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SCIENTIFIC CORRESPONDENCE

Clinical and morphological features including expression of βig-h3 and keratan sulphate proteoglycans in Maroteaux-Lamy syndrome type B and in normal cornea

Saeed Akhtar, Andrew Tullo, Bruce Caterson, Janet R Davies, Kelly Bennett, Keith M Meek

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Aim: To carry out a detailed morphological study of the cornea of a 16 year old female with a Maroteaux-Lamy syndrome (MLS).

Methods: Following a penetrating keratoplasty in July 1999, ultrastructural changes in the cornea were examined using electron microscopy. Proteoglycans were visualised using cuprolinic blue dye; and βig-h3 and keratan sulphate were detected by immunoelectron microscopy.

Results: The epithelial cells were degenerate and contained apoptotic nuclei. Proteoglycans were present in epithelial cells, intercellular spaces, and in swollen desmosomes. An abnormally large quantity of proteoglycans was present throughout the stroma. Keratocytes throughout the stroma had no cell organelles, were vacuolated, and contained a large quantity of abnormal proteoglycans. Labelling for βig-h3 was intense around electron lucent spaces in stroma. No labelling was seen in keratocytes or endothelial cells. In normal cornea, keratan sulphate labelling was regular throughout the stroma. In MLS VI type B cornea, keratan sulphate labelling was weak in the anterior stroma but very intense in the posterior stroma and in keratocyte lysosomes and vacuoles.

Conclusion: A deficiency of aryl sulfatase B results in the deposition of keratan sulphate proteoglycan and other proteoglycans in lysosomes, causing the death of keratocytes and an abnormal build-up of proteoglycans in the stroma. This might be responsible for the lateral aggregation of collagen fibrils and impaired fibrillogenesis in MLS VI. Degenerate swollen keratocytes, together with gross changes in epithelial, stromal, and endothelial cells, would be expected to increase light scattering significantly in these corneas.

Proteoglycans (PGs) form part of the corneal ground substance, where they normally bind water and cations by virtue of their polyanionic glycosaminoglycans (GAGs, previously known as mucopolysaccharides). Normally, cornea contains 4–4.5% GAG, of which 50% is keratan sulphate, 25% is chondroitin sulphate, and 25% is chondroitin-4-sulphate; it has recently been reported that there is no dermatan sulphate present.[1] Sulfatases catalyse the hydrolysis of sulphuric acid esters from a wide variety of substrates, including GAGs, glycolipids and steroids. GAGs (dermatan, heparan, keratan, and chondroitin sulphate) are byproducts of the catabolism of proteoglycans located in the cell coat or extracellular matrix; they are sequentially degraded in the lysosomal compartment by specific hydrolases.[2] As a result of a deficiency in enzyme activities, partially degraded GAGs accumulate within secondary lysosomes. A deficiency in the specific lysosomal sulfatase, which is involved in the degradation of GAGs, leads to rare clinical disorders termed "mucopolysaccharidoses" (MPS).[2–4] There are several MPS disorders recognised in humans, classified according to the specific deficiencies of the degradative lysosomal enzyme. The primary clinical ocular manifestation of the MPS diseases in humans and animals is corneal opacification.[5 6]

The clinical characteristics of Maroteaux-Lamy syndrome (MLS VI) are striking osseous and corneal changes without intellectual impairment until late. Dermatan sulphate (formerly chondroitin sulphate B) is secreted in urine. In all forms of the disease, leucocyte inclusions and a deficiency of aryl sulfatase B (N-acetylgalactosamine 4-sulfatase) are found. The stromal keratocytes and histocytes in MLS VI type B cornea are full of vacuoles and lysosomes containing cell organelle remnants.[1 2 7–9]

βig-h3 is an extracellular matrix protein that was first detected in a human lung adenocarcinoma cell line after stimulation by TGF-β.[10] Recently, specific missense mutations have been reported in the β*ig-h3* gene in four autosomal dominant corneal dystrophies—lattice type I, granular, Avellino, and Reis-Bücklers corneal stromal dystrophies.[11]

In the present paper, we report for the first time the presence of keratan sulphate proteoglycan and βig-h3 protein in epithelial cells, stroma, keratocytes, and endothelial cells in the cornea of a patient with Maroteaux-Lamy syndrome and in normal cornea.

METHODS

A patient was diagnosed as having MLS VI type B at 6 years of age. When she was 15, she was referred because of rapid deterioration of vision in both eyes as a result of corneal opacification. Left penetrating keratoplasty was carried out in July 1999, resulting in a significant improvement in vision, and allowing her return to normal schooling. However, 2 years after surgery, although the graft remained clear, vision deteriorated from optic nerve involvement due to raised intracranial pressure. Corneal tissue was obtained at the time of surgery. A normal corneal button was obtained 24 hours post mortem from a cadaver donor.

Corneal tissue of MLS type B was fixed immediately after excision in (i) 3% glutaraldehyde and 1% osmium tetroxide in 0.1 M phosphate buffer for morphological studies; (ii) 3% glutaraldehyde containing 0.05% cuprolinic blue (BDH Ltd, Dorset, UK) in a critical electrolyte concentration mode[12] for proteoglycan localisation (as described by Akhtar *et al*[13]); and (iii) 4% paraformaldehyde (PFA) in 0.1 M phosphate for 2 hours at 4°C and embedded at low temperature for immunoelectron microscopy (as described by Akhtar *et al*[14]). Rabbit polyclonal anti-human βig-h3 and mouse monoclonal anti-keratan sulphate (MAb 5-D-4, B Caterson, Cardiff) were visualised
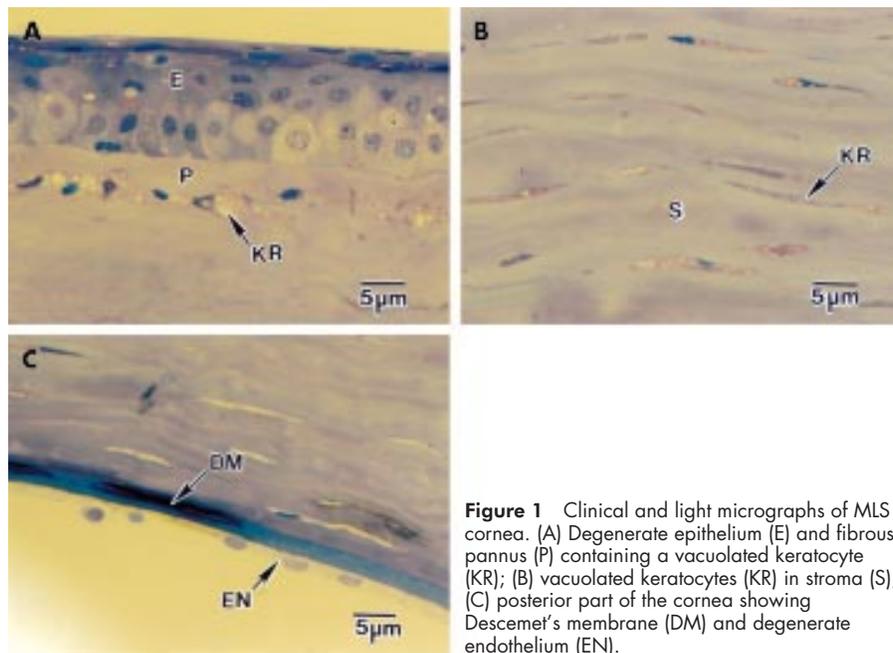
Figure 1 Clinical and light micrographs of MLS cornea. (A) Degenerate epithelium (E) and fibrous pannus (P) containing a vacuolated keratocyte (KR); (B) vacuolated keratocytes (KR) in stroma (S); (C) posterior part of the cornea showing Descemet's membrane (DM) and degenerate endothelium (EN).

with 10 nm immunogold conjugated anti-rabbit and anti-mouse second antibodies, respectively (Biocell, Cardiff, UK).

RESULTS

MLS cornea: morphology

Light microscopic observations showed that the epithelium was intact and thicker than in normal cornea (Fig 1A). Most of the epithelial cells were degenerate and contained apoptotic nuclei. Bowman's layer was replaced by a fibrous pannus, which contained vacuolated keratocytes (Fig 1A). Keratocytes in the middle and posterior stroma were also vacuolated (Fig 1B, C). Endothelial cells were degenerate and contained prominent nuclei (Fig 1C).

Electron microscopic observations showed that in the epithelium apoptotic nuclei (90%) contained condensed nuclear chromatin (Fig 2A) and blebbing in the nuclear membrane (Fig 2B). Intercellular spaces were filled with electron dense aggregates of PGs (Fig 2C). Most of the desmosomes were swollen, degenerate, and full of electron dense material (Fig 2D).

The PG filaments in the posterior stroma and, especially, in pre-Descemet's stroma (Fig 2E) were very large (approximately 200–240 nm), relative to the PG filaments (50–125 nm) in the anterior stroma. Collagen fibres in the lamellae, which contained keratocytes in extreme stages of degeneration, were replaced by granular material and sparse collagen fibrils (Fig 2F). Throughout the stroma, the keratocytes were degenerate and contained vacuoles and electron dense lysosomes (Fig 2F). These lysosomes were full of PG filaments, suggesting their inability to digest these PGs (Fig 2G). Only a few keratocytes had a prominent nucleus and rough endoplasmic reticulum.

Expression of βig-h3 and keratan sulphate

In normal cornea samples, moderate labelling of βig-h3 was observed throughout the cornea except keratocytes and endothelial cells. In diseased tissue, moderate labelling of βig-h3 was observed in the epithelium and in the normal looking parts of Bowman's layer. Labelling was dense on the granular material around electron lucent spaces (Fig 3A) and around the degenerate keratocytes (Fig 3B) present within the sparse collagen lamellae.

In normal cornea, labelling of keratan sulphate (MAb 5-D-4) was regular throughout the stroma (Fig 4A). In MLS corneal tissue, labelling of keratan sulphate was strong in basal epithelial cells, which were vacuolated and contained large quantities of PG filaments (as seen by cuprolinic blue staining). In anterior stroma, labelling of keratan sulphate was weak but very strong in posterior stroma. Labelling was very dense and in clumps on degenerate collagen fibrils, on fibrous material (Fig 4B) and around the electron lucent spaces (Fig 4C). In normal corneal keratocytes labelling was regular (Fig 4D), whereas in MLS tissue, vacuolated keratocytes (Fig 4E) were strongly labelled in their lucent vacuoles and on the electron dense material present in lysosomes (Fig 4E, F). Labelling was more dense in posterior keratocytes than in anterior keratocytes. Endothelial cells also showed some labelling (Fig 4G).

DISCUSSION

Our ultrastructural findings showed that in MLS corneal tissue, the epithelium was degenerate and contained perinuclear vacuoles, and Bowman's layer was replaced by vacuolated histocytes. This was similar to the observations of Kenyon et al[7] but contrary to the observations of Mollard et al,[2] who found the epithelium normal. Neither authors reported the presence of apoptotic nuclei in the epithelium, which we observed in most of the epithelial cells. We found degenerate keratocytes containing vacuolated inclusions with substrate storage in secondary lysosomes. We identified the storage material as proteoglycan and have shown for the first time that the secondary lysosomes and electron lucent vacuoles contain keratan sulphate. Proteoglycans were present in large quantities throughout the stroma but the cuprolinic blue stained filaments were relatively large (200 nm) in the posterior and pre-Descemet's stroma. As reported by others[2 7] we noticed disorganised collagen lamellae but unlike Kenyon et al[7] we did not find any abnormal Descemet's membrane. Mollard et al[2] suggested that the uniform spacing between collagen fibrils implied a correctly functioning endothelium, whereas Kenyon et al[7] found the endothelium to be vacuolated. The endothelium in our tissue was very degenerate.

βig-h3 has been found in basement membrane of skin and interstitial tissue.[15 16] Recently, it has been localised immunohistochemically in bullous keratopathy[17 18] and corneal dystrophies.[19] In our case, the amount of βig-h3 was increased as a result of the degeneration of the epithelium and because of
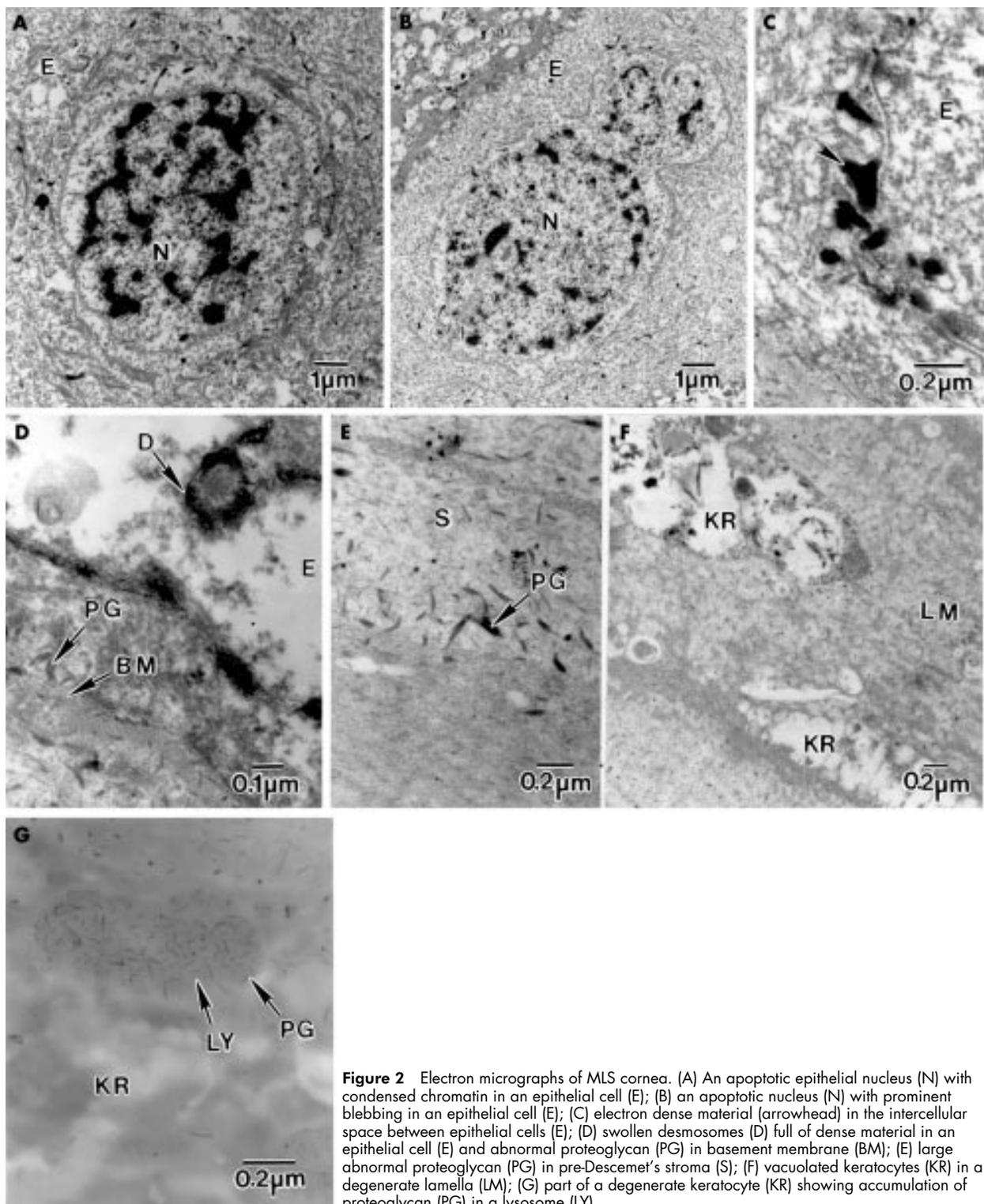
Figure 2 Electron micrographs of MLS cornea. (A) An apoptotic epithelial nucleus (N) with condensed chromatin in an epithelial cell (E); (B) an apoptotic nucleus (N) with prominent blebbing in an epithelial cell (E); (C) electron dense material (arrowhead) in the intercellular space between epithelial cells (E); (D) swollen desmosomes (D) full of dense material in an epithelial cell (E) and abnormal proteoglycan (PG) in basement membrane (BM); (E) large abnormal proteoglycan (PG) in pre-Descemet's stroma (S); (F) vacuolated keratocytes (KR) in a degenerate lamella (LM); (G) part of a degenerate keratocyte (KR) showing accumulation of proteoglycan (PG) in a lysosome (LY).

the oedematous condition of stroma in which widening of intercollagenous fibrillar spaces facilitates diffusion of macromolecules. βig-h3 is a protein of about 68 kD and therefore similar in size to albumin. Maurice[20] many years ago demonstrated the ability of albumin to diffuse across the stroma.

In normal cornea, we found keratan sulphate labelling was regular throughout the stroma, as reported by Bairaktaris et al.[21] Keratan sulphate labelling in MLS tissue was different from that seen in normal cornea. In MLS tissue, vacuolated epithelial cells which contained a large amount of proteogly-can (shown by cuprolinic blue staining) were strongly labelled for keratan sulphate. Only the posterior stroma showed very strong labelling. In the keratocytes, keratan sulphate labelling was highest in the lucent spaces and lysosomes.

MLS VI type B is a mucopolysaccharidosis that is a lysosomal storage disease involving a deficiency in aryl sulfa-tase B, which is the enzyme that removes the sulphate groups from the GAGs that are endocytosed by the corneal cells over time. This deficiency means that the sulphated GAGs, which
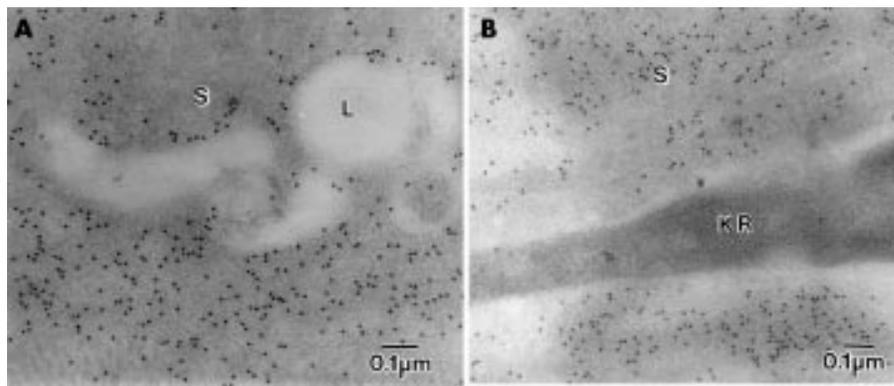
Figure 3 Immunolocalisation of βig-h3 in MLS type B cornea. (A) Very strong labelling around lucent spaces (L) in the stroma (S); (B) strong labelling around a keratocyte (KR) but not in the keratocyte.
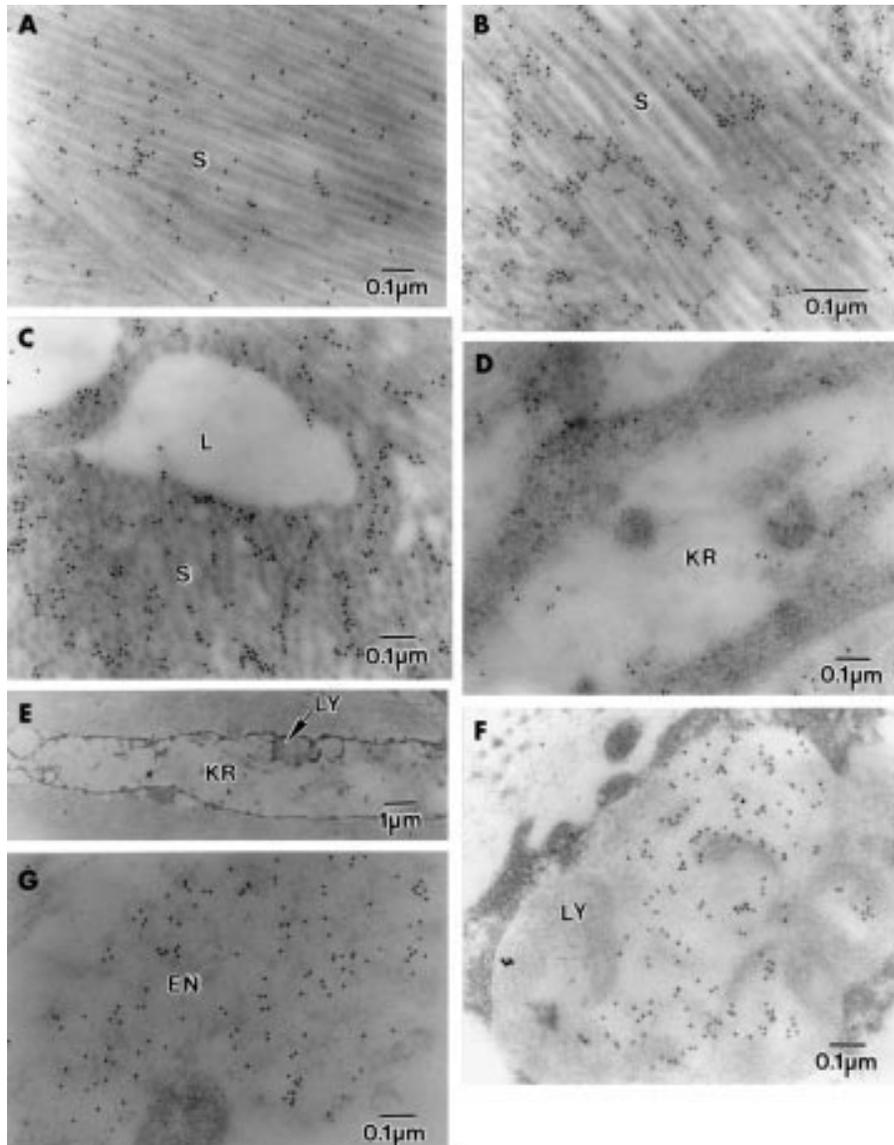

Figure 4 Immunolocalisation of keratan sulphate (KS) with MAB 5-D-4 in normal and MLS type B cornea. (A) Regular labelling of KS on collagen fibres in normal cornea; (B) strong labelling of KS in clumps on stromal collagen fibres (S) in MLS type B cornea; (C) very strong labelling of KS around lucent spaces (L) in the stroma (S) of MLS type B cornea; (D) moderate labelling of KS in a normal keratocyte (KR); (E) "empty" keratocyte (KR) in MLS type B cornea containing a lysosome (LY); (F) strong labelling of KS in the lysosome (LY) shown in (E); (G) labelling of KS in the endothelium (EN) of MLS type B cornea.

cannot be degraded and cleared from the cell, therefore accumulate in the lysosomes, eventually causing cell death. Monoclonal antibody 5-D-4 recognises oversulphated domains in keratan sulphate GAGs (the major GAG on proteoglycans in the cornea), so the heavy keratan sulphate labelling seen in the lysosomes and vacuoles represents the GAG that has accumulated over time. In the cornea, the candidate proteoglycans for 5-D-4 labelling are the so called "small leucine

rich proteoglycans" lumican and fibromodulin, each of which has three or four (lumican) or two or three (fibromodulin) keratan sulphate chains attached[22] and both of which act to regulate collagen fibrillogenesis. It is therefore an accumulation of one or both of these PGs that is visualised with monoclonal antibody 5-D-4.[23]

The greater quantity of keratan sulphate labelling in the posterior keratocytes and the posterior stroma may be due to

differences in the relative cell populations of the different regions. Since the accumulation of GAGs eventually kills the cells, over time the ability to supply the matrix with new PGs would be compromised.

Our data suggest that in this patient there was an intracytoplasmic accumulation of keratan sulphate, together with other GAGs, presumably due to the deficiency of aryl sulfatase B, which catabolises GAGs. This GAG accumulation seems to have led to degeneration and enlargement of keratocytes, followed by an abnormal build-up of proteoglycans in the stroma, disorganisation of collagen fibrils, and disruption of the parallel arrangement of collagen lamellae. A secondary effect of oedematous stroma involved degeneration of the epithelium, which contained cells with apoptotic nuclei, and accumulation of βig-h3 protein in the stroma. The gross changes in the epithelial, stromal, and endothelial cells and the disruption of the collagen would be expected to increase light scattering significantly and thus cause the cornea to become opaque.

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