer of the inner wall of SC in the aged human outflow system.^{32,33} The fine filamentous material described in these reports appears to correlate closely with the MAA binding fibrils observed in our study and laminin is known to be a basal lamina constituent. However, while the sialic acid of laminin from the

EHS murine tumour has been found to be present solely in the $\alpha(2,3)$ linkage,³⁴ it is not possible to draw a direct parallel with the human TM as both species and tissue variations abound in the structure and amino acid content of laminin. As yet no data exist for the precise nature of the sialic acid of human laminin and while double labelling using MAA and anti-laminin may provide further information, biochemical analyses would be necessary to confirm unequivocally that the $\alpha(2,3)$ sialyl groups herein demonstrated are located specifically on laminin.

The greater density of gold label observed with LFA compared with SNA suggests that sialic groups in linkages other than $\alpha(2,3)$ and $\alpha(2,6)$ may also be present on the SC endothelium – for example, $\alpha(2,8)$ as seen in neural cell adhesion molecules, for instance.³⁵ Ultrastructural localisation of a whole range of specific sialic acid residues, including comparative studies on the relative proportions and topography in normal, aged and glaucomatous TM, may offer further insights into the pathogenesis of POAG.

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