Corneal Endothelial Cell Apoptosis in Patients with Fuchs’ Dystrophy

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PURPOSE. To investigate whether apoptosis plays a notable role in degeneration of corneal endothelial cells in patients with Fuchs’ dystrophy.

METHODS. Forty-seven corneal buttons from 41 patients with Fuchs’ dystrophy were studied. Nucleus labeling, transmission electron microscopy (TEM), and TdT-dUTP terminal nick-end labeling (TUNEL) were used to detect apoptosis. TEM and TUNEL were performed on sections of all 47 corneal buttons, and nucleus labeling was performed on the last 10 corneas. Seven human donor corneas, two corneal buttons from two patients with keratoconus, and one corneal button from a patient with interstitial keratitis were used as negative controls for detection of apoptotic endothelial cells. Negative controls were studied by means of nucleus labeling, TUNEL, and TEM.

RESULTS. In the nucleus labeling assay, the average percentage of apoptotic endothelial cells was 2.65% in the Fuchs’ dystrophy group (n = 10) and 0.23% in the control group (n = 10; P = 0.0003). In the TUNEL assay, labeling of some endothelial cells was observed on 42 of 47 corneas in the Fuchs’ dystrophy group, whereas it was absent on most specimens of the control group. In TEM, most endothelial cell nuclei had a normal appearance, and apoptotic endothelial cells featuring condensed nucleus and decreased cell size could be observed exceptionally. Some apoptotic cells were found in the basal epithelial cell layer by means of nucleus labeling, TUNEL, and TEM in the Fuchs’ dystrophy group but not in the control group.

CONCLUSIONS. This study suggests that apoptosis plays an important role in endothelial cell degeneration in Fuchs’ dystrophy. Because of a lack of conclusive evidence of increased endothelial apoptosis by TEM, further studies are needed to ascertain this finding. (Invest Ophthalmol Vis Sci. 2000;41:2501–2505)

Fuchs’ dystrophy is a relatively frequent corneal condition affecting the corneal endothelium, categorized as an abnormality of neural crest final differentiation, and inherited in an autosomal dominant pattern with 100% penetrance and variable expressivity.1–3 It leads to corneal swelling through the loss of Na+,K+-ATPase pump sites within the endothelium.4–5 The primary cause of this endothelial dysfunction is unknown. Specular microscopy in patients with Fuchs’ dystrophy showed increasing cell size (indicating endothelial cell loss) together with progression of cornea guttata in advanced stages of the disease.6 Transmission electron microscopy (TEM) of corneal buttons from patients with advanced Fuchs’ dystrophy showed endothelial cell dystrophic changes, endothelial cell transformation to fibroblast-like cells, and, finally, death of many cells.1,7 Corneas with Fuchs’ dystrophy feature aberrant Descemet’s membrane synthesis by the endothelium with an abnormal posterior banded layer that may be followed by a nonspecific fibrous layer.1,7 The cause of the endothelial cell transformation with collagen deposition is unknown, and it is not clear whether it is a primary or secondary effect.

Apoptosis is thought to play a role in several corneal conditions, including keratocyte death after epithelial injury or excimer laser surgery, keratocyte degeneration in patients with keratoconus, dexamethasone-induced keratocyte death, and keratocyte cryopreservation injury.8–10 The aim of the present study was to investigate whether apoptosis plays a notable role in degeneration of corneal endothelial cells in patients with Fuchs’ dystrophy.

METHODS

Corneal Specimens

Forty-seven corneal buttons from 41 patients with Fuchs’ dystrophy were obtained at the time of corneal transplantation between 1994 and 1998. Patients with Fuchs’ dystrophy were characterized by an average age of 70.0 ± 11.3 years. There were 31 women and 10 men. Three different methods were used to detect apoptosis (i.e., nucleus labeling, TEM, and TdT-dUTP terminal nick-end labeling (TUNEL)). TEM and TUNEL were performed on sections of all 47 corneal buttons, and nucleus labeling was performed on flatmounts of the last 10 corneas.

The first 37 corneal buttons were routinely processed for histology and TEM. Corneal buttons were fixed in Carson...
solution for 24 hours after which they were divided into 2 pieces. For each corneal button, one half was routinely processed for light microscopy and TUNEL and the other half was processed for TEM. The last 10 corneal buttons were divided into 2 pieces. For each specimen, one half was fixed in 4% paraformaldehyde in phosphate-buffered saline for nucleus labeling and the other half was fixed in Carsonsolution for TUNEL and TEM.

Seven human donor corneas, two corneal buttons from two patients with keratoconus, and one corneal button from a patient with interstitial keratitis were used as negative controls for detection of apoptotic endothelial cells. These control specimens were processed with similar methods (nucleus labeling, TUNEL, TEM, and routine histology).

This study was carried out according to the tenets of the Declaration of Helsinki.

Assays

For nucleus labeling, the specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at room temperature. After washing in PBS, they were incubated in a solution of 10 μg/ml Hoechst 33258 (Sigma, Saint Quentin, France) in PBS for 15 minutes and then washed in PBS. Descemet’s membrane with the endothelium was dissected with a surgical blade under microscope and flatmounted in glycerol. The remaining corneal stroma and epithelium were placed in a petri dish with PBS. The specimens were examined using an epifluorescence microscope (Diaphot TDM; Nikon, Tokyo, Japan) with UV filters. Six to 12 photographs of each flattened specimen were taken using the same instrument. Photographs were analyzed by two observers in a blind fashion. The average number of endothelial cells analyzed per specimen was 667 (range, 511–1745). The number of apoptotic cells and the total number of cells were counted. Cells with nuclear condensation, dense nuclei, and satellites and cells with blebs were considered apoptotic. A strong coloration that corresponded to mitotic cells did not correspond to apoptotic cells, nor did cell fragments. The Wilcoxon rank sum test was used to compare the data.

Conventional histologic staining methods used in paraffin sections included hematoxylin, eosin, and safran, and periodic acid–Schiff.

Sections of paraffin-embedded corneal specimens were used for TUNEL. Fragmentation of DNA by activated endonucleases was localized in situ using an Apotag peroxidase kit (Oncor, Gaithersburg, MD). Corneal sections were first deparaffinized in xylene (2 × 5 minutes) and washed in 100° ethanol (2 × 5 minutes), 70° ethanol (5 minutes), and, finally, PBS. They were incubated with a 20 μg/ml proteinase K solution diluted 1:200 in PBS for 15 minutes at room temperature and washed in distilled water (2 × 2 minutes). They were processed using a microwave oven (700 W for 4 minutes followed by 500 W for 5 minutes) and then left at room temperature for 20 minutes. They were incubated with equilibration buffer for 5 minutes at room temperature and then with terminal deoxynucleotidyl transferase mixed with stop/wash buffer for 1 hour at 37°C followed by 30 minutes at 20°C. The terminal deoxynucleotidyl transferase enzyme is active at 37°C, whereas the stop/wash buffer is active at 20°C. After washing in PBS (3 × 5 minutes), specimens were incubated with anti–digoxigenin peroxidase conjugate diluted 1:100 in PBS for 30 minutes at room temperature and rinsed with PBS (2 × 5 minutes), after which color was developed in fast red peroxidase substrate. Specimens were then counterstained with hematoxylin and mounted. Negative controls consisted of corneal sections processed with no terminal deoxynucleotidyl transferase.

Samples for TEM were directly fixed in Carsonsolution, postfixed in 1% osmium tetroxide for 1 hour, and embedded in epoxy resin. Semi-thin cross sections were stained with toluidine blue and examined with a light microscope. Ultrathin cross sections were contrasted with uranyl acetate and lead citrate and examined with a transmission electron microscope (model EM10; Zeiss, Oberkochen, Germany).

RESULTS

Histology

All specimens (47/47) from the group of corneas with Fuchs’ dystrophy showed guttate excrescences. Descemet’s membrane thickness was always increased. It ranged from 14 to 46 μm in the group of corneas with Fuchs’ dystrophy and from 5 μm to 10 μm in the control group.

Nucleus Labeling Assay

Analysis of endothelium flatmounts showed apoptotic cells within the endothelial cell layer in the group of corneas with Fuchs’ dystrophy (Fig. 1A). Most specimens of the control group showed no apoptotic cells (Fig. 1B). The percentage of apoptotic endothelial cells was significantly higher (P = 0.0003) in the Fuchs’ dystrophy group (n = 10) than in the control group (n = 10). The average percentage of apoptotic endothelial cells was 2.65% ± 3.28% (mean ± SD) in the former group and 0.23% ± 0.54% in the latter group. The endothelial cell nuclei were oval, elongated, and irregularly spaced in the former group (Fig. 1A), and they were more round and more regularly spaced in the latter group (Fig. 1B). They appeared to be pushed into the areas between the guttae in the former group. No mitotic endothelial cells were observed in either group.

Analysis of the remaining corneal stroma and epithelium showed some apoptotic cells within the corneal epithelium. These apoptotic epithelial cells were more numerous in the Fuchs’ dystrophy group than in the control group.

TUNEL Assay

Labeling of some endothelial cells was observed on 42 of 47 corneas in the Fuchs’ dystrophy group (Fig. 2A), whereas it was absent in most specimens of the control group (Fig. 2B). Labeling of some epithelial cells and keratocytes was observed on 27 and 5, respectively, of 47 corneas in the Fuchs’ dystrophy group (Fig. 2C), whereas it was absent on most specimens of the control group (Fig. 2D). Negative controls showed no labeling (data not shown).

Transmission Electron Microscopy

All specimens (47/47) from the group of corneas with Fuchs’ dystrophy showed corneal injuries related to Fuchs’ dystrophy by means of TEM. Most endothelial cell nuclei had a normal appearance (Fig. 3A), and apoptotic endothelial cells featuring condensed nucleus and decreased cell size could be observed exceptionally (Fig. 3B). The endothelium was either normal, absent, or degenerated (i.e., fibroblast-like cells). Subepithelial fibrosis was observed in 21 of 47 corneas in the Fuchs’ dystrophy group. Some apoptotic cells with condensed chromatin...
were found in the basal epithelial cell layer of all the corneas in this group (Figs. 3C, 3D). Keratocytes showed no features of apoptosis.

In the control group, the ultrastructure of the corneal endothelium was normal. Epithelial basal cells had normal nuclei with no condensed chromatin (data not shown).

**DISCUSSION**

To the best of our knowledge, this is the first study suggesting that apoptosis plays a notable role in endothelial cell death in Fuchs' dystrophy. Although Fuchs' dystrophy is probably the most widely studied corneal dystrophy, the conventional methods used in previous studies (i.e., light microscopy and TEM) did not provide clear evidence of apoptotic endothelial cells in corneas with Fuchs' dystrophy. The explanation is certainly that the percentage of endothelial cells undergoing apoptosis is relatively low, so that it is unlikely that an apoptotic endothelial cell in cross section could be found. In fact, in the present study, we could not find any apoptotic endothelial cell by means of TEM in most specimens. Conversely, flat-mounted endothelium specimens clearly displayed apoptosis features in the nucleus labeling assay. It is notable that the number of endothelial cells analyzed in the nucleus labeling assay was dramatically higher (several thousands of observed cells per flat-mounted specimen with approximately 700 cells randomly photographed for statistical analysis) than that analyzed in TEM (approximately 10 cells/specimen). Apoptotic endothelial cells featured nuclear condensation, dense nuclei, and satellites. The average percentage of apoptotic endothelial cells was 2.65% in the Fuchs' dystrophy group \((n = 10; A)\) and 0.23% in the control group \((n = 10; B, P = 0.0005)\). Magnification, \(\times 800\).

**Figure 1.** Nucleus labeling assay. The Descemet’s membrane with the endothelium was dissected, flatmounted in glycerol, and examined using an epifluorescence microscope. Cells with nuclear condensation, dense nuclei, and satellites and cells with blebs were considered apoptotic (arrows). The average percentage of apoptotic endothelial cells was 2.65% in the Fuchs’ dystrophy group \((n = 10; A)\) and 0.23% in the control group \((n = 10; B, P = 0.0005)\). Magnification, \(\times 800\).

**Figure 2.** TUNEL assay. Labeling of some endothelial cells was observed on 42 of 47 corneas in the Fuchs’ dystrophy group (A), whereas it was absent on most specimens of the control group (B). Labeling of some epithelial cells was observed on 27 of 47 corneas in the Fuchs’ dystrophy group (C), whereas it was absent on most specimens of the control group (D). Arrowheads, apoptotic cells. Scale bars, 30 \(\mu m\).
volvement of apoptosis in endothelial cell death in Fuchs’ dystrophy. However, we must highlight that the absolute results of this assay are experiment-dependent, so that only comparisons of Fuchs’ dystrophy specimens with control specimens can be reliably carried out. Conversely, apoptotic figures found in the nucleus assay are convincing findings in favor of the presence of apoptotic cells. This latter assay is not experiment-dependent.

The presence of apoptotic cells in the epithelial basal cell layer of Fuchs’ dystrophy corneas was demonstrated by means of the three methods used to detect apoptosis. This feature was very uncommon in the control group. In TEM, these cells featured decreased cell size, condensed nuclei, and the presence of microvilli. They were found close to Bowman’s layer. This could indicate an increase in the level of apoptosis within the corneal epithelium in Fuchs’ dystrophy corneas. Although apoptosis is thought to play a notable role in epithelial cell final differentiation (i.e., transition from wing cells to superficial cells), basal epithelial cells are supposed to be resistant to apoptosis. The presence of apoptotic cells within the basal epithelial cell layer is certainly a pathologic feature of Fuchs’ dystrophy. Iwamoto and DeVoe previously reported that some nuclei in the basal epithelial cell layer showed marked clumping of the chromatin substance.18 Apoptosis within the epithelial basal cell layer can either be secondary to epithelial edema or primary (i.e., due to primary dysregulation of cell death in Fuchs’ dystrophy). Further studies investigating apoptosis in other corneal disorders with epithelial edema (such as aphakic and pseudophakic bullous keratopathy) are needed to determine whether corneal epithelial edema can induce apoptosis in the basal cell layer.

According to our data, there is no evidence that keratocytes degenerate through apoptosis in Fuchs’ dystrophy. However, the nucleus labeling assay did not permit an analysis of the corneal stroma. Keratocyte apoptosis was rare in the TUNEL assay. It was absent in TEM. In the TUNEL assay, of two keratoconic cornea specimens, one showed apoptotic keratocytes (data not shown). However, the number of keratoconic corneas included in this study was too small to draw conclusions on the role of apoptosis in keratocyte cell death in keratoconus.

Involvement of apoptosis in Fuchs’ dystrophy pathophysiology was recently suggested by other investigators. Two recent studies showed that the expression of proteins enhancing apoptosis was increased in corneal cells from patients with Fuchs’ dystrophy. Overexpression of bax and a lack of bel-2 (an antiapoptotic gene) expression were demonstrated in cultured keratocytes from fresh corneal buttons of Fuchs’ dystrophy patients. Reverse transcription-polymerase chain reaction analysis of the endothelial cells showed no bel-2 mRNA.19

**FIGURE 3.** TEM of corneal buttons from patients with Fuchs’ dystrophy. Most endothelial cell nuclei had a normal appearance (A), and apoptotic endothelial cells (B) featuring condensed nuclei and decreased cell size could be observed exceptionally. Some apoptotic cells with condensed chromatin were found in the basal epithelial cell layer (C, D). Arrow, apoptotic cell; n, normal nucleus; b, Bowman’s layer; d, Descemet’s membrane. Scale bars, 1 μm.
Overexpression of endothelial-monocyte activating polypeptide, a proapoptotic cytokine, was demonstrated in the endothelial cells from patients with Fuchs’ dystrophy. In vitro, human endothelial cells obtained from corneal buttons of patients with Fuchs’ dystrophy have only a limited growth capacity in cell culture, whereas transduction of these cells with the human papilloma virus E6/E7 oncogenes dramatically increases cell proliferation and improves cell morphology. Expression of these oncogenes could decrease the level of apoptosis of these cells.

Fuchs’ dystrophy pathophysiology is characterized by deposition of large amounts of wide-spaced collagen posterior to Descemet’s membrane, forming a posterior periodic collagenous layer with a slow, continuous loss of morphologically and physiologically altered endothelial cells, and a gradual decline in Na⁺,K⁺-ATPase pump site density on corneal endothelial cells. Which event is primary remains unknown. Considering that apoptosis plays a notable role in endothelial cell loss in Fuchs’ dystrophy, apoptosis could either be primary or secondary to the modification of the basement membrane (i.e., Descemet’s membrane) composition or to loss of contact between the endothelial cells and the basement membrane. In fact, apoptosis of adherent epithelial cells induced by loss of contact with basement membrane has been previously demonstrated in other models. However, Descemet’s membrane obtained from pseudophakic bullous keratopathy corneal buttons could not support growth of human corneal endothelium in vitro, whereas Descemet’s membrane with Fuchs’ dystrophy did. This would imply that endothelial cell apoptosis is not secondary to the modification of Descemet’s membrane composition in Fuchs’ dystrophy.

In conclusion, this study suggests that apoptosis plays an important role in endothelial cell degeneration in Fuchs’ dystrophy. Because of a lack of conclusive evidence of increased endothelial apoptosis by TEM, further studies are needed to ascertain this finding. Further investigations should also determine whether apoptosis is primary or secondary to the modification of Descemet’s membrane composition or to loss of contact between the endothelial cells and the basement membrane. If the hypothesis of endothelial cell apoptosis being primary in Fuchs’ dystrophy is right, the use of drugs inhibiting apoptosis (such as caspase inhibitors) could be considered in further in vitro studies. In fact, caspases are currently recognized as therapeutic targets for central nervous system diseases in which apoptosis is prominent.

Acknowledgments

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References