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P21, p27, bax, cathepsin and survivin pathways in macular dystrophy corneas

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Summary. The purpose of our study was to elucidate pathways of genetically programmed cell death (apoptosis) in corneas with macular dystrophy.

10 corneal buttons (10 patients) with macular dystrophy and 8 buttons (8 patients) from enucleated eyes with chorioideal melanoma (controls) were analysed histologically. Immunohistochemical analysis was performed to investigate the presence of p21, p27, bax, cathepsin and survivin proteins. The number of positive cells was determined by analysis of 100 cells and given in percentages.

The bax protein was present in 25.6% of epithelial cells in macular dystrophy corneas but was absent in controls. P21 and p27 were found in 35.7 and 87.5% of epithelial cells of macular dystrophy corneas, respectively, but again not in control tissue. In contrast, a lower percentage of cathepsin-positive (30.7% vs 58.8%) and survivin-positive cells (37.6% vs 52.1%) were present in epithelial cells of macular dystrophy corneas than in control epithelial cells. The difference reached statistical significance in the expression of p21 and p27 genes ($p<0.05$ in both).

P21 was positive in 3% of keratocytes, p27 in 1% of endothelial cells of macular dystrophy corneas but negative in controls (0%). Bax, cathepsin and survivin immunopositivity was not detected in keratocytes or endothelial cells of either group.

We conclude that the down-regulation of p21, p27 and cathepsin in epithelial cells of macular dystrophy corneas may be related to defense mechanisms against apoptotic cell death.

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Introduction

Corneal dystrophies are congenital, bilateral diseases of the cornea, in which corneal opacities -which are not of inflammatory origin- deteriorate visual acuity and progrediate with age. In macular dystrophy (MCD) the cornea is characterized by the accumulation of extracellular deposits in the stroma and Descemet's membrane, as well as by the intracellular storage of similar material in keratocytes and the corneal endothelium. Moreover, the presence of colloidal iron and alcial blue positive deposits is probably related to the several mutations in the carbohydrate 6-sulfotransferase (CHST6) gene coding corneal N-acetyl glucosamine 6-sulfotransferase, as the cause of MCD. Three subtypes of MCD (I, IA and II) have been distinguished based on the presence or absence of sulfated keratan sulfate (KS) in the patient's serum and cornea (Akama et al., 2000). Up to now the accumulation of these colloidal iron positive substances could not be explained in detail.

Our recent study has shown that apoptosis of epithelial cells and keratocytes is increased in macular dystrophy corneas (Szentmáry et al., 2007).

According to our present knowledge various pathways may be triggered and result in genetically programmed death of 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 appear to offer the potential to treat corneas of patients with macular dystrophy at molecular level.

In the human cornea, *p21* and *p27* are known as members of the so called p53 family, facilitating genetically programmed cell death through the mithochondrial pathway. The increase of p21 and p27 triggers apoptosis of the cells (Zieske, 2000; Yoshida et al., 2002; Zieske et al., 2004). These proteins regulate the cell cycle and participate in the regulation of cell proliferation in response to wounding of the corneal epithelium.

Bax is a proapoptotic protein which also participates in the mitochondrial pathway (Wilson et al., 1996). *Cathepsin*, which is a proteolytic enzyme, acts through the caspase cascade. Cathepsin B was found to be present in cytoplasmic granules, presumably lysosomes (Wasselius et al., 2003). Cathepsin V was found to have the features of cystine proteinase (Adachi et al., 1998). Survivin interacts in the cell cycle regulation to suppress Fas-mediated cell death (Suzuki et al., 2000; Giodini et al., 2002). Survivin translocation into the nucleus is dependent on Fas stimulation and also cell proliferation.

The purpose of this study was to elucidate the pathways of genetically programmed cell death (apoptosis) of corneas with macular dystrophy.

Materials and methods

The patient population comprised 10 eyes with macular dystrophy (10 patients, 70% males; age at the time of surgery 48 ± 18 years, range 24-69 years), who underwent central penetrating keratoplasty (PK) and 8 eyes (8 patients, 50% males) with choroideal melanoma following enucleation (controls; age 56 ± 16 years, range 24-81 years). The subtype of the macular dystrophy patients (study group) could not be determined for this restrospective study, as most of the patients were not available for genetical analysis. The control group (normal human corneas) was derived from enucleated eyes with melanoma malignum, in which the tumor was located at the posterior pole of the globe only.

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. No informed consent was obtained from patients enrolled in this retrospective study.

All the PKs were performed at our clinic between January 1992 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 p21, p27, survivin, cathepsin and bax positivity in macular dystrophy. All immunoperoxidase reactions were also performed in controls.

The sections were treated with proteinase K, and to inactivate endogenous peroxidase incubated in methanol and H_2O_2. All primary antibodies except survivin were products of DAKO (Carpinteria, USA) and were used at a dilution of 1:100 overnight at 37°C. Survivin was a product of Abcam (Cambridgeshire, UK), and was used at a dilution of 1:200 overnight at 37°C. The secondary anti-mouse IgG (product of DAKO) was used at a dilution of 1:100 the next day. Diaminobenzidine served as chromogen and methyl green as counterstain. P21, p27, survivin, cathepsin and bax indices were established by analysis of 100 cells and given in percentages at the epithelium and stroma (100 cells were assessed in each corneal layer). Due to the low number of endothelial cells in each sample, in the case of positivity of endothelial cells, the total number of positive endothelial cells was divided by the total number of endothelial cells in all samples, and then multiplied by 100.

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 1-3.

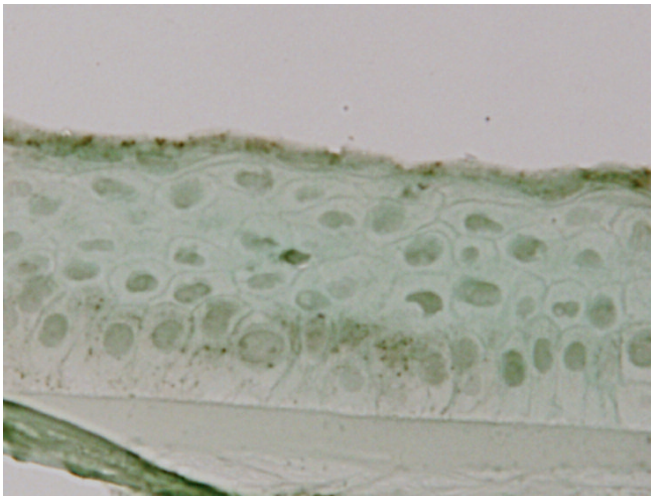

Fig. 1. Cathepsin positive epithelial cells in macular dystrophy (positive staining of intracytoplasmic granules). x 60

Table 1. Number of positive epithelial cells/100 cells (mean ± SD; minimum-maximum) following various immunohistochemical stainings.

	Control	Macular dystrophy	P value
P21	35.7±0.7 (10-60)	0±0 (0-0)	<0.05
P27	87.5±10.6 (80-95)	0±0 (0-0)	<0.05
Cathepsin	58.8±46.8 (0-100)	30.7±28.9 (0-60)	0.141
Survivin	52.1±27.4 (0-72)	37.6±31.9 (0-95)	0.228
Bax	0±0 (0-0)	25.6±35.0 (0-65)	0.180

P value indicates the comparison of positive cells in control and in macular dystrophy corneas following Mann-Whitney test.

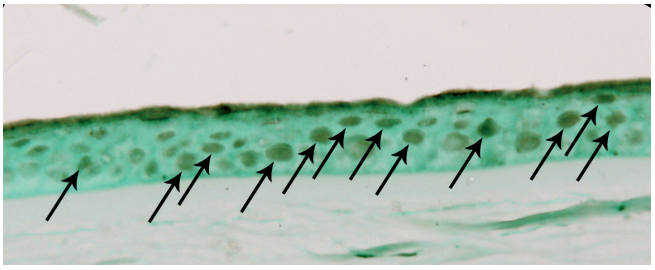

Fig. 2. Survivin positive epithelial cells (dark staining of cell nuclei) in control cornea (arrows). x 60

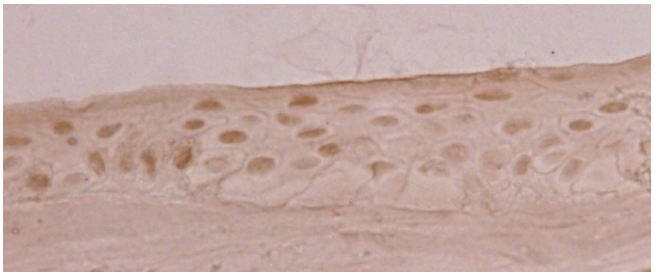

Fig. 3. Bax negative epithelial cells (no staining of cytoplasm) in macular dystrophy cornea. x 60

Immunohistochemical reactions showed various degrees of p21, p27, cathepsin, survivin and bax positivity in epithelial cells of control, as well as macular dystrophy corneas.

P21, p27, cathepsin, survivin and bax indices of the epithelial cells and the results of the statistical analysis are displayed in table 1. The number of p21 and p27 positive epithelial cells was significantly lower in macular dystrophy corneas compared to controls ($p<0.05$ in both). The number of cathepsin and survivin positive epithelial cells was lower, the number of bax positive epithelial cells was higher in macular dystrophy corneas as compared to controls, but the difference did not reach statistical significance ($p>0.141$).

P21 was positive in 3% of keratocytes of macular dystrophy corneas but absent in controls, p27 was positive in 1% of endothelial cells of macular dystrophy corneas but absent in controls. For cathepsin, survivin and bax neither keratocytes nor endothelial cells were positive in controls or macular dystrophy corneas.

Discussion

The most conspicious finding of this study is the total loss of p21 and p27 positive epithelial cells in macular dystrophy corneas compared to controls. Besides, the number of cathepsin and survivin positive epithelial cells was lower and the number of bax positive epithelial cells was higher, as compared to normal controls, without reaching statistical significance.

P21, p27, cathepsin and bax proteins trigger apoptosis. In contrast, survivin is known as an inhibitor of the apoptosis gene family. Taking into account that in our previous study (Szentmáry et al., 2007) we found apoptosis of a limited number of epithelial cells in some macular dystrophy corneas (15%), we may presume that the decreased presence of p21, p27 and cathepsin proteins is in part compensating the increased presence of bax and decreased presence of survivin (antiapoptotic protein) proteins of epithelial cells of these patients. The down-regulation of pro-apoptotic genes (p21, p27, cathepsin) points to the strong capacity of epithelial cells to protect themselves against apoptotic cell death.

We were also able to detect a very low number of p21 positive keratocytes and p27 positive endothelial cells in macular dystrophy corneas, but not in controls. As in our previous study (Szentmáry et al., 2007), we found a statistically significant increase in apoptotic keratocyte number in patients with macular dystrophy, and as in some cases apoptosis of endothelial cells (15%) was detected, we may suppose that the increased apoptotic activity may also be triggered by the increased presence of p21 in keratocytes and by the increased presence of p27 in endothelial cells. In addition, it seems that keratocytes fail to possess molecular defense mechanisms against factors, not determined in this study, triggering apoptosis.

In summary, in epithelial cells of macular dystrophy corneas, which do not accumulate intracellular acid mucopolysaccharides, we were able to prove that cells possess a strong capacity to protect themselves against apoptotic cell death.

In contrast, in stromal keratocytes and endothelial cells, which do accumulate acid mucopolysaccharides intracellularly in this stromal dystrophy, there is a lack of molecular defense mechanisms against apoptosis.

We conclude that the down-regulation of p21, p27 and cathepsin in epithelial cells of macular dystrophy corneas may be related to defense mechanisms against apoptotic cell death.

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