Objective: To investigate the potential role of apoptosis in the pathogenesis of Fuchs endothelial dystrophy of the cornea.

Methods: Twenty-one corneal buttons from patients with Fuchs dystrophy and 15 control corneas were studied. Apoptosis was assessed by the in situ end-labeling of double-stranded DNA breaks, and by immunohistochemical characterization of cellular markers associated with apoptosis (Fas, Fasl, Bcl-2, and Bax). Expression of Bcl-2 and Bax mRNA in the corneal stroma and endothelium was separately analyzed by a semiquantitative reverse transcriptase polymerase chain reaction. Furthermore, cultivated keratocytes generated from diseased corneal buttons and donor rims were exposed to camptothecin, an apoptotic inducer, for 6 and 24 hours. They were then examined for protein and messenger RNA (mRNA) expression of apoptotic regulatory molecules.

Results: DNA fragmentation was seen in the epithelium, stroma, and endothelium in 6 of 7 corneas with Fuchs dystrophy. A statistically significant difference was identified in the expression of Bax and its mRNA in the stroma, but not in the endothelium of Fuchs dystrophy corneas. Following exposure to camptothecin, keratocytes from patients with Fuchs dystrophy responded with an increased level of Bax and a low level of Bcl-2. This trend was distinctively different from the response of normal keratocytes.

Conclusions: The evidence in this study points to a disease-related disturbance in the regulation of apoptosis in Fuchs dystrophy. Our findings suggest that excessive apoptosis may be an important mechanism in the pathogenesis of Fuchs dystrophy.
MATERIALS AND METHODS

COLLECTION OF CORNEAS

This research has followed the tenets of the Declaration of Helsinki and has been approved by the Institutional Joint Committee on Clinical Investigation at the Johns Hopkins University (Baltimore, MD). Corneal buttons from patients with Fuchs dystrophy (n=21, 9 for immunostaining, 9 for mRNA study, and 3 for culture) were collected from patients who had undergone keratoplasty at the Wilmer Eye Institute, Baltimore. The average age of patients was 70.7 years and ranged from 36 to 88 years. The corneas were bisected with a razor blade; half of each cornea was used for experimental procedures, and the other half was used for routine histological examination. Three corneal buttons from patients with phakic bullous keratopathy, bacterial keratitis, and graft rejection were also used in the in situ end-labeling assay as controls.

Normal control corneas (n=12: 4 for immunostaining, 5 for mRNA study, and 3 donor rims for culture of keratocytes) were collected from the Maryland Eye Bank (Baltimore). The average age of donors was 55.8 years, and ranged from 46 to 66 years. The corneal buttons used in this study were graded to be in “good” or “very good” condition. The average death-to-preservation time of these buttons was 10.7 hours, and the average storage time in organ culture medium (Optisol; Bausch & Lomb, Rochester, NY) at 40°C was 5 days. Owing to practical difficulties, we did not use fresh corneas for normal controls. Apoptotic changes may occur in corneas during the storage period as shown in a previous study. Therefore, the baseline levels obtained from our control corneas may actually be higher than those of fresh normal corneas.

HISTOLOGICAL ANALYSIS

The corneas used for histological diagnosis were immediately fixed in 10% formaldehyde for at least 24 hours before processing. The eyes were then embedded in paraffin, separately fixed in 10% formaldehyde for at least 24 hours, and then bisected with a razor blade; half of each cornea was used for experimental procedures, and the other half was used for routine histological examination. Three corneal buttons from patients with phakic bullous keratopathy, bacterial keratitis, and graft rejection were also used in the in situ end-labeling assay as controls.

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IN SITU END-LABELING ASSAY

Detection of double-stranded DNA breaks (DNA fragmentation) in apoptotic cells was accomplished with the TACS Blue Label Detection kit (Trevena, Gaithersburg, Md) according to the manufacturer’s protocol, with modified tissue pretreatment to improve corneal stromal accessibility to the labeling reagent. Corneal sections with a thickness of 8 µm on superfrost slides (Fisher Scientific, Pittsburgh, Pa) were deparaffinized, dehydrated, and rehydrated. Slides were immersed in citrate buffer (0.01M; pH, 3.0), and boiled in a microwave for 5 minutes. After cooling them to room temperature, the slides were incubated for 10 minutes with 20 µg/mL of proteinase K and in situ labeled with dNTP mix (from the TACS Blue Label Detection kit) and terminal deoxynucleotidyl transferase in the presence of magnesium chloride at 37°C. The reaction was terminated with stop buffer. Streptavidin-horseradish peroxidase conjugate was then added to the tissue. The positive signal was visualized with TACS Blue Label, and the slides were counterstained with red counterstain C. The positive signal indicating DNA fragmentation could be recognized as a blue stain in a pink tissue background. To eliminate false-positive or false-negative results, staining was repeated, and both normal and diseased corneas were included in each experiment.

KERATOCYTE CULTURE

Cultivated keratocytes were generated from fresh corneal buttons and corneal donor rims according to a modified version of a previous protocol. Briefly, the epithelium and endothelium were dissected from the corneal stroma under a dissecting microscope. The stroma was cut into 2 × 2-mm slices, immersed in Eagle minimum essential medium (MEM) containing 2 mg/mL of collagenase (Life Science Inc, Gaithersburg, Md) and 0.5 µg/mL of hyaluronidase (Sigma Science Corp, St Louis, Mo), and incubated for 4 hours at 37°C in 5% carbon dioxide. Cells were washed once with 1% Penicillin-Streptomycin MEM containing 0.025% ethylenediamine-tetra-acetic acid. Cells were resuspended in 10% fetal bovine serum MEM and maintained at 37°C in 5% carbon dioxide. We used short-term keratocyte cultures (fewer than 4 subcultures) anticipating that the cells may maintain most of their original in vivo genetic characteristics.

KERATOCYTE RESPONSE TO CAMPTOTHECIN

Camptothecin (Sigma Science Corp) was dissolved in dimethyl sulfoxide to make a stock solution (1mM). It was then added to serum-free culture medium (Opti-MEM I; Life Science Inc) at a final concentration of 2µM and 6µM. Keratocytes from normal corneas and those with associated death domain proteins participate in the killing of targets such as virus-infected cells, cancer cells, and inflammatory cells at immune-privileged sites. Numerous studies have indicated a role for the death receptor family in autoimmune disorders such as Hashimoto thyroiditis and posterior uveitis. Fas has also been implicated in Alzheimers disease and aging. The Bcl-2 family of proteins responds to signals from diverse cytotoxic stimuli, including cytokine deprivation and DNA damage. These proteins are important signaling molecules in the maintenance of tissue homeostasis and in the protection against pathogens. The mutation or dysregulation of the Bcl-2 family members may lead to excessive apoptosis or cancer. In a typical cell,
Fuchs dystrophy were incubated with camptothecin for 6 and 24 hours, respectively, at 37°C in 5% carbon dioxide. They were then evaluated by immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR) for the expression of apoptotic regulatory proteins and mRNA.

IMMUNOHISTOCHEMISTRY

The expression of Fas, FasL, Bcl-2, and Bax in corneal buttons and keratocytes was evaluated by determining immunohistochemistry. Immunohistochemical staining was performed using an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif) technique. 30 Primary antibodies consisted of polyclonal rabbit antibodies recognizing epitopes of Fas and FasL (clones M-20 and V-20, respectively; Santa Cruz Biotech, Santa Cruz, Calif), Bcl-2, and Bax (Pharmingen, San Diego, Calif); and nonspecific rabbit IgG (Vector Laboratories, Burlingame, Calif) technique. 18 Primary antibodies were applied to corneal sections or keratocytes and incubated at room temperature for 1 hour. Biotin-labeled goat anti-rabbit immunoglobulin G (Vector Laboratories) was used as the secondary antibody. After incubation with the avidin-biotin-peroxidase complex (Vector Laboratories), slides were developed in 3,3’-diaminobenzidine and counterstained with 1% methyl green in methanol. Positively stained cells were then identified and graded arbitrarily according to the extent and intensity of the staining in the entire section. Cultivated keratocytes were counted in 3 representative ×40 fields or more than 200 cells, and the percentage of positive cells of total number of cells examined was recorded.

TOTAL RNA EXTRACTION AND SEMIQUANTITATIVE RT-PCR

Corneal endothelium with Descemet membrane was carefully separated from the stroma under a dissecting microscope, and the stromal tissue was then further cut into smaller pieces to maximize the yield of total RNA. Tissues were immediately immersed in 1 mL of RNA-STAT-60 (TEL-TEST Inc, Friendswood, Tex), and total RNA was extracted from corneal samples and/or pelleted keratocytes cultures according to the manufacturer’s instructions. The RNA extracts were treated with RQI RNase-free DNase (Promega Corp, Madison, Wis) and quantified using a spectrophotometer. The same amount of total RNA from each sample was used for reverse transcription. First-strand complementary DNA (cDNA) synthesis was accomplished with the Superscript II RNase HI-Reverse Transcriptase System (Life Technologies, Grand Island, NY) and the random primer (Promega, Madison, Wis).

A 347-base pair (bp) or 255-bp fragment in the coding region of Bcl-2 and Bax cDNA was amplified using AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, Calif). A total of 0.5 µg of cDNA was added to 4 n mol of each dNTP, 1.5 or 3.0 n mol of MgCl2, 3 pmol of phosphate-32–labeled forward primer, 3 pmol of reverse primer, 1 µL of GeneAmp, 10 × PCR buffer, and 0.5 U of AmpliTaq Gold polymerase (Perkin-Elmer Corp, Hayward, Calif). Water was added to adjust the total volume to 10 µL. The reaction mixture was then incubated in a Hybrid PCR Express thermocycler (Middlesex, England). Amplification involved denaturation at 94°C for 9 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, primer annealing at 54°C for 45 seconds, and chain elongation at 72°C for 1 minute. The final step was a 7-minute incubation at 72°C. An aliquot of each reaction mixture was then analyzed by electrophoresis on a 10% polyacrylamide gel, followed by ethidium bromide staining and autoradiography. Observation of a band of the predicted size on gel electrophoresis indicated the presence of mRNA in the original corneal sample. The negative control consisted of the omission of the RNA template or reverse transcriptase from the cDNA synthesis reaction for each sample. Intensity of each band was measured using the NIH image analysis system (National Institutes of Health, Bethesda, Md) and was recorded in digital form.

To verify that equal amounts of total RNA were added in each PCR reaction within an experiment and to assure a uniform amplification process, beta-actin mRNA was also transcribed and amplified for each sample. Sequences of the specific primers used for the current study were Bcl-2 forward, 5’CTAATTGCTGGCCTGCTCCCCCTTT3’; Bcl-2 reverse, 5’TGAACTCTGACCCCTGACCTGT3’; and Bax forward, 5’AACGTCTGGCTGACTGCTGCT3’; and Bax reverse, 5’TACAGTGCCGCACCTGCCTCG3’.

STATISTICAL ANALYSIS

Statistical analysis was conducted under the supervision of a statistician in the Division of Clinical Trials and Biometry at the Wilmer Eye Institute. Immunohistochemical analyses were evaluated by Fisher exact test. The t test was used to analyze digital densitometry data. A P value less than or equal to .01 was chosen as the limit of statistical significance.

RESULTS

proapoptotic and antiapoptotic family members (such as Bax and Bcl-2, respectively) seem to be in equilibrium. This equilibrium favors an equal concentration between Bax and one of its antagonists, such as Bcl-2.23 Any alteration in this balance may lead to the activation of cell death via an increase in Bax.

In this study, we evaluated the occurrence of programmed cell death in corneas with Fuchs dystrophy or other corneal disorders, and in normal eye bank corneas. We examined the expression of the apoptotic regulatory molecules Fas, Fasl, Bcl-2, and Bax in corneas with Fuchs dystrophy and in age-comparable normal eye-bank corneas. We also examined the messenger RNA (mRNA) expression of Bcl-2 and Bax in the corneal endothelium and stroma, respectively. To further investigate the regulation of apoptosis in Fuchs dystrophy, we cultured keratocytes derived from Fuchs dystrophy corneas and corneal donor rims. We then cocultured these keratocytes with camptothecin, a DNA synthesis inhibitor known to induce apoptosis in vitro.20 We evaluated the expression of apoptotic regulatory molecules in these keratocytes.

HISTOPATHOLOGICAL ANALYSIS

All of the diseased corneas included in this study displayed the classical pathological changes of Fuchs dys-
trophy. These corneal buttons were marked by epithelial and stromal edema, thickened Descemet membranes with posterior nodularity, and an attenuated endothelium. The control corneal tissue from eye-bank eyes displayed normal morphology.

**APOPTOSIS IN THE CORNEA**

The evidence of apoptosis in corneas with Fuchs dystrophy and in normal corneas was assessed by in situ DNA fragmentation (in situ end labeling). In corneas with Fuchs dystrophy, DNA fragmentation was seen in the epithelium and stroma in 5 of 7 samples, and in the endothelial cells in 6 of 7 samples. Figure 1 is a representative photomicrograph showing the staining in a diseased cornea (Figure 1, A and C) and in controls (Figure 1, B and D). Excessive apoptosis could be seen in the epithelium, and the staining could also be identified in the stromal and endothelial cells of the diseased cornea. In contrast, under the same staining condition, little or no positive staining was observed in the epithelium, stroma, or endothelium of the 4 control corneas. We examined 3 additional corneal buttons from patients with pseudophakic bullous keratopathy, bacterial keratitis, and graft rejection. Positive staining was observed in epithelial cells and in inflammatory cells infiltrating the stroma. The staining was not present in the keratocytes or endothelial cells of these corneas.

**EXPRESSION OF APOPTOTIC MOLECULES IN THE CORNEA**

To assess the role of apoptotic regulatory molecules in Fuchs dystrophy corneas, we examined the expression of Fas, FasL, Bcl-2, and Bax by immunohistochemistry. Both the intensity of the staining and the percentage of positively stained corneas were evaluated for each cellular marker and compared between Fuchs dystrophy and control eye-bank corneas. In Fuchs dystrophy corneas, intense Fas, FasL, and Bax staining was seen in the epithelium, endothelium, and stroma (often adjacent to Descemet membrane). Faint staining of Bcl-2 was seen occasionally in the epithelium and endothelium of these corneas. In contrast, only mild staining of Fas and/or FasL was seen in normal corneal epithelia and endothelia. Bcl-2 and Bax were mostly undetectable in normal corneas. Generally, the staining was found in the cytoplasm of corneal epithelial cells; however, the precise cellular location of the staining was somewhat difficult to determine because of the flattened morphology of endothelial cells and the compression of keratocytes by collagenous lamellae (Figure 2).

**Figure 3** summarizes the overall results of the immunohistochemical analysis. A statistical difference between Fuchs dystrophy and control corneas was present only in the group of stromal Bax expression (P = .007). A noticeable difference was also present in FasL stromal expression (P = .02); however, when evaluated by the intensity of the staining, a statistical difference was shown in groups of stromal FasL expression (P = .001), epithelial Bcl-2 expression (P = .01), epithelial Bax expression (P = .004), and stromal Bax expression (P < .001).

**EXPRESSION OF Bcl-2 AND Bax mRNA IN THE CORNEA**

Since immunohistochemical analysis indicated a significant difference in stromal Bax expression in Fuchs dys-
trophy corneas, we further compared the mRNA levels of Bcl-2 and Bax in normal and diseased corneas (Figure 4). Figure 4A is a representative polyacrylamide gel electrophoresis of DNA samples from RT-PCR of mRNA isolated from the stromal and endothelial layers of normal and Fuchs dystrophy corneas. The intensity of DNA bands, particularly bands with weak signals, is somewhat difficult to identify in the reproduction of the original autoradiographic image; therefore, they are reflected in Figure 4B using densitometric measurement of these bands. Scatter graphs were made according to the densitometry measurements of DNA bands for all of the samples examined. Statistically significant differences were identified in stromal levels of Bcl-2 ($P = .006$) and Bax ($P = .008$) between Fuchs dystrophy ($n=9$) and control corneas ($n=5$).

In the endothelium, the level of Bcl-2 and Bax mRNA expression was not appreciably different between normal and diseased corneas; however, significantly higher levels of Bcl-2 mRNA ($P = .006$) and Bax mRNA ($P = .008$) were identified in the stroma of diseased corneas when compared with those of normal controls.

**KERATOCYTE RESPONSES TO CAMPTOTHECIN**

As an antiapoptotic member of the Bcl-2 family, the cellular levels of Bcl-2 may increase when cells are exposed to cytotoxic stimuli; however, the elevated Bax mRNA and protein levels that we observed in the corneas with Fuchs dystrophy could occur at the late stage of DNA damage, or it could be the trigger that initiates apoptosis. To further delineate the role of the apoptotic regulatory molecules in Fuchs dystrophy, we used an in vitro approach. We stimulated cultivated keratocytes with camptothecin, an apoptotic inducer, and assessed protein and mRNA levels of apoptotic regulators.
The protein expression of Fas, FasL, Bcl-2, and Bax was up-regulated after stimulation of both normal and diseased keratocytes with 6 mm of camptothecin; however, no statistical difference in protein expression could be identified to distinguish the 2 groups. Expression of Bcl-2 and Bax mRNA in these keratocytes, on the contrary, clearly showed a disease-specific trend (Figure 5). In normal keratocytes, cellular Bcl-2 and Bax mRNA increased proportionately after camptothecin stimulation, with levels of Bcl-2 exceeding levels of Bax. In keratocytes with Fuchs dystrophy, there was no Bcl-2 response with low-dose camptothecin and a low-magnitude Bcl-2

![Figure 3. Immunohistochemical analysis of the expression of apoptotic molecules in corneas with Fuchs dystrophy (n=9) and control corneas (n=4). Expression was measured by the number of positively stained corneas/total number of corneas examined. This ratio was recorded as a percentage (y-axis) for each specific corneal layer (x-axis). The asterisk indicates a statistically significant difference (P<.01). A, Fas expression in the cornea. B, FasL expression in the cornea. C, Bcl-2 expression in the cornea. D, Bax expression in the cornea.

![Figure 4. A, A representative polyacrylamide gel electrophoresis of DNA samples from reverse transcriptase polymerase chain reaction of messenger RNA (mRNA) isolated from the stromal and endothelial (Endo) layers of normal and Fuchs dystrophy corneas. Lanes 1, 3, 5, 7, and 9: samples from endothelium of Fuchs dystrophy patients 1 through 5. Lanes 2, 4, 6, and 8: samples from corneal stroma of Fuchs dystrophy patients 1 through 4. Lane 10: samples from normal corneal stroma (n=2). Lanes 11 and 12: samples from normal corneal endothelium (n=3). Lane 13: negative control, the omission of RNA template from the complementary DNA (cDNA) synthesis reaction. B, Summary of the gel electrophoresis findings (A). Scatter graphs were made according to the densitometry measurements of DNA bands for all of the samples examined. Note that the scales are different for each of the 4 graphs because of the markedly different signal intensity. Statistically significant differences were identified in stromal levels of Bcl-2 (P=.006) and Bax (P=.008) between Fuchs dystrophy (n=9) and control groups (n=5). X-axis numbers indicate lanes 1 to 3. bp indicates base pair.

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response with high-dose camptothecin, which was contrary to the highly elevated levels of Bax mRNA. The response patterns in both groups were consistent at 6 and 24 hours after camptothecin exposure. Although the changes in mRNA levels only indirectly reflect the possible changes in protein level, given the sensitivity and quantitative nature of RT-PCR and the overall up-regulation of protein levels in these cells, the alteration in mRNA levels should be a trustworthy reference to changes in protein levels.

**COMMENT**

The results of our preliminary study suggest that aberrant responses of apoptotic regulatory molecules in the cornea may play an important role in the pathogenesis of Fuchs dystrophy. Although dysfunction of the corneal endothelium has been considered to be the cause of corneal decompensation in Fuchs corneal dystrophy, stromal keratocytes may also play a crucial role in the development of the disease.

As with the evaluation of cell viability, no single parameter can fully define cell death. Methods for studying cell death in tissue or individual cells include identifying cellular DNA fragmentation and analyzing apoptosis-associated proteins such as Bcl-2 homologues, caspases, and other signaling molecules. The key to the most accurate interpretation of apoptosis is the combination of multiple study methods with the careful interpretation of results. According to our study, distinctive pathological findings in the corneas with Fuchs dystrophy included DNA fragmentation in stromal and endothelial cells and an elevated expression of Bax mRNA and protein in the stroma. In addition, we noted alterations in expression of Bcl-2 and Bax mRNA following exposure to an apoptotic stimulus in keratocytes with Fuchs dystrophy.

Excessive apoptosis was identified in the epithelium of the cornea. This was likely due to the epithelial and stromal edema of decompensated corneas. The evidence of apoptosis in the corneal epithelial, stromal, and endothelial layers indicated that it is part of the pathological process of Fuchs corneal dystrophy; however, whether it is a result of end-stage disease or a triggering mechanism is still unclear. Therefore, we further studied the regulatory molecule of programmed cell death.

As discussed in the introduction, proapoptotic and antiapoptotic members of the family normally seemed to be in equilibrium. Thus, a lack of Bcl-2 production following camptothecin exposure would result in a relatively high level of cellular Bax and could subsequently activate the cell death process. The kerocyte responses to camptothecin in this study suggest that Bax may act as a trigger, rather than a passive by-product, for stromal apoptosis in Fuchs dystrophy. Expanding on these observations, we can speculate that various environmental stimuli to the cornea (such as hormonal changes with aging, inflammation, and other toxins) may lead to the development of Fuchs dystrophy by triggering excessive apoptosis in kerocytes.

Fuchs dystrophy has been considered a primary disorder of the corneal endothelium, based on the unique and early morphological changes of the endothelium and its surroundings. We did identify apoptosis in endothelial cells of Fuchs dystrophy corneas, which was consistent with the findings of a recent electron microscopy study that documented a higher percentage of endothelial cell apoptosis in Fuchs dystrophy corneas; however, our study indicates that the most remarkable differences between normal and Fuchs dystrophy corneas were found in kerocytes. Interestingly, Calandra et al revealed that Fuchs dystrophy corneas contained stromal collagens with altered biochemical properties, suggesting a possible abnormality in kerocytes.

The spectrum of possible functions of keratocytes is growing in light of recent research. Keratocytes are highly active cells involved in the turnover of the extracellular matrix and in the maintenance of corneal trans-
In conclusion, we have obtained strong preliminary evidence to indicate that a disturbance in the regulation of apoptosis may play a role in the pathogenesis of Fuchs dystrophy; however, this is only the first step in addressing many remaining questions. Are apoptotic changes found in this study unique to Fuchs dystrophy? What other genes may become insufficient to maintain normal endothelial cell function, subsequently, a prolonged degenerative process may eventually lead to the morphological and functional changes in the endothelial layer as seen in Fuchs dystrophy. The degeneration of the epithelium is the consequence of both keratocytes and endothelial cell decompensation. Although it is still unclear whether the functional changes in keratocytes precede changes in the endothelial cells, the aberrant response of Fuchs keratocytes to apoptotic stimuli leads us to consider this possibility.

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