Cornea

Histological and Confocal Microscopy Changes in Chronic Corneal Edema: Implications for Endothelial Transplantation

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PURPOSE. To report in vivo confocal microscopic (IVCM) features in corneal edema supported by histopathologic correlation.

METHODS. This was an observational study with evaluation of diagnostic technology. Twenty patients with clinically diagnosed corneal edema were involved, including 11 with Fuchs’ endothelial dystrophy (FED). All cases, in addition to a control group of six normal eyes, were examined with IVCM before keratoplasty. Four eyes were examined after surgery. Thirteen corneal samples obtained by penetrating keratoplasty were examined by light and/or electron microscopy. IVCM and histopathologic sections were then analyzed for correlation and proper interpretation. Seventeen patients underwent Descemet’s stripping endothelial keratoplasty (DSEK).

RESULTS. Subepithelial fibroblasts were seen histologically and with IVCM in 7 (53.8%) of 13 full-thickness corneal samples. IVCM alone detected these changes in 11 (55%) subjects before surgery, as well as after postoperative clinical improvement. Other IVCM features included absent (30%) or reduced (70%) subbasal corneal nerves, expanded hyperreflective keratocyte cell bodies, and processes with small vacuoles and large extracellular lacunae (95%), seen on IVCM only. Endothelial changes with polymegathism and reduced cell density were seen in non-FED cases.

CONCLUSIONS. This is the first study in which IVCM features of corneal edema have been compared in detail with histopathologic findings. Subepithelial fibroblasts, reduced subbasal corneal nerves, and stromal keratocyte morphology were well documented in this study. With increasing popularity of DSEK this work supports the role of IVCM in quantitative evaluation of corneal edema in early preoperative stages, as well as after surgery, when the cornea appear clinically, but not histologically, normal. (Invest Ophthalmol Vis Sci. 2011;52:8193–8207) DOI: 10.1167/iovs.11-8047

Corneal edema is a clinical condition characterized by increased corneal thickness due to excessive accumulation of water into (hydrophilic) stromal layers, usually secondary to decreased function or disease in the corneal endothelium. The important causes include late stages of Fuchs’ endothelial dystrophy (FED) and surgical trauma, commonly associated with cataract surgery (pseudophakic bullous keratopathy). Bullous keratopathy represents an advanced stage of chronic corneal edema, in which the corneal epithelium is affected and presents multiple cystic collections of water, termed bullae, of various sizes. This accumulation of water can be intraepithelial or subepithelial, causing separation of the basal epithelium from the underlying Bowman’s zone (BZ).

For many years, penetrating keratoplasty (PK) has been the definitive treatment for corneal edema with endothelial dysfunction. In PK, the whole cornea, including the edematous stroma and bullous epithelium, is replaced by donor tissue. With the advent of lamellar endothelial keratoplasty (EK) in the form of Descemet’s stripping endothelial keratoplasty (DSEK) or Descemet’s membrane endothelial keratoplasty (DMEK), the edematous stroma and epithelium are retained, and only the diseased endothelium is replaced. Clinical experience has substantiated the many advantages of these techniques over PK.1–4 However, it has also been observed that excessively or chronically edematous corneas do not clear in the same manner as early cases (Dua HS, personal observations, 2009). Certain changes, presumably permanent, appear to occur after longstanding corneal edema, raising the question of whether EK successfully restores normality and perhaps whether the indications for EK need to be refined in the light of changes induced by chronic edema.

It is important therefore to elucidate in detail the changes that occur in the cornea as a consequence of chronic edema. In vivo confocal microscopy (IVCM) has been a useful, noninvasive imaging tool for investigating cellular changes in various corneal disorders. In this study, we examined by IVCM the changes occurring in all layers of edematous corneas and in a subgroup that subsequently underwent EK. We were able to correlate these to histologic findings. Our purpose was to identify and document specific changes that would form the basis of further studies to evaluate the persistence or reversal of these changes after EK.

METHODS

In this observational case series, 20 patients (14 women and 6 men; age range, 56–85 years) with corneal edema, attending the Queen’s Medical Centre (Nottingham University Hospitals, United Kingdom) were included. The diagnosis of corneal edema was based on history, slit lamp examination, and central corneal thickness (CCT) measured by ultrasonic pachymetry (SP-3000 Pachymeter; Tomey Corporation, Nagoya, Japan).
The patients fell into two groups: those with Fuchs' endothelial dystrophy (FED; 11 patients, 9 women and 2 men) and non-Fuchs' patients (9 patients, 4 men and 5 women), which included five with pseudophakic bullous keratopathy (PKB) and four with conditions due to other causes (recurrent HSV endothelial/stromal keratitis \( n = 2 \), glaucoma with filtration surgery \( n = 1 \), and unknown \( n = 1 \)). None of the patients with PKB had an anterior chamber lens implant. Among the FED patients, five were also pseudophakic. Patients' and controls' demographics are given in Table 1.

All patients underwent routine assessment of visual acuity, pachymetry, and slit lamp examination. IVCM of the affected cornea was also performed in all patients (with Heidelberg Retina Tomogram II-Rostock Cornea Module; HRTII-RCM; Heidelberg Engineering GmbH, Heidelberg, Germany). This is a laser scanning confocal microscope (LSCM) using class I 670-nm red laser beam for illumination to yield high-contrast, digitized images of 400 × 400 μm each. The software driver (Eye Explorer, ver. 1.5.10.0; Heidelberg Engineering) of this confocal microscope allows for an automated z-scan with determination of focal depth within various corneal layers. Automated sequential acquisition of a series of 30 en face images at 2-μm intervals (covering 60 μm of tissue thickness) provided a volume scan (or z-scan) for each area of interest, which allowed examination of the area scanned from the surface (beginning) to the deepest layer (end). Oblique IVCM images of the areas examined were also obtained. Oblique images are the nearest the equipment can get to providing in vivo cross-sectional images resembling those obtained in histology sections.

The IVCM scanning was started at the central 3 to 4 mm of the cornea and then moved across to the superior, inferior, temporal, and nasal quadrants. This dynamic scanning was guided by a side-mounted digital camera attached to HRTII-RCM, the view of which is displayed next to the IVCM view on the screen to help ascertain the location of the cornea and then moved across to the superior, inferior, temporal, and nasal quadrants. This dynamic scanning was guided by a side-mounted digital camera attached to HRTII-RCM, the view of which is displayed next to the IVCM view on the screen to help ascertain the location of the cornea being scanned. Images were captured at each point through the thickness of the cornea, giving a total of 300 to 500 frames per cornea. When corneal grafting was completed, the tissue was marked for the 12-o'clock position so that histologic examination could be performed specifically in the quadrant of interest.

A control group of six people with normal corneas (five men and one woman; aged 21–72 years) were examined with IVCM, and their results were compared with those of the study group. IVCM examination was performed after surgery in a subgroup of four patients who underwent DSEK, to compare the results with preoperative findings. Two were examined in the early postoperative period, at months 7 and 8 (cases 11 and 7), and two in the late postoperative period, at months 20 and 26 (cases 9 and 18).

IVCM was performed with the patient’s eye under topical anesth-"
used with ImageJ to facilitate the tracing and length measurement of subbasal corneal nerves in IVCM images semimanually. An average of three to five images was chosen in each case. Details of the method are given in our previous publication.6 Thirteen corneal samples obtained by PK were processed for light microscopy. They were embedded in resin or wax blocks to obtain cross sections of 5 μm for hematoxylin and eosin (H&E) and 1 μm for toluidine blue staining. Three representative samples were further examined by electron microscopy (EM; cases 2, 16, and 17; Table 1). For light microscopy, they were examined with a digital scanner (Nanozoomer Digital Pathology scanner; NDP-C9600 series; Hamamatsu Photoins KK, Hamamatsu City, Japan), using its viewer software for high-magnification views and cell morphology. Calculation of nuclear length was performed using the software of the scanner, as illustrated in Figure 6. Nuclei of all the subepithelial cells in a given 40× field of the histology sections were measured. Twice as many nuclei of the corresponding corneal stroma were also randomly selected and measured. When two nuclei overlapped or lay very close to each other, they were avoided. The average nuclear size was determined and compared for the two sets of cells. Three patients, three fields each, were thus analyzed.

IVCM features were analyzed and correlated with histopathologic findings. Informed written consent was obtained from all patients. The study was approved by local Research Ethics Committee (REC no. 06/Q2403/46) and was consistent with the tenets of Declaration of Helsinki.

**RESULTS**

All patients presented with gradual deterioration of vision that was at its worst on waking in the morning. Three patients had painful bullous keratopathy. Best corrected visual acuity (BCVA) before surgery ranged between 6/9 (log MAR 0.17) and

<table>
<thead>
<tr>
<th>TABLE 2. Main IVCM Findings</th>
</tr>
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**A. Corneas with Edema**

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<tr>
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<th>Histology</th>
<th>IVCNM</th>
<th>Subepithelial Fibroblasts</th>
<th>Subbasal Nerve Density (mm/mm²)</th>
<th>BZ/Stromal Granularity</th>
<th>Keratocyte Changes</th>
<th>Endothelial Changes</th>
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**B. Corneal Edema with Corresponding Percentages and Light Microscopic Histologic Features**

<table>
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<th>Corneal Changes</th>
<th>n (%)</th>
<th>IVCNM Patterns</th>
<th>Histology Features</th>
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<td>Subepithelial fibroblasts</td>
<td>11 (55)</td>
<td>Bright round to oval figures, 10 to 20 μm in size, randomly distributed between basal epithelium and BZ.</td>
<td>Cells with elongated, slender nuclei 10.7 ± 4.1 μm (mean ± SD) in length between basal epithelium and BZ.</td>
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<td>Subbasal nerve density</td>
<td>14 (70)</td>
<td>4.47 ± 2.05 mm/mm² (mean ± SD)</td>
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<td>BZ/anterior stroma</td>
<td>20 (100)</td>
<td>Loss of normal reflective pattern (K-structures.)</td>
<td>No corresponding changes</td>
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<td>Keratocyte changes</td>
<td>19 (95)</td>
<td>Hyperreflective expanded cell bodies and processes; dark small paramural intracellular vacuoles (10–20 μm); large extracellular lacunae (40–100 μm) between the keratocytes.</td>
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<td>Endothelial changes</td>
<td>11 (55)</td>
<td>FED: classic strawberry-like changes.</td>
<td>FED, thickened Descemet’s membrane with inner excrescences on the endothelial side.</td>
</tr>
</tbody>
</table>

n/a, sample not available (in DSEK).

* Visible scar on slit lamp examination.
count fingers (CF; log MAR 1.7) in the affected eye, and the average central corneal thickness (CCT) was 679 ± 69 μm (range, 588–792). Slit lamp biomicroscopy revealed corneal scarring in two non-FED cases only. Thirteen patients underwent PK, of which six had a triple procedure (combined with lens extraction and implant). Seven patients underwent DESK, of which five were pseudophakic. Table 2A shows the histologic and IVCM findings in all subjects with corneal edema. Table 2B presents brief descriptive data of IVCM patterns in corneal edema.

Epithelial Bullae

On IVCM, epithelial bullae appeared as dark, round areas with well-defined margins. Most of these were intraepithelial, within the midepithelial layers (Figs. 1A–C). These changes were seen in 11 cases in this group, in which the basal layer was intact without evidence of separation from BZ. The larger spaces, located superficially, were ~100 μm. The size became smaller toward the basal layers. The appearance and change in size from superficial to deep layers is illustrated in Figures 1E–I.

Of those 11 cases, histology was available for 10. Histologic sections showed superficial bullae in case 1 only (Fig. 2A). Cases 2 and 3, which had FED, showed subepithelial separation without evidence of intraepithelial cystic spaces on light microscopy (Figs. 2B, 2C). Similar changes were not visible in the other samples.

Subepithelial Fibroblasts and Scarring

On histology, cells with elongated, thin nuclei resembling keratocyte nuclei were seen lying beneath the basal epithelium and above BZ (Fig. 3A). On IVCM, these cells appeared as bright dots in the subbasal plane (Fig. 3B).

In some eyes, the basal epithelial cells were separated from BZ by an accumulation of collagen “scar tissue,” with cells resembling keratocyte nuclei, as described above. These cells were termed keratocyte-derived cells (KDCs). The collagen scar tissue undulated and in places, extended into the plane of the basal epithelial layer (Fig. 3C). On IVCM, the corresponding area presented as islands of (basal) epithelial cells surrounded by hyperreflective (scar) tissue, within which were bright dots that corresponded to the nuclei of the cells (Fig. 3D). On oblique images the collagenous material presented as a continuous hyperreflective, undulating, subbasal sheet (Fig. 3E).
FIGURE 3. Light micrographs (left column) and IVCM images (right column) of cases with corneal edema. (A) Case 1: flat, elongated fibroblasts located between the epithelium and BZ (facing arrows). H&E. (B) A corresponding IVCM en face image of the same area taken before surgery, shows the subepithelial cells as bright oval figures. (C) Case 2: histology shows the deposition of collagen tissue with flat cells above BZ. The basal epithelium has an undulating profile corresponding to the variations in thickness of the collagen deposit (toluidine blue). (D) A corresponding IVCM en face image shows small islands of basal epithelial cells surrounded by bright collagen tissue containing brighter oval cells, as described in (B). (E, F, red line) A focal plane where both collagen tissue and basal cells would be imaged, thus giving the appearance of islands of cells surrounded by collagen tissue. (E) Oblique view of the same area clearly illustrating the subepithelial collagen layer of varying thickness with an undulating basal epithelium. (F, case 13) Histology section shows subepithelial collagen tissue with occasional flat cells. In addition, intraepithelial scar tissue is also seen, which is continuous with the subbasal scar tissue at two places (★). The epithelium is multilayered and thicker than normal. (G) En face IVCM image of the same area taken before surgery showing bright patches with oval cells in the midepithelium corresponding to the intraepithelial scar tissue. (H) Corresponding oblique IVCM image showing the intraepithelial scar tissue as bright lines sandwiching layers of epithelium (Ep). The subepithelial scar tissue (s) with flat cells is also visible. (I) Case 17: histology shows attenuation and break in BZ. This corresponds with hypercellular and abundant subepithelial scar tissue. (J) En face IVCM of the area shows a meshwork of hyperreflective strands corresponding to the collagen tissue. (K) On histology, the BZ is completely absent. There is excessive cellular scar tissue with islands of epithelium. (L) En face IVCM showing the bright patches of scar tissue surrounding epithelial islands. (A, F, I, K) H&E; (C) toluidine blue. Scale bar in IVCM images, 50 μm.
In two eyes (cases 3 and 13), in addition to subbasal scarring, there was one or more layers of scar tissue noted within the epithelium resembling reduplication of the basement membrane (Fig. 3F), and the epithelium itself was thickened (Fig. 3F). On IVCM, such layers were seen within the (mid)epithelium as hyperreflective patches or lines in en face or oblique sections, respectively (Figs. 3G, 3H). In places, there were breaches in the BZ with excessive deposition of scar tissue-collagen and increased presence of cells (Fig. 3I). On IVCM, scar tissue always presented as hyperreflective areas, whether it was beneath (Fig. 3E) or within (Fig. 3H) the epithelium. When the scar tissue was excessive, a meshwork of hyperreflective strands was seen on IVCM (Fig. 3J). Occasionally, isolated islands of epithelium were seen totally embedded within thick layer of fibrous tissue in histologic sections (Fig. 3K) and were clearly illustrated on IVCM (Fig. 3L).

On volumetric IVCM, the subepithelial KDCs appeared as round or oval, bright structures, 10 to 20 μm in size in en face images, between the basal epithelium and BZ (Figs. 4A–E). The KDCs were randomly distributed within a bright background and were more prominent in FED cases. Oblique views on IVCM were very useful in illustrating the exact location of these cells. They showed cells within BZ (Fig. 4F) or in fibrous tissue in histologic sections (Fig. 4K) and were clearly illustrated on IVCM (Fig. 3L).

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On EM, the BZ was noted to be thickened and irregular, with a layer of scar tissue containing horizontally oriented, elongated cell nuclei on its anterior surface. In places, the scar tissue extended anteriorly between the basal cells. The posterior demarcation of BZ appeared normal. In places, there were breaks in BZ with increased scar tissue at these sites (Fig. 5).

**Cell Morphometry**

In histopathologic sections, the cells located between epithelium and BZ had slender, elongated nuclei comparable in shape and size to the keratocyte nuclei seen in the anterior stroma (Fig. 6).

The length of subepithelial KDC nuclei was calculated in three cases. They ranged from 6 to 21.8 μm, with an average of 10.7 ± 4.1 μm (mean ± SD). In comparison, the keratocyte nuclei measured from 3.7 to 20.8 μm, with an average of 10.8 ± 3.8 μm (Fig. 6). The difference in average nuclear length between the two groups was not statistically significant ($P = 0.9$).

These cells were seen on histology and with IVCM in 7 (53.8%) of 13 full-thickness corneal samples. The remaining six samples did not show the cells on histology or by IVCM. IVCM alone illustrated the KDCs in four of the seven patients who...
FIGURE 6. Light micrographs of corneal sections show nuclear morphometry in cases 1 (A), 2 (B), and 13 (C). Subepithelial fibroblast nuclear size ranged from 6 to 21.8 μm, with an average of 10.7 ± 4.1 μm (mean ± SD). Keratocyte nuclear size ranged from 3.7 to 20.8 μm, with an average of 10.8 ± 3.8 μm. (A, C) H&E; (B) toluidine blue.
had undergone DSEK, and hence samples were not available for histologic examination. Thus, by IVCM subepithelial fibrous tissue was seen in 11 (55%) corneas, of which 9 did not show any visible clinical scarring on slit lamp examination (Table 2). Seven (63.6%) of the 11 FED corneas showed evidence of such subepithelial scar tissue on IVCM.

**Subbasal Corneal Nerves**

In 6 (30%) cases, no subbasal corneal nerves were demonstrated in the edematous central cornea by IVCM, whereas in the remaining 14 (70%), the mean central subbasal nerve density was significantly reduced to $4.47 \pm 2.05$ mm/mm$^2$ (mean ± SD). When the edema was sectorial, IVCM of the nonedematous part showed the presence of subbasal nerves that were not seen in the adjacent edematous area (Fig. 7).

**BZ and Anterior Stroma**

IVCM features of the BZ showed a marked deviation from normal. Apart from the associated scar tissue described above, in all cases, the fibrillary K-structures were absent, whereas in five cases, BZ presented a branching pattern of fine, dark lines (Figs. 8A, 8B). Furthermore, fine or coarse granularity with a variable degree of reflectivity was noted in the anterior stroma in 12 of the 20 cases (Figs. 8C, 8D).

**Stromal Keratocyte Changes**

On IVCM, stromal keratocytes showed hyperreflective expanded cell bodies and processes with darker small intracellular vacuoles, as well as extracellular lacunae, seen as larger, empty, round-to-oval spaces between the keratocytes (Fig. 9). The remarkable brightness of the cytoplasm in contrast to the surrounding extracellular matrix enabled sharp demarcation of the keratocyte borders, with enhanced visualization of interconnecting cell processes. The intercellular lacunae were approximately 40 to 100 μm in diameter, whereas the intracytoplasmic vacuoles were 10 to 20 μm and located in the vicinity of the keratocyte nuclei.

These changes were most obvious within midstromal layers at a focal depth of 200 to 400 μm in 19 (95%) of the cases. In the FED group, all corneas showed these morphologic changes.

Corresponding stromal changes could not be seen on histopathologic sections examined by light microscopy. Transmission electron microscopy was performed in three samples (cases 2, 16, and 17), and lipid material was seen within stromal keratocytes in one case (Fig. 10) without evidence of cytoplasmic vacuoles or extracellular lacunae.

**Stromal Corneal Nerves**

Tortuous curvilinear nerves were detected by IVCM in two cases within the midstromal (case 18) and deep layers (case 3; Fig. 11). The nerve width in these cases was 5 to 6 μm. Neuronal structures could not be detected with the histologic preparation and staining methods used in this study.

**DM and Endothelium**

On IVCM the classic strawberry-like pattern of DM and endothelium was seen in all cases of FED. This pattern comprised dark, round, 10- to 30-μm areas with bright centers, all within a bright background (Fig. 12A). They corresponded to the excrescences of thickened DM seen on light microscopy. Another co-existing pattern of bright, round, 15- to 35-μm shapes within dark circles was occasionally noticed (Fig. 12B). Oblique IVCM views illustrated these projections arising from DM (Fig. 12C). The normal endothelial pattern could not be identified on IVCM of FED patients.

On histology of all seven FED samples, the DM was thickened, with wartlike projections toward the endothelial side (Fig. 13A). In certain areas, these projections were seen buried within an overlay of outer collagenous material identified by color contrast between the two parts with H&E stain (Fig. 13B). This collagenous overlay caused the endothelial side to be flat rather than corrugated. Endothelial cells were attenuated and reduced in number.

In non-FED cases IVCM of endothelium showed loss of the normal honeycomb pattern, with features of polymegathism, thickened or ill-defined cell borders, prominent nuclei, and reduced cell density (Fig. 14A). All these features were seen in six (including three pseudophakic eyes) of the nine non-FED cases (12, 13, 16, 17, 18, and 19). Another pattern with occa-

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**Figure 7.** Slit lamp corneal micrograph (B) of case 17 shows marked edema in inferior half. IVCM image of edematous part (A) shows subepithelial fibrosis with no detectable subbasal nerves that are visible on IVCM of superior nonedematous area (C). IVCM focal depth 57 to 58 μm. Scale bar, 50 μm.

**Figure 8.** IVCM images of BZ and anterior stroma in corneal edema. Fine, arborizing, dark lines could be seen in en face (A) and oblique (B) views, replacing K-structures. Anterior stromal granularity was either finely (C) or coarsely hyperreflective (D) in appearance obscuring all keratocyte nuclei. Scale bar, 50 μm.
sional guttae, shown as dark, round spots, was noticed in case 17, without evidence of hyperreflective nuclei (Fig. 14B). The average endothelial cell density (ECD) was remarkably low ($512 \pm 105$ cells/mm$^2$; mean $\pm$ SD).

On histopathology endothelial cells were reduced in number in all non-FED samples (Fig. 15A). DM was thickened and multilaminated in case 17, mainly in the edematous part of the cornea where occasional buried guttate excrescences were also noted (Fig. 15B), confirming those seen on IVCM (Fig. 14B).

**Control Group**

IVCM findings in the control group showed normal patterns of basal epithelium, subbasal nerves, and BZ on en face and oblique views (Figs. 16A–C). These findings were compared to histologic features of anterior cornea studied in normal corneal tissue obtained from a donor cornea used in a DSEK procedure (case 18; Fig. 16D). Subbasal nerve density of $19.3 \pm 2.2$ mm/
mm² (mean ± SD) was significantly higher than the average density in the corneal edema study group (P = 0.001).

No evidence of subbasal fibrosis was detected, and stromal keratocyte nuclei were clearly demarcated with faint reflectivity of cell bodies and processes (Fig. 17A). Because of the markedly reduced reflectivity, lacunae and vacuoles could not be readily identified throughout the stromal layers. Straight midstromal nerves (Fig. 17B) with the normal honeycomb pattern of the endothelium (Fig. 17C) were clearly visible in the control group.

Postoperative Subgroup

Postoperative IVCM findings in four patients who underwent DSEK are summarized in Table 3.

Subepithelial Fibrosis

In three cases, IVCM showed persistence of subepithelial fibroblasts that were associated with fibrous bands in one case (patient 18, Table 1), despite improvement of BCVA to 6/9. The random distribution of these cells did not enable reliable cell density measurements. However, this value could be calculated in two cases and did not show significant change after surgery (Figure 18). The fourth case, patient 11, showed no evidence of subepithelial fibrosis 7 months after surgery, compared to just a few of these cells seen in the preoperative IVCM scan.

Subbasal Nerves

After DSEK, those nerves were detected in three cases and were not visible in one. The average central nerve density was 6.83 ± 3.35 mm/mm² (mean ± SD), which was not significantly better than the preoperative value of 4.47 ± 2.05 mm/mm². Patient 18, who had no detectable subbasal nerves before surgery, showed postoperative improvement to 3.2 ± 1.5 mm/mm², whereas a third case (patient 11) had an increase from 4.6 ± 1.8 to 9.8 ± 1 mm/mm² (mean ± SD). Nevertheless, all these postoperative values were significantly below the average density of subbasal corneal nerves in the control group.

BZ and Anterior Stroma

Postoperative IVCM did not reveal any significant change in the features of BZ and anterior stroma from that observed before surgery (Fig. 8).

Stromal Keratocyte Changes

After surgery, all four cases showed a variable degree of reduction in reflectivity of stromal keratocytes compared with preoperative IVCM (Fig. 19). This decrease was more obvious in cases 7 and 9 (Figs. 19E, 19F, respectively) where lacunae and vacuoles were absent, thus improving visibility of keratocyte nuclei that appeared well defined. This contrasted with cases 11 and 18 where the keratocytes were obscured by hyperreflective cell bodies and processes (Figs. 19G, 19H). Keratocyte nuclei that were obscured before surgery became more visible and well defined after surgery.

DISCUSSION

IVCM has been widely used as a noninvasive imaging tool to study histologic details of various corneal conditions. However, IVCM studies of corneal edema in FED and non-FED conditions have been limited in number and lack histopathologic correlation. In this study, we attempted to illustrate definitive pathologic changes in corneal edema by IVCM and to correlate them with corresponding histopathologic features. At the epithelial level, IVCM was able to illustrate cystic changes associated with epithelial edema. IVCM, through volume scans, showed these cysts to be located within the middle layers (i.e., intraepithelium rather than subepithelium) in 11 cases in this group, with an almost intact basal layer without evidence of separation from the basement membrane/BZ.

Histologic sections showed epithelial separation and bullae formation in two cases, with no clear evidence of intraepithelial cystic spaces on light microscopy. Epithelial cells were
otherwise histologically unremarkable in all layers. It is very likely that histologic processing of tissue samples results in tissue dehydration, which might explain the finding of fewer epithelial cysts in histologic sections, compared with the number seen with IVCM.

It is assumed in the literature that the accumulation of water and formation of cysts in the epithelium result in permanent changes. However, in our study, that water accumulation within epithelial layers did not represent or cause permanent disruption in these layers, since surface epithelium clinically resumes its normal appearance after successful DSEK, with subsequent improvement of corneal edema. This outcome was confirmed by showing unremarkable epithelium on IVCM in four post-DSEK cases.

Subepithelial Fibroblasts

This is the first study to document the presence of subepithelial fibroblasts in corneal edema with IVCM. Subepithelial fibroblasts, alone and in association with fibrous tissue, were found to be comparable in location, shape, size, and orientation in both IVCM and light microscopy of corresponding histologic cross sections (Figs. 3, 7). Their oval or rounded shapes on en face IVCM and their slender, elongated appearance in histologic cross sections suggest that these nuclei have a flat, discoid shape and are oriented parallel to the corneal surface (Figs. 3A, 3B).

Despite the random distribution of the nuclei, IVCM showed similarity between them and keratocyte nuclei in reflectivity, shape, size, and orientation, whereas cell morphometry on histologic sections confirmed this similarity in size. It is worth considering that cross sections in histology do not always go through the maximum dimension of the nucleus, a feature that partly explains the variation in nuclear length measurements shown in histopathology slides (Fig. 6).

The undulating profile of the basal epithelial layers caused by the accumulated subbasal collagen tissue accounted for the islands of cells and scar tissue visualized at the same focal plane on IVCM. The en face images of this arrangement presented as the characteristic “Swiss cheese” pattern (Figs. 3E, Fig. 18). Oblique views were even better in illustrating the exact plane of these cells within the anterior cornea (Figs. 3E, 4F).

On the volumetric IVCM scan, the bright structures representing en face images of cells and nuclei in the fibrous layer between BZ and the basal epithelium extended for 12 μm across the z (volume) scans. This observation suggests that the cellular fibrous tissue was approximately 12 μm thick and was

Figure 14. IVCM images of endothelium in non-FED-related corneal edema. Polymegathism, reduced cell density, and prominent nuclei are seen in (A). Occasional guttae represented by dark, round spots can be seen in (B, case 17) without visible nuclei. Scale bar, 50 μm.

Figure 15. Light micrographs of corneal cross sections show DM and endothelium in non–FED-related corneal edema. DM thickening is seen in (A), as well as embedded excrescences (arrows) in (B). (A) H&E; (B) with toluidine blue.

Figure 16. IVCM images and light micrograph of normal (control) cornea. Normal patterns of basal epithelium, subbasal nerves, and BZ with K-structures were seen on en face views (A, B) in controls. (C) An oblique view confirms intactness of anterior corneal layers. Histologic cross section in a normal cornea is shown in (D) stained with toluidine blue. Scale bar in IVCM, 50 μm.
distinguishable from the underlying BZ and the overlying basal epithelial cells.

The narrow septa that were seen to extend into the epithelium were very similar to the confocal images of “fingerprint lines” of basement membrane dystrophy, reported previously. In our cases of chronic corneal edema, the patients did not have basement membrane dystrophy. The lines therefore relate to a rucking or folding of the basement membrane with associated subbasal scar tissue and probably represent a pathology similar to that seen in epithelial basement membrane dystrophy.

In previous IVCM studies of FED, similar patterns were noticed by other investigators, but were presented with speculative and inaccurate interpretation due to lack of histopathologic correlation.

Of the 11 FED patients, 7 (63.6%) showed these cells on IVCM. None of these had clinically visible (slitlamp examination) corneal scarring before surgery. IVCM and histopathology correlated well in all the 13 full-thickness corneal samples with regard to the presence or absence of fibroelastic changes.

In chronic corneal edema, subepithelial fibrosis is mentioned in the literature as a late feature resulting from epithelial separation in progressive corneal edema. In this study, although all corneas with FED were devoid of clinically visible fibrotic changes, IVCM was able to detect the presence of subepithelial fibroblasts in 63.6% of them. This probably highlights the very early (subclinical) stage of subepithelial fibrosis in longstanding corneal edema. These cells initially produce a thin layer of collagenous material seen on light microscopy and EM sections, possibly causing the hyperreflective appearance surrounding the nuclear images on IVCM (Swiss cheese pattern).

When corneal edema persists, this pattern disappears as the sheet of fibrotic tissue between the basal epithelium and BZ becomes more continuous. Some fibrous septa extend into the overlying epithelial layers, resulting in epithelial irregularity, with patterns resembling reduplication of basement membrane (Figs. 3G, 3I). At this stage, the collagen fibers may present as a bright meshwork of interwoven fibers on IVCM (Fig. 3K), whereas epithelial islands may be seen totally surrounded by fibrous tissue both histologically and on IVCM (Figs. 3G, 3L, 3M). In an early electron microscopic study of FED, Iwamoto and De Voe mentioned similar findings of epithelial islands separated by continuations of subepithelial connective tissue.

The origin of these cells could be a fibroelastic transformation of stromal keratocytes that had migrated through breaks in BM or preexisting mesenchymal stem cells at the subepithelial level. Such transformation could be stimulated by epithelial separation and bullae formation associated with progressively increasing corneal edema. In a recent study Said et al. illustrated the migration of stromal keratocytes through breaks in BZ to populate the amniotic membrane transplanted on corneas with bullous keratopathy.

In this study, cell morphometry showed close similarity in nuclear size between these cells and anterior stromal kerato-
controls, which is comparable to the value of 21.6 mm/mm² than the value of 19.3 mm/mm².

In the present study, IVCM was a useful, in vivo, noninvasive, real-time imaging method for detecting the presence of subepithelial fibrous tissues—not the cells—in corneal edema and predict their time of accumulation relative to the duration of edema. In our study, IVCM was a useful, in vivo, noninvasive, real-time imaging method for detecting the presence of subepithelial fibroblasts, even in the early stages of corneal edema. The cells appear early in the pathogenesis of corneal edema, as they could be detected, even before endothelial transplant was indicated, especially in FED where the persistence of the cells after successful DSEK with clinical improvement of corneal edema and BCVA.

These nerves have been studied extensively with IVCM by other investigators. Recently Morishige et al.19 used second harmonic generation microscopy with fluorescent probes to detect the presence of subepithelial fibrous tissues—not the cells—in corneal edema and predict their time of accumulation relative to the duration of edema. In our study, IVCM was a useful, in vivo, noninvasive, real-time imaging method for detecting the presence of subepithelial fibroblasts, even in the early stages of corneal edema. The cells appear early in the pathogenesis of corneal edema, as they could be detected, even before endothelial transplant was indicated, especially in FED where the BCVA was 6/9 (data not presented).

FIGURE 18. IVCM images of corneas in two cases who underwent DSEK to show subepithelial fibroblasts before (A, C) and after surgery (B, D). Their densities are given in Table 3 and were not significantly different before and after surgery. Scale bar, 50 μm.

Kobayashi et al.,7 using HRTII-RCM, described certain IVCM patterns of BZ in the normal cornea, which they termed K-structures. These fibrillar structures were best seen with this type of confocal microscope machine and considered to represent one of the features of the intact normal cornea. In our study, BZ lost this reflective pattern in all cases of corneal edema, possibly due to the effect of excessive water content disrupting the normal arrangement of collagen fibrils.

This effect is thought to involve the anterior stromal layers as well, giving rise (in 60% of cases) to granularity of variable coarseness and reflectivity, which frequently obscured all anterior keratocyte nuclei. In five of the cases, BZ showed fine, dark lines of dendritic pattern, previously described by Jester et al.25 as basement membrane of basal epithelium. In this study, the oblique view showed this pattern throughout the whole BZ thickness, suggesting that it represents the BZ rather than the basement membrane, which is much thinner than this layer. After surgery, these changes remained irreversible despite remarkable improvement in BCVA, possibly indicating the presence of permanent degenerative stromal changes with undetermined or no clinical significance.

Keratocyte Changes

The morphologic changes of keratocytes on IVCM were seen in practically all cases of corneal edema. Lacunae and smaller intracellular vacuoles were visualized on IVCM in close proximity to keratocyte nuclei, along with hyperreflective expanded keratocyte cell bodies and processes.

All these changes were better visualized in midstromal rather than anterior stromal layers, possibly due to the higher keratocyte density with straight interwoven collagen fibrils in the anterior layers.24 Mustonen et al.8 mentioned the presence of stromal lacunae as an IVCM finding in 24% of corneas with FED, without giving further details of keratocyte morphology. They used a white-light slit scanning confocal microscope (SSCM) with poor resolution images compared with the yield of HRTII-RCM used in this study.

After surgery, stromal keratocytes were more visible (Figs. 19E, 19F) and tended to show less brightness, approaching the normal pattern seen in the control group (Fig. 17A). Thus, it can be inferred that these keratocyte changes are reversible.

Histologically, these changes could not be linked to distinct features on light microscopy in our study. EM revealed intracytoplasmic deposition of lipid material (Fig. 10). However, other EM studies of FED11,25 and bullous keratopathy20 have recorded changes consistent with the IVCM findings of our study. Iwamoto and De Voe, in 1971,11 described the keratocytes in one FED case to be fatter than normal, whereas, in more recent studies, Akhtar et al.26 and Yuen et al.25 mentioned intracellular vacuoles within degenerate keratocytes as well as electron-lucent lacunae throughout the stroma in bullous keratopathy.

The most likely cause of the increased reflectivity of the keratocytes observed in corneas with edema is keratocyte activation. Another possibility is that high water content within stromal extracellular matrix made the keratocytes with their interconnecting processes appear brighter or more hyperreflective than normal. Co-existing intracellular edema may explain the remarkable expansion and hyperreflectivity of stromal keratocytes in corneal edema. Keratocyte activation is a term commonly used to describe hyperreflective keratocytes in IVCM literature.27,28 The process of activation is well known in the literature to describe the response of stromal keratocytes to a variety of corneal injuries (physical, chemical, or infectious).29 This response in-
volves transformation of keratocytes into repair fibroblasts that was studied by several investigators such as Kitano and Goldman in a rabbit model, as well as Jester et al. in a cat model.

The first noticeable sign of activation is the increase in keratocyte size in association with other changes in cytoplasmic organelles and the nucleus. This sign is also associated with synthesis of substances such as integrins, fibronectin, and matrix metalloproteinases required for wound healing. As progressive stromal edema eventually involves the epithelium, it is possible that the epithelial injury induces keratocyte activation. Previous studies in the context of refractive surgery have shown that keratocyte activation (and subsequent scarring) occurs more with PRK than with LASIK, where the epithelium is intact. In this study, we demonstrated that the elongated keratocyte-derived cells, presumably fibroblasts, were located in the subepithelial region in 55% of the cases (63.6% of FED cases) and probably represent cells that have originated from activated stromal keratocytes. Recently, Morishige et al. suggested possible fibroblastic transformation of stromal keratocytes in 10 of 31 cases of corneal edema using immunofluorescent probes, thus giving further evidence of the existence of transformed keratocytes in the edematous corneal stroma.

Descemet’s Membrane and Endothelium

On light microscopy, Descemet’s membrane (DM) appeared thickened and multilaminated with guttate excrescences, as well as reduced endothelial cells in all FED corneal samples. This observation correlated well with IVM, which illustrated the classic strawberry-like pattern in all FED cases, with total obscuration of endothelial cell borders. This appearance is well described in the literature as hyporeflective, round images, 10 to 30 µm in diameter, with bright centers, all within a hyperreflective background. In this study, we noticed another co-existing pattern of DM guttae by IVM. They appeared as hyperreflective, round figures within dark circles ~10 to 40 µm in diameter. This variation in reflectivity patterns could be explained by the variable shapes and sizes of these excrescences as well as their tendency to be bare in some areas and buried in others.

In non-FED cases, the above patterns were not noticed on IVM. Endothelial cell changes including polymegathism and prominent hyperreflective nuclei as well as reduced cell density, were observed. This clear IVM imaging of endothelium could be explained by the almost normal thickness of DM with absence of warty projections seen in histologic sections of this subgroup. Further work is needed to compare morphologic changes in degenerated endothelium on IVM with those seen with scanning electron microscopy of endothelial sheets obtained via DSEK, to have better interpretation of IVM findings.

Our study was not designed to correlate the severity of changes with the duration of edema; however, three FED cases presented with symptomatic corneal edema within less than 1 year after successful cataract surgery, a fourth case in 2 years, and the fifth case after 5.5 years. In the non-FED cases, all those with PBK were symptomatic in the first postoperative year. However DM/endothelial changes comparable to those in FED were illustrated in case 17 only.

**CONCLUSION**

This is the first study in which IVM features of FED and non-FED corneal edema were compared in detail with histopathologic findings. It demonstrates the versatility and utility of IVM in detecting fine and early pathologic changes associated with this condition in a noninvasive, real-time manner. Histologic changes related to subepithelial fibroblasts, reduced subbasal corneal nerves, and stromal keratocyte morphology were well documented in this study. With increasing popularity of
DSEK, this work supports the role of IVCM in quantitative evaluation of histologic features in corneal edema, both before surgery and throughout the post-DSEK follow-up period for better correlation with clinical outcomes. The persistence of histologic changes with regard to subbasal nerves, subepithelial fibroblasts, and hyperreflective keratocytes, in the host cornea after successful endothelial transplantation is important. It suggests that even though the edema clears and corneal transparency improves, it does not return to a completely normal state. The implications of the persistent changes are unclear at this stage, but longer term follow-up of a larger number of patients will improve our understanding of these changes.

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