Objective: To determine if keratocyte populations are different in corneas with Fuchs dystrophy compared with control corneas.

Methods: Eleven corneas excised during penetrating keratoplasty for Fuchs dystrophy and 5 control corneas of eyes enucleated for choroidal melanoma were examined using light microscopy. Twenty control corneas age-matched to the corneas with Fuchs dystrophy were examined using confocal microscopy in vivo. The number of keratocytes in a full-thickness column of central stroma with frontal area of 1 mm², determined using histologic and confocal methods, was compared between corneas with Fuchs dystrophy and controls.

Results: By histology, the mean (SD) number of cells in a full-thickness column of stroma in Fuchs dystrophy (12 215 [1394] cells) was less than in control corneas (15 628 [710] cells; \(P < .001\)). The mean (SD) number of keratocytes in the anterior 10% of the stroma of corneas with Fuchs dystrophy (682 [274] cells) was less than in the control corneas measured using histology (1858 [404] cells; \(P < .001\)) and confocal microscopy (1481 [397] cells; \(P < .001\)).

Conclusions: Keratocytes are depleted by 54% to 63% in the anterior 10% of the stroma of corneas that require penetrating keratoplasty for Fuchs dystrophy. Keratocyte loss might contribute to anterior stromal changes that persist and degrade vision after endothelial keratoplasty.

The central cornea was examined in vivo using a Tandem Scanning confocal microscope (Tandem Scanning Corporation, Richmond, Virginia); eyes scheduled for surgery were examined prior to surgery (except for 2 control corneas from 2 subjects with choroidal melanoma). The examination procedure has been described in detail previously.\textsuperscript{14,16,17}

**HISTOLOGY**

After removal of the corneas with Fuchs dystrophy for PK and from enucleated eyes with choroidal melanoma, the central cornea was fixed in 10\% buffered formalin. The corneal button was embedded in paraffin and 4-µm-thick serial sagittal sections were cut and stained with 4',6-diamidino-2-phenylindole. Fifteen serial sections spaced 8 µm apart were photographed at an original magnification of ×20 (Olympus DP70 camera operating on a BX60 transmission light microscope; Olympus America Inc, Center Valley, Pennsylvania). Images of a reticle were captured at the same magnification for calibration of horizontal and vertical dimensions. Additional sections were stained with hematoxylin-eosin for examination by light microscopy. The diagnosis of Fuchs dystrophy was confirmed by an ophthalmic pathologist based on the presence of guttae, thickening of Descemet membrane, and endothelial cell loss.

**KERATOCYTE DENSITY AND STEREOLOGY**

From confocal images, cell nuclei, which appeared as bright objects, were identified using a custom automated program.\textsuperscript{14} From histologic images, 4',6-diamidino-2-phenylindole-stained nuclei in the corneal stroma were identified by one observer using a point-and-click method (Figure 1). Volumetric cell density was calculated using stereologic methods from the number of cells in a predefined area of the confocal and histologic images, as described previously.\textsuperscript{16,18} The sum of confocal depth of field and thickness of a keratocyte nucleus was 11.9 µm.\textsuperscript{19} In histologic images of all corneas, cell densities were calculated using stromal thickness measured by confocal microscopy in vivo\textsuperscript{16,17} to avoid shrinkage artifacts caused by fixation; the other 2 dimensions were corrected for tissue shrinkage of 5.7\%, as described previously in control corneas.\textsuperscript{16,18} We assumed that the frontal diameter of a keratocyte nucleus was 16 µm in histologic samples.\textsuperscript{16,20}

Keratocyte density was estimated in 5 layers of stroma, the anterior 10\%, 10\% to 33\%, 33\% to 66\%, 66\% to 90\%, and the posterior 10\% of stromal thickness (Figure 2).\textsuperscript{16} By confocal microscopy, cell density was estimated from 2 manually selected frames from each layer. Each histologic section was divided into the same layers of stroma based on the stromal thickness in the section.

**ABSOLUTE NUMBER OF KERATOCYTES**

Keratocyte density decreases in edematous corneas because cells are distributed throughout a larger volume of tissue. To elimi-
were not examined by confocal microscopy preoperatively. vivo, which was not available for 2 control corneas because they because this did require a knowledge of stromal thickness in density could only be estimated in 3 control corneas by histology in vivo, the number of keratocytes could be calculated in all 5 control corneas by histology. In contrast, keratocyte den-

Figure 2. Division of full-thickness stroma into 5 layers. By confocal microscopy, mean keratocyte densities were determined from 2 manually selected frames (frontal section) from each of the 5 layers. By histology, each sagittal section was divided into the same 5 layers of stroma, and mean cell density in each layer was determined from 15 full-thickness sections that were each spaced by 8 µm.

nate this effect of corneal edema, we estimated the absolute number of cells in a full-thickness column of stroma with a frontal area of 1 mm². For confocal microscopy data, the absolute number of cells in a full-thickness column of stroma was the product of full-thickness weighted mean keratocyte density and central stromal thickness in vivo.¹⁶,²¹

For histologic data, the absolute number of keratocytes in a full-thickness column of stroma was derived by counting all the cells in a section of full-thickness stroma and using stereologic methods to adjust for a frontal area of 1 mm².¹⁶,²¹ Because the latter did not require knowledge of stromal thickness in vivo, the number of keratocytes could be calculated in all 5 control corneas by histology. In contrast, keratocyte density could only be estimated in 3 control corneas by histology because this did require a knowledge of stromal thickness in vivo, which was not available for 2 control corneas because they were not examined by confocal microscopy preoperatively.

DEPTH OF HYPOCELLULAR ZONE

The thickness of the anterior stromal hypocellular zone in corneas with Fuchs dystrophy was determined from the depth of key structures in confocal scans.¹⁶,¹⁷ Images were reviewed by 2 experienced observers (J.W.M., S.V.P.) to identify the surface epithelium, subbasal nerve plexus, and first image with normal-appearing countable keratocyte nuclei. In control corneas, the Bowman layer is not visible but the image of the most anterior and highest density of keratocyte nuclei corresponds to the boundary between the Bowman layer and the cellular stroma (Figure 3). In corneas with Fuchs dystrophy, we identified the boundary between the Bowman layer and stroma by the presence of sparse bright objects that were reminiscent of keratocyte nuclei but were morphologically abnormal and possibly represented degenerate keratocytes (Figure 3). This image was always deep to the subbasal nerves, and the distance between this image and the first keratocyte nuclei with normal morphology was defined as the hypocellular zone. Images were also examined to identify activated keratocytes, which morphologically have visible cell bodies and processes.

Figure 3. Confocal images of the anterior stroma in Fuchs dystrophy and control corneas. Few keratocytes were visible at the anterior boundary of the stroma in Fuchs dystrophy (A), although in control corneas this region of stroma has the highest density of stromal cells (B). The stromal boundary was approximately 15 µm posterior to subbasal nerves in both eyes. In patients with Fuchs dystrophy, this boundary was identified by the appearance of remnants of cells and cellular debris, whereas in control corneas, it was identified by the high concentration of cell nuclei. Cell density was also reduced 25 µm posterior to the stromal boundary in Fuchs dystrophy (C) compared with controls (D).

STATISTICAL ANALYSIS

Keratocyte density and the number of keratocytes determined by histology in corneas with Fuchs dystrophy were compared with those determined by histology in the control corneas after enucleation and to those determined by confocal microscopy in vivo in the 20 age-matched control corneas. Densities were compared between groups using generalized estimating equation models to account for possible correlation between fellow eyes of the same subject.²² P values were adjusted for multiple comparisons by using the Bonferroni technique, and \( P < .05 \) was considered significant.

Keratocyte density and the number of keratocytes determined using confocal microscopy were compared with those determined using histology; for corneas with Fuchs dystrophy, we used generalized estimating equation models, and for control corneas, we used paired \( t \) tests. Correlations were assessed using the Pearson correlation coefficient, with significances calculated by using generalized estimating equation models.

RESULTS

CLINICAL AND HISTOPATHOLOGIC FINDINGS

By slitlamp examination, all 11 eyes with Fuchs dystrophy had central confluent guttae with variable amounts of stromal edema; epithelial edema was clinically noted in only 1 eye. The mean (SD) best-corrected visual acuity was 0.49 (0.10) logMAR (Snellen equivalent, 20/62; range, 0.34–0.66 logMAR). The mean (SD) central corneal thickness by ultrasonic pachymetry was 642 (54) µm (range, 575 to 766 µm). Seven eyes had a cataract and
By histology, full-thickness keratocyte density in corneas with Fuchs dystrophy (mean [SD], 23 872 [3340] cells/mm²; n=11) was lower than that in control corneas (mean [SD], 33 376 [2430] cells/mm²; n=3; P < .001). Keratocyte densities were lower in all layers of stroma in corneas with Fuchs dystrophy than in corresponding layers of control corneas (P < .001), with the exception of the middle third of the stroma (P = .99; Table; Figure 4). By histology, the mean (SD) number of keratocytes in a full-thickness column of stroma of corneas with Fuchs dystrophy (12 215 [1394] cells; n=11) was lower than that of control corneas (15 628 [710] cells; n=5; P < .001; Table). The mean (SD) number of keratocytes in the anterior 10% of the stroma of corneas with Fuchs dystrophy (682 [274] cells; n=11) was lower than in the control corneas (1858 [404] cells; n=5; P < .001; Figure 1; Table).

The mean (SD) full-thickness cell density determined by histology in the 11 corneas with Fuchs dystrophy was not different from full-thickness cell density determined by confocal microscopy in the 20 age-matched control corneas (21 463 [4429] cells/mm²; P=.13). In corneas with Fuchs dystrophy, the number of keratocytes was decreased only in the anterior 10% of the stroma compared with the number of keratocytes in the same layer of the age-matched, control corneas (mean [SD], 1481 [397] cells; n=20; P < .001). In eyes with Fuchs dystrophy, best-corrected visual acuity correlated with the absolute number of keratocytes in a full-thickness column of stroma (r = −0.76; P = .007; n=11) but not with the number of keratocytes in the anterior 10% of the stroma (r = −0.47; P = .14; n=11). There were no correlations between central corneal thickness and the absolute number of keratocytes in a full-thickness column of stroma (r = 0.28; P = .40; n=11) or the number of keratocytes in the anterior 10% of the stroma (r = 0.13; P = .71; n=11). With a sample size of 11, the minimum detectable correlation was ±0.69 (or r² = 0.48; α = .05, β = .20).

### FUCHS DYSTROPHY VS CONTROLS

By histology, full-thickness keratocyte density in corneas with Fuchs dystrophy (mean [SD], 23 872 [3340] cells/mm²; n=11) was lower than that in control corneas (mean [SD], 33 376 [2430] cells/mm²; n=3; P < .001). Keratocyte densities were lower in all layers of stroma in corneas with Fuchs dystrophy than in corresponding layers of control corneas (P < .001), with the exception of the middle third of the stroma (P = .99; Table; Figure 4). By histology, the mean (SD) number of keratocytes in a full-thickness column of stroma of corneas with Fuchs dystrophy (12 215 [1394] cells; n=11) was lower than that of control corneas (15 628 [710] cells; n=5; P < .001; Table). The mean (SD) number of keratocytes in the anterior 10% of the stroma of corneas with Fuchs dystrophy (682 [274] cells; n=11) was lower than in the control corneas (1858 [404] cells; n=5; P < .001; Figure 1; Table).

In corneas with Fuchs dystrophy, the weighted mean (SD) keratocyte density for the full-thickness stroma determined by automated assessment of confocal images was 21 507 (3915) cells/mm² and did not differ from that determined by histology (23 872 [3340] cells/mm²; P = .11). By the confocal method, keratocyte density was overestimated in the anterior 10% of the stroma (P < .001) and underestimated in the posterior third of the stroma (P = .02, 66%–90%; P < .001, posterior 10%) compared with the histologic method (Figure 5).

In control corneas, the weighted mean (SD) keratocyte density for the full-thickness stroma determined by automated assessment of confocal images was 29 200 (6534) cells/mm² and did not differ from that determined by histology (33 376 [2430] cells/mm²; P = .25), although our sample size was small (n = 3). There were no differences in keratocyte density between the confocal and histologic methods within the different layers of stroma (P > .50; Figure 5).
The mean (SD) thickness of the anterior stromal hypocellular zone in corneas with Fuchs dystrophy was 15.6 (8.5) µm (range, 4.9-32.0 µm). The first morphologically normal keratocyte nuclei were a mean (SD) of 27.4 (9.6) µm (range, 9.6-41.8 µm) deep to the subbasal nerves, which were identified in all eyes. None of the images of corneas with Fuchs dystrophy contained activated keratocytes.

**COMMENT**

The major finding in this study was that keratocytes were depleted from the anterior stroma of corneas in advanced Fuchs endothelial dystrophy, forming an anterior hypocellular zone. Decreased stromal cellularity was strongly associated with worse visual acuity in eyes with Fuchs dystrophy; however, this finding should be interpreted with caution because 7 of the 11 eyes had cataracts, which would confound visual acuity. We also confirmed our suspicion that with the degraded confocal image conditions in corneas with Fuchs dystrophy, the automated estimation of keratocyte density by our software is not accurate.

**KERATOCELLULAR LOSS IN FUCHS DYSTROPHY**

Fuchs endothelial dystrophy is generally accepted to be a primary disorder of the corneal endothelium, although the exact pathophysiology of the condition is unknown. Changes in the stroma of corneas with Fuchs dystrophy are typically assumed to be secondary to endothelial dysfunction. To our knowledge, keratocyte populations in Fuchs dystrophy have not been studied using histologic methods. We found absolute keratocyte loss, and not simply redistribution from corneal edema, in Fuchs dystrophy compared with controls. By histology, the number of keratocytes in the anterior 10% of the stroma of 11 corneas with Fuchs dystrophy was reduced by 63% compared with the normally high-density anterior layer in the 5 control corneas. This loss might be somewhat underestimated because we assumed that swelling was uniform, whereas, in fact, the anterior stroma swells less than the posterior stroma.23,24

Control histologic data were only available in a small number of corneas in this study, and these corneas were not age-matched. To overcome these limitations, we compared keratocyte density determined by the histologic method in the corneas with Fuchs dystrophy to keratocyte density determined by the confocal method in 20 age-matched control corneas; the number of keratocytes in the anterior 10% of the stroma was reduced by 54% in Fuchs dystrophy, confirming that anterior keratocytes were indeed depleted in corneas with advanced Fuchs dystrophy. The difference in keratocyte density between the 3 control corneas examined by histology and the 20 control corneas examined by confocal microscopy could be explained by differences in age16 and by the large variability of keratocyte density in the control population.21,25

**MECHANISM OF CELL LOSS**

The mechanism of cellular depletion from the anterior stroma of corneas with Fuchs dystrophy is unknown, but it is plausible that the keratocytes undergo apoptosis triggered by cytokine release from the epithelium, a known response to mechanical and viral epithelial injuries.26-28 Similarly, in corneas with advanced Fuchs dystrophy, it is conceivable that epithelial cell disruption or bullae formation result in cytokine release causing apoptosis of keratocytes. There is evidence of apoptosis in all cellular layers of the cornea in Fuchs dystrophy.9,20,23 Li et al23 suggest that keratocytes were hypersensitive to apoptotic stimuli and that changes in keratocytes might precede endothelial and epithelial changes. Szentmary et al23 found apoptotic cells in all layers of corneas with Fuchs dystrophy and pseudophakic corneal edema, indicating that kerato-
Keratocyte apoptosis might be a response to stromal edema of any cause. Indeed, if keratocyte loss were a result of apoptosis triggered by epithelial injury, one would expect to find anterior keratocyte depletion associated with any cause of chronic corneal edema and not only Fuchs dystrophy. Alternative mechanisms of keratocyte loss in Fuchs dystrophy could include starvation of the stromal cells or cell death resulting from a chronically abnormal environment. The latter are less likely because we would expect increased cell death throughout the corneal stroma and not predominantly in the anterior stroma, and because endothelial permeability does not decrease in Fuchs dystrophy.31,32

CONFOCAL MICROSCOPY IN FUCHS DYSTROPHY

Confocal microscopy enables visualization of keratocyte and other cell nuclei in vivo as bright objects. Our automated program14 for estimating keratocyte density is rapid and eliminates the subjectivity introduced by manual analysis but requires keratocyte nuclei to have a consistent appearance and corneas to have normal or subnormal backscatter (haze).25 In the corneas with advanced Fuchs dystrophy, although automated assessment of confocal images indicated anterior keratocyte loss compared with controls, cell density was overestimated compared with histologic analysis. Manual review of the confocal images indicated that the program erroneously counted small bright objects and other diffuse localized areas of increased reflectivity (Figure 3). In the posterior stroma, the automated program underestimated keratocyte density compared with histologic methods, and we suspect that loss of image contrast caused by increased anterior stromal haze contributed to the underestimate. This program, and most likely other programs designed by using images of control corneas, cannot be used in corneas with advanced Fuchs endothelial dystrophy without modification to overcome these limitations.

Qualitative examination of the confocal images confirmed a sparse population of keratocytes in the anterior stroma of corneas with Fuchs dystrophy; the high density of cell nuclei found in control corneas was not visible in any of the corneas with Fuchs dystrophy (Figure 3), and the mean thickness of the anterior hypocellular stroma was 16 μm. Mustonen et al12 found a range of appearances of the anterior stroma including normal keratocyte nuclei, activated keratocytes, and diffuse haze in confocal images of corneas with different severities of Fuchs dystrophy. This suggests that the anterior stroma of corneas with Fuchs dystrophy undergoes different stages of cellular changes, or that some corneas behave differently compared with others. Further studies by confocal microscopy are needed to determine if a sequence of events in the anterior stroma is responsible for the changes that degrade vision.

CLINICAL RELEVANCE

Endothelial keratoplasty has surpassed PK as the treatment of choice for Fuchs dystrophy, and although EK results in better uncorrected visual acuity compared with PK, many eyes fail to attain a best-corrected visual acuity of 20/20 or have resulting glare and poor contrast.3,4,34 Scatter or aberrations from the host cornea after EK may affect vision;3,4 thus, understanding the changes and biology of the host cornea might provide insight into improving visual outcomes. Increased scatter in the anterior stroma of corneas with Fuchs dystrophy is not fully understood, although it is clinically recognized as subepithelial haze and contributes to increased forward scatter, which affects vision.4 Increased scatter after keratorefractive surgery has been attributed to increased reflectivity from activated keratocytes,11 although our results suggest that increased scatter in Fuchs dystrophy was not directly from keratocytes; we found no evidence of keratocyte activation, and the number of keratocytes in the anterior stroma was decreased. The increased scatter possibly originates from the extracellular matrix and might be related to the deposition of abnormal proteins or changes in the proteoglycan properties of the anterior stroma.7,33,36 Some have also suggested that increased scatter arises from fibril-free regions (lakes) of stroma caused by the death of keratocytes.24 The temporal relationship of matrix changes to keratocyte loss is unknown, but the loss of keratocytes may also impair the subsequent repair of the abnormal matrix.

The sequence of cellular and extracellular changes in the anterior stroma prior to EK, and whether or not keratocytes repopulate the anterior stroma after restoration of endothelial function, are unknown and warrant further investigation. Correlation of these changes to visual function might provide a better understanding of visual outcomes after EK, and the opportunity to modulate the stromal changes in the future could improve outcomes of this procedure.

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