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P53, CD95, cathepsin and survivin pathways in Fuchs' dystrophy and pseudophakic bullous keratopathy corneas

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Summary. Our purpose was to elucidate the pathways of apoptosis of corneas with Fuchs' dystrophy and pseudophakic bullous keratopathy. Sixteen corneal buttons (14 patients, median age 73 years) with Fuchs' dystrophy, 13 with pseudophakic bullous keratopathy (PBK) (13 patients, median age 69 years) and 8 buttons (8 patients, median age 59 years) from enucleated eyes with chorioideal melanoma (controls) were analysed histologically. Immunohistochemical analysis was performed to investigate the expression of p21, p27, p63, survivin, CD95, cathepsin, bax, bcl-2 and Ki67.

Positive immunohistochemical reactions were detected in epithelial cells of the corneas, but keratocytes and endothelial cells were not positive in any of the groups or stainings. The number of p27 and survivin positive epithelial cells was significantly lower (p=0.048 and 0.041) and the number of cathepsin positive epithelial cells was significantly higher (p=0.004) in Fuchs' dystrophy corneas compared to controls. In pseudophakic bullous keratopathy, p21 and p27 positive epithelial cells were present in a significantly lower (p=0.02 and 0.005) number than in controls.

We conclude that genetically programmed cell death is related to the p27, cathepsin and survivin pathways in Fuchs' dystrophy and to the p21 and p27 pathways in pseudophakic bullous keratopathy.

Key words: Apoptosis, Fuchs' dystrophy, Pseudophakic bullous keratopathy, Survivin, Cathepsin

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Introduction

Recent studies have shown that apoptosis of the corneal cells may have a role in the pathogenesis of Fuchs' endo-epithelial dystrophy (Borderie et al., 2000; Li et al., 2001) and in pseudophakic bullous keratopathy (Szentmáry et al., 2005). In our recent work we have found a significantly increased number of apoptotic epithelial cells, keratocytes and endothelial cells in Fuchs' dystrophy corneas, and a significantly increased number of apoptotic keratocytes in pseudophakic bullous keratopathy corneas compared to normal human controls (Szentmáry et al., 2005).

According to our present knowledge, various pathways may be triggered and result in genetically programmed death of the cells, such as the CD95 dependent (FAS), p53 and cathepsin ways (Barazzone et al., 1998; Gansauge et al., 1998). Detection of these pathways may facilitate the pharmacological control of stromal-epithelial interactions with different corneal diseases, and may offer the potential to treat corneas of patients with Fuchs' dystrophy or pseudophakic bullous keratopathy.

The purpose of this study was to elucidate the pathways of genetically programmed cell death (apoptosis) of corneas with Fuchs' dystrophy and pseudophakic bullous keratopathy.

Materials and methods

The patient population comprised 16 eyes with Fuchs' dystrophy (14 patients, 6 males) and 13 eyes with pseudohakic bullous keratopathy (PBK) (13 patients, 10 males) who underwent central penetrating keratoplasty (PK) and 8 eyes (8 patients, 4 males) with choroideal melanoma following enucleation (controls). We compared 16 excised corneal buttons with Fuchs'

dystrophy and 13 corneal buttons with PBK (age at the time of surgery 73/ 69 years, range 55-88/ 38-85 years), to 8 corneal buttons of 8 patients with chorioideal melanoma (age 59 years, range 24-81 years) considered as controls.

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

All the PKs were performed at our clinic between January 2001 and December 2003. 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.

Histological changes were analysed in 4 µm thick sections using light microscopy after haematoxylin-eosin (HE) staining.

Immunoperoxidase reactions were performed to examine p27, p63, CD95, survivin, cathepsin, bax and bcl-2 expression in Fuchs's dystrophy, and to examine p21, p27, CD95, cathepsin, bcl-2 and Ki67 expression in pseudophakic bullous keratopathy. All immunoperoxidase reactions were also performed in controls.

All antibodies except survivin were products of DAKO (Carpinteria, USA) and were used in a dilution of 1:100 overnight at 37°C. Survivin was a product of Abcam (Cambridgeshire, UK), and was used in a dilution of 1:200 overnight at 37°C. Then, the sections were treated with proteinase K, and to inactivate endogenous peroxidase, incubated in methanol and H_2O_2. The secondary anti-mouse IgG (product of DAKO) was used in a dilution of 1:100 the next day. Diaminobenzidine served as chromogen and methyl green as counterstain. P21, p27, p63, survivin, CD95, cathepsin, bax, bcl-2 and Ki67 indices were established by analysis of 100 cells and given in percent.

For statistical analysis, the software package SPSS/PC version 13.0 was used. Comparisons between variables were performed using nonparametric tests (Mann-Whitney U test for unpaired samples, Wilcoxon test for paired samples). A P-value of less than 0.05 was considered statistically significant.

Results

Sample images for the different immunohistochemical reactions are shown in Figures 1-7.

Positive immunohistochemical reactions were

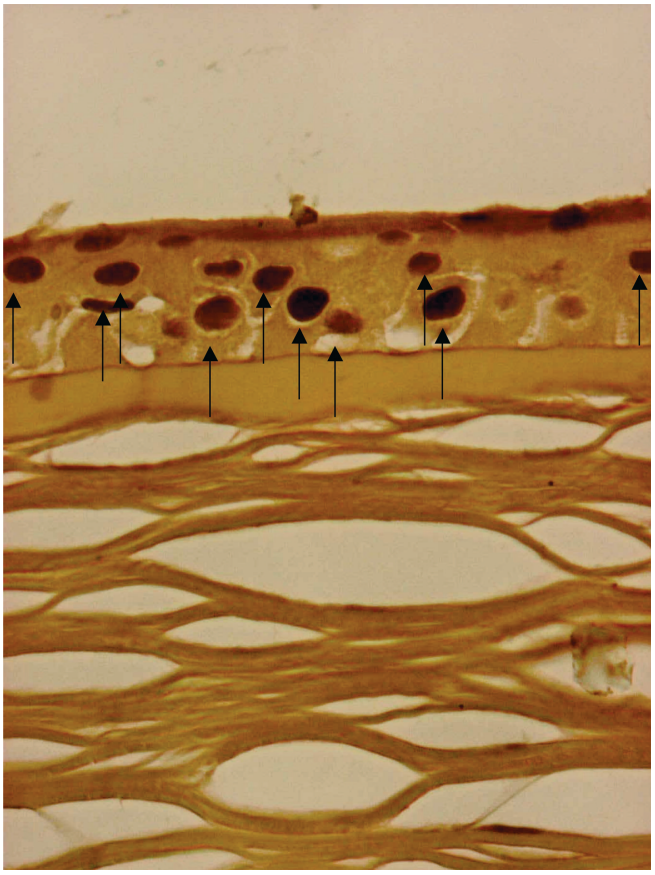

Fig. 1. The dark staining of cell nuclei indicate p63 positive epithelial cells in contol cornea. x 125

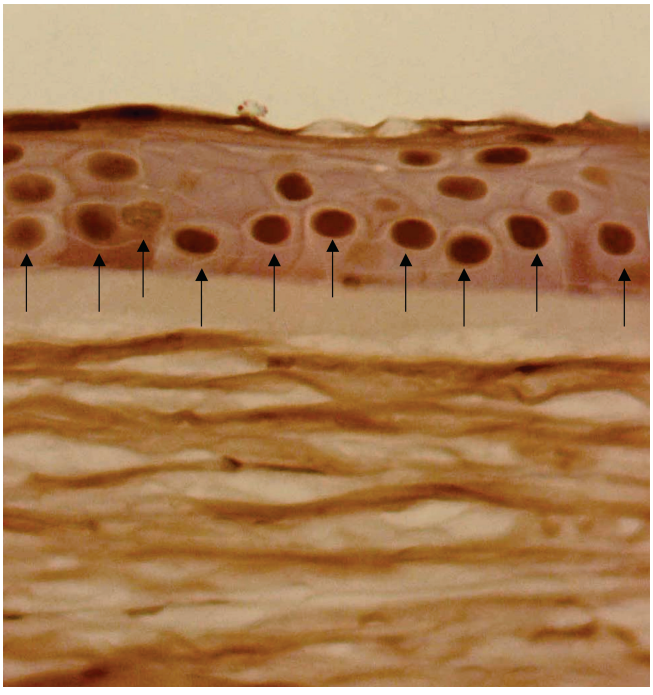

Fig. 2. The dark staining of cell nuclei indicate p27 positive epithelial cells in Fuchs' dystrophy. x 125

detected in epithelial cells of the corneas, keratocytes and endothelial cells were not positive in any of the groups or stainings.

P21, p27, p63, CD95, cathepsin, survivin, bax, bcl-2 and Ki67 indices of the epithelial cells are displayed in Table 1. The result of the statistical analysis is displayed in Table 2.

The number of p27 and survivin positive epithelial cells was significantly lower (p=0.048 and 0.041), and the number of cathepsin positive epithelial cells was significantly higher (p=0.004) in Fuchs' dystrophy corneas compared to controls. In Fuchs's dystrophy, p63 was less frequent, CD95 and bax were more frequently expressed than in controls, however, the difference did not reach statistical significance (p>0.4).

In pseudophakic bullous keratopathy, p21 and p27 positive epithelial cells were present in a significantly lower (p=0.02 and 0.005) number than in controls. The number of Ki67 positive epithelial cells was lower, the number of CD95, cathepsin and survivin positive epithelial cells was higher in PBK corneas as compared

Table 2. The numbers show the p values following statistical analysis (significant p values are displayed in bold).

	Fuchs' vs. control	PBK vs. control	Fuchs' vs. PBK
P21	-	**0.02**	-
P27	**0.048**	**0.005**	**<0.001**
P63	0.573	-	-
CD95	0.475	0.29	0.55
Cathepsin	**0.004**	0.84	**0.001**
Survivin	**0.041**	0.09	**0.001**
Bax	0.606	-	-
Bcl-2	0.617	1.0	-
Ki67	-	0.588	-

The number of positive epithelial cells in Fuchs' dystrophy and pseudophakic bullous keratopathy (PBK) corneas was compared to the number of positive cells in controls, and it was also compared in Fuchs' dystrophy and PBK (Mann-Whitney U test for unpaired samples, Wilcoxon test for paired samples).

Table 1. Number of positive epithelial cells/100 cells (median (minimum-maximum)) in Fuchs' dystrophy and pseudophakic bullous keratopathy corneas following immunohistochemical stainings.

	Fuchs' dystrophy	Bullous keratopathy	Control
P21	-	0 (0-30)	40 (10-60)
P27	53.2 (47.9-81.2)	0 (0-0)	87.5 (80-95)
P63	137 (0-62.5)	-	47.45 (24-70)
CD95	0 (0-23)	0 (10-40)	0 (0-3)
Cathepsin	100 (0-100)	80 (0-98)	77.5 (0-100)
Survivin	9 (0-51)	77.5 (50-92)	60.5 (0-72)
Bax	0 (0-21.2)	-	0 (0-0)
Bcl-2	0 (0-1)	0 (0-0)	0 (0-0)
Ki67	-	7.5 (0-40)	30.5 (1-60)

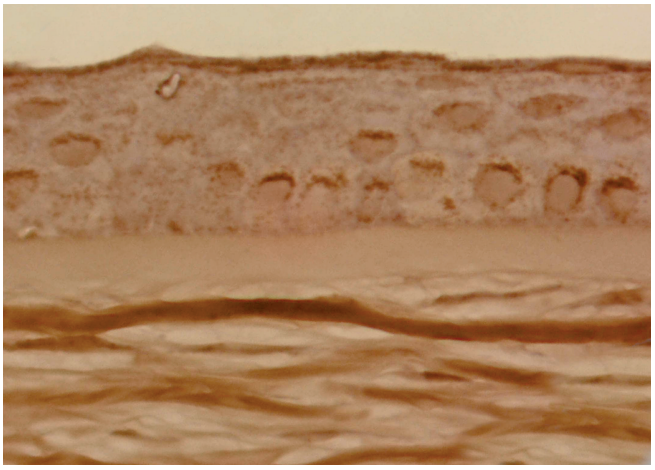

Fig. 3. Cathepsin positive epithelial cells (positive staining of cytoplasmic granules) in Fuchs' dystrophy. x 125

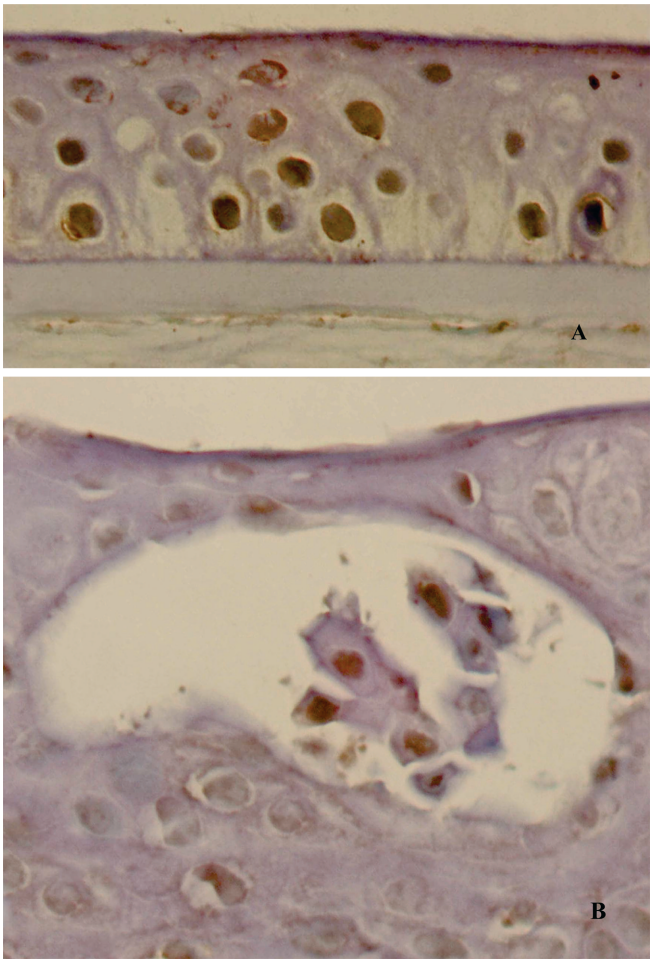

Fig. 4. The dark staining of cell nuclei indicate the presence of survivin in epithelial cells of pseudophakic bullous keratopathy corneas without **(A)** and with **(B)** severe epithelial edema. x 125

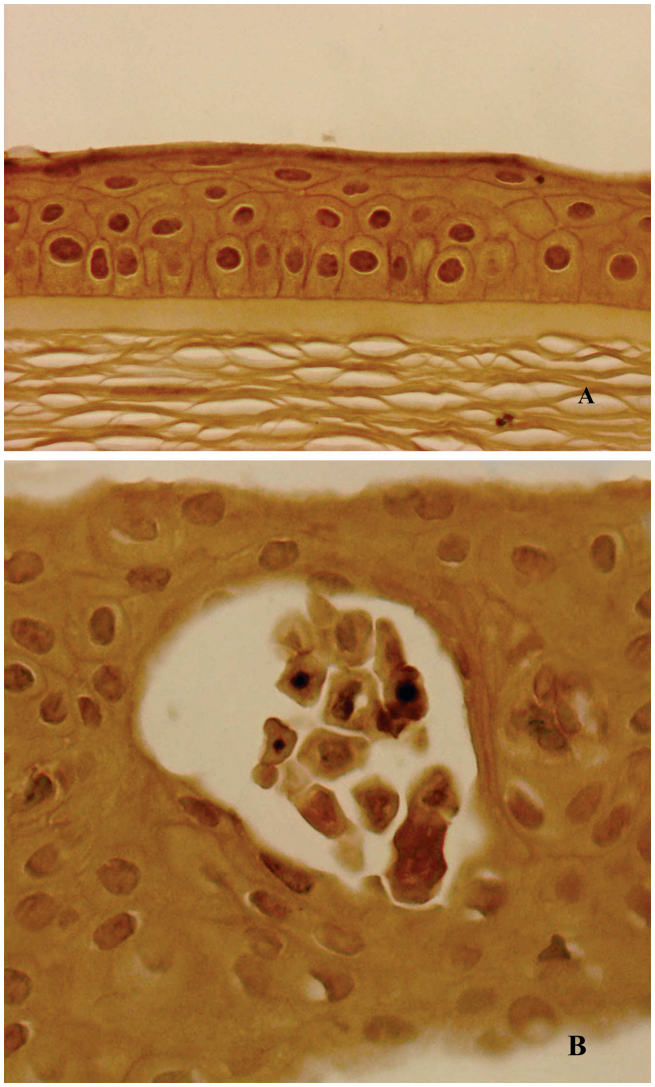

Fig. 5. The dark staining of cell nuclei indicate CD95 (FAS receptor) positive epithelial cells in pseudophakic bullous keratopathy corneas without **(A)** and with **(B)** severe epithelial edema. x 125

to controls, however the difference did not reach statistical significance ($p>0.09$).

Bcl-2 was not expressed in Fuchs' dystrophy, pseudophakic bullous keratopathy or in control corneas.

Comparing the number of positive epithelial cells in Fuchs' dystrophy and PBK, the expression of p27 and cathepsin was significantly higher ($p<0.001$, $p=0.001$), the expression of survivin was significantly lower ($p=0.001$) in Fuchs' dystrophy than in PBK.

Discussion

The most conspicuous finding of this study is the significantly increased number of cathepsin positive epithelial cells, the significantly decreased number of p27 and survivin positive epithelial cells in *Fuchs' dystrophy*, and the significantly decreased number of p21 and p27 positive epithelial cells in pseudophakic bullous keratopathy compared to control corneas.

Comparing the number of positive epithelial cells in Fuchs' dystrophy and PBK, the expression of p27 and cathepsin was significantly higher, the expression of survivin was significantly lower in Fuchs' dystrophy.

There were no changes of the p53, CD95, cathepsin and survivin pathways in stromal keratocytes and in endothelial cells in Fuchs' dystrophy and PBK corneas.

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 have been presumed to increase secondary to epithelial and stromal edema and swelling, and 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. Although none of the above theories have been proven or precluded (Borderie et al., 2000; Li et al., 2001; Szentmáry et al., 2005). The reason for the increased apoptosis of epithelial cells, keratocytes and endothelial cells in pseudophakic bullous keratopathy have not been explained in detail, yet.

In the human cornea *p63* was identified as a keratinocyte stem cell marker (Pellegrini et al., 2001). P21 and p27 are known as members of protein families which regulate the cell cycle and participate in the regulation of cell proliferation in response to wounding of the corneal epithelium. These proteins are expressed in basal cells of the central and peripheral region of cornea and limbus, their expression is not present 24 hours after epithelial scraping. P21, p27 and p63 are all members of the so called p53 family facilitating genetically programmed cell death through the mithochondrial pathway. The increase of p21, p27 and p63 triggers apoptosis of the cells (Yoshida et al., 2002; Zieske, 2000; Zieske et al., 2004).

In between the various pathways that may trigger apoptosis, *cathepsin*, which is a proteolytic enzime, acts through the caspase cascade. Cathepsin B was shown to be present in the epithelium, in stromal cells and in the endothelium of the cornea. At all locations it was found to be present in cytoplasmic granules, presumably lysosomes (Wasselius et al., 2003).

Survivin is normally active only in embryos or rapidly dividing cells, such as those of the immune system. It is thought to attach to the "mitotic spindle", a foundation in the nucleus of cells that pulls newly divided chromosomes into the two daughter cells created during cell division (Giodini et al., 2002). Survivin translocation into the nucleus is dependent on Fas stimulation and cell proliferation. Survivin is also known as a member of the inhibitors of the apoptosis gene family, it interacts in the cell cycle regulation to suppress

Fas-mediated cell death (Suzuki et al., 2000).

Our results show that genetically programmed cell death may be related to the increased expression of cathepsin in Fuchs' dystrophy, and therefore, the apoptotis of the cells is mediated through the caspase cascade. As a consequence, the expression of survivin and p27 genes significantly decreases in Fuchs' dystrophy: survivin and p27 may interact in the cell cycle regulation to suppress cathepsin-mediated cell death in the disease.

The decrease of p27 expression in both Fuchs' dystrophy and PBK corneas, and the decrease of p21 expression in PBK corneas shows that the activity of the p53 pathway is decreased in both corneal diseases. Nevertheless, the fact that p27 expression was significantly higher, and the expression of survivin was significantly lower in Fuchs' dystrophy compared to PBK, shows that the increased apoptotic activity of the cells in Fuchs' dystrophy is somewhat compensated by the decrease of survivin, and the increased apoptotic activity of the cells in PBK is somewhat compensated by the decreased activity of the p53 pathway of the cells.

Fas (CD95), *Fas ligand*, *bax*, *bcl-2* are expressed in all three major cell types of the cornea and could have important functions in normal corneal physiology and in the pathophysiology of corneal disease (Wilson et al., 1996). The receptor of the Fas molecule, which is also called by the name CD95, is a death receptor, activation of which results in apoptosis of the cell (Barazzone et al., 1998).

Bcl-2 is an antiapoptotic protein. It can be localized to the nuclei and nuclear envelope of corneal epithelial cells, keratocytes and endothelial cells, and may play a critical role in modulating apoptotic cell desquamation in the human corneal epithelium (Yamamoto et al., 1996).

Bax is a proapoptotic protein which participates in the mitochondrial pathway. 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 compared to controls. In the same study, following exposure of Fuchs' dystrophy corneas to camptothecin, an apoptotic inducer, keratocytes responded with an increased level of bax and low level of bcl-2. This trend was distinctly different from the response of normal keratocytes (Li et al., 2001).

In our study, CD95 was detected more frequently in Fuchs' dytsrophy and PBK corneas compared to controls, but without significant increase. The same was true for the expression of bax in Fuchs' dystrophy. The numer of bcl-2 positive cells remained unchanged in both corneal pathologies. Related to these three proteins, we may say that the pathways related to CD95, bax and bcl-2 do not determine the increased apoptotic death of the cells in Fuchs' dystrophy and PBK.

The proliferation of the cells was also not significantly increased in PBK, detected by Ki67 as a proliferation marker, so the increased apoptotic death of the cells does not induce a subsequent increased proliferation as a compensatory mechanism in pseudophakic bullous keratopathy.

A previous study has shown that in corneal epithelial cells, osmotic stress increases the expression of bax and caspase-3 and suppresses the expression of bcl-2 (Luo et al., 2007). In the same study, the relationship between hyperosmolar stress and the induction of apoptosis in the ocular surface epithelium has been proven.

Both in Fuchs' dystrophy and PBK, osmotic stress of the cells in all layers of the cornea is present due to the decreased function of the endothelial cells. Similar to the above study, we were able to prove increased expression of bax in Fuchs' dystrophy and the caspase cascade in both Fuchs' dystrophy and PBK, and no expression of bcl-2 in Fuchs' dystrophy and in PBK. We can add to the above study the fact that significantly increased expression of the caspase cascade could only be proven in Fuchs' dystrophy, but the activity of the bax and bcl-2 expression was not significantly changed related to osmotic stress.

Recent studies on the molecular genetic background of corneal dystrophies have led to better understanding of the pathogenesis, and to revision of the classification of inherited corneal diseases (Dighiero et al., 2001; Auw-Hedrich and Witschel, 2002; Niel et al., 2003). Fuchs' dystrophy appears to have autosomal dominant inheritance. Recently, missence mutations in the gene encoding the α2 chain of type VIII collagen have been found in such patients (Biswas et al., 2001), but the exact genetic background and all probable chromosome disorders or gene mutations have not yet been identified. None of the previous studies could elucidate the genetic background of the increased apoptotis of the cells in Fuchs' dystrophy.

We conclude that genetically programmed cell death is related to the p27, cathepsin and survivin pathways of epithelial cells of Fuchs' dystrophy and to the p21 and p27 pathways of epithelial cells of pseudophakic bullous keratopathy corneas. P53, CD95, cathepsin and survivin pathways are not altered in stromal keratocytes and endothelial cells in both diseases.

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