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Cell proliferation and apoptosis in stromal corneal dystrophies

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Summary. The aim of our study was to evaluate corneal cell proliferation and apoptosis in cases of granular, macular and lattice dystrophy, and to provide evidence which may help to clarify whether apoptosis is a pathogenic factor in any of these dystrophies. The study group comprised 39 eyes (from 33 patients) which had undergone penetrating keratoplasty (PK) for stromal dystrophies: these comprised 12 eyes (from 9 patients, 55.5% males) with granular dystrophy, 13 eyes (12 patients, 33.3% males) with macular dystrophy, and 14 eyes (13 patients, 61.5% males) with lattice type I dystrophy. A further 4 corneal buttons from enucleated eyes of 4 patients with choroideal melanoma served as controls. Immunocytochemical analysis of Ki67 (DNAcon Kit, DakoCytomation A/S, Glostrup, Denmark) was used for evaluation of cell proliferation. Apoptosis was detected by use of the TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labelling) assay method (Apoptag reagent, Q-Biogene, Strasbourg, France). Statistical comparisons were made using the Mann-Whitney test.

No Ki67-positive cells were detected in the study-group or control corneas. In control corneas no apoptotic activity was found. In the study group the mean (normalised) apoptotic keratocyte number was 1.1±1.7 in granular dystrophy and 0.5±1.1 in lattice type I dystrophy ($p = 0.36$, 0.63 respectively). Compared to the controls, the difference was statistically significant only for macular dystrophy (1.6±1.2; $p = 0.01$).

Keratocyte apoptosis seems to be a concomitant or pathogenic factor in macular dystrophy. However, the pathways that are triggered to result in increased apoptotic cell death remain to be clarified.

Key words: Granular dystrophy, Macular dystrophy, Lattice dystrophy, Apoptosis, Proliferation

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Introduction

Corneal dystrophies are characterised by corneal opacities which are most often bilateral and progressive, occur after birth, and are not inflammatory. In stromal dystrophies, such as granular, macular and different types of lattice dystrophies, metabolically generated and deposited material causes dysfunction, sometimes with a severe loss of visual acuity (Rodrigues and Krachmer, 1988; Seitz et al., 2004). The disorders were extensively investigated and described by histological, immunohistochemical and even electron microscopical methods in the past century. Interest has been extremely focused again on this field in the past few years by the possible subclinical detection of the disease even in relatives of patients with molecular genetical analysis (Takács et al., 1998; Dighiero et al., 2001; Auw-Hadrich and Witschel 2002; Klinworth, 2003; Niel et al., 2003).

Clinically, the subepithelial corneal deposits also cause irregularity and/or lack of corneal epithelium which results in pain. As the patients suffer from severe loss of visual acuity by the age of 40 to 60, corneal transplantation is usually required. Recently, phototherapeutic keratectomy (PTK) has also been introduced, and has proved to be an intervention which is effective in the improvement of visual acuity for a significant period of time in these patients (Campos et al., 1993; Orndahl and Fagerholm, 1998; Seitz et al., 2002).

The postoperative wound-healing processes and their regulatory mechanisms either in normal or in corneal dystrophy corneas are not known in detail. Introducing apoptosis research in corneal pathology helps to understand the pathogenesis of such disorders. Recent investigations have disclosed TUNEL-positive keratocytes in different types of corneal dystrophies such as keratoconus (Kim et al., 1999) and Fuchs' dystrophy (Borderie et al., 2000; Li et al., 2001), and have emphasised the role of apoptotic processes in the pathogenesis of such conditions.

However, the apoptotic cell processes have not been investigated in corneal stromal dystrophies.

The purpose of our present study was to investigate the proliferative and apoptotic activity in cells of corneas with granular, macular and lattice dystrophy, and to provide evidence which may help to clarify whether apoptosis is a pathogenic factor in any of these dystrophies.

Materials and methods

The study group comprised 39 eyes (from 33 patients) which had undergone penetrating keratoplasty (PK) for stromal dystrophies. The group comprised 12 eyes (from 9 patients, 5 (55.5%) males) with granular dystrophy, 13 eyes (12 patients, 4 (33.3%) males) with macular dystrophy, and 14 eyes (13 patients, 8 males) with lattice type I dystrophy. In the lattice dystrophy group there were two sets of patients with family relationships: 2 eyes were those of 2 brothers; and a further 3 eyes were of 2 brothers from a second family. For the patients with granular and macular dystrophy, no family relationships were known. A further 4 corneal buttons from enucleated eyes of 4 patients (2 (50%) males) with choroideal melanoma served as controls.

The PKs were performed at the Department of Ophthalmology of Semmelweis University, Budapest and at the Department of Ophthalmology of Medical and Health Science Center of Debrecen University, between January 1981 and December 2003. The enucleations were performed at the Department of Ophthalmology, Semmelweis University.

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 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 from the enucleated eyes. All corneal buttons were processed through graded alcohol and finally embedded in paraffin wax.

Histological analysis was performed on 4 μm thick sections. The types of different dystrophies were determined with the help of trichrome, colloidal iron and congo red (CR) histochemical reactions. For general histological examination HE (haematoxilin-eosin) and PAS (periodic acid Schiff) stainings were used. For each section, the minimum and maximum thickness of the epithelium, and the diameters parallel and perpendicular to the surface epithelium of stromal deposits in 3 locations (in the subepithelial, central and posterior stromal zones), were measured at 160x magnification.

Immunocytochemical analysis of Ki67 (DNAcon Kit, DakoCytomation A/S, Glostrup, Denmark) was used for evaluation of cell proliferation. Apoptosis was detected on separate samples by use of the TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labelling) assay method (Apoptag reagent, Q-Biogene, Strasbourg, France), as previously described and applied in human tumours (Szende et al., 2001; Farczadi et al., 2002) and in animal models (Toronyi et al., 2002).

The number of TUNEL and Ki67-positive cells was determined in the epithelial, stromal and endothelial layers of each corneal button. To achieve a normalised figure for comparison purposes the total number of cells in five non-overlapping, full-thickness columns extending from the anterior epithelial surface to the endothelial cell layer were manually counted for each layer of each specimen. The width of each column was defined by the microscope field at 125x magnification (Fig. 2b). Identification of Ki67- and TUNEL-positive cells was confirmed by optical examination at 300x magnification. Apoptosis may however also occur as a consequence of injury to the peripheral epithelium at the time of trephination; in a recent study, Kim et al. (1999) determined how close to the full-thickness trephine cut such apoptosis could be detected. In accordance with their recommendations, our observations on the corneal dystrophy and normal corneas were made at least 500 mm from the edge of the corneal buttons. For each specimen, the mean proliferative and apoptotic cell number values of 5 different fields of each corneal layer were calculated. If any Ki67- or TUNEL-stained cells were detected within the cornea of a single section, the cornea was counted as positive for that factor.

For statistical analysis, the software package SPSS (version 10.0 for Windows) was used. Only one eye of each patient was used at random in each group for statistical analysis. 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.

We also examined whether there may be a relationship between the number of apoptotic cells and the dimensions of the stromal deposits in each group of patients. As a first step, for each cross-section sample the total corneal area covered by such deposits was calculated. For each deposit (usually of irregular shape), the area was approximated as the product of the two extreme dimensions (parallel and perpendicular to the surface epithelium), and the areas for all deposits in the sample were summed. Next, for each dystrophy (granular, macular and lattice dystrophy patients), three subgroups (reflecting the severity of the occlusion) were formed according to the total area of deposits of the examined corneal section. For statistical analysis of this data the S-Plus 2000 software package was used; comparisons between groups were performed using the Poisson regression with overdispersion model (Agresti 2002a). The parameters of the model were estimated with the quasi-likelihood method (Agresti, 2002b). A p-value less than 0.05 was considered to indicate statistical significance.

Results

Sample images of corneas with granular, macular

and lattice dystrophy are shown in Figures 1, 2, and 3. Histological findings, including quantitative measures, for the corneal buttons of the different dystrophies are summarised in Tables 1-3.

No Ki67-positive cells were detected in any of the study and control group corneas. In normal corneal buttons no apoptotic activity was found in epithelial cells, keratocytes or in endothelial cells. For the study-group corneas, apoptotic activity was detected as described below.

In epithelial cells: apoptotic activity was found in 2 macular-dystrophy corneas (15%; eyes nos. 4 and 10; two TUNEL-positive cells in each corneal button). In granular- and lattice-dystrophy corneas, no apoptotic epithelial cells were found.

In stromal keratocytes: apoptotic activity was

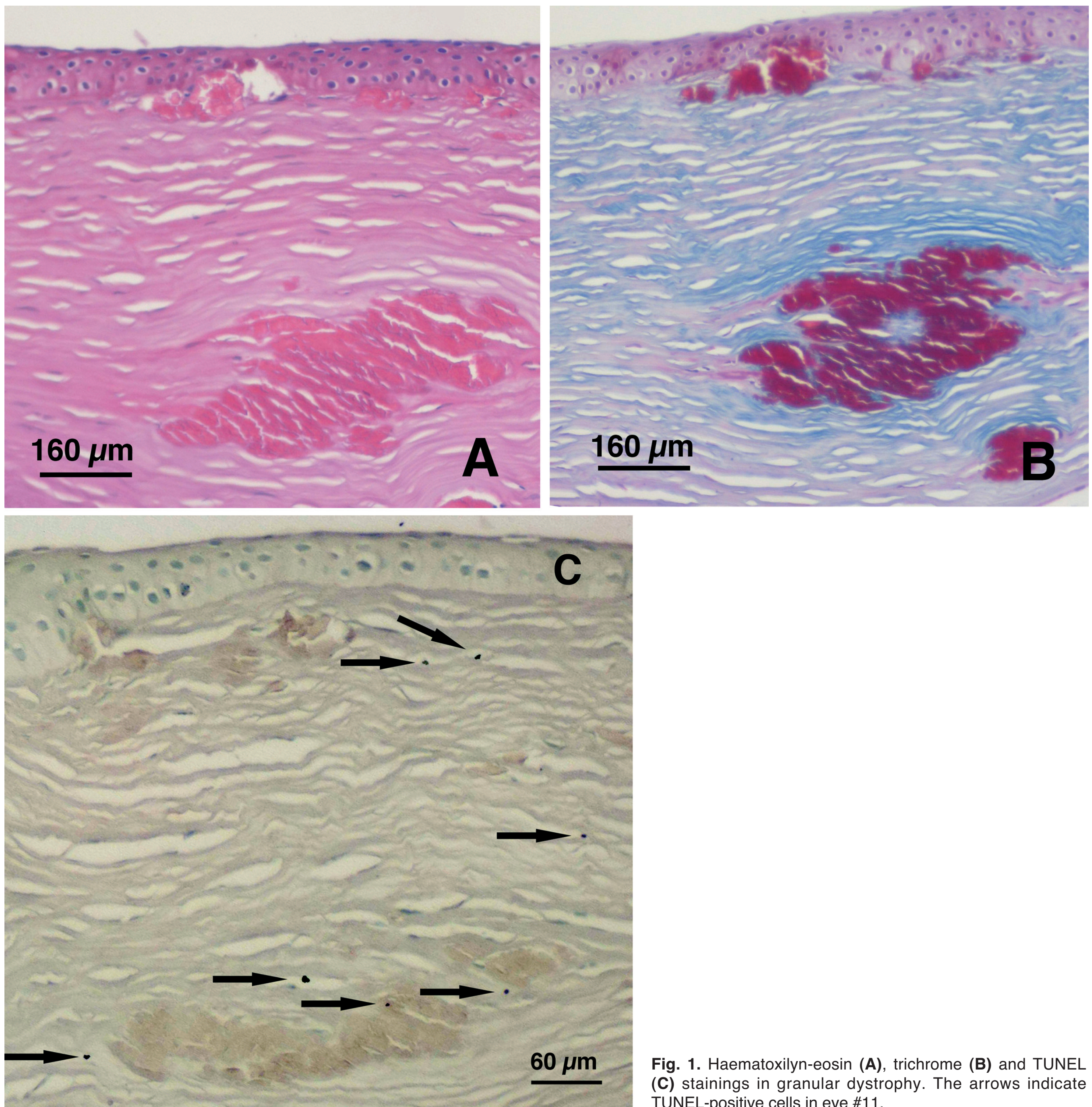

Fig. 1. Haematoxilyn-eosin **(A)**, trichrome **(B)** and TUNEL **(C)** stainings in granular dystrophy. The arrows indicate TUNEL-positive cells in eye #11.

detected by the TUNEL assay in 4 granular-dystrophy corneas (33%), in 10 macular-dystrophy corneas (77%) and in 2 lattice-dystrophy corneas (14%). In granular and lattice type I dystrophy the TUNEL positive keratocytes were detected mostly in the upper third of the corneal stroma. The mean apoptotic keratocyte numbers in each group, as determined by the TUNEL assay, are shown in Table 4. For the corneas with macular dystrophy, there was a statistically significant difference (p = 0.01) in the mean (normalised) apoptotic keratocyte numbers as compared to normal human corneas. The mean apoptotic keratocyte numbers in corneas with granular and lattice dystrophy were in each case higher compared to the control group, but statistical

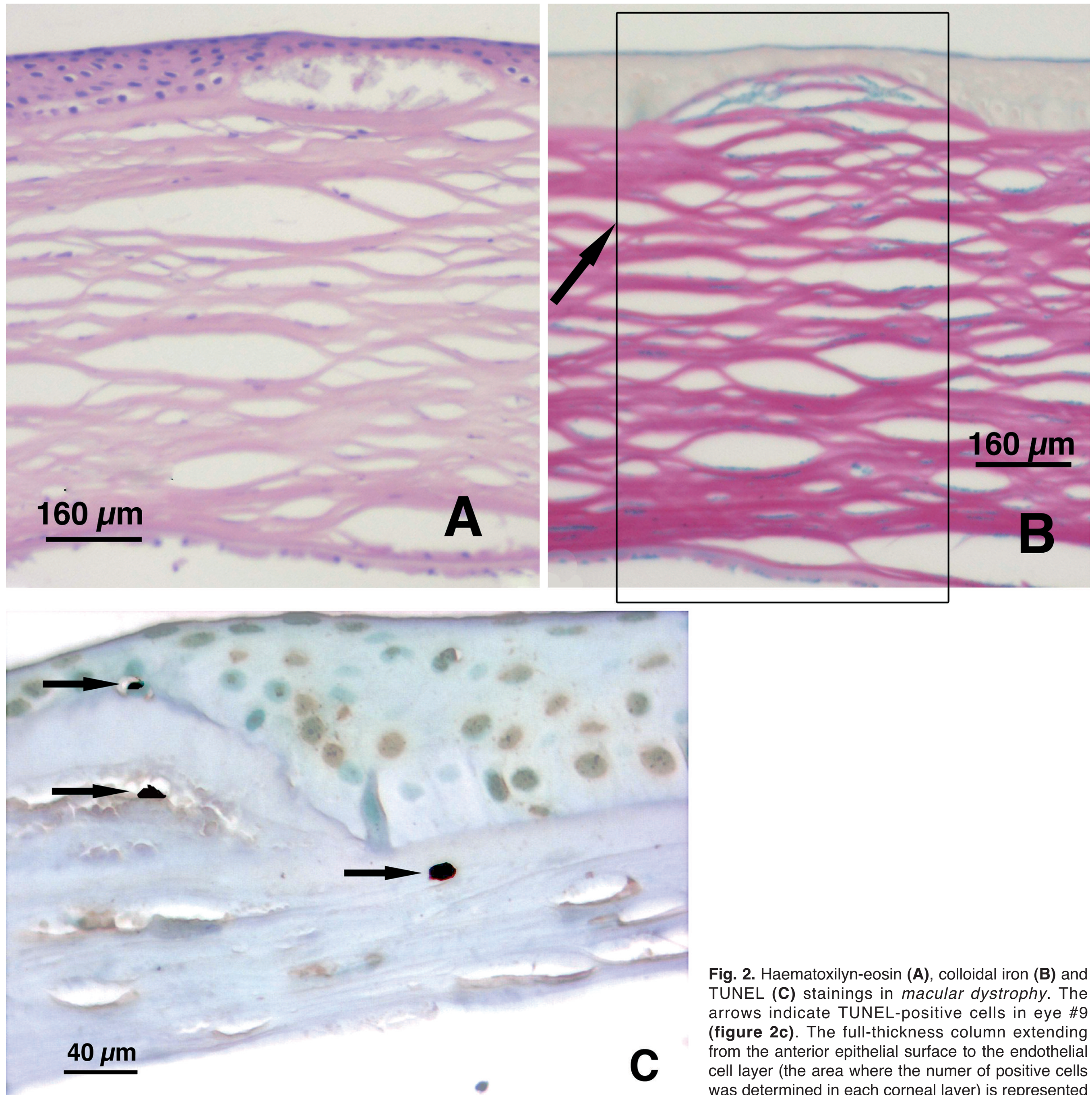

Fig. 2. Haematoxilyn-eosin **(A)**, colloidal iron **(B)** and TUNEL **(C)** stainings in *macular dystrophy*. The arrows indicate TUNEL-positive cells in eye #9 **(figure 2c)**. The full-thickness column extending from the anterior epithelial surface to the endothelial cell layer (the area where the numer of positive cells was determined in each corneal layer) is represented on **figure 2b** (arrow).

significance for the differences was not demonstrable (p = 0.36, 0.63 respectively).

In endothelial cells, apoptotic activity could be detected in 1 cornea with granular dystrophy (8%; eye no. 4) and in 2 corneas with macular dystrophy (15%; eyes nos. 5 and 13; one TUNEL-positive cell in each corneal button). In the lattice-dystrophy corneas no apoptotic endothelial cells could be shown.

In granular and lattice type I dystrophy, in the majority of corneas with TUNEL-positive cells, the epithelium showed more irregularity of thickness and/or the subepithelial and stromal corneal deposits were greater in size and number than for the contralateral eye (Tables 1, 3). Nevertheless, with the Poisson regression with overdispersion model, in the *granular dystrophy* group a relation between deposit size and apoptotic cell number could not be shown (p = 0.06). However by removal of the "outlier" eye no. 6, the p value indicated high statistical significance (p = 0.006).

In eyes with *lattice corneal dystrophy*, using the

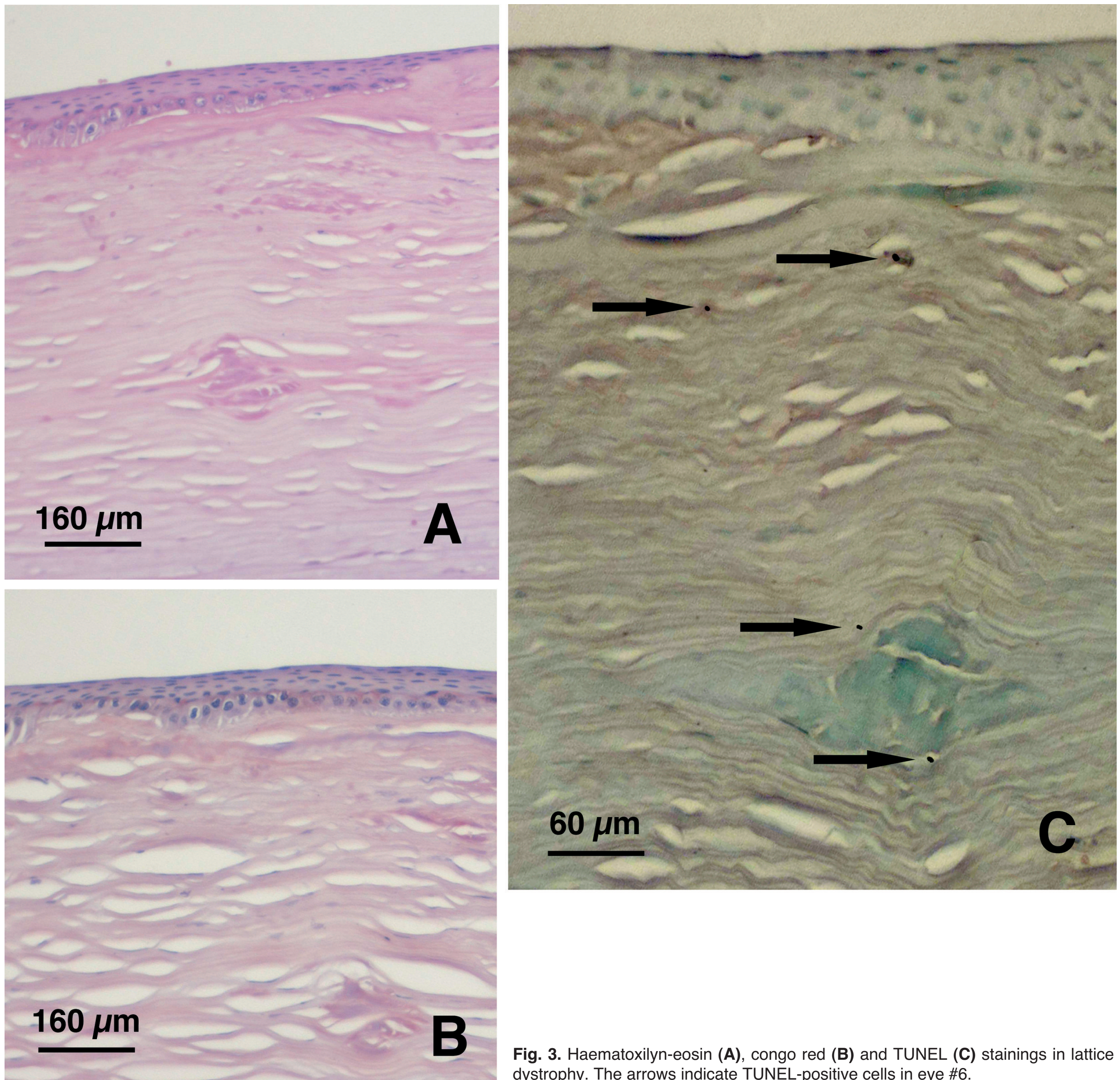

Fig. 3. Haematoxilyn-eosin **(A)**, congo red **(B)** and TUNEL **(C)** stainings in lattice dystrophy. The arrows indicate TUNEL-positive cells in eye #6.

same statistical model the above correlation of deposit size and apoptotic cell number could not be shown (p = 0.14).

In eyes with *macular dystrophy* we did not find any relationship between the increased number of apoptotic keratocytes and the size of the stromal deposits (Table 2). In eyes nos. 1 and 4 in this dystrophy group the apoptotic activity of the subsequently-operated eye was lower, while at the same time the epithelium was thinner and the size and number of subepithelial and stromal corneal deposits was greater. The Poisson regression with overdispersion model failed to indicate statistical significance (p = 0.59).

Discussion

The most conspicuous finding of this study was the presence of apoptotic keratocytes in stromal corneal

Table 1. Corneal buttons with granular dystrophy. Epithelial thickness, details of stromal deposits (no. of deposits of similar size shown in parenthesis), and mean apoptotic keratocyte numbers (per field of view).

Eyes with granular dystrophy	Year of PK	Epithelial thickness (min.-max.; mm)	Details of stromal deposits (Dimensions parallel and perpendicular to surface epithelium; mm)(no. of deposits)			Apoptotic keratocyte number per field of view (mean ± SD; n = 11)
			Subepithelial stroma	Central stroma	Posterior stroma	
1	1992	7-27	Continuous x 27-53	120 x 67; 53 x 67	-	3.2±2.5
2	1983	7-33	Continuous x 13	-	-	0.2±0.4
3	2001	14-20	333 x 67	-	-	0.0±0.0
4	1999	14-47	333 x 53; 67 x 133 (2); 133 x 33	-	-	1.8±0.8
5	1981	20-27	1333 x 27	-	-	0.0±0.0
6	1981	20-27	Continuous x 13	100 x 67 (2); 67 x 67 (3)	-	0.0±0.0
7	1983	20-33	67 x 133 (6)	67 x 133 (8)	67 x 133 (4)	0.0±0.0
8	1981	20-33	Spots	Spots	-	*
9	1981	20-47	1200 x 67; 1333 x 67	-	-	0.0±0.0
10	1992	27-47	133 x 67	267 x 13; 67 x 13 (5)	133 x 67	0.0±0.0
11	1992	27-47	67 x 133 (5)	200 x 167 (3)	-	4.2±3.4
12	1983	47-80	333 x 67	13 x 20	-	0.0±0.0

Eyes of the same patient: eyes nos. 2 and 9; nos. 7 and 12; nos. 10 and 11. PK: penetrating keratoplasty. * : No data available for this sample.

Table 2. Corneal buttons with macular dystrophy. Epithelial thickness, details of stromal deposits (no. of deposits of similar size shown in parenthesis), and mean apoptotic keratocyte numbers (per field of view).

<table>
<tr><th rowspan="2">Eyes with macular dystrophy</th><th rowspan="2">Year of PK</th><th rowspan="2">Epithelial thickness (min.-max.; μm)</th><th colspan="3">Details of stromal deposits (Dimensions parallel and perpendicular to surface epithelium; mm) (no. of deposits)</th><th rowspan="2">Apoptotic keratocyte (mean ± SD; n = 12)</th></tr>
<tr><th>Subepithelial stroma</th><th>Central stroma</th><th>Posterior stroma</th></tr>
<tr><td>1</td><td>2000</td><td>7-27</td><td>400 x 7</td><td colspan="2">Colloidal iron positivity in spots</td><td>1.8±0.8</td></tr>
<tr><td>2</td><td>2003</td><td>14-20</td><td colspan="3" rowspan="4">Colloidal iron positivity in spots</td><td>0.8±1.0</td></tr>
<tr><td>3</td><td>2001</td><td>20-33</td><td>0.0±0.0</td></tr>
<tr><td>4</td><td>1999</td><td>20-40</td><td>4.2±3.4</td></tr>
<tr><td>5</td><td>2003</td><td>20-40</td><td>3.0±1.0</td></tr>
<tr><td>6</td><td>1994</td><td>20-40</td><td>467 x 100</td><td colspan="2">Colloidal iron positivity in spots</td><td>3.2±2.5</td></tr>
<tr><td>7</td><td>1998</td><td>27-33</td><td colspan="3">Colloidal iron positivity in spots</td><td>0.6±0.8</td></tr>
<tr><td>8</td><td>1983</td><td>27-40</td><td>2667 x 100; 200 x 70 (2)</td><td colspan="2" rowspan="3">Colloidal iron positivity in spots</td><td>3.0±1.0</td></tr>
<tr><td>9</td><td>2002</td><td>27-47</td><td>533 x 33; 200 x 20; 133 x 7</td><td>1.8±0.8</td></tr>
<tr><td>10</td><td>2000</td><td>33-47</td><td>200 x 20</td><td>0.4±0.5</td></tr>
<tr><td>11</td><td>2001</td><td>33-53</td><td>Colloidal iron positivity in spots</td><td>233 x 7</td><td>Colloidal iron positivity in spots</td><td>0.0±0.0</td></tr>
<tr><td>12</td><td>1982</td><td>33-53</td><td colspan="3" rowspan="2">Colloidal iron positivity in spots</td><td>*</td></tr>
<tr><td>13</td><td>2001</td><td>40-47</td><td>3.0±1.0</td></tr>
</table>

Eyes of the same patient: eyes nos. 1 and 4; PK: penetrating keratoplasty; *: no data available for this sample.

dystrophies. This apoptotic activity was present in granular and lattice type I dystrophies, and in macular dystrophy in a statistically significantly increased number compared to normal. To the authors best knowledge this is the first paper to investigate the apoptotic cell alterations in stromal corneal dystrophies compared to normal human corneas.

The epithelium may contain a limited number of apoptotic and proliferating cells (maximum 1-2 cells/ section) even in normal healthy corneas. Recent investigations have disclosed TUNEL-positive keratocytes in different types of corneal dystrophies, such as keratoconus (Kim et al., 1999) and Fuchs' dystrophy (Borderie et al., 2000; Li et al., 2001), and have emphasised the role of apoptotic processes in the pathogenesis of such conditions.

In corneas with *granular* and *lattice type I dystrophy* with TUNEL-positive cells, the epithelium generally showed more irregularity of thickness, and/or the subepithelial and stromal corneal deposits were greater in size and number; however our findings were not completely consistent. Using the Poisson regression with overdispersion model, a statistical correlation between deposit size and apoptotic cell number could not be shown in these two dystrophies (p = 0.06; p = 0.14). We think nevertheless that an increase in the size of subepithelial stromal deposits and in irregularity of thickness of the epithelium may result in increased apoptotic activity of keratocytes in the two dystrophies, which may form part of the progression of the disease over a period of time. It is known that corneal dystrophies may have periods of progression alternating with periods of remission (Kim et al., 1999). It is thus possible that the increased apoptotic activity indicates a period of progression in the above granular and lattice dystrophy corneas.

In eyes with *macular dystrophy* we did not note any apparent relationship between the increased number of apoptotic keratocytes and the size of the stromal deposits and irregularity of thickness of the epithelium (Table 2), and no relation could be shown either using the Poisson regression with overdispersion model (p = 0.59). In 10 of these corneas (77%) TUNEL-positive cells could be detected, and we found a statistically significant increase in the mean (normalised) apoptotic keratocyte number (p = 0.01). Moreover, in some cases with macular dystrophy, apoptosis of epithelial cells (in eyes nos. 4 and 10) and endothelial cells (eyes nos. 5 and 13) were

Table 3. Corneal buttons with lattice type I (Haab-Dimmer) dystrophy. Epithelial thickness, details of stromal deposits (no. of deposits of similar size shown in parenthesis), and mean apoptotic keratocyte numbers (per field of view).

Eyes with Haab-Dimmer dystrophy No.	Year of PK	Epithelial thickness (min.-max.; μm)	Details of stromal deposits (Dimensions parallel and perpendicular to surface epithelium; mm) (no. of deposits)			Apoptotic keratocyte number per field of view (mean ± SD; n = 11)
			Subepithelial stroma	Central stroma	Posterior stroma	
1	1993	0-47	Continuous x 13-20	-	-	*
2	2003	7-20	Continuous x 13-20	33 x 20 (2)	-	0.0±0.0
3	1993	7-20	Continuous x 13-20	200 x 47; 267 x 27; 20 x 27; 13 x 20 (3)	67 x 27	0.0±0.0
4	1994	7-20	Continuous x 7-13	13 x 20 (3)	-	0.0±0.0
5	1992	7-20	Continuous x 13-20	67 x 13; 33 x 67	-	0.0±0.0
6	1981	7-27	Continuous x 27-53	67 x 27; 13 x 20	-	3.2±2.5
7	1995	7-33	Continuous x 13-20	133 x 67 (2); 40 x 27, 13 x 20 (3)	-	*
8	1994	13-27	Continuous x 13-20	13 x 20	-	0.0±0.0
9	2003	13-33	Continuous x 7-13	27 x 33 (4); 13 x 20 (6)	-	0.0±0.0
10	1982	13-60	Continuous x 27-33	-	-	0.0±0.0
11	2002	20-33	Continuous x 20-27	13 x 20	-	0.0±0.0
12	1983	20-60	Continuous x 13-20	-	-	0.0±0.0
13	1994	27-33	Continuous x 7-13	200 x 67; 67 x 67; 33 x 27; 27 x 20	-	2.2±1.9
14	1998	27-60	Continuous x 13-20	13 x 20 (2)	-	*

Eyes of the same patient: eyes nos. 3 and 13. Eyes of brothers: nos. 6 and 12; and nos. 3, 8 and 13. PK: penetrating keratoplasty. * : No data available for these samples.

Table 4. Apoptotic keratocyte numbers in corneas with granular, macular and lattice dystrophy (mean of numbers in 5 different fields of view; Mean ± SD, minimum, maximum) and p-values (Mann-Whitney test) for comparisons with numbers in normal human corneas. For statistical analysis only one eye of each patient was used in each group.

	Normal cornea (n=4)	Granular dystrophy (n=8)	Macular dystrophy (n=11)	Lattice dystrophy (n=10)
Number of TUNEL-positive keratocytes	0.0±0.0 (0.0-0.0)	1.1±1.7 (0.0-4.2)	1.6±1.2 (0.0-3.2)	0.5±1.1 (0.0-3.2)
p values		p=0.36	p=0.01	p=0.63

also detected. Taking all this into account, we suggest that apoptosis is definitely a concomitant and possibly a pathogenic factor in macular dystrophy, although the pathways that are triggered to result in increased apoptotic cell death remain to be clarified.

It is well known that patients with stromal dystrophies often have to be treated for recurrent corneal erosions (Campos et al., 1993; Seitz et al., 2002).

We suggest that in our stromal dystrophy patients chronic disruption of the irregular epithelium may have led to an increase in the amount of cytokines (interleukin-1, FAS ligand protein, bone morphogenic proteins 2 and 4, bcl2 protein (Nagy et al., 1996; Wilson et al., 1996, 2001; Mohan et al., 1997, 1998; Wilson, 2000) which triggered apoptosis of epithelial cells, keratocytes or both. The fact that in our study TUNEL-positive keratocytes were detected generally in the upper third of the corneal stroma in granular and lattice type I dystrophy also speaks in favour of the above hypothesis (Mohan et al., 2003). However, the presence of apoptotic epithelial and endothelial cells and significantly increased numbers of apoptotic keratocytes in corneas with macular dystrophy supports the supposition that abnormal production of fibrillogranular material, or some other dysfunction of these cells, may result in increased genetically programmed cell death in this dystrophy.

Clinically, corneal transplantation is the gold standard treatment option in patients with corneal dystrophies. Recently, phototherapeutic keratectomy (PTK) has been used with good results in improving visual acuity and in the treatment of recurrent corneal erosions (Campos et al., 1993; Rapuano, 1997; Orndahl and Fagerholm 1998; Seitz et al., 2002). It is well known that in healthy eyes corneal opacities ("haze") following excimer laser treatment may result from the corneal wound healing response (Kang et al., 2002; Csutak et al., 2004) which is initiated by keratocyte apoptosis (Mohan et al., 2003). Taking into account the fact that the number of apoptotic cells increases in advanced cases of stromal corneal dystrophies, especially in macular dystrophy, careful consideration should be given before laser treatment of these patients, because of the possibly elevated likelihood of haze formation.

In advanced cases of stromal dystrophies these pathological processes are also present in the deeper stroma. Therefore, for such patients PK would be the final and stable solution. It is also well described that during a lifetime, the number of endothelial cells constantly decreases (Rodrigues and Krachmer, 1988). Taking into account that in granular and macular dystrophy patients there is an increased loss of endothelial cells by apoptosis, we may conclude that corneal decompensation occurs earlier in these patients as compared to healthy subjects. This hypothesis also speaks in favour of performing a full-thickness corneal transplantation in these diseases rather than a corneal excimer laser treatment.

The normal epithelium may contain a limited number of proliferating cells (max. 1-2 cells/section) even in normal healthy corneas. With deposition of metabolic substances and increased number of apoptotic cells in corneas with stromal dystrophies, however, we were expecting a slightly increased proliferation of the epithelial cells. The fact that the number of proliferating cells is not increased in these corneas may also be related to the disfunction of the cells in these dystrophies.

We conclude that, besides changes in the distribution and size of corneal deposits, increased apoptotic activity of the corneal cells may also form part of the progression of granular and lattice type I dystrophy; and this seems definitely to be a concomitant, and possibly a pathogenic factor in macular dystrophy. However, the pathways that are triggered to result in increased apoptotic cell death and the genetic factors still remain to be clarified in stromal corneal dystrophy patients.

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