Histopathological and immunohistochemical studies of lenticules after epikeratoplasty for keratoconus

H Nakamura, F Riley, H Sakai, W Rademaker, B Y J T Yue, D P Edward

Aims: To examine histopathological and immunohistochemical changes in lenticules and host of corneal buttons from patients who previously underwent epikeratoplasty for keratoconus.

Methods: 12 penetrating keratoplasty specimens from patients with keratoconus who had previously undergone epikeratoplasty, eight keratoconus, and seven normal corneas were examined. Immunostaining for Sp1, α1-proteinase inhibitor (α1-PI), and α2-macroglobulin (α2M) were performed.

Results: In nine of the 12 lenticules, the keratoconus-like disruptions were found in Bowman’s layer. Peripheral and anterior keratocyte repopulation of the lenticules was observed in all cases. Keratocyte repopulation in the anterior and mid-stromal regions of the lenticules appeared related to the time since epikeratoplasty. Sp1 nuclear staining of the basal and wing epithelial cells was more intense in lenticules and keratoconus corneas than in normal corneas. Lenticular, host, and keratoconus keratocytes showed positive Sp1 staining, whereas staining was absent in normal corneas. Compared to normal corneas, α1-PI and α2M immunostaining was lower in the lenticules, host, and keratoconus specimens.

Conclusions: The epithelial cells and keratocytes repopulated in the lenticules retain keratoconus-like biochemical abnormalities such as upregulation of Sp1 and downregulation of α1-PI and α2M. The authors speculate that both keratocytes and the corneal epithelium may participate in the development of keratoconus.

Materials and Methods

Twelve corneal buttons were obtained from patients who had previously received epikeratoplasty for management of keratoconus at the time of penetrating keratoplasty from the King Khaled Eye Specialist Hospital, Riyadh. The grafts were done because of varying degrees of uncorrected refractive problems following the epikeratoplasty. Seven normal human eyes from donors (ages 22–83 years old) were obtained from the Illinois Eye Bank, Chicago, or from the National Disease Research Interchange, Philadelphia, PA, within 24 hours of death. None of the donors had any known ocular diseases, and their corneas were clear and unremarkable. As another set of controls, eight corneal buttons from

Abbreviations: α1-PI, α1-proteinase inhibitor; α2M, α2-macroglobulin; ECM, extracellular matrix
patients (ages 22–70 years) with typical clinical features of keratoconus but without undergoing epikeratoplasty surgery previously were obtained following transplantation from the Cornea Service at the University of Illinois at Chicago.

Corneas excised from normal human eyes and keratoconus buttons were fixed in 10% buffered formalin, processed, and embedded in paraffin. Immunohistochemistry was performed on deparaffinised 5 μm sections using the indirect immunoperoxidase technique. The primary antibodies used in the study included (a) a polyclonal rabbit anti-Sp1 antibody (PEP 2, diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), (b) polyclonal goat antibodies specific for α1-PI (1:100, ICN Biomedicals, Irvine, CA, USA), and (c) α2M (1:100, ICN Biomedicals). The chromogen used for the anti-Sp1 was fast red TR/naphthol AS-MX phosphate (Sigma, St Louis, MO, USA). For α1-PI and α2M, 3,3-diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen.

The staining intensity in each experiment was scored by three masked observers on a scale of 0 to 4, with 0 indicating no staining and 4 the most intense staining. Experiments were repeated three times.

Histopathological changes in the epikeratoplasty specimens were evaluated on haematoxylin and eosin stained slides. Alterations in Bowman’s layer of the lenticules were evaluated only in the central regions of the specimens to exclude breaks seen along suture tracks in the peripheral regions of the specimens.

To confirm the labelling intensity evaluated by the masked observers we performed image analysis on the selected images presented in the paper demonstrating immunostaining with the three antibodies (see figs 3, 5, and 6). The image file was converted from RGB to grey scale image using Adobe Photoshop 7.01. Imaging Processing Tool kit 3.0 (Reindeer Games, Inc), incorporated in the Adobe Photoshop 7.01, was used to measure the intensity of staining. The intensity, judged as 256 shades of grey with 0 representing black and 256 representing white, was measured in at least six basal epithelial cells, wing cells, keratocytes, and/or six areas (25 μm diameter circle) in the stromal matrix. Background intensity was also taken in the empty space. The staining intensity of each cell type or the stromal matrix tissue was obtained by subtracting the background from the measured intensity values. Statistical analysis was performed using two tailed unpaired Student’s t test to compare the staining intensity of KC or EpiK cornea with normal control. Values of p<0.05 was considered to be significant.

RESULTS
The salient clinical features of the 12 patients who had previously received epikeratoplasty as a treatment for keratoconus are summarised in table 1.

By histopathology, the keratoconus-like breaks in Bowman’s layer were found in nine of the 12 failed lenticules (table 2, fig 1).

Keratocyte repopulation was observed in all lenticules at all levels of the peripheral stroma (table 2). Keratocytes were identified morphologically by the spindle-shaped nature of their nuclei, which are quite distinct from other cells such as leucocytes that might be resident in the corneal stroma. The

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age at PKP</th>
<th>Period after epiK (year)</th>
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<td>F</td>
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<td>cryofrozen</td>
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<td>2</td>
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<td>5</td>
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PKP, penetrating keratoplasty; epiK, epikeratoplasty.

Table 2  Histopathology results

<table>
<thead>
<tr>
<th>Case</th>
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<th>Keratocyte repopulation</th>
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A, anterior; M, middle; P, posterior.
cells with spindle-shaped nuclei accounted for most of the cells within the lenticules. Rare cells consistent with leucocytes in the stroma were also noted. In the central region, repopulation was also observed in all lenticules in the posterior portion. Keratocytes that repopulated the posterior portion of the lenticules were mainly seen adjacent to Bowman’s layer of the host corneas. In some areas, keratocytes appeared to migrate through the Bowman’s break of the host tissues (fig 2). However, repopulation in the anterior and mid-stromal region of the central lenticule was noted in only five lenticules. In these cases, the patients had a significantly longer history of epikeratoplasty than the others (5.8 (SD 2.8) vs 2.7 (1.4) years; p<0.05, Mann-Whitney U test, tables 1 and 2).

Immunohistochemical experiments showed positive Sp1 staining in the nuclei of basal corneal epithelial cells and wing cells in the lenticules, as well as keratocytes in both the lenticules and host stromas. The staining intensity in the epithelial cells of lenticules and that in the stromal cells in the lenticules and host corneas were statistically higher than that in normal human corneas (p<0.05, figs 3 and 4). Keratoconus corneas, used as positive controls, also showed strong nuclear staining of comparable intensity for Sp1 in both corneal epithelial cells and keratocytes.

Compared to that in normal human corneas, immunostaining for both α1-PI and α2M was considerably weaker in epithelial cells of the lenticules, and keratocytes in the lenticules and host stromas (figs 5 and 6). Each of these differences was statistically significant (p<0.05, figs 7 and 8). A similar reduction in staining intensity was likewise observed in keratoconus corneas. Staining intensity in the stromal lamellae for α1-PI in the lenticules and host corneas was also statistically lower than that in normal corneas (p<0.05, figs 6 and 7). The staining for α2M was also reduced but the difference was not statistically significant from the normal controls (figs 6 and 8). In the epikeratoplasty specimens the immunoreactivity with three antibodies in

Figure 1 Lenticules from patients who underwent epikeratoplasty for keratoconus (cases 5, 10, and 11). Arrow indicates the focal disruption in Bowman’s layer. The disruption or break resembles that described in keratoconus corneas (bar indicates magnification).

Figure 2 The central portion of the lenticules and host corneas from cases 3 and 6. Arrow indicates Bowman’s layer of the host cornea. Arrowheads indicate keratocytes repopulated in the lenticules. Asterisk denotes a break observed in Bowman’s layer of host. The symbol ^ indicates disruption of Bowman’s layer in the lenticule. In case 3, a spindle cell appeared to extend through the break in Bowman’s layer from the host stroma to the lenticule. The repopulated keratocytes were situated adjacent and right above the host Bowman’s layer. In case 6, keratocyte repopulation was observed throughout the anterior and posterior regions of the lenticules (bar indicates magnification).

Figure 3 Immunostaining for Sp1 in corneas from a 61 year old normal individual and a 22 year old keratoconus patient, as well as a lenticule of a patient after epikeratoplasty for keratoconus (case 12). In both the lenticule and keratoconus corneas (positive controls), the nuclear immunolabelling was stronger in basal corneal epithelial cells, wing cells, and keratocytes than cells in the normal cornea (chromagen fast red TR/naphthol AS-MX phosphate). Image analysis confirmed significant differences in labelling intensity between normal control (epithelium: basal: 35.8 (SD 4); wing: 32 (4); stromal cell: 9 (3)) and epikeratoplasty (epithelium, basal: 64 (7) (p = 0.000008); wing: 59 (8) (p = 0.001); stromal cell: 82 (4) (p = 0.000001)), and keratoconus (epithelium: basal: 55 (4) (p = 0.001); wing: 55 (3) (p = 0.00001); stromal cell: 81 (2) (p < 0.00001)).
the lenticular stroma and the host was comparable (figs 6, 7, and 8). As expected, the staining intensity was comparable in the keratoconus and epikeratoplasty specimens.

Image analysis confirmed the staining differences that were observed on semiquantitative assessment of labelling intensity (see fig 3, 5, and 6).

**DISCUSSION**

To the best of our knowledge, this is the first demonstration of keratoconus-like breaks in Bowman's layer in lenticules from eyes that previously underwent epikeratoplasty as a treatment for keratoconus. The breaks observed in our study occurred with or without keratocyte repopulation and closely resembled those described previously in keratoconus. Both the presence of Bowman's breaks and absence of Bowman's layer have been reported previously in lenticules after epikeratoplasty for myopia,24 25 28 aphakia,24 28 and keratoconus.28 However, those large breaks, at times with almost total loss of Bowman's layer, differed from the focal breaks or fragmentation observed in our cases in the failed lenticules and in typical keratoconus corneas.10–12 Absence of large fragments of Bowman's layer is unusual in typical keratoconus and if seen may be associated with hydrops or associated contact lens related complications. The incidence of Bowman's layer disruption was also much higher in our cases (nine of 12 cases) than that in previous reports (eight of a total of 21 cases in the literature).23–28

This is also the first demonstration of remarkable keratocyte repopulation of the posterior stroma in the central lenticule. Keratocyte repopulation in lenticule had been reported to be slow process from peripheral region, and to take 48 months to complete.29 In our cases, the central posterior repopulation was noted in all 12 cases examined including seven lenticules which were examined at the time of less than 4 years after epikeratoplasty (fig 2 and table 2). Since all of the lenticules in this series were cryofrozen before the epikeratoplasty, the donor keratocytes would not be expected to survive through the cryofreezing process.30 Therefore, we suggest that the cells present in the lenticules were host keratocytes, and that the keratocytes in the posterior region of the lenticule migrated from underlying host tissue through breaks in the host Bowman's layer.

As previously reported in eyes with and without keratoconus,22 25 29 30 we noted that keratocytes repopulated the lenticules in the periphery, and that keratocyte repopulation was not predominant in the superficial and central region of the lenticules (table 2). In five lenticules, repopulation in the anterior or mid-stromal portions of central region was observed and the extent of keratocyte repopulation in these regions, interestingly, seemed to correlate with the length of time since epikeratoplasty. The time dependence supports a previous conclusion29 that the anterior and middle lenticular keratocyte repopulation perhaps takes place gradually from the periphery, along stromal lamella.

Immunohistochemical experiments demonstrated that in the epithelium overlaying the lenticules, Sp1 staining was increased and staining for inhibitors z1-PI and z2M was decreased. These have been shown to be changes that are specific to keratoconus corneas.16–18 The epithelium of the lenticules thus displayed a keratoconus-like phenotype, which is not surprising as the epithelium was derived from

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**Figure 4** Staining intensity for Sp1 in the corneal epithelial cells and keratocytes in the lenticules (n = 12), host stromas (n = 12), and normal human (n = 4) and keratoconus (n = 3) corneas as scored by three masked observers. The scores were analysed by Mann-Whitney U tests. *p* < 0.01 compared with normal human specimens; †p* < 0.05.

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**Figure 5** Immunostaining for α1-proteinase inhibitor in corneas from 93 year old (top) and 78 year old (bottom) normal individuals, and a 70 year old keratoconus patient, as well as lenticules of patients after epikeratoplasty for keratoconus (cases 3 and 12). Arrow indicates Bowman's layer of the host cornea. Arrowheads indicate keratocytes. Note that the intensity of the brown positive staining is lower in the epithelium, keratocytes, and stromal lamellae in the lenticules and host corneas than that in normal corneas. Staining is also weaker in keratoconus corneas compared to normal controls (chromagen 3-3' diaminobenzidine). Image analysis confirmed significant differences in labelling intensity between normal control (epithelium: basal cell in host: 19 (2) (p = 0.00000001); wing: 53 (4) (p = 0.0000001); stromal ECM (ECM) 72 (5)); and epikeratoplasty case 3 (epithelium: basal: 63 (8) (p = 0.00000001); wing: 53 (4) (p = 0.0000001); ECM 72 (5)); stromal cell in lenticule: 31 (6) (p = 0.0000001); ECM in lenticule: 41 (2) (p = 0.00000001); cell in host: 19 (2) (p = 0.0000001) and keratoconus (epithelium: basal: 43 (4) (p = 0.00000001); wing: 49.3 (10) (p = 0.00000001); stromal cell: 37 (8) (p = 0.0000001); ECM 12.5 (3) (p = 0.0000001)). Image analysis for case 12 also showed highly significant differences from control.

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**Figure 6** Immunostaining for z1-PI and z2M was
the host. The role of the corneal epithelium in development of keratoconus was proposed in the 1960s\textsuperscript{10,11,39} based on electron microscopic studies. Further evidence was provided when biochemical abnormalities including upregulation of degradative enzymes and downregulation of inhibitors were prominently observed in the keratoconus epithelium.\textsuperscript{16-18} A study by three dimensional scanning electron microscopy\textsuperscript{12} further indicated that the changes in the Bowman's layer precede those in the corneal stroma and that the corneal epithelium may be an important factor at the early stage of keratoconus development. The current finding of alterations in the epithelium overlying the lenticules is consistent with this theory. It is possible that the corneal epithelium overlying the lenticules, with imbalanced enzyme and inhibitor levels, may cause disruption in Bowman's layer typically seen in keratoconus specimens.

In the lenticular stroma, upregulation of Sp1 was demonstrated in the repopulated keratocytes. Downregulation of α1-PI and α2M was also observed. These results indicate that the repopulated keratocytes retained the biochemical abnormalities ascribed to keratoconus. The rarity of recurrent cases of keratoconus after penetrating keratoplasty seems to argue against the corneal epithelial theory. Indeed, if the epithelial cells were the sole causative factor,\textsuperscript{10,11,39} frequent recurrence of keratoconus would occur as the epithelial cells are expected around 5 days to migrate from the recipient into the donor cornea.\textsuperscript{7} It is possible that the corneal epithelial involvement is necessary but not sufficient. We speculate that perhaps both the disruption of the Bowman's layer\textsuperscript{10,11,12,39} and a predisposed defect in keratocytes are required for the disease development. The current study, showing defects in Bowman's layer, delayed keratocyte repopulation, and biochemical abnormalities ascribed to keratoconus in the repopulated keratocytes in failed lenticules 15 months to 9 years after epikeratoplasty, supports this possibility.
In summary, we demonstrated keratoconus-like disruptions in Bowman's layer in nine of the 12 lenticules. The keratocytes appeared to repopulate the lenticules from the peripheral and underlying host tissues. By immunohistochemistry, Sp1 nuclear staining in both epithelium and stroma was increased in the lenticules and keratoconus than that in normal corneas. In contrast, immunostaining for z1-PI and z2M in epithelial cells, keratocytes, and stromal lamellae was less intense in the lenticule, host corneas, and keratoconus specimens than in normal cornea. These findings suggested that the epithelial cells and keratocytes that repopulated the lenticules retained keratoconus-like biochemical abnormalities resulting in some of the changes noted in the lenticules.

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