Abnormalities of Stromal Structure in the Bullous Keratopathy Cornea Identified by Second Harmonic Generation Imaging Microscopy

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Purpose. To identify structural alterations in collagen lamellae and the transdifferentiation of keratocytes into myofibroblasts in the corneal stroma of bullous keratopathy (BK) patients and to examine the relation of such changes to the duration of stromal edema or the underlying cause of BK.

Methods. Six normal human corneas and 16 BK corneas were subjected to second harmonic generation (SHG) imaging microscopy to allow three-dimensional (3-D) reconstruction of collagen lamellae. Expression of α–smooth muscle actin (αSMA) was examined by immunofluorescence analysis and conventional laser confocal microscopy.

Results. Collagen lamellae were interwoven at the anterior stroma and uniformly aligned at the posterior stroma, whereas αSMA was not detected throughout the entire stroma of the normal cornea. Nine (56%) and 7 (44%) of the 16 BK corneas showed abnormal collagen structure at the anterior and posterior stroma, respectively. Expression of αSMA was detected in the anterior or posterior stroma of 7 (44%) and 6 (38%) of the 16 BK corneas, respectively. Disorganization of collagen lamellae and myofibroblastic transdifferentiation were detected only in corneas with a duration of stromal edema of at least 12 months. Corneas with BK as a result of birth injury showed abnormal collagen structure at the posterior stroma, whereas those with BK resulting from laser iridotomy did not.

Conclusions. Changes in the structure of the entire stroma were detected in BK corneas with a duration of stromal edema of at least 12 months, suggesting that such changes may be progressive. In addition, the underlying cause of BK may influence structural changes at the posterior stroma. (*Invest Ophthalmol Vis Sci.* 2012;53:4998–5003) DOI:10.1167/iovs.12-10214

Bullous keratopathy (BK) is one of the main conditions for which keratoplasty is performed.[1–4] The preferred surgical procedure for BK has changed recently from penetrating keratoplasty to endothelial keratoplasty such as Descemet's stripping (automated) endothelial keratoplasty (DSAEK)[5–8] or Descemet's membrane endothelial keratoplasty (DMEK).[9–11] This change has improved visual outcome[6,12] and reduced the frequency of graft rejection.[13,14] The Eye Bank Association of America Statistical Report (provided in the public domain at http://www.restoresight.org) has revealed that endothelial keratoplasty has accounted for up to 30% of total corneal grafting since 2008, underlining the facts that the number of endothelial keratoplasty operations is increasing and that the indications for such surgery are expanding worldwide.[15]

Clinical investigations have shown that endothelial keratoplasty provides patients with a favorable postoperative visual acuity.[13,14] A recent study, however, found that 77% of eyes treated with DMEK and 23% of those treated with DSAEK achieved a visual acuity of 20/25 or better 12 months after surgery.[16] This means that 23% of DMEK eyes and 77% of DSAEK eyes did not manifest a favorable visual acuity after surgery. The rationale for endothelial keratoplasty is based on the supposition that the clarity of the edematous cornea can be recovered and visual acuity improved if stromal edema is removed. The discrepancy between this supposition and clinical outcome may suggest that subclinical stromal changes may affect the postoperative visual acuity of patients undergoing endothelial keratoplasty.

With the use of second harmonic generation (SHG) imaging microscopy, we previously detected subepithelial fibrosis and fibroblastic cells in corneal specimens from individuals with stromal edema.[17] We have also detected myofibroblasts in corneal specimens of BK patients by immunofluorescence analysis of α–smooth muscle actin (αSMA).[18] These pathologic changes were observed only in specimens from individuals for whom the duration of stromal edema was at least 12 months.[17,18] In vivo laser confocal microscopy has also revealed subepithelial fibrosis-like changes or the presence of fibroblastic or myofibroblastic cells in the anterior stroma of post-DSAEK BK patients with a preoperative duration of stromal edema of at least 12 months.[19] These various observations suggest that pathologic changes at the anterior stroma in individuals with BK are progressive and related to the duration of stromal edema.

Further improvement of postoperative visual acuity after DSAEK surgery would be facilitated by characterization of the condition of the entire edematous corneal stroma. Macroscopic changes such as Descemet's folding in the posterior stroma of the BK cornea necessitate evaluation of the condition of the posterior stroma and its relation to that of the anterior stroma. We have now evaluated the structure of collagen lamellae and the presence of myofibroblasts in the stroma of normal and BK corneas. Our observations revealed abnormalities in the structure of collagen lamellae as well as expression of the myofibroblast marker αSMA in BK corneas with a duration of

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stromal edema of at least 12 months. Such changes were detected in both the anterior and posterior stroma, with those in the posterior stroma possibly being related to the underlying cause of BK.

METHODS

Specimens

The study was approved by the Institutional Review Board of Yamaguchi University Hospital and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects. Specimens of six normal corneas (three male and three female; mean age ± SD, 63.7 ± 8.0 years; age range, 48–69 years) were obtained from Sight Life (Seattle, WA). Corneal buttons from 16 individuals with BK (6 male and 10 female; mean age ± SD, 63.9 ± 17.9 years; age range, 44–85 years) were obtained at the time of penetrating keratoplasty. Of the 16 BK cases, 6 were the result of intraocular surgery (3 of glaucoma surgery and 3 of cataract surgery), 4 were caused by laser iridotomy, 3 were attributable to birth injury, and 3 were due to other causes. The age and duration of stromal edema for each patient were determined from clinical charts (Table).

Tissue Preparation and Immunofluorescence Analysis

All corneal buttons were transferred to 4% paraformaldehyde immediately after collection. The tissue was fixed overnight at 4°C, after which two smaller (2- to 3-mm^2) blocks were dissected from the central region of each button and washed with PBS. One of the two blocks was for SHG imaging microscopy and the other for immunofluorescence analysis of αSMA. For the latter, each corneal block was permeabilized by exposure to acetone for 5 minutes at −20°C, washed with PBS, incubated first overnight at 4°C in 50% TD buffer (TD buffer: 137 mM NaCl, 25 mM Tris HCl [pH 7.4], 0.7 mM Na2HPO4, 5 mMKCl) containing 3% BSA and then for 72 hours at 4°C with FITC–conjugated mouse monoclonal antibodies to αSMA (1:100 dilution; Sigma, St. Louis, MO) and Syto59 dye (1:1000; Molecular Probes, Carlsbad, CA) in 50% TD buffer, and finally washed three times for a total of 3 to 4 hours with PBS. Corneal blocks for both SHG imaging microscopy and αSMA immunofluorescence analysis were mounted in 50% glycerol in PBS. For observation of the structure of the anterior or posterior stroma, the epithelial or endothelial side, respectively, of each tissue block was positioned next to the objective lens. The corneal blocks stained for αSMA and nuclei were observed with a laser confocal microscope (LSM 710 NLO; Carl Zeiss, Jena, Germany).

SHG Imaging Microscopy

Specimens were examined with an Axiovert 200 microscope (Zeiss), equipped with a 40× (numerical aperture, 1.2) water-immersion objective lens (Zeiss), with a working distance of 200 μm. Two-photon second harmonic signals from collagen were generated with a mode-locked titanium:sapphire laser (Chameleon; Coherent, Santa Clara, CA). The optimal wavelength for the generation of second harmonic signals from human corneal collagen was previously found to be 800 nm.[20] Forward scatter signals or transmitted signals that passed through the tissue were collected with the use of a condenser lens (numerical aperture, 0.55) and a narrow band-pass filter (400/50) positioned in front of the transmission light detector. Backscatter signals were collected by the microscope objective and detected over wavelengths from 377 to 430 nm with the use of the Zeiss LSM 710 META detector. With the multitrack mode of LSM 710 META, we obtained sequential, en face, second harmonic and single-photon fluorescence signals from the same optical slice. All samples were scanned with a 1-μm step size in the *z*-axis to generate three-dimensional (3-D) data sets extending from the surface of Bowman's layer or Descemet's membrane to a depth of 200 μm into the stroma. Twelve-bit, 512 × 512 images were recorded. The 3-D data sets were reconstructed with the use of the Zeiss LSM Image Examiner. A minimum of three 3-D data sets was collected from different randomly scanned regions of each corneal block.

TABLE. Characteristics of Study Subjects

Cause of BK	*n*	Mean Age ± SD (y)	Stromal Edema Duration (mo)	
			Range	Mean ± SD
Intraocular surgery	6	68.0 ± 16.6	9–39	18.0 ± 11.0
Laser iridotomy	4	78.0 ± 4.0	12–19	15.8 ± 3.3
Birth injury	3	46.0 ± 2.0	15–60	33.7 ± 23.5
Other	3	68.0 ± 15.6	6–16	11.0 ± 5.0
Total	16	63.9 ± 17.9	6–60	19.1 ± 13.4
Controls	6	63.7 ± 8.0		

RESULTS

With the use of SHG imaging microscopy, we obtained SHG signals derived from collagen fibers and lamellae in the corneal stroma (Fig. 1). Optical section images revealed distinct fiber-like structures with random orientations at the anterior stroma of the normal cornea (Fig. 1A). Such short fibers of the same orientation formed narrow and short lamellae, as previously described.[20] No obvious differences in collagen structure at the anterior stroma were apparent between any of the BK corneas examined and the normal corneas (Fig. 1B), again consistent with our previous observations.[17] At the level of Bowman's layer, a dot-like pattern of weak signals was detected in the normal cornea (Fig. 1C), whereas abnormal fibrous structures, indicative of subepithelial fibrosis, were apparent in some BK corneas (Fig. 1D), as previously described.[17] Wide lamellae consisting of long collagen fibers with the same orientation were detected at the posterior stroma of the normal cornea (Fig. 1E). This lamellar structure was also observed in some BK corneas (Fig. 1F), whereas abnormal linear structures corresponding to "cracked" lamellae were apparent in others (Fig. 1G). In other focal planes at the posterior stroma, distinct shortened and disorganized collagen lamellae were observed in some BK corneas (Fig. 1H).

To examine the 3-D structure of stromal collagen, we piled up the individual SHG images in order to reconstruct 3-D images and then obtained projection images of collagen lamellae in normal human corneas and corneas affected by BK (Fig. 2). Collagen lamellae at the anterior stroma of the normal cornea were interwoven and adhered to Bowman's layer (Fig. 2A). In contrast, those in the posterior stroma of the normal cornea were aligned parallel to Descemet's membrane (Fig. 2D). The structure of collagen lamellae in the anterior and posterior stroma of some BK corneas resembled that in the corresponding regions of the normal cornea (Figs. 2B, 2E). However, in other BK corneas, the structure of stromal collagen lamellae appeared altered. In the anterior stroma, abnormal SHG signals indicative of ectopic collagen fiber formation were thus detected above and below Bowman's layer (Fig. 2C). This finding of subepithelial fibrosis is consistent with our previous observations.[17] The interwoven structure of collagen lamellae was maintained in the BK corneas with subepithelial fibrosis. In the posterior stroma of some BK corneas, collagen lamellae adjacent to Descemet's membrane appeared to be highly packed or misaligned with the membrane (Fig. 2F).

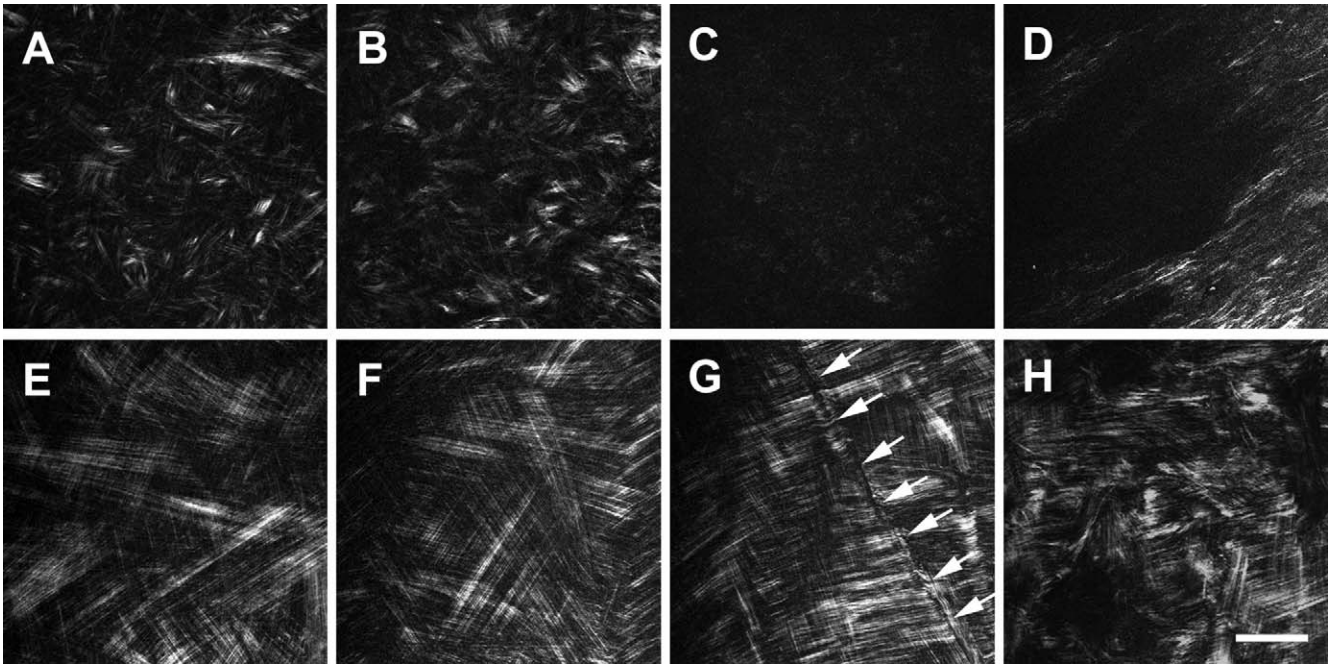

Figure 1. Representative images of the cornea obtained by SHG imaging microscopy. Images were derived from the anterior stroma (**A**, **B**), Bowman's layer (**C**, **D**), and the posterior stroma (**E**–**H**) of the normal cornea (**A**, **C**, **E**) or of BK corneas without (**B**, **F**) or with (**D**, **G**, **H**) collagen structural abnormalities. *Arrows* indicate "cracked" collagen lamellae. Scale bar, 50 μm.

Immunofluorescence analysis of αSMA expression revealed the absence of αSMA-positive cells in both the anterior and posterior stroma of all normal corneas examined (Figs. 3A, 3D). Similarly, αSMA-positive cells were not detected in the anterior or posterior stroma of some BK corneas (Figs. 3B, 3E). In contrast, other BK corneas manifested αSMA-positive cells in the anterior or posterior stroma (Figs. 3C, 3F), indicative of the transdifferentiation of keratocytes into myofibroblasts. No obvious morphologic differences were apparent between αSMA-positive cells in the anterior stroma and those in the posterior stroma.

To evaluate the impact of stromal edema on stromal pathologic changes, we examined the relation between the duration of stromal edema and either the presence of structural abnormalities of stromal collagen lamellae or αSMA expression in stromal cells (Fig. 4). In the case of the anterior stroma, abnormal collagen structure and αSMA-positive stromal cells were detected only in BK corneas affected by stromal edema for at least 12 months, providing further support for our previous observations.[17,18] With regard to the posterior stroma, abnormalities in the structure of collagen lamellae and αSMA-positive cells were also detected only in BK corneas with a duration of stromal edema of at least 12 months.

Finally, we evaluated the relation between structural abnormalities of stromal collagen lamellae and the underlying conditions for BK (Fig. 5). We excluded three BK corneas from this analysis because the underlying causes of BK were unknown or multiple, thus leaving three corneas with BK as a result of birth injury, four with BK caused by laser iridotomy, and six with BK attributable to intraocular surgery. For the patients with BK resulting from birth injury, collagen structural abnormalities in the anterior stroma were detected in the two

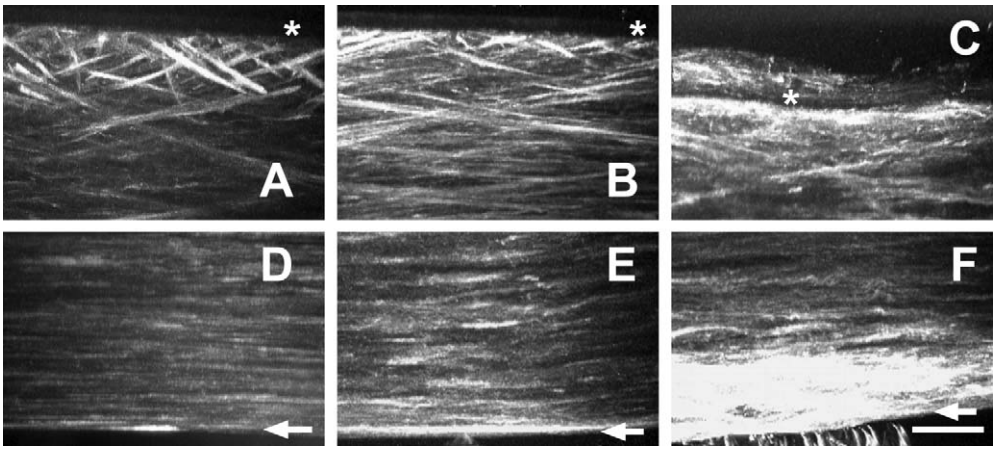

Figure 2. Representative projection images of the corneal stroma obtained by SHG imaging microscopy. Images were derived from the anterior (**A**–**C**) or posterior (**D**–**F**) stroma of the normal cornea (**A**, **D**) or of BK corneas without (**B**, **E**) or with (**C**, **F**) structural abnormalities of collagen lamellae. *Asterisks*: (**A**) through (**C**) indicate Bowman's layer. *Arrows*: (**D**) through (**F**) indicate Descemet's membrane. Scale bar, 50 μm.

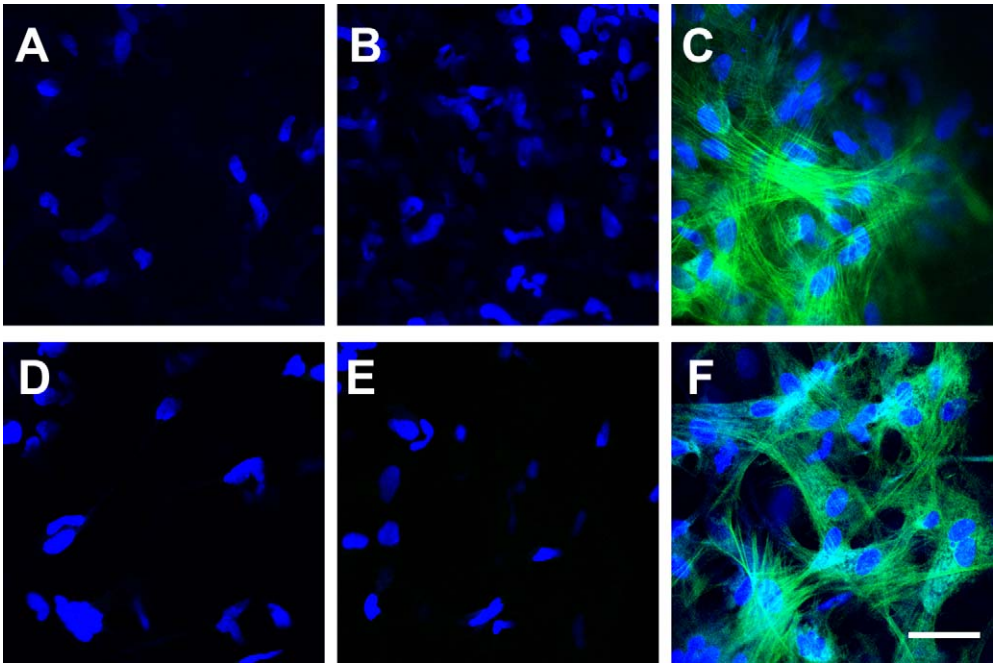

Figure 3. Immunofluorescence analysis of αSMA expression in stromal cells of the cornea. Images were derived from the anterior (**A**–**C**) or posterior (**D**–**F**) stroma of the normal cornea (**A**, **D**) or of BK corneas without (**B**, **E**) or with (**C**, **F**) cells positive for αSMA expression (*green fluorescence*). *Blue fluorescence* indicates nuclei. Scale bar, 50 μm.

corneas with the longest duration of stromal edema, whereas those in the posterior stroma were detected in all three subjects. For the patients with BK caused by laser iridotomy, collagen structural abnormalities in the anterior stroma were detected in the two corneas with the longest duration of stromal edema, whereas no such abnormalities were apparent in the posterior stroma of any of these four subjects. For the patients with BK attributable to intraocular surgery, collagen structure abnormalities in the anterior stroma were detected in the two corneas with the longest duration of stromal edema, whereas those in the posterior stroma appeared not to be related to the duration of stromal edema. It should be emphasized, however, that these observations were made with only a small number of subjects for each underlying cause of BK.

Discussion

Our present results have revealed that pathologic structural changes are apparent throughout the entire corneal stroma of

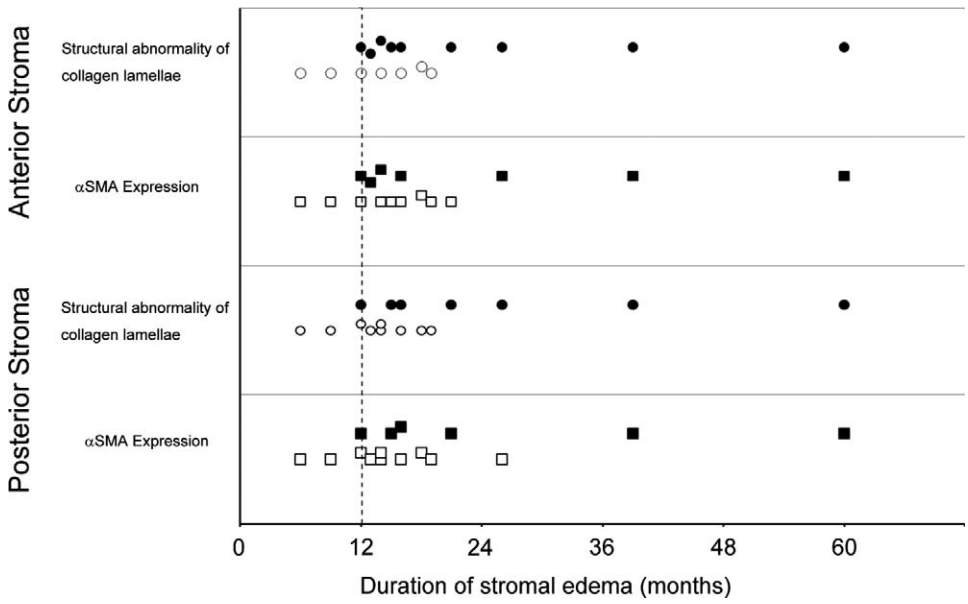

Figure 4. Relation between the duration of stromal edema and either structural abnormalities of collagen lamellae or αSMA expression in the anterior or posterior stroma of corneas affected by BK. *Open and closed symbols* represent corneas without or with the pathologic changes, respectively.

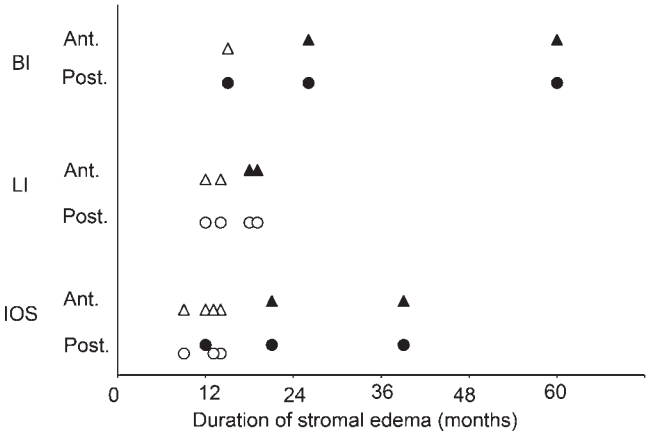

Figure 5. Relation between the duration of stromal edema and structural abnormalities of collagen lamellae in the anterior or posterior stroma of BK corneas according to underlying condition. BI, birth injury; LI, laser iridotomy; IOS, intraocular surgery; Ant, anterior; Post, posterior. *Open and closed symbols* represent corneas without or with abnormalities, respectively.

individuals with BK at ~12 months after the onset of stromal edema, indicating that BK may be a progressive disease and that stromal edema may give rise to such pathologic changes. Furthermore, the underlying cause of BK may differentially affect structural changes in the posterior stroma, suggesting that it might be necessary to take the cause of BK into account when deciding on a treatment strategy.

Pathologic and immunofluorescence analyses previously detected changes described as posterior collagenous proliferation[21] or a posterior collagenous layer[22,23] in the posterior stroma of corneas affected by BK. We have now detected structural disorganization of collagen lamellae at the posterior stroma of the BK cornea. As we described previously,[17] SHG imaging microscopy is able to detect collagen structural alterations in larger regions of the corneal stroma compared with conventional microscopic analysis of tissue sections. Furthermore, a great advantage of SHG imaging microscopy is that it can detect the structure of collagen lamellae.

As we had described previously for the anterior stroma,[17] we have now shown that the structure of collagen lamellae is altered in the posterior stroma of the BK cornea in a manner apparently related to the duration of stromal edema. The pathophysiologic basis for such disorganization of collagen lamellae at the posterior stroma is unclear. However, the separation of collagen fibers induced by stromal edema may allow the deposition of extracellular matrix between the fibers over time, giving rise to the observed disorganization of collagen lamellae. Indeed, abnormal accumulation of extracellular matrix in the stroma of the BK cornea has been described.[23] In contrast to collagen lamellae at the anterior stroma of the normal cornea, which are well packed and dense, those at the posterior stroma are rough and not densely packed, characteristics that might facilitate the accumulation of extracellular matrix and consequent changes in lamellar structure.

We examined the relation of the occurrence of changes in the structure of collagen lamellae in the corneal stroma to the underlying causes of BK. Such changes occurred frequently in cases of BK related to birth injury but not in those of BK caused by laser iridotomy. Corneas damaged by birth injury experience physical deformation of the stroma and loss of endothelial cells, resulting in the observed structural disorganization of collagen lamellae. In contrast, laser iridotomy does not result in direct physical injury to the cornea. Although several mechanisms of laser iridotomy–induced BK have been proposed,[24,25] the underlying pathogenesis remains unknown. The fact that laser iridotomy does not result in physical deformation of the cornea may explain the absence of changes to the structure of collagen lamellae in the posterior stroma of corneas with BK related to this procedure. Although the number of cases of laser iridotomy–induced BK in the present study was limited, all four such corneas, with a duration of stromal edema between 12 and 19 months, did not show an altered structure of collagen lamellae in the posterior stroma, whereas all three corneas with BK resulting from birth injury, with a duration of stromal edema of at least 15 months, did. Further investigation with cases matched for duration of stromal edema will be required to confirm this difference. The number of subjects in the present study was relatively small, in part because the number of BK patients undergoing penetrating keratoplasty is declining as a result of the transition of the preferred surgery from penetrating keratoplasty to endothelial keratoplasty.

Our present results confirm our previous findings of pathologic changes such as subepithelial fibrosis and fibroblastic-myofibroblastic transdifferentiation of keratocytes at the anterior stroma of BK corneas with a duration of stromal edema of at least 12 months.[17,18] Such changes to the anterior stroma were detected in the cases of laser iridotomy–induced BK with the longest duration of stromal edema, whereas pathologic changes were not detected at the posterior stroma of any of the subjects in the laser iridotomy group. This difference may indicate that pathologic changes at the anterior stroma are influenced by several factors such as tear fluid exposure following epithelial erosion,[18] whereas the posterior stroma is exposed to a stable environment after the development of edema.

Previous studies have described the formation of retrocorneal fibrous membrane in many cases of BK.[22,23] We did not detect such a structure in the present study, however. Retrocorneal fibrous membrane has been found to contain many types of collagen including types I, III, IV, V, VI, XII, and XIV.[22,23,26] Although all types of collagen are able to generate SHG signals, an oriented structure is important for the generation of a strong signal. SHG signals derived from Bowman's layer are weak (Fig. 1C), even though Bowman's layer contains several types of collagen. Electron microscopy has revealed that the collagen in Bowman's layer is not aligned but instead is amorphous without orientation,[27] likely explaining why SHG signals derived from Bowman's layer are weak. The structure of collagen in retrocorneal fibrous membrane is unknown, but if the collagen is not aligned or oriented, then it would not be expected to generate a strong SHG signal. Future studies combining immunostaining of sectioned samples with SHG imaging microscopy may provide new information on retrocorneal fibrous membrane.

Our previous[17,18] and present observations have shown that collagen structural alterations at the anterior stroma and subepithelial lesions are present in the BK cornea, even though BK is considered to be an endothelial disease with a pathogenesis attributable to endothelial decompensation. Na^+- and K^+-dependent ATPase activity has been found to be decreased in the epithelium of the BK cornea,[28] suggesting that epithelial function is impaired and that the excess water content of the corneal stroma associated with stromal edema may be derived from both aqueous humor and tear fluid. Such an impairment of corneal epithelial function may be related to the occurrence of epithelial erosion, which is thought to be a contributing factor to anterior stromal scarring in BK.[18] Further investigation is thus warranted into the role of epithelial changes in BK and into whether epithelial protection should be considered for the BK cornea before surgery.

The possibility that epithelial erosion might affect the development of pathologic changes at the anterior stroma suggests that such erosion should be prevented in individuals with BK before the performance of endothelial keratoplasty, as pointed out in our previous study.[18] On the basis of our results, we propose that (1) BK patients who are candidates for endothelial keratoplasty should undergo the procedure within 12 months after the onset of clinical stromal edema (the earlier, the better); (2) the cornea of such patients should be treated to prevent corneal epithelial erosion by application of oil ointment until the surgery is performed; and (3) BK patients with a clinical course of more than 12 months should be informed that the recovery of postoperative visual acuity may be unsatisfactory or delayed because of pathologic changes to their anterior stroma.[19] In addition to postoperative care, preoperative care may thus be important to achieve a favorable postoperative visual acuity in candidates for endothelial keratoplasty.

In conclusion, we have demonstrated structural alterations in the entire corneal stroma of individuals with BK. In addition to the duration of stromal edema,[17–19] the underlying cause of BK may influence such pathologic changes. Such factors should be taken into account in determination of the timing of endothelial keratoplasty.

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