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Epithelial cell, keratocyte, and endothelial cell apoptosis in Fuchs' dystrophy and in pseudophakic bullous keratopathy

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PURPOSE. *To elucidate the pathomechanism of Fuchs' dystrophy and pseudophakic bullous keratopathy (PBK) by examining cell apoptosis in different corneal layers.*
METHODS. *The authors studied corneal buttons obtained from 21 eyes following central penetrating keratoplasty: 14 corneal buttons (13 patients, age 70.8 ± 10.0 years) with Fuchs' dystrophy, and 7 buttons (7 patients, age 69.6 ± 10.2 years) with PBK. Four buttons from enucleated eyes with choroidal melanoma served as controls. Histologic changes were examined using light microscopy with hematoxylin-eosin (HE) staining. The average numbers of apoptotic cells per field of view (125x magnification) in separate samples of the epithelial, stromal, and endothelial layers were determined using the TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling) assay.*
RESULTS. *In 11 of the Fuchs' dystrophy corneas and 2 of the PBK corneas, apoptotic activity was detected. In the control corneas no apoptotic activity was found. Compared to the controls there was a statistically significant difference in the mean (normalized) apoptotic cell numbers for all three layers ($p=0.01$ in each case) in the Fuchs' dystrophy corneas, and for the stromal layer ($p<0.01$) in PBK corneas. The apoptotic cell numbers for the epithelial and endothelial layers of the latter were higher, but the difference was not statistically significant ($p=0.07$, 0.07).*
CONCLUSIONS. *Apoptosis may play a role in the pathomechanism of Fuchs' dystrophy and in keratocyte death in corneas with PBK. (Eur J Ophthalmol 2005; 15: 17-22)*

KEY WORDS. *Fuchs' dystrophy, Apoptosis, Pseudophakic bullous keratopathy*

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INTRODUCTION

Fuchs' dystrophy is an autosomal dominantly inherited disease of the cornea, characterized by a pleomorphic corneal endothelium and irregularity of the thickened Descemet's membrane. Despite its relatively common occurrence, its pathomechanism is not well understood. The abnormal functioning of the endothelial cells is manifested by corneal swelling and deposition of collagen and extracellular matrix in the Descemet's membrane (1). Several alterations of corneal function have been found in corneas with Fuchs' dystrophy: endothelial pump dysfunction (2), abnormality of endothelial cell final differentiation at the perinatal period (3), aberrant fibrinogen metabolism (4, 5), chromosome changes in keratocytes (6), and altered mitochondrial ionic metabolism (1). Recently, the pre-

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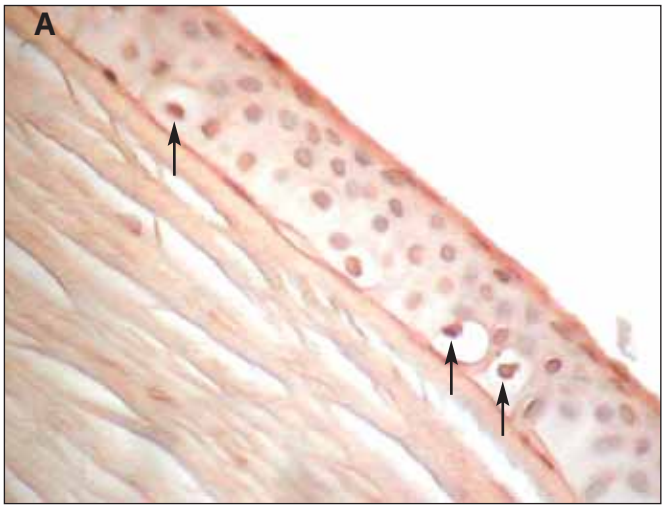

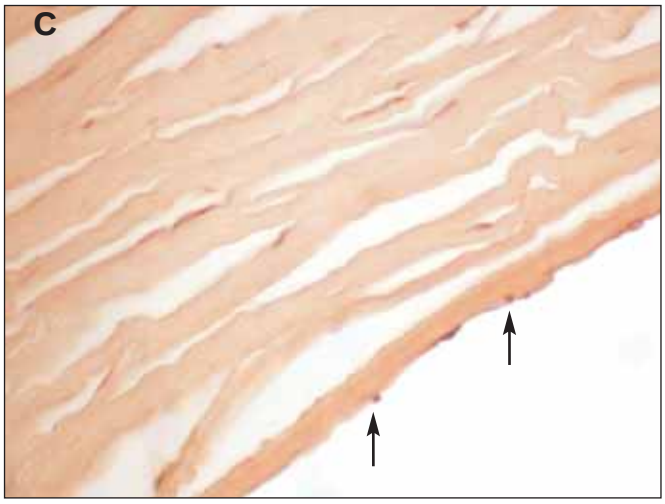

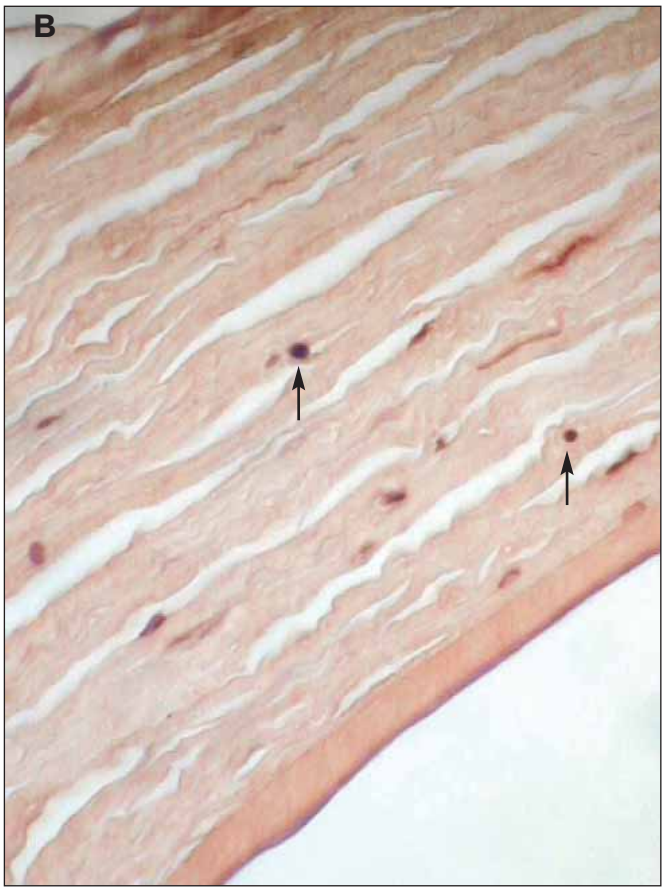

Fig. 1 - *Apoptosis of epithelial cells* **(A)**, *keratocytes* **(B)**, *and endothelial cells* **(C)** *in Fuchs dystrophy (arrow) by use of the TUNEL assay method. Original magnification 40x.*

viously accepted theory about changes in endothelial cell permeability in such corneas seems to have been disproved (7, 8), and the primary reason for the dysfunction of the endothelial cells remains unknown.

Apoptosis is genetically programmed cell death, characterized by cell shrinkage, chromatin condensation, and DNA fragmentation in the cells. It is assumed to participate in the normal corneal cell turnover, but with low activity (perhaps one apoptotic epithelial cell is seen in the typical field of view at 40x magnification) (9, 10). Recent studies have shown that apoptosis of keratocytes and endothelial cells may have a role in the pathogenesis of Fuchs' endo-epithelial dystrophy compared to corneas with pseudophakic bullous keratopathy, keratitis, graft rejection, keratoconus, and human donor corneas (11, 12).

The purpose of this study was to elucidate the role of epithelial cell, keratocyte, and endothelial cell apoptosis in the pathogenesis of Fuchs' dystrophy and pseudophakic bullous keratopathy.

METHODS

The patient population comprised 20 patients (21 eyes) who underwent central penetrating keratoplasty and 4 patients (4 eyes) with choroidal melanoma following enucleation. We compared 14 excised corneal buttons from 13 patients with Fuchs' dystrophy (age at the time of surgery 70.8 ± 10.0 years, range 54.5 to 84 years; 3 male) and 7 corneal buttons from 7 patients with pseudophakic bullous keratopathy (age 69.6 ± 10.2

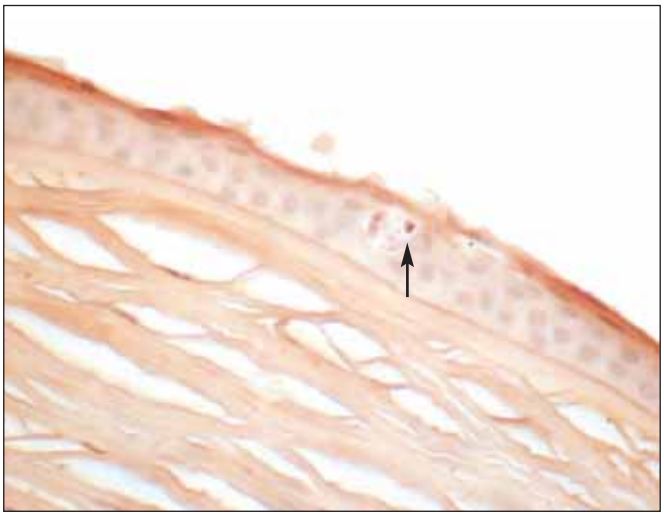

Fig. 2 - *Epithelial cell and nucleus shrinkage in Fuchs' dystrophy by use of the TUNEL assay method. Original magnification 40x.*

years, range 60 to 82 years; 3 male) to 4 corneal buttons of 4 patients with choroidal melanoma (age 67.2 ± 15.9 years, range 57 to 91 years; 2 male) considered as controls. The mean age of the patients with Fuchs' dystrophy (p=0.6) and with PBK (p=0.4) did not differ significantly from control patients.

The study was carried out in conformance with the tenets of the Declaration of Helsinki; Institutional Review Board/Ethics Committee approval was not required.

All the penetrating keratoplasties were performed at our clinic between January 2001 and January 2003, by a total of seven different surgeons. A hand-held trephine was used.

All corneal buttons and enucleated eyes were fixed in 4% paraformaldehyde plus 1% glutaraldehyde. Thereafter a hand-held trephine was used for trephination of corneal buttons of the enucleated eyes. All corneal buttons were processed through graded alcohol and finally embedded in paraffin wax.

Histologic changes were analyzed in 4-mm-thick sections using light microscopy after hematoxylin-eosin (HE) staining. Apoptosis was detected on separate samples by use of the TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling) assay method as described by Szende et al (13). The number of apoptotic cells was determined in epithelial, stromal, and endothelial layers of each corneal button. To achieve a normalized figure for comparison purposes, total number of cells in 15 nonoverlapping, full-thickness columns extending from the anterior epithelial surface to the endothelial cell layer were manually counted for each layer of each specimen. The diameter of each column was the 125x microscopic field. The mean apoptotic cell number values of 15 different fields of each corneal layer of each specimen were calculated.

For statistical analysis, the software package SPSS (version 10.0 for Windows NT) was used. Comparisons between groups or variables were performed using a nonparametric test (Mann-Whitney test for unpaired samples). A p value less than 0.05 was considered to indicate statistical significance.

RESULTS

Histology

All corneas with Fuchs' dystrophy showed epithelial and stromal edema. As a result of intracellular and intercellular edema of the basal cells, bullous separation of the epithelial cell layer from Bowman's membrane was always present. There was an increase of interstitial fluid separating the stromal collagen fibrils from each other and with formation of focal stromal vacuolar fluid spaces. The Descemet's membrane thickness was always increased, with presence of posterior nodularity, and epithelial cells were found to be attenuated and elongated.

All corneas with pseudophakic bullous keratopathy showed epithelial and stromal edema. Bullous separation of the epithelial cell layer from the Bowman's membrane was always present. The increased amount of interstitial fluid separated the stromal collagen fibrils from each other, with formation of focal stromal vacuolar fluid spaces. Descemet's membrane thickness was always increased, and a decreased number of endothelial cells was found.

All corneal buttons of enucleated eyes with choroidal melanoma were without pathologic findings.

TUNEL assay

Sample images for the different corneal layers are shown in Figure 1. In 11 of the 14 corneas with Fuchs' dystrophy and in 2 of the 7 corneas with pseudopha-

kic bullous keratopathy apoptotic activity was detected by the TUNEL assay in epithelial cells, keratocytes, or endothelial cells. The TUNEL positive cell nuclei were condensated or nucleus and cell shrinkage indicated apoptotic activity (Fig. 2). The typical site of accumulation of apoptotic cells was at the basal cell layer of the epithelium and around vacuoles in the stroma.

In corneal buttons of enucleated eyes with choroidal melanoma no apoptotic activity was found.

The mean apoptotic cell numbers in each layer, as determined by the TUNEL assay, are shown in Table I. There was a statistically significant difference in the mean (normalized) apoptotic cell numbers of the epithelial ($p=0.01$), stromal ($p=0.01$), and endothelial ($p=0.01$) layers in the corneas with Fuchs' dystrophy, and of stromal layer ($p<0.01$) of corneas with PBK compared to control corneas. The mean apoptotic cell numbers of epithelial and endothelial layers of corneas with pseudophakic bullous keratopathy were in each case higher compared to the control group although statistical significance for the differences was not demonstrable (both $p=0.07$).

DISCUSSION

Although Fuchs' dystrophy is a common corneal dystrophy, previous studies did not explain the role of apoptosis of epithelial cells, keratocytes, and endothelial cells in the pathomechanism of the disease. The density of apoptotic epithelial cells and keratocytes has been presumed to increase secondary to epithelial and stromal edema and swelling; endothelial programmed cell death was supposed to be a result of the modification of the Descemet's membrane in such corneas. Increased apoptotic activity might also be a primary dysfunction of both epithelial and endothelial cells in these patients, however, none of the above theories has been proved or precluded (11, 12).

Li et al have shown increased expression of proteins enhancing apoptosis in corneas with Fuchs' dystrophy: In cultured keratocytes of fresh corneal buttons from such corneas overexpression of the bax gene and lack of bcl-2 (antiapoptotic gene) has been detected compared to a control group with the diagnosis of pseudophakic bullous keratopathy, bacterial keratitis, and graft rejection. The above defect is supposed to act as a trigger for programmed cell death in these corneas (12).

Borderie at al demonstrated increased amount of apoptotic cells in the basal epithelial cell layer and endothelial cell layer by means of nucleus labeling and TUNEL assay in corneas with Fuchs' dystrophy compared to a control group of human donor corneas, corneal buttons with keratoconus, and a corneal button with interstitial keratitis (11).

Contradicting the above studies keratocyte apoptosis

TABLE I - MEAN APOPTOTIC CELL NUMBER VALUES OF 15 DIFFERENT FIELDS OF VIEW OF EACH CORNEAL LAYER IN CORNEAS WITH FUCHS' DYSTROPHY AND PSEUDOPHAKIC BULLOUS KERATOPATHY

Corneal layer	Fuchs' dystrophy (n = 14)	Pseudophakic bullous keratopathy (n = 7)	Normal corneas (n = 4)
Epithelium	0.5±0.6 (0–2.7) p=0.01	0.3±0.6 (0–1.8) p=0.07	0
Stroma	0.1±0.3 (0–0.9) p<0.01	0.2±0.2 (0–0.6) p=0.01	0
Endothelium	0.3±0.4 (0–1.3) p=0.01	0.1±0.1 (0–0.3) p=0.07	0

Values are mean±SD (minimum–maximum); p values compared to normal human corneas

has been proved to be the mode of cell death in keratoconus (14) and in human corneal allograft rejection (15).

The interleukin-1 cytokine is known to be the master regulator of the corneal wound healing response, which activates apoptosis of keratocytes and corneal fibroblasts via the FAS ligand system in case of epithelial or stromal injury (9, 10). A recent study has shown that mRNA for interleukin-1 alpha is abundant in corneas from patients with pseudophakic bullous keratopathy compared to corneas with Fuchs' dystrophy (16). However, no data support increase in keratocyte apoptosis in corneas with pseudophakic bullous keratopathy.

In the present study all the corneas displayed the classical pathologic features of Fuchs' dystrophy, pseudophakic bullous keratopathy, or normal human corneas.

Compared to the controls there was a statistically significant difference in the mean (normalized) apoptotic cell numbers for all three layers ($p=0.01$ in each case) in the Fuchs' dystrophy corneas, and for the stromal layer ($p<0.01$) in pseudophakic bullous keratopathy corneas on cross section. The apoptotic cell numbers for the epithelial and endothelial layers of the latter were higher, but the difference was not statistically significant ($p=0.07$, 0.07) (Tab. I).

Conventional methods used in previous studies did not provide clear evidence of apoptotic endothelial cells in Fuchs' dystrophy (2). Therefore, Borderie et al examined apoptosis of endothelial cells on flat mounts of the cornea, to avoid the loss of endothelial cells at cross-sectional preparation (11). We suggest that although cross sectional preparation results in endothelial cell loss, equal proportion of endothelial cells were lost in the Fuchs' dystrophy, pseudophakic bullous keratopathy, and control groups and therefore the endothelial cell layer was comparable in each group. We suggest that apoptosis may play a role in cell death in Fuchs' dystrophy and in keratocyte death in pseudophakic bullous keratopathy. However, the density of apoptotic keratocytes may also increase secondary to stromal edema and swelling in both pathologies.

The recent establishment of the molecular genetic background of corneal dystrophies has led to better understanding of the pathogenesis and to revision of the classification of inherited corneal diseases (17-19). Fuchs' dystrophy appears to have autosomal dominant inheritance. Recently missense mutations in the gene encoding the ?2 chain of type VIII collagen have been found in such patients (20), but the exact genetic background and all probable chromosome disorders or gene mutations have not yet been identified. We do not know whether there are alterations of the genetic program encoding the apoptotic activity of keratocytes of patients with pseudophakic bullous keratopathy.

We conclude that epithelial cell, keratocyte, and endothelial cell apoptosis may play role in the pathogenesis of Fuchs' dystrophy and in keratocyte death in corneas with pseudophakic bullous keratopathy. The molecular genetic background and the detailed regulatory mechanisms of both corneal pathologies remain to be clarified.

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