Ultrastructural and Molecular Analysis of Bowman’s Layer Corneal Dystrophies: An Epithelial Origin?

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PURPOSE. Two mutations (R555Q and R124L) in the BIGH3 gene have been described in anterior or Bowman’s layer dystrophies (CDB). The clinical, molecular, and ultrastructural findings of five families with CDB was reviewed to determine whether there is a consistent genotype:phenotype correlation.

METHODS. Keratoplasty tissue from each patient was examined by light and electron microscopy (LM and EM). DNA was obtained, and exons 4 and 12 of BIGH3 were analyzed by polymerase chain reaction and single-stranded conformation polymorphism/heteroduplex analysis. Abnormally migrating products were analyzed by direct sequencing.

RESULTS. In two families with type I CDB (CDBI), the R124L mutation was defined. There were light and ultrastructural features of superficial granular dystrophy and atypical banding of the “rod-shaped bodies” ultrastructurally. Patients from three families with “honeycomb” dystrophy were found to carry the R555Q mutation and had characteristic features of Bowman’s dystrophy type II (CDBII).

CONCLUSIONS. There is a strong genotype:phenotype correlation among CBDI (R124L) and CDBII (R555Q). LM and EM findings suggest that epithelial abnormalities may underlie the pathology of both conditions. The findings clarify the confusion over classification of the Bowman’s layer dystrophies. (Invest Ophthalmol Vis Sci. 2000;41:3286–3292)

A mong the corneal dystrophies of Bowman’s layer (CDB) two main forms, Reis-Bücklers1,2 and Thiel-Behnke dystrophies3 are recognized. Their subclassification has in the past been a source of confusion. However, Küchle and colleagues4 suggested that the two represent distinct conditions and proposed the designations CDB type I (CDBI; synonyms: geographic or “true” Reis-Bücklers’ dystrophy) and CDB type II (CDBII; synonyms: honeycomb or Thiel-Behnke dystrophy). In CDBI, symptoms are of early onset and show marked progression, with a more profound effect on visual acuity than granular dystrophy. Histopathologic examination reveals deposits staining intensely red with Masson trichrome at the level of Bowman’s layer. Ultrastructural examination reveals “rod-like bodies” similar to those in granular dystrophy. CDBII presents with frequent recurrent erosions of early onset within the first years of life. Bowman’s layer is replaced by a densely fibrous, paucicellular layer of variable thickness between the epithelium and stroma. On electron microscopy, this material demonstrates twisted “curly fibers” measuring 9 to 15 nm in diameter.

The identification of mutations in the BIGH3 (or transforming growth factor, β-induced [TGFβI]) gene on chromosome 5q31 in a variety of the corneal dystrophies has facilitated a reevaluation of their clinical classifications.5 Mutations of two “hotspots” at amino acids 124 and 555 of the encoded protein, keratoepithelin, have been shown in several BIGH3-related dystrophies. Among the anterior dystrophies, including Reis-Bücklers’ and Thiel-Behnke dystrophies, three mutations have been described: R555Q in “honeycomb” dystrophy,6 R124L in a geographic form, and in a single Sardinian family a trinucleotide deletion, ΔF540.6–8 We have undertaken a detailed analysis of families with Bowman’s layer dystrophies. Our results suggest a strong genotype:phenotype correlation, which allows a reevaluation of the clinical nomenclature.

MATERIALS AND METHODS

Patient Details

Ethical approval was obtained from Manchester Health Commission, Research Ethics Committee (Central), reference CM/96/043. All work conformed to the Declaration of Helsinki. With informed consent, DNA was obtained from all individuals, both affected and unaffected (totals of 14 and 5, respectively).

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who were examined. The most recent keratoplasty host button of each patient (families 1 and 2, penetrating keratoplasty [PK]; families 3–5, lamellar keratoplasty [LK]) was prepared for light and electron microscopy.

**Light and Electron Microscopy**

Tissue for LM was fixed in 10% formalin in 0.1 M phosphate buffer and for EM in 2.5% glutaraldehyde in 0.1 M phosphate buffer. A segment of cornea from one patient (family 1, individual 1.3) was fixed in 2.5% glutaraldehyde containing 0.05% cuprolin blue. For LM, tissue was dehydrated through a graded series of ethanol, cleared with xylene, embedded in paraffin wax, cut into 5-μm sections, and mounted onto 5-aminopropyltriethoxysilane-coated glass slides. All corneas were stained with hematoxylin and eosin (H&E), Masson’s trichrome, Congo red, and periodic acid-Schiff (PAS) stains. All corneas with toluidine blue, and thin sections with uranyl acetate and lead citrate.

Resin-embedded blocks were cut into semi-thin (1 μm) and thin (65 nm) sections. Semi-thin sections were stained with toluidine blue, and thin sections with uranyl acetate and lead citrate. Thin sections were placed onto nickel grids, and transmission EM was carried out using a JEOL 1010 instrument (London, UK).

**Nucleic Acid Preparation and PCR Analysis**

Genomic DNA was extracted by conventional methods from blood lymphocytes.

Exons 4 and 12 of \( BIGH3 \) were amplified as previously reported.\(^5\) Genomic DNA (4 ng) was suspended in a 5 μl reaction containing 10 pmol of both primers, 40 μM each dATP, dGTP, dTTP, dCTP, 5 μM dATP, and 1× PCR buffer (containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1–1.5 mM MgCl\(_2\), and 0.1% gelatin), overlaid with mineral oil. The samples were heated to 96°C (10 minutes) and cooled to 51°C, and 0.15 units Taq polymerase was added. The samples were processed as follows: 92°C (30 seconds), 51°C (30 seconds), 72°C (30 seconds) ×35 cycles, and 72°C (10 minutes). For SSCP, an equal volume of formamide stop solution was added to the amplified products. Gels were run at 350 V (4°C) and silver stained according to standard protocols. Direct sequencing was performed by dye terminator cycle sequencing (Perkin Elmer, Applied Biosystems Ltd., Foster City, CA) using a fluorescent sequencer (ABI 373).

**RESULTS**

**Clinical Manifestations**

**CDBI. Family 1.** A mother (1.1) and two daughters (1.2 and 1.3) of a previously reported Dutch family\(^1\) were subjected to repeat penetrating keratoplasty (Table 1; Figs. 1A, 1B). All individuals examined underwent their first PK in the third decade of life. In all, reduced visual acuity and recurrent corneal erosions began in the first decade. Corneal opacification was bilateral, geographic, and asymmetric. Corneal opacification was predominantly subepithelial, although stromal dot-like lesions were also present. There was epithelial irregularity but normal corneal thickness. After keratoplasty erosions continued and after about a year, superficial opacification began to recur at the graft margins, spreading centrally.

**Family 2.** Patient 2.1 was a simplex patient who had recurrent erosions from the age of 6 months. Visual acuity was reduced to 6/60 at 10 years at which time he underwent right PK followed 2 years later by left PK. The clinical findings were of confluent, asymmetrical, subepithelial opacification that was not homogeneous.

**CDBI. Family 3.** The proband, patient 3.1, presented in 1984 aged 27 years and underwent left penetrating keratoplasty (PK) in 1986 (Fig. 1C). In 1993 visual acuities were right 6/36 and left 6/18, and superficial honeycomb changes were noted. She underwent right PK in 1996. Her son, 3.2, presented in 1987 at the age of 5 years with early morning erosions. Bilateral honeycomb changes were noted, and by 1999 visual acuities had fallen to right 6/18 and left 6/12.

**Family 4.** The proband, 4.1, presented at age 38 years with three of her five children (4.2–4.4) in 1981 with acuities of 6/24 in each eye and recurrent erosions since childhood. She had honeycomb reticular opacity of both corneas and underwent left PK in 1992 and right PK in 1994. Patient 4.2 presented at the age of 11 years with photophobia and recurrent episodic soreness and honeycomb corneal opacities and has undergone bilateral PK. Patient 4.3 presented at age 5 years with mild corneal signs and recurrent sore eyes.

### Table 1. Symptoms and Treatment of Families 1–5

<table>
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<tr>
<th>Family</th>
<th>Patient</th>
<th>Erosions</th>
<th>Visual Failure</th>
<th>Treatment Right</th>
<th>Treatment Left</th>
<th>BIGH3 Mutation</th>
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By 1996 acuities were reduced, troublesome erosions. He has undergone bilateral PTK, improving vision to 6/9 with cessation of erosions. Patient 4.4 presented in his first year with sore sticky eyes and by 1992 had “typical honeycomb” changes in both corneas. He has now undergone bilateral PTK with cessation of erosions. Patient 4.5, the brother of 4.1, presented at age 43 years with bilateral superficial “cobweb” corneal opacities and reported recurrent ocular

FIGURE 1. (A) CDB type I (patient 1, family 2) with early demonstration of a diffuse, geographic superficial corneal opacification. (B) CDB type I (patient 1, family 1) showing recurrence of disease within graft and extending almost to limbus. As in (A), an extensive superficial corneal opacification is visible. (C) CDB type II. The “honeycomb” appearance of subepithelial reticular opacities are seen, maximal centrally.

FIGURE 2. Light microscopy. (A) Montage showing full-thickness cornea after repeat penetrating keratoplasty (patient 1, family 1). There is widespread subepithelial deposition of material staining red with Masson trichrome, which only involves the stroma where it extends along the graft margins (arrows). The epithelium is poorly attached, being partially dehiscent (Masson trichrome). High-magnification (patient 1, family 1) image demonstrating subepithelial material above Bowman’s membrane, extending around pannus, and present between and within basal epithelial cells. Superficial epithelial cells show poor cell–cell attachment (Masson trichrome). (B) Penetrating keratoplasty specimen (patient 1, family 5). There is a subepithelial band of hyaline material of variable thickness. Similar material is also seen within the epithelium above the basal layer. In one area, overlying a thicker area of deposit, basal epithelial cells show poor attachment to substratum (Masson trichrome).
irritation from infancy. By age 52 years, his acuities were right 6/12 and left 6/18. Left PTK improved visual acuity to 6/9 with cessation of erosions.

Family 5. Patient 5.1 presented with a history of recurrent sore eyes since infancy with recent deterioration of vision. Examination revealed typical honeycomb anterior corneal dystrophy. Her father, two of three siblings, two of five offspring, and four of seven grandchildren are also affected. She has undergone multiple surgical procedures. Patient 5.2, her daughter, presented late with gross visual failure, a history of recurrent erosions and widespread amorphous corneal opacification underlying grossly irregular honeycomb subepithelial fibrosis. Extensive PTK at this late stage did not significantly improve corneal clarity.

Light and Transmission Electron Microscopy

CDBI. LM revealed, in family 1, an irregular, partially dehiscent epithelium with separation of cells in the wing cell layer and focal detachment from the substratum. Condensed basal epithelial cells were seen, with small intraepithelial deposits of material that was eosinophilic on H&E staining and red with Masson trichrome. In patient 2.1, the epithelium was intact, but intraepithelial deposits were also present. In all patients, Bowman’s layer was absent in areas (Figs. 2A, 2B). The granular dystrophy-type material was widely dispersed in thin bands beneath the epithelium, above and below what remained of Bowman’s layer. Deposits did not demonstrate features of amyloid and did not stain with PAS. In patient 2.1, who had undergone his first PK, the deposits were also in middle and deep stroma.

On EM, accumulations of electron dense material with the morphologic characteristics of granular dystrophy deposits were seen in locations corresponding to LM observations. These were moth-eaten, rod-shaped, and trapezoidal (Figs. 3A through 3G). In two patients from family 1, there was additionally periodic 9- to 10-nm cross-banding periodicity. These are also present in (B) and (H). (E) A basal epithelial cell containing a vesicle (V) adjacent to the nucleus (N) that is distinct from the subepithelial layer of deposits (SD). (F) A series of interconnected vesicle-like structures (V), lying between epithelial cells (E) and in continuity with subepithelial deposits (SD). (G) Inset from (F). These deposits are associated with irregularly arranged collagen fibrils, finer fibrillar material, and abnormal proteoglycans (PG and arrows).

Figure 3. Electron microscopy, CDBI. (A) Low magnification showing darkly staining epithelial cells (Ep) overlying a band of electron-dense rod-shaped, subepithelial deposits (SD), which are situated above Bowman’s layer (Bo). In the basal aspect of one epithelial cell, a vesicle is seen (box and Fig. 2B). (B) Inset from (A). A membrane-bound vesicle (V) containing trapezoidal and rod-shaped electron-dense deposits. It is situated close to underlying subepithelial deposits but with no clear continuity seen in this plane. (C) An area in which deposits with a ‘moth-eaten’ appearance are seen. (D) In this field, there are many trapezoidal deposits that exhibit 9- to 10-nm cross-banding periodicity. These are also present in (B) and (H). (E) A basal epithelial cell containing a vesicle (V) adjacent to the nucleus (N) that is distinct from the subepithelial layer of deposits (SD). (F) A series of interconnected vesicle-like structures (V), lying between epithelial cells (E) and in continuity with subepithelial deposits (SD). (G) Inset from (F). These deposits are associated with irregularly arranged collagen fibrils, finer fibrillar material, and abnormal proteoglycans (PG and arrows).
CDBII. LM revealed an irregular epithelium with focal areas of vacuolation and partial dehiscence of basal cells from the underlying substratum. There was a variably thick band of hyaline, avascular, paucicellular connective tissue beneath the epithelium, largely obliterating Bowman’s layer (Fig. 2C). This material was eosinophilic on H&E staining, did not stain red with the Masson trichrome stain, was not Congophilic, and did not stain with PAS. The deposits were limited to the subepithelial region, with an abrupt transition to anterior stroma (e.g., Fig. 2C), but in one case (family 3, patient 1), small collections of this hyaline material were seen between cells in the wing cell epithelial layer. On EM in all families the epithelium appeared degenerate with vacuolated cells, aggregates of cytokeratin filaments and areas of poor attachment to substratum (Fig. 4A). A broad band of subepithelial material was composed of irregular aggregates of curved (“curly”) fibrils, 9 to 10 nm diameter. Intermingled with these fibrils at irregular intervals were groups of normal collagen fibers (Fig. 4B). The abnormal material abutted against basal epithelial cells where basal lamina material was absent and extended between epithelial cells (Fig. 4C). In such areas hemidesmosomes were absent or poorly formed. In other areas there were large numbers of hemidesmosomes overlying apparently intact basal laminar material. In one case (4.I) collections of curly fibers were seen totally enclosed by epithelial cells but within the extracellular compartment (Figs. 4C, 4D).

Molecular Analysis

SSCP/heteroduplex analyses of the entire published coding sequence of the BIGH3 gene identified shifts in exons 4 (families 1 and 2) and 12 (families 3–5). Neither shift was found in 200 unrelated, unaffected individuals or in unaffected family members. In families 1 and 2, DNA sequence analysis revealed a G-to-T transition at nucleotide 417, causing an arginine-to-leucine substitution at amino acid 124. In families 3 to 5, DNA sequence analysis revealed a G-to-A transition at nucleotide 1711, causing an arginine-to-glutamine substitution at amino acid 555 (Fig. 5A).

DISCUSSION

We have analyzed five families with “Bowman’s layer” dystrophies. Our findings at the molecular level concur with others in demonstrating two forms of Bowman’s layer dystrophies, associated with R124L (CDBI) and R555Q (CDBII) mutations of the BIGH3 gene.6–8 Both show a strong phenotypic homogeneity. In CDBI (families 1 and 2) there is a superficial, confluent form of granular dystrophy. The onset of corneal erosions was in infancy, and visual failure followed during childhood. Surgical intervention was necessary in all individuals, in some in the second decade of life, and was followed by recurrence within the graft. In CDBII, the onset of erosions was also within the
first years of life, but visual deterioration was considerably slower than in CBDI. In our families, PTK successfully treated the erosions for prolonged periods, thereby delaying the need for surgical intervention.

In both CBDI and CDBII there were epithelial abnormalities on LM and EM. In CBDI, epithelial dehiscence, lack of cell contact with abnormal hemidesmosome/basal lamina implies an abnormality of cell attachment. In recurrent dystrophy, deposits were between the epithelium and donor Bowman’s membrane with intraepithelial involvement seen on LM and EM. Electron-dense, rod-shaped deposits were found within small vesicles inside and between epithelial cells, in some cases directly connected to deposits in Bowman’s layer. Beneath the deposits, both Bowman’s layer and the stroma (of the donor tissue) were normal. In CDBII, small collections of hyaline material were seen between epithelial cells on LM, and in one case collections of curly fibers were seen totally enclosed by epithelial cells.

These observations suggest that the epithelium is likely to be responsible for the production of the abnormal material, which aggregates as “rod-shaped” deposits or “hyaline material,” supporting the work of others. This implies that the designation of these conditions as Bowman’s layer dystrophies is incorrect because these are more likely to be dystrophies of epithelial origin. This is in keeping with similar findings for classical granular dystrophy.14

In CDBI the rod-shaped bodies are described as indistinguishable from those in granular dystrophy. However, we found a periodic, 9-nm cross-banding, which has not been described. CBDI and granular dystrophy both result from BIGH3 mutations (R124L and R555W, respectively). We suggest that both mutations cause aggregation of a mutant keratoepithelin protein, which is supported by the observation that antibodies to this protein bind to deposits in BIGH3-related dystrophies. However, the materials show distinct patterns of accumulation and, although they share ultrastructural features, this distinct banding pattern suggests that the material is not identical.

Our findings confirm that CDBII, or honeycomb dystrophy, is caused by the R555Q BIGH3 mutation. We have clinical and ultrastructural details of seven further families with this phenotype. This is, in our experience, the commoner condition, accounting in total for 9 of 11 Bowman’s dystrophy families. Ultrastructural examination of patients with CDBII who underwent surgery demonstrated the presence of curly fibrils as described by others.

Recent reports suggest that the R124L and R555W BIGH3 mutations do not account for all forms of Bowman’s layer dystrophies. Other BIGH3-related mutations may cause similar phenotypes, including a ΔF540 mutation described in a Sardinian family. In addition there remain other families with an anterior corneal dystrophy, whose exact phenotype is not clearly defined, which does not map to 5q31. These include families, which, confusingly, have also been designated CDBII or Thiel-Behnke dystrophy and show genetic linkage to chromosome 10q24.

The identification of mutations in the BIGH3 gene in a variety of anterior and stromal corneal dystrophies has enabled their morphologic manifestations to be correlated with the underlying molecular defect. Previous literature on Bowman’s layer dystrophies has been confused in the application of eponymous titles to different phenotypes. The histopathologic and ultrastructural appearances that we report, together with their clinical correlations, should help to clarify this confusion and allow their reevaluation as disorders of epithelial origin.

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References


