Charles University in Prague

First Faculty of Medicine

Biology and Pathology of the Cell

Mgr. Stanislava Merjava

FENOTYPICKÁ CHARAKTERIZACE ZDRAVÉ LIDSKÉ ROHOVKY A JEJÍ ZMĚNY PŘI ZADNÍ POLYMORFNÍ DYSTROFIÍ ROHOVKY

PHENOTYPICAL CHARACTERIZATION OF THE HEALTHY HUMAN CORNEA AND THE ALTERATIONS CAUSED BY POSTERIOR POLYMORPHOUS CORNEAL DYSTROPHY

Ph.D. Thesis

Supervisor: Mgr. Katerina Jirsova, PhD.

Prague 2011

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 6.5.2011

Jméno, Příjmení: Stanislava Merjavá
Podpis:

Identifikační záznam:

MERJAVÁ, Stanislava. *Fenotypická charakterizace zdravé lidské rohovky a její změny při zadní polymorfní dystrofii rohovky [Phenotypical characterization of the healthy human cornea and the alterations caused by posterior polymorphous corneal dystrophy].* Praha, 2011. 118 s., 7 příloh. Disertační práce (PhD.) Univerzita Karlova v Praze, 1. lékařská fakulta, Laboratoř biologie a patologie oka, Ústav dědičných metabolických poruch. Vedoucí práce Mgr. Jirsová, Kateřina, PhD.

ACKNOWLEDGEMENTS

First of all, I give my whole-hearted thanks to my beloved Michal, my mother and my whole family for their love, support and understanding. Without them I would have never finished my doctoral thesis.

My great thanks belong to my supervisor Dr. Katerina Jirsova for her scientific approach, understanding, and for the opportunity to perform and finish my Ph.D. studies in the Laboratory of the Biology and Pathology of the Eye. Great thanks also go to my colleagues: Dr. Viera Vesela, Kristyna Brejchova, Sarka Kalasova, Lubica Dudakova, Ales Neuwirth, Dr. Aref Al-Fakih, Dr. Petra Liskova as well as to colleagues from the Ocular Tissue Bank: Dr. Brettschneider, Jarka Kortusova, Dr. Denisa Zlacka, Dr. Ivana Krabcova and Klara Kopecka. They supported and helped me in the friendliest manner over the years.

Special thanks go to the Department of Ophthalmology, First Medical Faculty of Charles University and the General Faculty Hospital in Prague for all the pathological samples which were used in my research. Especially I have to thank Prof. Martin Filipec (Lexum European Eye Clinic, Prague, Czech Republic) for collecting samples over the years from patients with posterior polymorphous corneal dystrophy, and all the valuable clinical information pertaining to them.

Last but not least, I would like to thank Eva Malinova, Dr. Zuzana Zemanova and Prof. Kyra Michalova from the Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, General Faculty Hospital and First Faculty of Medicine, Charles University in Prague, for giving me valuable advice regarding the fluorescence in situ hybridization preparation.

This work was supported by the grant from the Grant Agency of the Czech Republic 201898301/031040, the Czech Ministry of Education, Youth and Sports research project MSM 0021620806/20610011, by the Project 260501 from Charles University in Prague and by the grant from the Ministry of Health of the Czech Republic MZOVFN2005.

ABSTRAKT

Cíle: Cílem práce bylo charakterizovat pomocí vybraných protilátek zdravou lidskou rohovku a rohovku od pacientů se zadní polymorfní dystrofií rohovky (ZPDR). I když je toto onemocnění považováno za ojedinělé, v České republice se nachází jeden z největších souborů pacientů s tímto postižením. To velmi dobře umožnilo sledovat změny na úrovni klinické, buněčné i molekulární.

Materiál a metody: K experimentům byl použit soubor 25ti kontrolních rohovek a soubor rohovek od 16ti pacientů se ZPDR. Imunocyto- a imunohistochemicky byly detekovány epiteliální (cytokeratiny) a mezoteliální márkry (mesothelin, kalbindin 2 a protein HBME-1) ve všech vrstvách kontrolních rohovek. Výskyt jednotlivých márkrů byl potvrzen i molekulárními metodami (RT-PCR a Western blot). U rohovek se ZPDR byly sledovány změny v expresi cytokeratinů a ve složení extracelulární matrix (kolagenu IV a VIII). Pro objasnění původu abnormálních endotelových buněk u pacientů se ZPDR po transplantaci rohovky, které způsobují relaps onemocnění, byly současně použity dva metodické postupy; nepřímá fluorescenční imunohistochemie a fluorescenční in situ hybridizace.

Výsledky: V rohovkách pacientů se ZPDR byly charakterizovány změny na úrovni exprese cytokeratinů v abnormálním endotelu (silná pozitivita pro cytokeratiny 7, 19, 8 a 18, slabší pozitivita pro cytokeratiny 1, 3/12, 4, 5/6, 10, 10/13, 14, 16 a 17) a změny v lokalizaci jednotlivých řetězců kolagenů IV a VIII. I když ZPDR postihuje především zadní vrstvu rohovky (endotel a Descemetovu membránu), změny byly detekovány i na úrovni bazální membrány epitelu a zadní části stromy. Byl objasněn původ abnormálních buněk endotelu, které způsobují relaps onemocnění u pacientů se ZPDR. Tyto abnormální buňky migrují na štěp z netransplantované periferní části pacientovy rohovky.

Dále bylo charakterizováno cytokeratinové spektrum v jednotlivých vrstvách zdravé lidské rohovky, limbu a spojivky. Za klíčový nález považuji přítomnost cytokeratinu 8 v bazální vrstvě epitelu limbu svědčící o významu tohoto cytokeratinu v procesu diferenciace buněk při obnově rohovkového epitelu. Ve zdravém endotelu rohovky byly detekovány márkry epitelu (cytokeratin 8 a 18) a mezotelu (mesothelin, kalbindin 2 a protein HBME-1).

Závěr: Charakterizace zdravé lidské rohovky je předpokladem pro detailní určení změn, ke kterým dochází u patologických stavů. Rozšíření znalostí o abnormálních nálezech u rohovek pacientů se ZPDR může vést k zpřesnění diagnostiky a prognózy klasické léčby pacientů s tímto onemocněním a může být základem pro nové terapeutické postupy.

Klíčová slova: *rohovka; zadní polymorfní dystrofie rohovky; endotel; epitel; cytokeratin; kolagen*

ABSTRACT

Purpose: The aim of this work was to characterize the healthy human cornea and the cornea of patients suffering from posterior polymorphous corneal dystrophy (PPCD) using different antibodies. Despite the fact that PPCD is a very rare disorder, one of the largest groups of PPCD patients in the world comes from the Czech Republic. This offers us the opportunity to investigate the changes on the clinical, cellular and molecular levels.

Material and Methods: A collection of 25 control corneas as well as 16 pathological corneas from PPCD patients were used. Epithelial (cytokeratins) and mesothelial markers (mesothelin, calbindin 2, HBME-1 protein) were detected in all layers of the healthy corneas using immunocyto- and immunohistochemistry. The expression of all markers was confirmed using molecular methods as well (RT-PCR and Western blot). Changes in the expression of cytokeratins and changes in the extracellular matrix structure (collagen IV and VIII) were studied in the PPCD corneas. Combined fluorescent immunohistochemistry with fluorescence in situ hybridization were used in order to characterize the origin of abnormal cells on the posterior graft surface, which cause the recurrence of the PPCD after penetrating keratoplasty surgery.

Results: Changes in the cytokeratin expression (strong positivity for cytokeratins 7, 19, 8 and 18; weaker positivity for cytokeratins 1, 3/12, 4, 5/6, 10, 10/13, 14, 16 and 17) and changes in the localization of individual collagen IV and VIII chains were described in the PPCD corneas. Although PPCD affects primarily the Descemet membrane and the endothelium, changes in the basal membrane of the epithelium and posterior stroma were also detected. The exact origin of the abnormal endothelial cells, which cause the recurrence of PPCD in some cases, was established. These abnormal cells migrate into the donor graft from the non-transplanted peripheral part of the recipient cornea.

A whole spectrum of cytokeratins was described in the individual layers of the healthy human corneal, limbal and conjunctival epithelium. I considered a strong signal for cytokeratin 8 in the basal layer of the limbal epithelium to be a key finding, which could play a role in the differentiation processes by corneal epithelial renewal. Epithelial (cytokeratins 8 and 18) and mesothelial markers (mesothelin, calbindin 2 and HBME-1 protein) were detected in the human corneal endothelial cells.

Conclusions: Characterization of the healthy human cornea is a prerequisite for characterization of pathologies. Knowledge about changes in PPCD corneas could be helpful for more precise diagnosis and prognosis; moreover it could be a basis for new therapeutical procedures.

Key words: *cornea; posterior polymorphous corneal dystrophy; endothelium; epithelium; cytokeratin; collagen*

CONTENTS

ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2 protein
ABZ	Anterior banded zone
AD	Autosomal dominant inheritance
AEC	3-amino-9-ethylkarbazol
AE1	Anti-cytokeratins 11, 14, 19 antibody
AE3	Anti-cytokeratins 5, 7, 8 antibody
AR	Autosomal recessive inheritance
BM(s)	Basement membrane(s)
BME	Basement membrane of the epithelium
BSA	Bovine serum albumin
CAM 5.2	Anti-cytokeratins 8 and 18 antibody
cDNA	Complementary DNA
CD 31	Platelet endothelial cell adhesion molecule
CEP X	α satellite DNA probe hybridizes to the centromere of human chromosome X
CEP Y	α satellite DNA probe hybridizes to the centromere of human chromosome Y
CK(s)	Cytokeratin(s)
COL8A2	Gene for α2 collagen VIII chain
CSRP2BP	Cysteine-rich protein 2-binding protein
C/EBPδ	CCAAT enhancer binding protein delta
DAPI	4.6-diamidino-2phenylindoldihydrochlorid
DM	Descemet membrane
DNA	Deoxyribonucleic acid
FACIT	Fibril-associated collagens with interrupted triple helices
FECD	Fuchs endothelial corneal dystrophy
FISH	Fluorescence in situ hybridization
FITC	Fluorescein-isothiocyanate
GAG(s)	Glycosaminoglycan(s)
GAPDH	Glyceraldehyde-3-phospate dehydrogenase
GFAP	Glial fibrillary acid protein
HBME-1	Hector Battifora mesothelial cell-1 protein
HRP	Horse-radish peroxidase
CHED	Congenital hereditary endothelial dystrophy
IF(s)	Intermediate filament protein(s)
IgG	Immunoglobulin G
LESC(s)	Limbal epithelial stem cell(s)
mRNA	Messenger ribonucleic acid
OMIM	Online Mendelian Inheritance in Man
OTB	Ocular Tissue Bank
PBS	Phosphate buffered saline
PCL	Posterior collagenous layer
PNBZ	Posterior non-banded zone
PPCD	Posterior polymorphous corneal dystrophy
p	Short arm of the chromosome
q	Long arm of the chromosome
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

SABcomplex/AP	Streptavidin-biotin complex/Alkalic phosphatase
SAGE	Serial analysis of gene expression
SLC4A11	Sodium bicarbonate transporter-like protein 11
TAC(s)	Transit amplifying cell(s)
TCF8	Transcription factor 8 (ZEB1)
TM	Trabecular meshwork
TNFR2	Tumor necrosis factor receptor 2
TRADD	TNFR1-associated death domain protein
TRITC	Tetramethylrhodamin-isothiocyanate
VFN	Všeobecná fakultní nemocnice (General Faculty Hospital)
VSX1	Visual system homeobox gen 1
ZEB1	Human zinc finger E-box binding homeobox 1 gene (formerly TCF8)
XECD	X-linked endothelial corneal dystrophy

1 AIMS OF THE STUDY

The main aim of my doctoral thesis was to better characterize a healthy adult human cornea in comparison with pathological ones. As this is a wide theme I addict my attention to one particular corneal disease - posterior polymorphous corneal dystrophy (PPCD). Despite the fact that PPCD is a very rare disorder, one of the largest groups of PPCD patients in the world comes from the Czech Republic. This offers us a great opportunity to investigate its clinical, biological, molecular and genetical aspects. The aims of my PhD thesis were divided into two main sections and the detailed structure of the partial aims of this dissertation is hence as follows:

1. The characterization of healthy adult human corneas without any ocular injury or illness. Particular themes of interest were:

- to determine cytokeratin and collagen expression in adult human cornea, limbus and conjunctiva
- to characterize the basal cell layer of the limbus
- to determine the phenotype of normal adult corneal endothelial cells; to evaluate the expression of mesothelial markers (mesothelin, calbindin 2 and HBME-1 protein) as well as simple epithelia markers (cytokeratins 8 and 18) in these cells, and to discuss their function

2. The exploitation and extension of my diploma thesis: “The characterization of the corneal changes of posterior polymorphous corneal dystrophy patients”.
Particular themes of interest were:

- to characterize the morphology of corneal endothelial cells of patients with PPCD and compare them with cells of normal corneal endothelium
- to determine the spectrum of cytokeratins expressed in cells on the posterior surface of the cornea in PPCD patients
- to determine the changes in presence and localization of different chains of collagen IV and VIII in patients with PPCD
- to determine the origin of cells causing the recurrence of PPCD on explanted corneal buttons

Based on these aims the results of my doctoral thesis are divided into two main sections. First, a healthy human cornea is characterized and then differences in corneas with PPCD when compared to healthy ones are showed.

Since the vast majority of the achieved results have been published in scientific per-reviewed journals, I find it sufficient to present the results in the form of the original appended articles.

2 LITERAL INTRODUCTION

2.1 Corneal anatomy and physiology

The cornea is a transparent and avascular tissue which forms the outermost surface of the eyeball where it covers the iris, pupil and anterior chamber. Its refractive power is 40 to 44 diopters, which is about two-thirds of the total refractive power of the eye (Olsen, 1986). Transparency, together with surface smoothness, contour and refractive index determine the optical properties of the cornea. The curvature of the corneal surface is not constant due to the fact that it is approximately 0.5 mm thick at the center, and its thickness increases gradually toward the periphery, where it is about 0.7 mm thick (Mishima, 1968). The cornea is one of the most heavily innervated and most sensitive tissues in the body. The density of nerve endings in the cornea is thus about 300 to 400 times greater than that in the skin (Rozsa and Beuerman, 1982). The cornea is supplied with glucose by diffusion from the aqueous humor and by oxygen primarily via diffusion from tear fluid. Apart from its optical function the corneal surface also protects the internal space of the eye against the penetration of potentially pathological agents.

The cornea consists of six different individual layers: the multilayered epithelium and its basal membrane; the Bowman layer; the corneal stroma; the Descemet membrane; and the corneal endothelium (Fig. 1). Epithelial and endothelial cells are metabolically active.

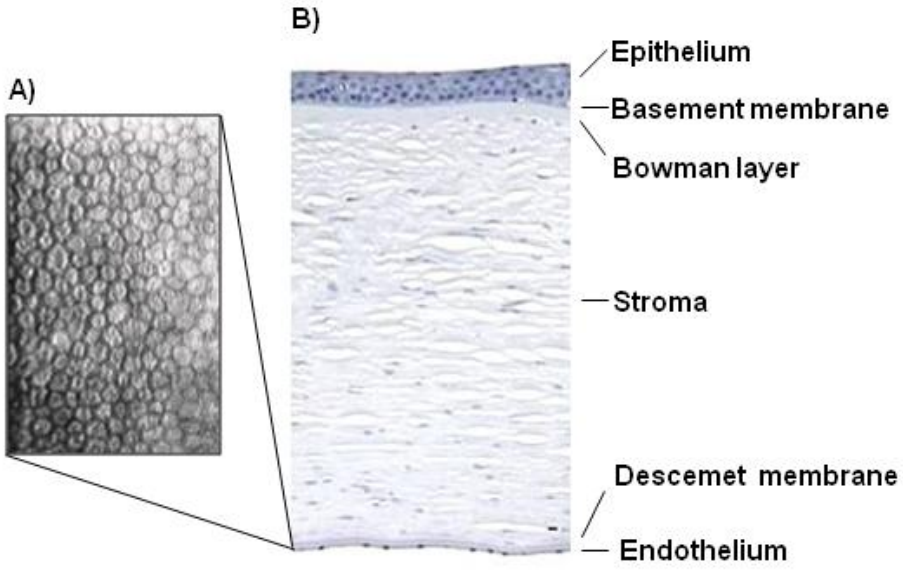

Figure 1: Monolayer of flat hexagonal endothelial cells (Specular microscopy) (A), and the corneal section, with well visible particular corneal layers stained with Harris haematoxylin (B). Scale bar represents 10 µm.

2.1.1 Corneal epithelium

The corneal epithelium is approximately 50 μm thick and is composed of nonkeratinized, stratified squamous epithelial cells. It consists of five to six layers: a monolayer of mitotically active columnar basal cells, two or three layers of wing cells and two or three layers of terminally differentiated superficial cells. The differentiation process requires about 7 to 14 days, when the superficial cells are desquamated into the tear film (Hanna et al., 1961). In the peripheral epithelium, specialized dendritic Langerhans cells are present, which are implicated in antigen processing and contribute to the immunological features of the cornea (Gillette et al., 1982). The epithelium provides a barrier to external biological and chemical insults and protects the ocular surface from microbial attack (Sack et al., 2001). The vertebrate corneal epithelium develops from the surface ectoderm.

2.1.2 Basement membrane of the epithelium

The basement membrane of the epithelium (BME) is 40 to 60 nm thick membrane. Basal cells of the epithelium participate on its development. Type IV collagen and laminin, both of which are secreted by corneal epithelial cells, are major components of BME (Kefalides, 1971). The minor components are fibronectin, fibrin, collagen VII, proteoglycans and heparan sulfate (Berman et al., 1983; Timpl, 1989). The presence of the BME fixes the polarity of the epithelial cells, provides a matrix on to which cells can migrate, and plays a prominent role in epithelial wound healing and in cell adhesion, differentiation and regeneration (Leinonen et al., 1994).

2.1.3 Bowman layer

The Bowman layer is an acellular membrane-like zone between the BME and corneal stroma. It is 12 μm thick and consists predominantly of proteoglycans and collagen I and III, which are secreted by stromal keratocytes (Beuerman and Pedroza, 1996; Marshall et al., 1991). The Bowman layer is compact and immune, but it does not regenerate after injury. Many mammals do not have this layer but still exhibit a well-organized epithelial structure, therefore its physiological role remains unclear. It has been previously discussed that the Bowman layer may be a visible indicator of ongoing stromal-epithelial interactions in humans, but may have no critical function in corneal physiology (Wilson and Hong, 2000).

2.1.4 Corneal stroma

The corneal stroma represents more than 90% of the corneal thickness. The physical strength, stability of shape and transparency of the cornea depend on stromal anatomic and biochemical properties. The stroma consists predominantly of an extracellular matrix: collagens and glycosaminoglycans (GAGs), keratocytes (stromal fibroblasts) and nerve fibers. Collagens assemble into collagen fibers, which are highly uniform in diameter (22.5 to 35 nm). The distance between the collagen fibers is also highly uniform (41.5 ± 0.5 nm). This regular arrangement (uniform diameter and

interfibrillar space) are less than half of the wavelength of visible light (400 to 700 nm) and together with a continuous slow production and degradation are major determinants of corneal transparency (Maurice, 1957; Scott, 1998). Collagen fibers constitute more than 70% of the dry weight of the cornea and form 200 – 300 lamellae parallel with the surface (Hamada et al., 1987). Various GAGs are presented between collagen fibers in the corneal stroma. Except for hyaluronan, all of them bind to core protein and form proteoglycans, which could modulate collagen fibrillogenesis (Iozzo, 1998). The most abundant GAGs in the cornea are keratan sulfate, chondroitin sulfate and dermatan sulfate. They can absorb and retain a large amount of water and hence maintain corneal hydratation.

2.1.5 Descemet membrane

The Descemet membrane (DM) is the basement membrane (BM) of the corneal endothelium and is a product of these cells. DM protects the cornea from aqueous humor penetrating into the stroma and consequent stromal oedema. In healthy human cornea DM consists of two morphologically separated regions: the anterior banded zone (ABZ - average thickness of 3 μm) formed during intrauterine life and the posterior non-banded zone (PNBZ) which is synthesized after birth (thickness increases with age from 2 to 10 μm), (Johnson et al., 1982). The ABZ is mostly composed of wide-spaced collagen, which is characterized by ultrastructural labeling as collagen type VIII and in horizontal sections forms a hexagonal lattice (Sawada, 1982). The PNBZ is a broad layer of amorphous extracellular matrix (Levy et al., 1996b; Sawada et al., 1990). Besides collagen VIII, collagen IV, laminin and fibronectin are present in DM (Ben-Zvi et al., 1986).

2.1.6 Endothelium

The endothelium is a monolayer of flat, mostly hexagonal cells which are highly metabolically active. They contain a large nucleus and abundant cytoplasmic organelles. After the birth there are approximately 4000 – 6000 endothelial cells/mm^2 (Nishida, 2005), which do not proliferate and are normally arrested in the G_1-phase of the cell cycle (Joyce et al., 1996). Their density decreases with age (Laule et al., 1978), (0.6% per year). The existence of stem cells for corneal endothelium is still being discussed (McGowan et al., 2007). Endothelial damage is repaired by the migration and enlargement of the remaining endothelial cells. The most important physiological function of the corneal endothelium is to regulate the water content of the corneal stroma. Na+/K+ -dependent ATPase, Na+/H+ and HCO_3^- exchangers are expressed in the basolateral membrane of cells and are essential for maintaining corneal transparency through its dehydration (Waring et al., 1982).

According to a newly accepted concept the corneal endothelium and keratocytes originate from the neural crest and the lateral plate mesoderm, which together form the periocular mesenchyme. A broad spectrum of transcriptional factors is required for the appropriate differentiation of the periocular mesenchyme to keratocytes and

corneal endothelial cells (Cvekl and Tamm, 2004; Gage et al., 2005; Kidson et al., 1999; Reneker et al., 2000; Sowden, 2007).

In accordance with the crucial participation of the neural crest in its development, the human corneal endothelial cells express neural cell markers such as neuron-specific enolase, S-100 protein, neuron cell adhesion molecule and neurofilaments, (Foets et al., 1992a, 1992b; Hayashi et al., 1986; Shamsuddin, et.al., 1986). In addition, the expression of epithelial cell markers – cytokeratins (CKs) 8 and 18 – in normal human corneal endothelial cells has been discussed (Cockerham et al., 2002; Foets et al., 1990; Kasper et al., 1992). Of the mesenchymal cell markers, vimentin is present in human corneal endothelial cells, but this protein is observed in a number of different cell types of mesodermal origin, including different epithelia as well as the vascular endothelium, and thus cannot be considered as a protein specific only to mesothelial cells (Blose and Meltzer, 1981, Celis et al., 1983; Foets et al., 1990; Hayashi et al., 1986; Risen et al., 1987). On the other hand, the absence of markers typical to the vascular endothelium, such as factor VIII, vascular cell adhesion protein and platelet endothelial cell adhesion molecule (CD 31) in corneal endothelium clearly demonstrates that the term endothelium, although widely used for the posterior cells of the cornea, is a misnomer (Foets et al., 1992b; Shamsuddin, et.al., 1986; Scheef et al., 2007; Treseler et al., 1985).

2.2 Mesothelial markers

Because of the partially shared origin of mesothelial cells and human corneal endothelial cells, and since they occupy a similar location as do typical mesothelial cells in the pleura or peritoneum (the basal membrane on one side and the aqueous humor of the anterior chamber on the other), one may expect a sharing of some phenotypical markers between the two cell types due to their similar roles, at least in terms of fluid movement between a tissue and a cavity. In accordance with the crucial participation of the lateral plate mesoderm on the development of the human corneal endothelium, we have determined the presence and expression of three proteins typically expressed in the mesothelium: mesothelin, calbindin 2 and Hector Battifora mesothelial cell-1 protein (HBME-1) in healthy human cornea, limbus and conjunctiva (Ko et al., 2001; Miettinen and Kovatich, 1995; Ordonez, 2003a). Besides the presence of calbindin 2 in mouse neurons, none of the above mentioned proteins have been described in the cornea up to now (Felipe et al., 1999).

Mesothelin: is a 40 kDa membrane-bound peptide, which is physiologically expressed in mesothelial cells lining the pleura, pericardium and peritoneum; moreover its limited immunoreactivity is manifested in the surface epithelium of the ovary, the tunica vaginalis, the rete testis, and the epithelia of the trachea, tonsil and Fallopian tube. Its biological function is not known (Chang and Pastan, 1996; Chang et al., 1992; Ordonez, 2003a). However, mesothelin is highly expressed in malignant mesotheliomas as well as in various carcinomas and adenocarcinomas; it may be released from cell membranes, and a soluble form has been detected in the serum of mesothelioma patients (Hellstrom et al., 2006; Ordonez, 2003a, 2003b).

Calbindin 2 (calretinin): is a 29 kDa calcium-binding protein. Together with calbindin 1, calmodulin, parvalbumin, S-100 protein and troponin C, calbindin 2 is a member of the large troponin C superfamily (Baimbridge et al., 1992; Persechini et al., 1989). This protein was originally cloned from a chick retina, and it is abundantly expressed in the neurons of the central and peripheral nervous systems, including murine corneal sensory neurons (Felipe et al., 1999; Rogers, 1987; Rogers et al., 1990). Outside of neurons, it is typically found in mesothelial and mesothelioma cells as well as in a variety of other cell types (Al Moghrabi et al., 2007; Doglioni et al., 1996; Ko et al., 2001; Marchevsky, 2008). The function of calbindin 2 is not fully understood, but it is thought to act as a buffer to prevent abnormal intracellular calcium increases (Rogers, 1987; Rogers et al., 1990). Its presence protects neuronal cells against various types of injury (Lukas and Jones, 1994). This protein was localized by Gotzos et al. (1992) in both the cytoplasm and the cell nuclei in phase G1 and during mitosis in human adenocarcinoma cells, where it associates with the kinetochor and polar microtubules. This localization indicates a role in spindle microtubule stability and chromosome separation (Gotzos et al., 1992).

HBME-1 protein: the protein to which the HBME-1 antibody reacts is still unidentified. The HBME-1 antibody reacts strongly to mesothelial cells, especially in the pleura and peritoneum. The antibody was raised against cultured mesothelioma cells, and because it neither immunoblots nor immunoprecipitates, the antigen has remained unidentified (personal communication H. Battifora in Miettinen and Kovatich, 1995). Due to its overexpression in various neoplastic epithelial cells, this unknown protein nonetheless serves as a useful marker in the differential diagnosis of epithelial mesothelioma on the one hand, and sarcoid mesothelioma or adenocarcinoma on the other (Dahlstrom et al., 2001; Miettinen and Kovatich, 1995). It is located both in the cytoplasm and plasmatic membrane of mesothelioma cells, where a thick brush pattern indicates the presence of this protein in some of the microvilli (Batifora and McCaughey, 1995; Miettinen and Kovatich, 1995; Ordonez, 1997).

2.3 Limbus

The highly vascularized and innervated limbus constitutes the transition zone between the cornea and sclera. It contains a reservoir of pluripotential stem cells - limbal epithelial stem cells (LESCs), which together with basal cells of the corneal epithelium participate in the renewing of the corneal epithelium throughout life (Davanger and Evenson, 1971; Dua et al., 2009; Chang et al., 2008; Thoft and Friend, 1983; Tseng, 1989). LESCs exhibit a high proliferative capacity and are located in the basal layer of the limbus, undergoing asymmetric self-renewal cell division, where one cell remains undifferentiated and stays as the stem cell while other fast-dividing progenitor cells, named transit amplifying cells (TACs), begin to divide and differentiate into the suprabasal and superficial cells of the corneal epithelium (Hall and Watt, 1989; Lehrer et al., 1998; Morrison et al., 1997; Schlötzer-Schrehardt and Kruse, 2005).

LESCs are involved in corneal epithelial renewal which is important for maintenance of the integrity of the ocular surface (Davanger and Evenson, 1971; Tseng, 1989). Despite an enormous effort by all researchers, there is currently no single marker that can be used to definitively identify LESCs. The major stem cell associated markers in ocular or non-ocular tissues in the past decade were categorized into at least three groups: 1) nuclear proteins (for example transcription factor p63 or C/EBPδ: CCAAT enhancer binding protein delta); 2) cell membrane or transmembrane proteins including integrins (integrin β1, α6 etc.), receptors (Notch1) and drug resistance transporters (ABCG2: ATP-binding cassette sub-family G member 2); and 3) cytoplasmic proteins such as cytokeratins (CK19), nestin, and α-enolase. In addition, a variety of differentiation markers (CK3/12, E-cadherin, connexin 43, etc.) have also been proposed to distinguish the stem cells from differentiated cells. Moreover, morphological characteristics such as small cell size and a high nucleo-cytoplasmic ratio can be used in combination with putative stem cell markers (Budak et al., 2005; De Paiva et al., 2005; Chen et al., 2004; Notara et al., 2010; Schlötzer-Schrehardt and Kruse, 2005; Watanabe et al., 2004).

2.4 Conjunctiva

The conjunctiva is a mucous membrane consisting of a stratified non-keratinizing epithelium which varies in thickness and appearance from the eyelid margin to the limbus (Nelson and Cameron, 2005). Conjunctival epithelium contains Langerhans cells, melanocytes and goblet cells producing ocular mucin that constitutes the innermost layer of the tear film (Hogan et al., 1971).

2.5 Intermediate filaments

Intermediate filaments (IFs) are intracellular cytoskeleton proteins (10 – 12 nm in diameter), (Fuchs and Cleveland, 1998). At least five classes of IFs are distinguishable, each of which is expressed in a highly specific and conservative manner (Tab. 1). Mesenchymal cells are characterized by the expression of vimentin (Franke et al., 1978), muscle cells by desmin (Lazarides and Hubbard, 1976), glial cells by glial fibrillary acid protein (GFAP), (Bignami and Dahl, 1974), neuronal cells by a triplet of neurofilaments (Weber et al., 1983), and epithelial cells by cytokeratins (CKs).

IF class		Expression
I	Cytokeratin type I	Epithelial cells
II	Cytokeratin type II	Epithelial cells
III	Vimentin	Mesenchymal cells
	Desmin	Muscle cells
	GFAP	Astrocytes
	Peripherin	Neurons of the peripheral nervous system
IV	Neurofilament proteins	Neurons
	Alpha-internexin	Neurons of the central nervous system
	Nestin	Neuroepitehlial stem cells
V	Lamins	Nuclear lamina

Table 1: Classes of intermediate filaments (according to Pitz and Moll, 2002).

2.5.1 Cytokeratins

CKs are the typical IF proteins of epithelia, expressed in a tissue-specific and differentiation-dependent manner. Previously, CKs were divided into two subfamilies according to their electrophoretic migration properties: type I (“acidic”) and type II (“neutral to basic”), (Moll et al., 1982). Recently, the “Keratin Nomenclature Committee” established the novel consensus nomenclature for mammalian cytokeratin genes and proteins, where hair CKs and special epithelial CKs designations were implemented:

a) the type I cytokeratins: CK9-CK10, CK12-CK28 and CK31-CK40
b) the type II cytokeratins: CK1-CK8 and CK71-CK86 (Schweizer et al., 2006).

Each one of the CKs is a product of a distinct gene (Upasani et al., 2004). In humans 54 functional genes exist and they are clustered at two different chromosomal sites: chromosome 17q21.2 (type I cytokeratins, except CK18) and chromosome 12q13.13 (type II cytokeratins including CK18), (Hesse et al., 2004; Rogers et al., 2005). Since CKs belong to the family of IFs they share common protein-structural characteristics. They contain a central rod domain of ~310 amino acids with α-helical conformation flanked

by non-helical globular head and tail domains of variable length (Parry et al., 2007). The protein products of this gene family all form alpha-helical coiled-coil dimmers that can rapidly assemble into 10 nm wide filaments without the need for any cofactors or associated proteins. CKs always consist of equimolar amounts of type I proteins and type II proteins (Steinert, 1990).

CKs are very stable, relatively resistant to degradation and very antigenic (Morgan et al., 1987). Their function is not only to keep a cell's shape and to save the cell from mechanical stress (Oshima et al., 1996), but they also play a role in differentiation or the functional specialization state, and protection from apoptosis, stress and injury (Caulin et al., 2000; Zatloukal et al., 2000). Typically, cytokeratin filaments insert upon desmosomes and hemidesmosomes. Thus, they contribute to stability between epithelial cells themselves, but also to the basement membrane attachment and to the connective tissue compartment of a given epithelium (Moll et al., 2008). They play a role in the intracellular transduction of signals and malignant transformation (Oshima et al., 1996; Traub et al., 1994). Some CKs (CKs 5, 7, 8/18, 19 and 20) exhibit characteristic expression patterns in human tumors; therefore, they are of major importance in the immunohistochemical diagnosis of carcinomas (Moll et al., 2008).

Moreover, mutations in the distinct CK genes cause a lot of human autosomal-dominant familial diseases. In the hereditary blistering skin disease epidermolysis bullosa simplex, a spectrum of point mutations of CK5 and CK14 were detected (Coulombe et al., 1991; Lane et al., 1992). In Meesmann's corneal dystrophy, mutations in CK3 and CK12 cause intraepithelial microcysts in the corneal epithelium (Irvine et al., 1997). Other hereditary disorders are pachyonychia congenital type I (Jadassohn-Lewandowsky form) and pachyonychia congenital type II (Jackson-Lawler form) where mutations in genes for CK6, CK16 or CK17, respectively were described (Bowden et al., 1995; McLean et al., 1995). Mutations in CK1 and CK10 cause the blistering disorder bullous congenital ichthyosiform erythroderma and mutations in CK4 and CK13 are associated with white sponge nevus (Chipev et al., 1992; McLean et al., 1994; Richard et al., 1995; Rothnagel et al., 1992; Rugg et al., 1995). No mutations of the human CK19, CK7, CK20 and CK15 genes causing a disease have yet been found (Moll et al., 2008; Owens and Lane, 2004).

Simple-epithelial cytokeratins

Simple epithelium expresses predominantly CKs 8, 18, 7, 17 and 19; additionally, CKs 7 and 19 are characteristic of glandular epithelium (Leube et al., 1986; Moll et al., 2008; Ramaekers et al., 1987).

CK8 and CK18 are the phylogenetically oldest CKs and are the first CKs expressed during embryogenesis, as early as in pre-implantation embryos (Blumenberg, 1988; Jackson et al., 1980). The absence of CK8 causes mid-gestational lethality or colorectal hyperplasia and inflammation in mice (Baribault et al., 1993; Jackson et al., 1980). Both are major components of the intermediate filaments of simple or single layered

epithelia found in the liver, mammary gland, including corneal epithelial cells, vascular endothelium and in the pleural and peritoneal mesothelium (Franke et al., 1981; Kasper et al., 1992; Oshima et al., 1996; Pronk et al., 1993; Stosiek et al., 1990).

Providing mechanical strength in the single layered epithelia and interacting with desmosomes are the basic, but not the only, functions of CK8 and CK18. The total amount of cellular CK8 and CK18 is kept at a stable level under physiological conditions, while the expression of both often retains or increases during carcinogenesis, which may result in increased invasive potential and tumorogenicity (Moll et al., 1982; Oshima et al., 1996; Raul et al., 2004; Trask et al., 1990). Moreover, CKs 8 and 18 have a cytoprotective role against chemical insults (Bauman et al., 1994).

CK8/18 are very important in the regulation of apoptosis, where they regulate Fas targeting in the surface membrane; bind the cytoplasmatic domain of tumor necrosis factor receptor 2 (TNFR2) and moderate the TNF-dependent activation of JNK and NFκB transcription factors; and sequester TRADD (TNFR1-associated death domain protein) and inhibit the activation of TNFR1-induced apoptosis (Caulin et al., 2000; Gilbert et al., 2001; Hsu et al., 1995; Inada et al., 2001; Liu et al., 1996).

Furthermore, their role in the regulation of cell cycle was described (Galarneau et al., 2007; Ku et al., 2002; Magin et al., 2007; Toivola et al., 2001). They bind DNA, RNA and molecules that are important in signal transduction (Liao, 1995; Liao and Omary, 1996; Omary, 1992; Traub, 1994). The CK8/18 pair plays an important role in cell-cycle regulation through phosphoserine-binding protein 14-3-3, which is a key regulator in signal-transduction/cell-cycle checkpoint control (Aitken, 1995). During the S/G2/M phases, when cytokeratins become hyperphosphorylated, 14-3-3 binds to CK8/18 and, due to cdc25 (dual-specificity phosphatase), remains free to dephosphorylate the cdc2/cyclin B complex, thus cells come through the M-phase checkpoint (Fu et al., 2000; Ku et al., 1998; Liao and Omary, 1996; Omary et al., 1998). The key role of CK8 in cell-cycle regulation was demonstrated and confirmed in CK8 null mice, where hepatic cells exhibited an altered cellular redistribution of 14-3-3 protein into the nuclei and its binding to cdc25. All this leads to cell-cycle deregulation and finally, in the G2-phase, to cell-cycle arrest (Ku et al., 2002; Toivola et al., 2001).

CK7 and CK19 are “additional” (secondary) and also widely distributed simple-epithelial CKs together with CK17, typical for basal myoepithelial cells (Leube et al., 1986). CK7 and CK19 typically occur in simple ductal epithelia such as bile and pancreatic ducts (Ramaekers et al., 1987), moreover CK19 was proposed as a marker for stem cells (Lauweryns et al., 1993b; Notara et al., 2010). Another simple-epithelia CK with restricted distribution is CK20, which is specific to gastrointestinal epithelia, urothelium and Merkel cells (Moll et al., 1992, 1995).

Stratified-epithelial cytokeratins

The stratified-epithelial CKs are comprised of CK1, CK2e, CK2p, CK3 to CK6, and CK9 to CK17. The basal cell layer of the stratified epithelium expresses CKs 5 and 14, while the cells of the superficial layers express CKs 1 and 10 or 4 and 13, depending on the state of epithelial keratinization and differentiation (Moll et al., 1982; Morgan et al., 1987). Stable basal cells also express CK15, which has been proposed as a potential marker of stem cells in the hair follicle bulge as well as in limbal epithelial stem cells (Liu et al., 2003; Lloyd et al., 1995; Moll et al., 1993; Porter et al., 2000; Waseem et al., 1999). CK6 and CK16 are also expressed in several stratified epithelia occurring physiologically in mucosal epithelia. Their upregulation is correlated with increased proliferative activity occurring as a result of inflammatory, hyperplastic, or neoplastic conditions (Fransseen et al., 2004; Pitz and Moll, 2002; van der Velden et al., 1999).

Corneal cytokeratins

The typical marker of corneal epithelium is CK3 which forms a dimmer with CK12 and both are believed to be cornea-specific (Lauweryns et al., 1993a; Moll et al., 1982). Moreover, in the corneal epithelium CKs 4, 7, 8, 11, 13, 14, 16, 18 and 19/5 have been previously detected together with pancytokeratin and group of CKs AE1/AE3 (Cockerham et al., 2002; Kasper et al., 1992; Ross et al., 1995).

In the adult human corneal endothelium, no CKs have been detected and have been confirmed with the exception of the CK pair 8/18 (Kasper et al., 1992; Wollensak and Witschel, 1996).

Limbal and conjunctival cytokeratins

CK19 has been described as one of the major components in the conjunctival epithelium (Elder et al., 1997; Kasper et al., 1988; Pitz and Moll, 2002; Schlotzer-Schrehardt and Kruse, 2005). It exhibits the opposite direction in its labeling gradient than CK3 does. CK19 is located in all layers of the conjunctival and limbal epithelium and its presence decreases in the peripheral part of the cornea, and finally disappears in the central corneal epithelium (Kasper et al., 1988; Lauweryns et al., 1993a; Pitz and Moll, 2002). Moreover, CK15 was detected in the basal cells of the limbus and conjunctiva (Lloyd et al., 1995).

2.6 Collagens

Collagen is the major component of the extracellular matrix and the most abundant protein in mammals making up about 25 to 35% of the whole-body protein content. More than 28 types of collagen have been described in literature up to now, which differ in their structure, function and tissue distribution (Myllyharju and Kivirikko, 2001).

The collagen superfamily can be divided on the basis of polymeric structures or other features (Myllyharju and Kivirikko, 2001; Prockop and Kivirikko, 1995):

a) Collagens that form fibrils: I, II, III, V, XI, XXIV, XXVII
b) Collagens that form networks: IV, VIII, X
c) Fibril-associated collagens with interrupted triple-helices (FACIT): IX, XII, XIV, XVI, XIX, XX, XXI
d) Collagens that form filaments: VI
e) Collagens that form anchoring fibrils: VII
f) Collagens with transmembrane domains: XIII, XVII, XXIII, XXV
g) The family of collagens: XV, XVIII, XXII, XXVI
h) "Non-collagenous" collagens: conglutinin, collectin-43, etc.

Collagen molecules consist of three polypeptide chains, called pro-α chains (left-handed helix), which are wrapped around each other and form right-handed superhelix (triple-helix) called protocollagen. The final structure is a rope-like rod (Prockop and Kivirikko, 1995). All three pro-α chains are identical or even three different pro-α chains can contribute to protocollagen formation (Marshall et al., 1993). A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of these collagen subunits. The primary structure consists of a repetitive sequence (*Gly-X-Y*)n, where every third amino acid is glycine, X is often alanin or proline and Y is frequently hydroxyproline (Ihanamäki et al., 2004). Collagen pro-α chains are synthesized in the rough endoplasmic reticulum and a number of post-translational modifications like hydroxylation and glycosylation are involved in their synthesis before they associate into triple-helix formation (protocollagen), where they have propeptide extensions at both their N- and C- terminal ends (Michelacci, 2003). The protocollagen molecules are transported through Golgi apparatus to the extracellular matrix, and during this transport they aggregate laterally (Bonfanti et al., 1998). After secretion the N and C propeptides are cleaved by specific proteinases (Prockop et al., 1998). The collagen molecules assemble spontaneously into fibrils (10 – 300 nm), which are stabilized by covalent crosslinks and then into collagen fibers (0.5 – 3 μm), (Kadler et al., 1996; Smith-Mungo and Kagan, 1998).

Mutations in the distinct collagen genes cause a lot of human disorders; for example osteogenesis imperfecta, osteoporosis, chondrodysplasias, Ehlers-Danlos syndrome and others (rewieved in Prockop and Kivirikko, 1995). In the autosomal

recessive or X-linked Alport syndrome mutations in α3, α4, α5 and α6, collagen IV chains were detected (Lemmink et al., 1997). This disease is tightly connected with posterior polymorphous corneal dystrophy (Ihanamäki et al., 2004). Moreover, changes in collagen IV localization were described in keratoconus and bullous keratopathy but without mutation in distinct genes (Deng et al., 2001; Kenney et al., 1997, Ljubimov et al., 1996; Tuori et al., 1997).

Corneal collagens

At least 12 different types of collagens are expressed in the normal human cornea (Tab. 2), where they constitute more than 70% of the dry weight.

Localization in the human cornea	Collagen type
BME	IV
	VI
	VII
	XII
	XVIII
Bowman layer, stroma	I
	III
	IV
	V
	VI
	VII
	XII
	XIII*
	XV
	XVIII°
DM	IV
	V
	VIII
	XVIII

Table 2: Collagens expression and localization in the healthy human cornea (* only in 2/3 of the posterior stroma, ° only in the Bowman layer), (according to Jirsova et al., 2008).

The major structural component of the BME is collagen IV (Hudson et al., 1993; Kefalides, 1973; Ljubimov et al., 1995). To date, six α chains of collagen IV (α1 – α6) have been identified (Leinonen et al., 1994). Trimers of α1 (IV) and α2 (IV) chains ([α1]2α2 and [α1]3) are ubiquitous and form the major component of BMs, whereas chains α3 (IV), α4 (IV), α5 (IV) and α6 (IV) represent only a minor component of BMs with restricted tissue distribution (Sanes et al., 1990; Timpl, 1989). The BMEs of the cornea and conjunctiva contain different types of collagen IV chains. Whereas the α5 (IV) chain is present in the corneal BME, the α2 (IV) chain is present in the conjunctival BME (Kabosova et al., 2007; Ljubimov et al., 1995; Tuori et al., 1997). Type IV collagen

provides not only an architectural network, but the different NC1 domains of its α chains also regulate cellular behavior including antiangiogenic or antitumor properties (Floquet et al., 2004). It has been shown that the α1 and α2 chains of collagen IV favor cell migration, whereas the α3 chain limits the invasive phenotype (Ortega and Werb, 2002). Among collagen IV a collagen VII and XVIII were detected in human BME. Collagen VII forms anchoring fibrils which penetrate the BME and course into the stroma, where they form anchoring plaques together with collagen I (Gipson et al., 1987).

Collagen fibers in normal human corneal stroma are rich for collagen I, but also contain relatively large amounts of collagen V and VI (Lee and Davison, 1984; Marshall et al., 1991; Newsome et al., 1981). Collagen VI is a non-fibrillar collagen, which forms by lateral aggregation special 'microfibrillar' structures between other fibrils, due to can participate in their stabilization (Lee and Davison, 1984; Zimmermann et al., 1986). Collagen III is present in low proportions in the corneal stroma but increases during wound healing, inflammation and several other pathological conditions. Moreover collagens IV, XII, XIII and XIV were described in corneal stroma (Ljubimov et al., 1995; Michelacci, 2003; Sandberg-Lall, 2000).

The major structural component of the DM is collagen VIII, which forms a hexagonal lattice on the anterior part of DM (Sawada et al., 1990). Type VIII collagen is a non-fibrillar, short chain collagen. It consists of two collagenous polypeptides, α1 (VIII) and α2 (VIII) chains, which form two distinct homotrimeric or heterotrimeric proteins (Greenhill et al., 2000; Illidge et al., 2001). Its exact function, apart from its structural properties (in DM it creates a matrix underneath the endothelial cells that can resist compression while maintaining an open porous structure), is still unclear; however, studies suggest its importance in cell differentiation and determining endothelial cell phenotype (Sage, 1987; Shuttleworth, 1997; Yamaguchi et al., 1989). Moreover, it was suggested that collagen VIII may play a role as a peri- or subcellular matrix environment that permits or stimulates cell proliferation (Hopfer et al., 2005). Among collagen VIII, collagens IV, V and VI were detected in DM (Marshall, 1991).

In human corneal epithelium no collagens were detected and in corneal endothelial cells only collagen VIII was present (predominantly α1 chain), (Gottsch et al., 2005).

2.7 Corneal dystrophies

According to the traditional classification which is based on the anatomic location of major changes, three groups of corneal dystrophies are recognized: anterior corneal dystrophies affecting the epithelium and Bowman layer, stromal dystrophies and posterior dystrophies affecting the DM and the endothelium (Aldave and Sonmez, 2007; Pieramici and Afshari, 2006). Endothelial corneal dystrophies result from primary endothelial dysfunction leading to corneal opacifications and oedema. This group includes Fuchs endothelial corneal dystrophy (FECD), posterior polymorphous corneal dystrophy (PPCD), congenital hereditary endothelial dystrophy (CHED) and X-linked endothelial corneal dystrophy (XECD), (Tab. 3). All are thought to represent defects of neural crest terminal differentiation (Bahn et al., 1984). Common features present in this group of dystrophies include altered morphology of endothelial cells, secretion of an abnormal posterior collagenous layer (PCL) in the posterior side of DM and consequent corneal decompensation (Levy et al., 1996a).

Posterior dystrophy	Inheritance	OMIM	Locus/gene
Fuchs endothelial corneal dystrophy (FECD)			
FECD1 (early-onset)	AD	136800	1p34.3-p32/ *COL8A2*
FECD2 (late-onset)	AD	610158	13pter-q12.13/ Unknown
FECD3 (late-onset)	AD	613267	18q21.2-q21.32/ Unknown
FECD4 (late-onset)	AD	613268	20p13-p12/ *SLC4A11*
FECD5 (late-onset)	AD	613269	5q33.1-q35.2/ Unknown
FECD6 (late-onset)	AD	613270	10p11.2/ *ZEB1*
FECD7 (late-onset)	AD	613271	9p24.1-p22.1/ Unknown
Posterior polymorphous corneal dystrophy (PPCD)			
PPCD1	AD	122000	20p11.2/ Unknown
			20p11.21/ *VSX1*
PPCD2	AD	609140	1p34.3-p32/ *COL8A2*
PPCD3	AD	609141	10p11.2/ *ZEB1*
Congential hereditary endothelial dystrophy (CHED)			
CHED1	AD	121700	20p11.2/ Unknown
CHED2	AR	217700	20p13-p12/ *SLC4A11*
Harboyan syndrome	AR	217400	20p13-p12/ *SLC4A11*
X-linked endothelial corneal dystrophy (XECD)			
XECD	X-linked	300779	Xq25/ Unknown

Table 3: Summary of posterior dystrophies with their inheritance (AD – Autosomal dominant, AR – Autosomal recessive), OMIM number (Online Mendelian Inheritance in Man) and genes implicated in the dystrophy (p – short arm, q – long arm).

2.7.1 Fuchs endothelial corneal dystrophy

FECD is the commonest, progressive, bilateral degeneration of the cornea. It has been estimated that 4% of the population over the age of 40 is affected. Posterior excrescences known as "*cornea guttata"*, prominating through the corneal endothelium to the anterior chamber are characteristic features of this dystrophy. In these prominences a collagen IV and VIII were detected (Levy et al., 1996b; Merjava, unpublished data), (Fig. 2). Transmission and scanning electron microscopy revealed abnormal structures in some endothelial cells in FECD, due to which it was suggested that endothelial cells here may undergo fibroblastic or, rarely, epithelial metaplasia (Hidayat and Cockerham, 2006; Waring et al., 1978). The occurrence and reactivity of pancytokeratin (AE1/AE3), CK7, 20 and CAM 5.2 (CK pair 8/18) antibodies in the abnormal endothelial cells supports this theory (Hidayat and Cockerham, 2006).

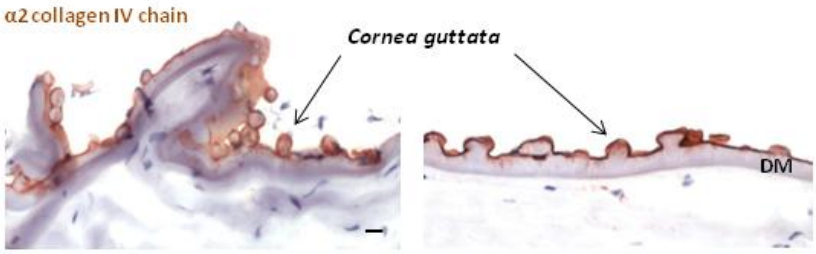

Figure 2: Excrescences known as "cornea guttata" positive for α2 collagen IV chain observed in the Fuchs endothelial corneal dystrophy on the Descemet membrane (DM), (S. Merjava, unpublished data). Scale bar represents 10 μm.

The exact mechanism of FECD pathophysiology remains unknown. An investigation of FECD using the serial analysis of gene expression (SAGE) in diseased tissue demonstrated a significant decrease in the expression levels of several genes associated with oxidation and apoptosis, and significantly higher levels of oxidative DNA damage and apoptosis in FECD endothelium were detected compared to controls, providing evidence that oxidative stress plays a key role in FECD pathogenesis (Jurkunas et al., 2010). Moreover, alterations in the aqueous humor proteome composition in FECD were reported (Richardson et al., 2010).

FECD is genetically heterogeneous, exhibiting an autosomal dominant inheritance pattern. Mutations in the gene encoding the α2 collagen VIII chain (*COL8A2;* OMIM *120252) on chromosome 1 have been found to cause a rare form of early-onset FECD, while the more common late-onset FECD has been localized to loci on chromosomes 9, 13, 18, 20 and 5 (Biswas et al., 2001; Gottsch et al., 2005; Liskova et al., 2007a; Sundin et al., 2006a; 2006b; Riazuddin et al., 2009). Moreover, five missense mutations were reported in the *ZEB1* gene (OMIM *189909) in a cohort of late-onset FECD patients. As *ZEB1* has been previously shown to underlie PPCD3 it has been suggested that PPCD and FECD are allelic variants of the same disease continuum, and that genetic interaction between genes that cause corneal dystrophies can modulate the expressivity of the phenotype (Riazuddin et al., 2010).

2.7.2 Posterior polymorphous corneal dystrophy

PPCD is a bilateral, autosomal dominant disorder affecting primarily the corneal endothelium and DM (Hogan and Bietti, 1969). The epithelization and proliferation of the pathologic endothelium in PPCD corneas are the most common findings at the cellular level, and it has been characterized in detail using light, electron and *in vivo* confocal microscopy (Boruchoff and Kuwabara, 1971; Krachmer, 1985; Patel et al., 2005). Epithelization was also confirmed by the detection of CKs, typical epithelial proteins, using whole antikeratin antiserum (Rodrigues et al., 1980).

2.7.2.1 Clinical findings

The first clinical description of PPCD is attributed to Koeppe in 1916 (Koeppe, 1916), however the first clinico-pathologic study was published by Morgan and Paterson in 1967. PPCD is characterized biomicroscopically (slit-lamp examination) by vesicular lesions (0.25 to 1 mm in diameter), bands and geographic opacities at the level of posterior DM and the endothelium (Cibis and Tripathi, 1982; Laganowski et al., 1991; Morgan and Paterson, 1967). Two general patterns of lesions: (1) a focal vesicular and band-shaped pattern; (2) a geographic pattern, were observed (Hirst and Waring, 1983). Using more detailed observations with specular microscopy (Fig. 3) small endothelial cells compacted together, normal-sized cells with a typical endothelial mosaic pattern, or abnormal endothelial cells that are enlarged and pleomorphic, have been observed (Hirst and Waring, 1983).

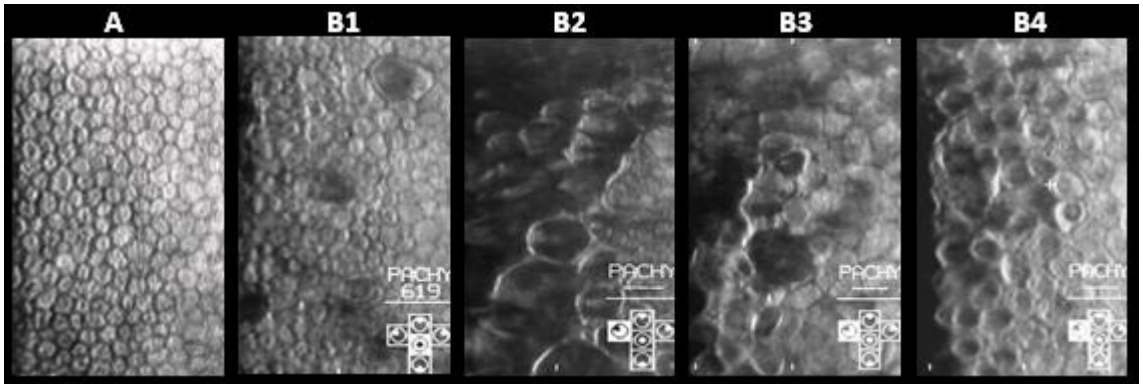

Figure 3: Specular microscopy: a monolayer of flat hexagonal cells of the healthy human cornea (A), abnormal endothelial cells and lesions typical for PPCD (B1-4). (Photos provided by MUDr. Liskova, archive of OTB VFN, Prague).

Although the disease has been reported to be non-progressive in most affected subjects, in some patients secondary changes such as corneal oedema or glaucoma may lead to visual impairment and necessitate surgical management such as keratoplasty and glaucoma surgery (Cibis et al., 1977). The aberrant endothelium grows over the trabecular meshwork blocking the iridocorneal angle, which may lead to iridocorneal adhesion, iris atrophy and increasing the intraocular pressure, thus

causing secondary glaucoma (Cibis et al., 1977; Krachmer, 1985). In most cases PPCD becomes symptomatic in middle age, however cases of PPCD manifestating as corneal oedema at birth, or within a few weeks after birth, have also been described (Héon et al., 2002; Levy et al., 1996a; Liskova et al., 2010).

2.7.2.2 Laboratory observations

The thickness of the corneal epithelium appears normal in most cases when examined by light microscopy; occasionally it shows evidence of intracellular oedema. The Bowman layer varies in thickness and thin fibrocellular tissue (fibrous pannus) between the epithelium and Bowman layer may be observed (Feil et al., 1997; Grayson, 1974). The corneal stroma shows the usual changes characteristic of oedema. DM is of irregular thickness (thinner or thicker) or shows a multilaminar pattern (Johnson and Brown, 1978). A large amount of wide-spaced collagen is deposited posterior to DM forming an abnormal PCL (Waring, 1982). Morphological characterization of the endothelial cells is typically not homogeneous. In some cases the endothelium is not present. It may also be discontinuous or arranged in more than one layer (two or three). The endothelial cells are enlarged, flattened or damaged and epithelial in appearance (Feil et al., 1997; Hogan and Bietti, 1969, Johnson and Brown, 1978).

As the significant pathological abnormalities are limited to the posterior cornea, examination using electron microscopy focused mainly on DM and the endothelium. Focal fusiform nodular protrusions of connective tissue were noted in DM. Also described was the formation of an abnormal PCL (Johnson and Brown, 1978; Waring, 1982). Collagens I, III – VI, VIII, laminin, tenascin and fibronectin were detected using immunoelectron microscopy in this PCL (Gottsch et al., 2005; Levy et al., 1995b). In patients with PPCD (as in the other endotheliopathies) three types of PCL (banded, fibrillar and fibrocellular) have been described (Waring, 1982). Epithelial-like abnormal endothelial cells with prominent microvilli, abundant keratofibrils, desmosomes and sparse microorganelles (all characteristics of epithelial cells) have been documented in many publications (Boruchoff and Kuwabara, 1971; Feil et al., 1997; Krachmer, 1985; Matsumoto et al., 1988; Richardson and Hettinger, 1985; Rodrigues et al., 1981). Other investigators have described fibroblast-like transformation of the endothelial cells characterized by dilated endoplasmic reticulum (Johnson and Brown, 1978; Waring, 1982). Epithelization of the endothelial cells in PPCD was confirmed in cell cultures, where the outgrowth of the PPCD explants began within 24 – 48 hours, compared to control endothelium which required 4 – 6 days (Rodrigues et al., 1981).

2.7.2.3 Inheritance and models of PPCD

The transmission in most of the families is autosomal dominant (Cibis et al., 1977). At least four different genes are implicated in PPCD, of which three are known. PPCD1 is linked to chromosome 20, and the visual system homeobox gene 1 (*VSX1*; OMIM *605020) was reported as disease-causing (Héon et al., 1995; 2002). Evidence

also exists that in the linked families another undiscovered PPCD gene at 20p11.2 is implicated, suggesting possible microheterogeneity (Aldave et al., 2009; Gwilliam et al., 2005). PPCD2 and some forms of FECD are caused by the gene encoding the α2 collagen VIII chain (*COL8A2*; OMIM *120252) on chromosome 1 (Biswas et al., 2001). Finally, the human zinc finger E-box binding homeobox 1 gene (*ZEB1* also known as *TCF8*; OMIM *189909) on chromosome 10 is implicated in PPCD3 (Krafchak et al., 2005; Liskova et al., 2007b; Vincent et al., 2009).

Two animal models for PPCD have been described. ZEB1 heterozygous and null mice showing a part formed multilayered endothelium as a result of proliferation, ectopic expression of epithelial genes in this layer and in keratocytes, including the basement membrane component α3 collagen IV chain, corneal thickening, and corneolenticular and iridocorneal adhesion (Liu et al., 2008). Most recently, a spontaneous mutant named "PPCD1 mouse" has been described with similar features as those observed in human PPCD corneas. Locus for this PPCD1 mouse phenotype was mapped to a 6.1 Mbp interval on chromosome 2, which is syntenic to the human chromosome 20 PPCD1 interval. Duplication leading to haploinsufficiency of *CSRP2BP* (Cysteine-rich protein 2-binding protein) gene has been postulated to be responsible for the mouse PPCD1 phenotype and it has been suggested that *CSRP2BP* haploinsufficiency may lead to human PPCD1 as well (Shen et al., 2010).

2.7.2.4 Prevalence and treatment

The exact prevalence of PPCD is unknown. Based on published data it can be estimated that there are approximately 300 – 400 patients with this disorder worldwide. More than a hundred PPCD patients come from the Czech Republic (prevalence 1/100 000), most of them originating geographically from an area around the town of Klatovy (Fig. 4).

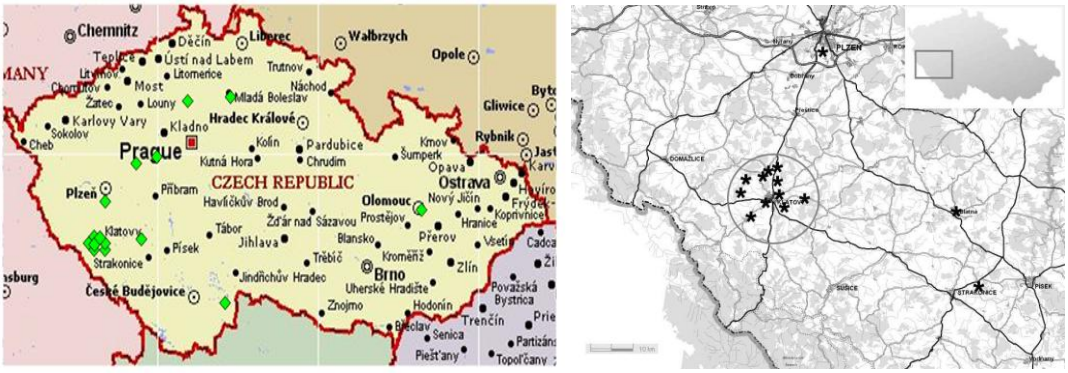

Figure 4: Occurrence of PPCD patients in the Czech Republic. Geographical origin of 14 families from the west and southwest area of the Czech Republic. 11 families originate geographically from an area around the town of Klatovy (20 km in diameter), (Maps provided by MUDr. Liskova).

In Czech patients PPCD is generally more progressive and secondary changes like corneal oedema and secondary glaucoma, which may further lead to total blindness, are observed in a large percentage of them. Penetrating keratoplasty is the gold standard treatment for patients with decreased visual acuity and symptoms associated with corneal oedema. Newer lamellar transplant techniques, however, are beginning to replace full thickness cornea transplants. These innovative treatments include deep lamellar endothelial keratoplasty and more recent Descemet membrane stripping endothelial keratoplasty (Pieramici and Afshari, 2006; Studeny et al., 2010).

The disease may recur rarely in the graft. The first report on the recurrence of PPCD after penetrating keratoplasty was published by Boruchoff et al. (1990). A few years later Sekundo et al. (1994) described repopulation of the posterior surface of three donor corneas by the host pathological endothelium using light and electron microscopy.

2.7.2.5 Cytokeratins and collagens in PPCD corneas

Rodrigues et al. (1980) were the first who detected CKs in the unusual abnormal epithelial-like cells in the corneal endothelial layer of patients suffering from PPCD (Rodrigues et al., 1981). The detection of epithelial membrane antigen, pancytokeratin, CKs AE1/AE3, CK7, 20 and CAM 5.2 (CK8 and 18) together with vimentin followed (Cockerham et al., 2002; Moroi et al., 2003). Only a diffuse positivity was observed for monoclonal antibody 2B4.14.1; a protein typical for normal human corneal endothelium (Feil et al., 1997; Ross et al., 1995). These data suggest that the corneal endothelium in patients with PPCD undergoes a metaplasia from a normal phenotype to a transitional phenotype and in the end to an abnormal epithelial phenotype (Ross et al., 1995). Changes in the epithelium of PPCD patients when compared to control epithelial cells for previously mentioned antibodies were not detected (Cockerham et al., 2002).

A detailed characterization describing changes in the collagen composition in corneas of patients suffering from PPCD has not been published. Only an ectopic expression of the α3 collagen IV chain in pathological endothelium was found in one PPCD patient with a known disease-causing mutation in the *ZEB1* gene, as well as in the abnormal endothelium of *ZEB1* null mice (Krafchak et al., 2005; Liu et al., 2008).

2.7.3 Congenital hereditary endothelial dystrophy

CHED is a rare, bilateral corneal disorder manifesting as cloudy corneas at birth or in early childhood. Histological features of CHED-affected corneas include diffuse epithelial and stromal oedema, defects in the Bowman layer, a degenerated corneal endothelium with multinucleated cells or melanin deposits, and a thickened DM (Ehlers et al., 1998; Kirkness et al., 1987; Mullaney et al., 1995; Rodrigues et al., 1975). Signals for pancytokeratin AE1, cytokeratin CAM 5.2 (CK pair 8/18) and CK7 antibodies were detected in the abnormal endothelial cells of CHED patients (Cockerham et al., 2002). Autosomal dominant (CHED1; OMIM #121700) or recessive (CHED2; OMIM #217700) forms are known (Rodrigues et al., 1995). Autosomal dominant CHED1 was mapped to the pericentromeric region of chromosome 20, a region that lies within the region linked to PPCD1 (Toma et al., 1995). The locus for CHED2 has been mapped to chromosome 20p13 and mutations within the sodium bicarbonate transporter-like protein 11 gene (*SLC4A11;* OMIM *610206 *)* were identified as disease-causing (Hand et al., 1999; Mohamed et al., 2001; Vithana et al., 2006). CHED may be associated with deafness (Harboyan syndrome), nystagmus and congenital glaucoma (Abramowicz et al., 2002; Desir et al., 2007; Pedersen et al., 1989).

2.7.4 X-linked endothelial corneal dystrophy

XECD (OMIM #300779) is the most recently described endothelial corneal dystrophy. The disease has been identified in a single large Austrian pedigree. An X-linked inheritance pattern was suggested and linkage to a 4.73-centimorgan region on Xq25 was demonstrated. Resembling moon craters in the endothelium, corneal opacification, oedema and subepithelial band keratopathy were described in male or female, respectively (Schmid et al., 2006).

3 MATERIAL AND METHODS

3.1 Material used

The study followed the ethical standards of the Ethics Committee of the General Faculty Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki.

3.1.1 Control tissues

In total 25 corneo-scleral discs (11 – 17 mm in diameter, 16 male and 9 female, aged from 16 to 82, mean age of 57.6 ± 19.2 years), not acceptable for transplantation because of a positive serology of the donor or their endothelial quality, two trabecular meshwork (TM) specimens, one anterior segment and, additionally, two Descemet membrane-endothelium lamellae with a stromal rim (Studeny et al., 2010), were used. The time between death and storing in liquid nitrogen did not exceed more than 24 hours as standard. All samples were obtained from the Ocular Tissue Bank Prague, General Faculty Hospital, Prague, Czech Republic. Cadaverous tissues obtained from the Institute of Forensic Medicine and Toxicology, General Faculty Hospital and First Faculty of Medicine, Prague, were used as positive controls.

3.1.2 Pathological tissues

Collection of our PPCD patients reaches 16 cases (7 men and 9 women; mean age 39.9 ± 20.1 years at the time of their first keratoplasty). All patients were included in analysis of corneal surviving after first penetrating keratoplasty. All but one eye requiring corneal transplantation developed stromal and epithelial oedema. None of the examined patients suffered from secondary glaucoma or any other ophthalmic disorder. Three of the PPCD patients exhibited a keratoconus pattern on videokeratography (without the typical thinning of the cornea); three had bullous keratopathy. Two corneas were obtained from members of the family previously mapped to 20p11.2 (Gwilliam et al., 2005), while nine were from seven members of five families originating in the same small geographic area within the Czech Republic (Fig. 5). Preliminary unpublished genotyping data in these families also suggest linkage to the PPCD1 locus. One cornea was from a patient with an identified disease-causing mutation in the *ZEB1* gene (Liskova et al., 2007b). The diagnosis of PPCD was based on the presence of characteristic bilateral vesicular lesions, bands and geographic opacities observed on slit-lamp microscopy together with positive family history.

Four of our patients were re-transplanted after first penetrating keratoplasty (two men and two women; mean age 40.8 ± 18.0 years at the time of re-operation). The time to removal of the original donor button upon re-operation ranged from 3 to 8 years, and the main indication for re-operation was endothelial rejection, decompensation of the graft and recurrence of PPCD. Fortunately, one of these four

patients was sex-mismatched, i.e. the donor cornea was gained from a person of a different gender, so that we were able to use indirect fluorescent immunohistochemistry and consequently fluorescence in situ hybridization (FISH) analysis of the sex chromosomes.

One TM specimen was obtained during glaucoma surgery performed on an eye two years after keratoplasty in a patient with PPCD. All pathological corneal explants and TM specimen were obtained from the Department of Ophthalmology, First Medical Faculty of Charles University and General Faculty Hospital in Prague.

3.2 Preparation of the samples

Eye balls of the control donors were dried and corneo-scleral discs (with limbus and conjunctiva) were incised using trepan (11 – 12 mm in diameter) (Fig. 5). Corneal discs were dissected and used for the preparation of cryosections, imprints on Millicell or Supor membranes and paraffin-embedded sections. Pathological corneal explants 7 – 8 mm large were used for cryosections only.

Figure 5: Trepanation of corneo-scleral disc from whole globe.

3.2.1 Cryosections

Tissues (corneas, TM and other tissues) for cryosection preparation were dissected, snap frozen in liquid nitrogen, embedded in an Optimal Cutting Temperature Compound and stored at -70°C. Tissue slices 7 µm thick were cut radially to evaluate tissue from the central to the peripheral parts of the cornea, or as tangential sections of the limbus, allowing evaluation of the complex architecture of the limbal crypts (for the characterization of the limbal area only), (Chen et al., 2004). Slides containing four slices per slide were kept under -20°C until they were used for indirect fluorescent or enzymatic immunohistochemistry.

3.2.2 Millicell membranes and Supor membranes

Corneal epithelium, endothelium, conjunctival epithelium and peritoneal cells were used for impression cytology on Biopore Millicell membranes (MILLICELL® - CM, PICM 01250, Millipore, Bedford MA) or Supor® - 200 membranes (PALL Corp., Michigan, USA). The membranes were used for indirect fluorescent immunocytochemistry (Millicell and Supor), RT-PCR, qRT-PCR (RNA was isolated from cells on the Millicell membranes) and Western blot (proteins were obtained from the Millicell membranes). The membranes were kept under -70°C.

3.3 General techniques used

3.3.1 Histochemistry

Slides from each control and PPCD patients were stained with common haematoxylin and the eosin method for morphological assessment by light microscopy.

3.3.2 Fluorescent immunohistochemistry and immunocytochemistry

After fixation in cold acetone, rinsing in phosphate buffered saline (PBS), permeabilization in TRITON X-100 (only in case of immunocytochemistry) and blocking in blocking solution, the slides and membranes were incubated with primary antibodies diluted in PBS containing bovine serum albumin (BSA) for 1 hour at room temperature. The primary antibodies which were used are in appended table (Tab. 4). After washing in PBS the slices were incubated with appropriate secondary antibodies; fluorescein isothiocyanate-conjugated (FITC) and rhodamine-conjugated (TRITC) anti-mouse or anti-goat IgG (all from Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 hour at room temperature. After rinsing in PBS the slices were mounted with Vectashield - propidium iodide or 4.6-diamidino-2phenylindoldihydrochlorid (DAPI) (Vector Laboratories, Inc. Burlingame, USA) to counterstain the DNA within the nuclei.

Individual alterations in the immunohistological protocols are mentioned and described in detail in appended publications, and were mostly done according to manufacturer instructions.

3.3.3 Double-staining on radial and tangential sections

Double-staining was performed on six different corneo-scleral samples. A mixture of mouse anti-CK8 antibody with goat anti-CK3, anti-CK15, anti-integrin α6 and anti-vimentin antibodies (Tab. 4) was applied to the sections in one step, followed by a mixture of FITC-conjugated donkey anti-mouse IgG and TRITC-conjugated donkey anti goat IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA). All antibodies were diluted only in PBS as described by manufacturers.

Antigen	Company	Clone	Antibody type	Dilution
calbindin 2 (calretinin)	Santa Cruz	N-18	Goat	1/75
cytokeratin 1	Santa Cruz	N-20	Goat	1/250
cytokeratin 2	Acris	Ks 2.342.7.4	Mouse (Ms)	1/50 - 1/10
cytokeratin 3	Santa Cruz	C-14	Goat	1/50
cytokeratin 3/12	RDI	AE5	Ms	1/400
cytokeratin 4	Sigma-Aldrich	6B10	Ms	1/200
cytokeratin 5	Santa Cruz	RCK103	Ms	1/400
cytokeratin 5/6	Dako	D5/16B4	Ms	1/40
cytokeratin 6	Santa Cruz	LL020	Ms	1/20
cytokeratin 7	Dako	OV-TL12/30	Ms	1/50
cytokeratin 8	Millipore	4.1.18	Ms	1/400
cytokeratin 8/18	Novocastra	5D3	Ms	1/75
cytokeratin 9	Acris	multi-epitope cocktail	Ms	1/50 - 1/15
cytokeratin 10	Santa Cruz	RKSE60	Ms	1/50
cytokeratin 10/13	Dako	DE-K13	Ms	1/25
cytokeratin 14	Chemicon	LL002	Ms	1/50
cytokeratin 15	Chemicon	LHK15	Ms	1/100
cytokeratin 15	Santa Cruz	A-13	Goat	1/400
cytokeratin 16	Acris	LL025	Ms	1/30
cytokeratin 17	Acris	Ks 17.E3	Ms	1/25
cytokeratin 18	Dako	DC10	Ms	1/50
cytokeratin 18	Sigma	CY-90	Ms	1/800
cytokeratin 19	Dako	RCK108	Ms	1/50
cytokeratin 20	Acris	Q2	Ms	1/50 - 1/15
HBME-1	Dako	HBME-1	Ms	1/50
integrin α6	Santa Cruz	C-18	Goat	1/40
mesothelin	Santa Cruz	K1	Ms	1/50
vimentin	Santa Cruz	C-20	Goat	1/100

Table 4: Primary antibodies used for fluorescent immunohisto- and immunocytochemistry.

3.3.4 Enzymatic immunohistochemistry and immunocytochemistry

After fixation in cold acetone, rinsing in PBS or Tris buffered saline, permeabilization in TRITON X-100 (only in case of immunocytochemistry) and blocking in blocking solutions, slides and membranes were incubated with primary antibodies for 1 hour at room temperature (Tab. 5). After washing, the slices were incubated with appropriate biotinylated secondary antibodies and then the tertiary complex was applied. In the case of the UltraTech HRP AEC kit (streptavidin/horseradish peroxidase), (Immunotech, France), the staining was visualized by 3-amino-9-ethylcarbazol (AEC), (Immunotech). In the case of SABcomplex/AP (alkalic phosphatase), (DakoCytomation, Glostrup, Denmark), the staining was detected by a mixture of naphtol, levamizol, Fast Red and veronal acetate buffer (all from Sigma, St.

Louis, USA). After rinsing the slides were counterstained with Harris haematoxylin and mounted in an Aquatex medium (Merck KGaA, Germany).

Individual alterations in immunohistological protocols are mentioned and described in detail in the appended publications and were done according to manufacturer instructions.

Antigen	Company	Antibody type	Dilution
calbindin 2 (calretinin)	Santa Cruz	Goat	1/75
collagen IV α1 chain	Wielisa	Mouse (Ms)	1/50
collagen IV α3 chain	Wielisa	Ms	1/50
collagen IV α5 chain	Wielisa	Ms	1/50
collagen IV α2 chain	Chemicon	Ms	1/300
collagen IV α4 chain	Dr. Sado, Japan	Rat	1/25
collagen IV α6 chain	Dr. Sado, Japan	Rat	1/50
collagen VIII α1 chain	Dr. Greenhill, New Zealand	Ms	1/15
collagen VIII α2 chain	Dr. Greenhill, New Zealand	Ms	1/1000
cytokeratin 8	Millipore	Ms	1/400
cytokeratin 8/18	Novocastra	Ms	1/75
cytokeratin 18	Dako	Ms	1/50
cytokeratin 18	Sigma	Ms	1/800
HBME-1	Dako	Ms	1/50
mesothelin	Santa Cruz	Ms	1/50

Table 5: Primary antibodies used for enzymatic immunohisto- and immunocytochemistry.

3.3.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells on Millicell membranes using an Rneasy Plus Microkit (Qiagen, Hilden, Germany) or by means of TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA were reverse transcribed into cDNA using SuperScript II Reverse Transcriptase or SuperScript III/RNase OUT Enzyme Mix according to the manufacturer's instructions (Invitrogen, Carlsbad, USA) on MyCycler (BIO-RAD, CA, USA). Subsequently, equal amounts of cDNA were amplified with the specific oligonucleotides for CK8, CK18, the houskeeping gene glyceraldehyde-3-phospate dehydrogenase (GAPDH) and β-actin. The number of PCR cycles for the different primer pairs, the protocol for each cycle and primers sequences are described in detail in the appended publications.

3.3.6 Western blot

After lysis of cells in the lysis buffer (0.2% Triton X-100, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and protease inhibitors in PBS) the protein concentration was determined using the commercial BCA Protein Assay Kit (Pierce, Rockford, USA) and absorbance was measured on the Helios Gamma spectrophotometer (Thermo

Spectronic, UK). After SDS-poly-acrylamide electrophoresis (Laemmli, 1970), the proteins were transferred to nitrocellulose membranes (Serva Electroforesis GmbH, Heidelberg, Germany) and probed with primary antibodies against CK8, CK18, mesothelin, calbindin 2 and β-actin. The secondary ImmunoPure® Peroxidase conjugated antibody (Pierce Biotechnology, Rockford, USA) was applied and positive reactions were visualized using an enhanced chemiluminescent technique with the SuperSignal® West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology) and a Syngene membrane documentation system Chemigeniius-Q and GeneSnap program (Synoptics Ltd., Cambridge, UK). Individual protocols and antibody dilutions are described in detail in the appended publications.

3.3.7 Fluorescence in situ hybridization of the sex chromosomes

Detection of gonosomes was performed immediately after immunohistochemical staining with the CK19 antibody and all signals were evaluated simultaneously using a quadrate bandpass DAPI/FITC/ORANGE/AQUA filters (360/490/560/426 nm).

After fixation and rinsing in increasing ethanol grade the detection of gonosomes was performed. Directly labeled α satellite VYSIS DNA probes CEP X (DXZ1) Spectrum Aqua Probe and CEP Y (DYZ3) Spectrum Orange Probe (both from Abbott, Des Plaines, IL, USA) were used and the reaction was run on a Thermobrite (Abbott Molecular, Des Plaines, IL, USA). Cell nuclei were counterstained with DAPI (Vectashield, Vector Laboratories, Inc. Burlingame, USA). The hybridization protocol and washing procedures are described in detail in the appended publications.

3.3.8 Microscopic techniques

The specimens were examined by light and fluorescent microscopy using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) and Zeiss AX10 Imager Z1 microscope (Carl Zeiss GmbH, Jena, Germany) at a magnification of 100 – 1000x. Images were taken using a Vosskühler VDS CCD-1300 camera, (VDS Vosskühler GmbH, Germany), a CCD ProgRes MF camera and a JENOPTIK ProgRes C12plus camera (both from Jenoptik, Laser Optik Systeme GmbH, Jena, Germany). A LUCIA 4.8, NIS Elements image analysis system (Laboratory Imaging, Czech Republic) and photo software Isis MetaSystem (MetaSystem, Altlussheim, Germany) were used for picture analysis. The percentage of positive cells was calculated. The intensity of cell staining was graded using a scale in accordance with Diebold et al. (1997).

4 RESULTS

4.1 Characterization of corneal, limbal and conjunctival epithelium

The most intense staining present throughout the corneal epithelium was observed for CK3, CK5 and CK14; CK19 was found only at the corneal periphery. CK4 and CK10/13 revealed mild to moderate positivity, mostly in the superficial layers of the corneal epithelium. The suprabasal cell layers of all examined areas showed a strong positivity for CK16. A heterogeneous staining pattern with a centrifugal decrease in signal was observed for CK8 and CK18. CK5/6, CK14 and CK19 were present in the limbus, where a positive signal for CK3 was observed in the suprabasal and superficial cells only. CK15 appeared in the basal and suprabasal layers of the limbus. The perilimbal conjunctiva showed strong immunostaining for CK10/13, CK14 and CK19. A moderate signal for CK7 was detected in the superficial layers of the conjunctiva, as well as mRNA for CK7 was found in conjunctival epithelium using semi-quantitative RT-PCR. qRT-PCR confirmed CK6 and CK18 expression in the corneal and conjunctival epithelium (all results from qRT-PCR were obtained by Mgr. Ales Neuwirth, Institute of Molecular Genetics AS CR, Prague). None of the detected CKs were expressed by keratocytes in the stroma. For more detailed results, please see appended publication 1.

4.2 Detection of CK8 in the limbal basal cells

We have detected CK8 positivity in limbal basal cells in 60% of the cadaverous corneo-scleral samples. Positive basal cells formed a single line or separated clusters. The signal for CK8 became weaker towards the surface of the limbal epithelium. The central corneal epithelium was positive for CK8 predominantly in the superficial and suprabasal layers, but some heterogeneous positivity was detected in the basal layer of several samples as well. In each specimen that contained positive limbal basal cells, the epithelium of the cornea was positive as well. Similarly, in most specimens in which CK8 was absent from the limbal basal cells, the epithelium of the central cornea was negative as well.

Colocalization of CK8 with vimentin and CK15 in the limbus was also found. CK3 showed only occasional positivity in some of the surface limbal cells. The expression of integrin α6 in the basal membrane was absent or decreased under the CK8-positive clusters. CK8 expression in the cornea, limbus and conjunctiva was confirmed using RT-PCR. For more detailed results, please see appended publication 2.

4.3 Detection of CK8 and CK18 in the healthy human endothelium

Approximately 50% of the corneal endothelial cells were positive for CK8 (Chemicon), CK18 (Sigma) and the CK pair 8/18 (Novocastra) in the endothelium when acetone was used for fixation. Four and 52% CK18-positive cells were observed using immunofluorescent and enzymatic immunohistochemistry, respectively, when the CK18 antibody provided by Dako was used. No signal was detected when 4% formalin or 10% paraformaldehyde was used as a fixative, irrespective of the antibody used.

CK8 and CK18 proteins and mRNAs were detected in the endothelium of all tested corneas by Western blot or semi-quantitative RT-PCR, respectively. For more detailed results, please see appended publication 3.

4.4 Detection of mesothelial markers (mesothelin, calbindin 2, HBME-1 protein) in the healthy human cornea

A strong signal for mesothelin was present in the corneal epithelium, while less intense staining was visible in the endothelium. Similarly, higher and lower mRNA levels were detected using qRT-PCR in the corneal epithelium and endothelium, respectively. HBME-1 antibody strongly stained the corneal endothelium and stromal keratocytes. A marked positivity was present in the corneal stromal extracellular matrix, while no staining was present in the sclera. Calbindin 2 was detected using immunohistochemistry and Western blot in the corneal epithelium, endothelium and stroma. Both cytoplasmic and nuclear staining for calbindin 2 were clearly visible on the epithelial and endothelial imprints. Intranuclear dots, probably representing an association with kinetochor and polar microtubules, were more readily detectable in superficial epithelial cells than in endothelial cells. qRT-PCR confirmed calbindin 2 expression in epithelial and endothelial cells (all results from qRT-PCR were obtained by Mgr. Ales Neuwirth, Institute of Molecular Genetics AS CR, Prague). For more detailed results, please see appended publication 4.

4.5 Cytokeratin expression in PPCD samples

All used PPCD corneal specimens display areas of typical endothelial morphology, as well as areas consisting of two to six cell layers thickness with both flat endothelial-like cells and epithelial-like polygonal cells with round nuclei and a large cytoplasm. Both of these morphologically distinct cell types showed strong immunostaining for CK7, CK19, CK8 and CK18, while weaker positive signals were observed for CK1, CK3/12, CK4, CK5/6, CK10, CK10/13, CK14, CK16 and CK17. PPCD endothelium was completely negative for CKs 2e, 9, 15, and 20. Focal positivity was detected in PPCD trabecular meshwork for CK4, 7 and 19. CK8 and CK18 were the only CKs expressed in the control endothelium. PPCD and control corneal epithelium displayed similar staining patterns. A distinct positivity for CK3/12, 4, 5/6, 10/13, 14, 16 and 17 was

observed in aberrant PPCD endothelium for the first time. For more detailed results, please see appended publication 5.*

4.6 Collagen expression in the PPCD samples

More than 50% of the PPCD specimens exhibited positivity for α1 and α2 collagen IV chains in the BME and the posterior stroma, while no staining was detected in these areas in control specimens. The signal for the α1 and α2 collagen IV chains was more intense in DM of PPCD corneas compared to controls and it was shifted from the stromal side (in control tissue) to the endothelial side of DM (in the patients). A less intensive signal in PPCD corneas for the α3 and α5 chains in DM and an accumulation of α3, α4 and α5 in the posterior stroma in diseased corneas were the only differences in staining for the α3 – α6 collagen IV chains. The α1 collagen VIII chain was detected on both the endothelial and stromal sides of DM in 90% of patients with PPCD, compared with a prevailing localization on the stromal side of DM in control corneas. A change in the localization of the α2 collagen VIII chain in DM from vertically striated features in control specimens to double line positivity in the DM of PPCD corneas and positive staining in the PCL of four patients were also detected. In three PPCD patients a fibrous pannus (abnormal layer located between the BME and Bowman layer), positive for α1, α2, α3, α5 collagen IV chains and α1 collagen VIII chain, was observed. For more detailed results, please see appended publication 6.*

4.7 Detection of CK19 and X and Y chromosomes in endothelial cells of PPCD patient after repeat penetrating keratoplasty

The pathological endothelium of the failed PPCD explant revealed strong positivity for CK19 using indirect fluorescent immunohistochemistry. In most CK19-positive cells, both X and Y chromosomes were simultaneously detected using FISH. The results clearly showed that the original abnormal endothelial cells of the patient (XY), had, within 3.5 years, totally overgrown the posterior corneal surface of the graft (XX). For more detailed results, please see appended publication 7.

* Results published in Diploma thesis: S. Merjava, "*The characterization of the corneal changes of posterior polymorphous corneal dystrophy patients*", 2007. Faculty of Science, Charles University, Prague. (in Czech)

5 DISCUSSION AND CONCLUSIONS

The work and aims of my PhD thesis were predominantly addressed at describing the changes which occurred in patients suffering from PPCD. To do that the healthy human cornea had to be characterized first to have the possibility of comparing the situation under normal and pathological circumstances.

5.1 Phenotypical characterization of the healthy human cornea

Our results show that the control corneal, limbal and conjunctival epithelium express a wide spectrum of cytokeratins and that the corneal epithelium can be characterized as a primary nonkeratinizing stratified epithelium (no CK10 and weak CK1 positivity, strong CK3, 4, 5, 13 and 14 positivity), however the weak expression of some simple epithelial CKs (CKs 8 and 18) was also observed. Moreover, we are reporting for the first time the presence of CK6 in the corneal epithelium, which was confirmed using qRT-PCR as well (*please, see appended publication 1*).

Interestingly we have shown a high expression of CK8 in the basal cells of the control limbus as well, which is retained during differentiation and migration of the limbal cells to the central cornea. It is very difficult to identify LESCs because no direct methods have been established up to now; similarly, no specific molecular markers have been discovered. Although CK expression alone is not sufficient to identify stem cells or progenitor TACs, the expression profile of several key cytokeratins (CK19, CK15) together with other known potential markers (ABCG2, p63, vimentin etc.) can be used for LESCs characterization (Budak et al., 2005; De Paiva et al., 2005; Schlötzer-Schrehardt and Kruse, 2005; Watanabe et al., 2004). The possibility that CK8 may be a new marker for LESCs could be considered, but CK8 is present in abundance in half of the basal limbal epithelial cells; moreover, it is still present in elongated cells projecting from CK8-positive clusters up to the cells in the central corneal epithelium. As stem cells represent less than 10% of the total limbal basal cell population (Lavker et al., 1991), it is clear that CK8 is not a marker specific to LESCs only. The expression of CK8 is very important for normal cell signaling and cell-cycle regulation as well as for the migratory and invasive ability of cells (Ku et al., 2002; Raul et al., 2004; Toivola et al., 2001). The obtained data support our hypothesis that CK8 could still play some unidentified role in the activation of corneo-limbal cells and their proliferation and migration, but the exact relation between CK8 expression and the renewal of cells in the corneo-conjunctival area remains to be elucidated (*please, see appended publication 2*).

Besides the unambiguous presence of CK8 and CK18 in the control corneal epithelium (Kasper et al., 1992), their expression in corneal endothelium has been a matter of some controversy. In studies by Foets et al. (1990), Kasper et al. (1992) and Wollensak and Witschel (1996), an occasional positivity for CKs 8 and 18 in the control endothelium was observed. In contrast, no CK8 or CK18 expression was detected in

other studies (Cockerham et al., 2002; Kramer et al., 1992; Levy et al., 1995a). To provide evidence that CK8 and CK18 are expressed in the adult human corneal endothelium, we have used different fixation and processing methods prior to immunohistochemical analysis. In addition, we have confirmed our positive results obtained by immunohistochemistry at the mRNA level by RT-PCR and at the protein level by Western blot. Finally, our findings clearly demonstrated that most endothelial cells express CKs 8 and 18, and based on these results we can imply that the corneal endothelium shares some features with simple epithelia (*please, see appended publication 3*). Such knowledge may lead to a better understanding of the development and differentiation processes in the posterior corneal layers, including the type of progenitor cells involved.

Since the development of the avian corneal endothelium was well established in the past, many investigators at first accepted a neural crest origin of the corneal endothelial cells in man as well (Bahn et al., 1984, Hayashi et al., 1986; Johnston et al., 1979). Alternatively, some investigators have postulated that human corneal endothelium is only derived from mesenchymal tissues originating in the mesoderm (Risen et al., 1987). Later, the mesectoderm, the newly named ectomesenchyme, was proposed as the tissue from which the corneal endothelium together with DM develops, and whose exact origin was discussed (Sevel and Isaacs, 1988; Weston et al., 2004). The current, newly accepted concept is that the corneal endothelium and keratocytes originate from both the neural crest and lateral plate mesoderm, which together form the periocular mesenchyme (Gage et al., 2005; Sowden, 2007).

The fact that the mammalian endothelium originates from both neural and mesodermal cells (Gage et al., 2005; Sowden, 2007), may cause that the human corneal endothelium exhibits the distinctive phenotypical heterogeneity (Foets et al., 1990; Foets et al., 1992a; Hayashi et al., 1986). Neuronal markers (neurofilaments, neural cell adhesion molecule, neuron specific enolase and S-100 protein), epithelial cell markers (CKs 8 and 18) as well as mesenchymal cell marker vimentin were detected in human corneal endothelial cells (Foets et al., 1990; Foets et al., 1992a; 1992b; Hayashi et al., 1986; Risen et al., 1987; Shamsuddin et al., 1986).

Because of the partially shared origin of mesothelial cells and human corneal endothelial cells, we have demonstrated that three other proteins, expressed constitutively in mesothelial cells, are expressed in the human cornea. In our recent study we clearly demonstrate that mesothelin, HBME-1 protein and calbindin 2, which are considered to be reliable markers of healthy and neoplastic mesothelium (Marchevsky, 2008; Miettinen and Kovatich, 1995), are abundantly expressed in the human cornea and extend the phenotypical heterogeneity of human corneal endothelial cells (*please, see appended publication 4*).

5.2 The alterations caused by posterior polymorphous corneal dystrophy

The phenotypic diversity of the corneal endothelium is manifested by its instability in some endothelial pathologies, including PPCD or FECD, in which abnormal endothelial cells acquire characteristics of “fibroblast-like” or mostly “epithelial-like” cells (Boruchoff and Kuwabara, 1971; Hidayat and Cockerham, 2006; Johnson and Brown, 1978). As the largest group of PPCD patients in the world comes from the Czech Republic we had a very good opportunity to investigate the whole spectrum of changes which occurred in PPCD explants compared to control corneas.

By investigating CK expression, we focused on characterizing these epithelial-like cells with the aim of improving our knowledge of PPCD pathogenesis. Besides immunostaining for CK7 and CK8/18, which had been previously observed in aberrant PPCD endothelium (Cockerham et al., 2002), we demonstrated that the abnormal endothelium of PPCD patients expresses a mixture of CKs, with CK7 and CK19 predominating. Interestingly, we also detected CK3/12, the expression of which is normally restricted to the corneal epithelium (Moll et al., 1982), in all of the examined PPCD patients, suggesting that the aberrant endothelium formed during PPCD shares features of the corneal epithelium. The expression of the basal cell marker CK14 and the stratification marker CK4 may correlate with the ability of the pathologically altered endothelium to form multilayered structures, while the expression of the hyperproliferation-associated markers CK6 and CK16 (van der Velden et al., 1999) may correlate with the proliferative capacity of these aberrant PPCD cells. Due to the weak positive signals for CK1 and CK10, markers for terminal differentiation and cornification (van der Velden et al., 1999) found in a few PPCD cells, we conclude that the altered cells are not already transformed into a distinct differentiated epithelial phenotype.

In addition to the endothelium, we detected the epithelization of superficial cells in a PPCD trabecular meshwork, probably reflecting the capacity of the abnormal cells to migrate outwards from the cornea and to overgrow the surrounding tissues. This finding has a clinical implication because the overgrowth of abnormal cells may lead to closing of the iridocorneal angle and to an increase of intraocular pressure, which causes a secondary glaucoma (Cibis et al., 1977; Krachmer, 1985), (*please, sees appended publication 5*).

We can conclude that in terms of CK composition, the aberrant PPCD endothelium shares features of both simple (CK7, 8, 18, 17, 19) and squamous stratified (CK4, 13) epithelium with a proliferative capacity (CK6 and 16). The pattern of CK expression found in the cells on the posterior surface of PPCD corneas is most probably related to a metaplastic process during which endothelial cells are shifted to endo-epithelial and epithelial phenotypes. The broad CK spectrum expressed in our PPCD patients is more likely a sign of the deranged maturation of an emerging metaplastic epithelium.

Despite extensive research of PPCD, the exact mechanism leading to the transformation of PPCD endothelium into cells with epithelial characteristics is still unknown and remains to be elucidated. It is not known if the alteration in CK expression is a more-or-less direct consequence of genetic changes or a secondary response to a more general deregulation, independent of genetic mutations. A non-genetic explanation for the altered CK expression is suggested by the fact that there was no difference in CK composition between patients with mutations in different genes. On the other hand, one can imagine that deregulation of transcription factors can lead to the overexpression of broad CK spectrum. Another possible explanation could be that changes in BM composition may further lead to alterations in cytokeratin expression in adjacent cells (Kurpakus et al., 1992). Hence we have evaluated collagen IV and VIII composition in pathological PPCD corneas to be the main components of the BM and corneal extracellular matrix.

Although morphological as well as functional changes in the endothelium and DM are the main features of PPCD (Boruchoff and Kuwabara, 1971; Hogan and Bietti, 1969; Rodrigues et al., 1980), we have also described changes in the composition of the BME and the anterior and posterior part of the stroma. The most striking difference identified was the presence of the α1 and α2 collagen IV chains in the BME of the central cornea and the posterior part of the stroma in PPCD corneas. Moreover, more intensive staining for α1 and α2 collagen IV chains and their localization on the endothelial side of DM were observed in diseased corneas when compared to their presence mostly on the stromal part of DM in control specimens.

Because the localization of the α1 (IV) and α2 (IV) chains in cornea is normally restricted to the BM of the limbus and conjunctiva (Kabosova et al., 2007), i.e. areas with cells showing marked proliferative activity, their occurrence on the endothelial side of DM in PPCD patients may play a role in stimulating the proliferative activity of the aberrant endothelium. Additionally, an accumulation of the α1 (IV) and α2 (IV) chains as well as the α1 (VIII) and α2 (VIII) chains was observed in PCL. As collagen VIII is expressed in rapidly proliferating cells, such as different tumor cells and endothelial cells during angiogenesis (Paulus et al., 1991; Sage and Iruela-Arispe, 1990), it may happen that the proliferation of endothelial cells of PPCD patients is induced by the collagen VIII deposited in the PCL as well (*please, see appended publication 6*).

We do not detect any correlation between changes in collagens IV or VIII expression and mutations in different genes. It was demonstrated previously that mutations in *COL8A2* in PPCD corneas result in changes in the basement membrane composition (aberrant formation of DM), (Biswas et al., 2001) and that mutations in the *TCF8* gene lead to the abnormal expression of α3 collagen IV chains (Krafchak et al., 2005). But our cases did not show pathogenic mutations in the *COL8A2* gene responsible for PPCD2 either. All of our patients show a linkage to the PPCD1 locus on chromosome 20p11.2 (Gwilliam et al., 2005), except for one patient with a mutation in

ZEB1 gene (Liskova et al., 2007b). Changes in the collagen IV and VIII localization were detected without respect to the type of mutations in individual PPCD patients.

Despite the high mitotic potential of diseased endothelium in PPCD, there are only a few reports on the recurrence of this disorder. So far only 12 cases have been clearly documented in literature, of which only three were examined by light and electron microscopy (Boruchoff et al., 1990; Krachmer et al., 1985; Sekundo et al., 1994). On the basis of morphology it has been suggested that PPCD recurrence is caused by the migration of the host endothelium (Sekundo et al., 1994). However no proof has so far been provided for this hypothesis, thus the possibility still remains that the donor endothelium undergoes metaplasia triggered by unknown mediators present in aqueous humor. In order to find out the exact origin of these cells we have examined a sex-mismatched corneal button explanted from a PPCD patient by a combination of indirect fluorescent immunohistochemistry and FISH. The combination of these two methods allowed us to show that in PPCD, proliferation and migration of the original pathological endothelium from the host periphery into the donor graft may significantly contribute to corneal graft failure (*please, see appended publication 7*). There arises a question why not to transplant the cornea in its whole diameter with its peripheral part as well. However, this is not possible due to high number of antigen presenting cells located at the peripheral part of the cornea (Gillette et al., 1982). Some improvements may be reached by posterior lamellar techniques, which include deep lamellar endothelial keratoplasty and the more recent Descemet stripping endothelial keratoplasty (Pieramici and Afshari, 2006; Studeny et al., 2010). These methods have less postoperative complications, are less stressful for the patient, and most importantly, almost the whole endothelium with DM is replaced, greatly decreasing the possibility of recurrence.

The pathology of PPCD is both very complex and polymorphous and despite enormous effort, the exact originating mechanisms of this illness remain unknown. Nevertheless, research and methodological progress is developing rapidly and in the next few years the mystery of PPCD will certainly be uncovered.

6 SUMMARY OF THE ACHIEVEMENTS

The aims of the dissertation (chapter 1) were fulfilled and the major contributions can be summarized in the following points:

- A whole spectrum of cytokeratins was detected in the adult human cornea, limbus and conjunctiva which allow us to better discern between healthy and pathological tissue. Corneal epithelium was characterized as primary nonkeratinizing stratified epithelium with the expression of some simple epithelial markers.
- The strong expression of CK8 in limbal epithelial basal cells, which is maintained during the differentiation and migration of the limbal cells towards the central corneal epithelium, was described as a typical feature of a normal human corneo-scleral disc.
- CK8 and CK18 (typical simple epithelia markers) were detected in adult human corneal endothelium of all specimens at both the protein and mRNA levels. This finding may contribute to the relatively easy transformation of an endo to epithelial phenotype. Moreover, we have shown that the results are highly dependent on the different fixation solutions and methodological processes used.
- Proteins typical to the human mesothelial cell phenotype – mesothelin, calbindin 2 and HBME-1 protein, were detected in the human cornea, especially in the endothelial cells. This extends the phenotypical heterogeneity of the corneal endothelium.
- The spectrum of cytokeratins expressed in the abnormal endothelial cells on the posterior surface of the cornea in PPCD patients was determined. In terms of CK composition, the aberrant PPCD endothelium shares features of both simple and squamous stratified epithelium with a proliferative capacity. This abnormal CK expression may be related to the altered composition of collagen extracellular matrix of DM.
- The increased expression of the α1, α2 collagen IV chains and α1 collagen VIII chain, and the change in their localization in DM, which may contribute to the increased endothelial proliferative capacity observed in PPCD patients, were described in PPCD corneas.
- The origin of cells causing the recurrence of PPCD after keratoplasty surgery was established, it is caused by the overgrowth of the original diseased host endothelium into the donor graft.
- The protocol for combined fluorescent immunohistochemistry with FISH was successfully prepared and could be used in future laboratory projects.

7 ISSUES FOR FUTURE RESEARCH

Finally, the following steps can be taken to extend the work presented in this dissertation:

- The exploitation of our results in the search for a specific LESCs marker could be useful for the selective and specific isolation of LESCs and their cultivation for future transplantations.
- The phenotypical heterogeneity of the human corneal endothelium can further be enhanced by the detection of additional mesothelial, epithelial or neuronal markers. The achieved results could improve the methodological processes in the isolation, cultivation and grafting of endothelial cells.
- Molecular techniques should be included for the evaluation of the exact mechanism leading to epithelial metaplasia in patients with PPCD. The cultivation and characterization of the abnormal endothelial cells from PPCD patients should be helpful.
- The screening of oncogenes and tumor suppressor genes in the abnormal endothelial cells, as well as the characterization of the spectrum of proteins in the anterior chamber liquid of PPCD patients, could elucidate why these abnormal cells show some of the features of tumor cells (abnormal proliferation, migration, multilayer structures formation).
- Mutations in genes or a complex of genes which are implicated in PPCD pathogenesis and which cause the illness should be determined using direct sequencing, next-generation sequencing or comparative genomic hybridization.

8 REFERENCES

Abramowicz MJ, Albuquerque-Silva J, Zanen A. Corneal dystrophy and perceptive deafness (Harboyan syndrome): CDPD1 maps to 20p13, J Med Genet 2002;39:110-112.

Aitken A, Jones D, Soneji Y, Howell S. 14-3-3 proteins: biological function and domain structure. Biochem Soc Trans 1995;23:605-611.

Aldave AJ, **Sonmez B**. Elucidating the molecular genetic basis of the corneal dystrophies. Arch Ophthalmol 2007;125:177-186.

Aldave AJ, Yellore VS, Vo RC, et al. Exclusion of positional candidate gene coding region mutations in the common posterior polymorphous corneal dystrophy 1 candidate gene interval. Cornea. 2009;28:801-807.

Al Moghrabi H, Elkeilani A, Thomas JM, Mai KT. Calretinin: an immunohistochemical marker for the normal functional endometrial stroma and alterations of the immunoreactivity in dysfunctional uterine bleeding. Pathol Res Pract 2007;203:79-83.

Bahn CF, Falls HF, Varley BS, et al. Classification of corneal endothelial disorders based on neural crest origin. Ophthalmology 1984;91:558-563.

Baimbridge KG, Celio MR, Rogers JH. Calcium-binding proteins in the nervous system. Trends Neurosci 1992;15:303-308.

Baribault H, Price J, Miyai K, Oshima RG. Mid-gestational lethality in mice lacking keratin 8. Genes Dev 1993;7:1191-1202.

Battifora H, McCaughey WT. Tumors of the serous membranes. 3rd ed. Washington D.C. American registry of pathology; 1995:72.

Bauman PA, Dalton WS, Anderson JM, Cress AE. Expression of cytokeratin confers multiple drug resistance. Proc Natl Acad Sci USA 1994;91:5311-5314.

Ben-Zvi A, Rodrigues MM, Krachmer JH, Fujikawa LS. Immunohistochemical characterization of extracellular matrix in the developing human cornea. Curr Eye Res 1986;5:105-117.

Berman M, Manseau E, Law M, Aiken D. Ulceration is correlated with degradation of fibrin and fibronectin at the corneal surface. Invest Ophthalmol Vis Sci 1983;24:1358-1366.

Beuerman RW, Pedroza L. Ultrastructure of the human cornea. Microsc Res Tech 1996;33:320-335.

Bignami A, **Dahl D**. Astrocyte-specific proteins and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. J Comp Neurol 1974;153:27-38.

Biswas S, Munier FL, Yardley J, et al. Missense mutations in COL8A2, the gene encoding the α2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. Hum Mol Genetics 2001;21:2415-2423.

Blose SH, **Meltzer DI.** Visualization of the 10-NM filament vimentin rings in vascular endothelial cells in situ: close resemblance to vimentin cytoskeletons found in monolayers in vitro. Exp Cell Res 1981;135:299-309.

Blumenberg M. Concerted gene duplications in the two keratin gene families. J Mol Evol 1988;27:203-211.

Bonfanti L, Mironov AA, Martinez-Menárguez JA, et al. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. Cell 1998;95:993-1003.

Boruchoff SA, Werner MJ, Albert DM. Recurrence of posterior polymorphous corneal dystrophy after penetrating keratoplasty. Am J Ophthalmol 1990;109:323-328.

Boruchoff SA, Kuwabara T. Electron microscopy of posterior polymorphous degeneration. Am J Ophthalmol 1971;72:879-887.

Bowden PE, Haley JL, Kansky A, et al. Mutation of a type II keratin gene (K6a) in pachyonychia congenita. Nat Genet 1995;10:363-365.

Budak MT, Alpdogan OS, Zhou M, et al. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. J Cell Sci 2005;118:1715-1724.

Caulin C, Ware CF, Magin TM, Oshima RG. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. J Cell Biol 2000;149:17-22.

Celis JE, Larsen PM, Fey SJ, Celis A. Phosphorylation of keratin and vimentin polypeptides in normal and transformed mitotic human epithelial amnion cells: behavior of keratin and vimentin filaments during mitosis. J Cell Biol 1983;97:1429-1434.

Cibis GW, Krachmer JH, Phelps CD, Weingeist TA. The clinical spectrum of Posterior polymorphous dystrophy. Arch Ophthalmol 1977;95:1529-1537.

Cibis GW, Tripathi RC. The differential diagnosis of Descemet´s tears and Posterior polymorphous dystrophy bands: A clinicopathologic study. Ophthalmology 1982;89:614-620.

Cockerham GC, Laver NV, Hidayat AA, McCoy DL. An immunohistochemical anylysis and comparison of Posterior polymorphous dystrophy with Congenital hereditary endothelial dystrophy. Cornea 2002;21:787-791.

Coulombe PA, Hutton ME, Letai A, et al. Point mutation in human keratin 14 genes of epidermolysis bullosa simplex patients: Genetic and functional analysis. Cell 1991;66:1301-1311.

Cvekl A, Tamm ER. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. Bioessays 2004;26:374-386.

Dahlstrom JE, Maxwell LE, Brodie N, et al. Distinctive microvillous brush border staining with HBME-1 distinguishes pleural mesotheliomas from pulmonary adenocarcinomas. Pathology 2001;33:287-291.

Davanger M, Evenson A. Role of the pericorneal structure in renewal of corneal epithelium. Nature 1971;229:560-561.

Deng Y, Liu S, Cai R. Immunohistochemistry research on keratoconus. Yan Ke Xue Bao 2001;17:65-67,75.

DePaiva CS, Chen Z, Corrales RM, et al. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem cells 2005;23:63-73.

Desir J, Moya G, Reish O, et al. Borate transporter SLC4A11 mutations cause both Harboyan syndrome and non-syndromic corneal endothelial dystrophy, J Med Genet 2007;44:322-326.

Diebold Y, Calonge M, Fernández N, et al. Characterization of epithelial primary cultures from human conjunctiva. Graefe's Arch Clin Exp Ophthalmol 1997;235:268-276.

Doglioni C, Dei Tos AP, Laurino L, et al. Calretinin: a novel immunocytochemical marker for mesothelioma. Am J Surg Pathol 1996;20:1037-1046.

Dua HS, Miri A, Alomar T, Yeung AM, Said DG. The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. Ophthalmology 2009;6:856-863.

Ehlers N, Modis L, Moller-Pedersen T. A morphological and functional study of Congenital Hereditary Endothelial Dystrophy. Acta Ophthalmol Scand 1998;76:314-318.

Elder MJ, Hiscott P, Dart JK. Intermediate filament expression by normal and diseased human corneal epithelium. Hum Pathol 1997;28:1348-1354.

Feil SH, Barraquer J, Howell DN, Green WR. Extrusion of abnormal endothelium into the posterior corneal stroma in a patient with Posterior polymorphous dystrophy. Cornea 1997;16:439-446.

Felipe CD, Gonzalez GG, Gallar J, Belmonte C. Quantification and immunocytochemical characteristics of trigeminal ganglion neurons projecting to the cornea: effect of corneal wounding. Eur J Pain 1999;3:31-39.

Floquet N, Pasco S, Ramont L, et al. The antitumor properties of the α3(IV)-(185-203) peptide from NC1 domain of type IV collagen (tumstatin) are conformation-dependent. J Biol Chem 2004;279:2091-2100.

Foets BJ, van den Oord JJ, Desmet VJ, Missotten L. Cytoskeletal filament typing of human corneal endothelial cells. Cornea 1990;9:312-317.

Foets BJ, van den Oord J, Engelmann K, Missotten L. A comparative immunohistochemical study of human corneotrabecular tissue. Graefes Arch Clin Exp Ophthalmol 1992a;230:269-274.

Foets BJ, van den Oord JJ, Volpes R, Missotten L. In situ immunohistochemical analysis of cell adhesion molecules on human corneal endothelial cells. Br J Ophthalmol 1992b;76:205-209.

Franssen ME, Boezeman JB, van de Kerkhof PC, van Erp PE. Monitoring hyperproliferative disorders in human skin: flow cytometry of changing cytokeratin expression. Cytometry Part B (Clinical Cytometry) 2004;57B:32-39.

Franke WW, Schmid E, Osborn M, Weber K. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci USA 1978;10:5034-5038.

Franke WW, Schiller DL, Moll R, et al. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. J Moll Biol 1981;153:933-959.

Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 2000;40:617-647.

Fuchs E, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. Science 1998;279:514-519.

Gage PJ, Rhoades W, Prucka SK, Hjalt T. Fate maps of neural crest and mesoderm in the mammalian eye. Invest Ophthalmol Vis Sci 2005;46:4200-4208.

Galarneau L, Loranger A, Gilbert S, Marceau N. Keratins modulate hepatic cell adhesion, size and G1/S transition. Exp Cell Res 2007;313:179-194.

Gilbert S, Loranger A, Daigle N, Marceau N. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. J Cell Biol 2001;154:763-773.

Gillette TE, Chandler JW, Greiner JV. Langerhans cells of the ocular surface. Ophthalmology. 1982;89:700-710.

Gipson IK, Spurr-Michaud SJ, Tisdale AS. Anchoring fibrils form a complex network in human and rabbit cornea. Invest Ophthalmol Vis Sci 1987;28:212-220.

Gottsch JD, Zhang Ch, Sundin OH, et al. Fuchs corneal dystrophy: aberrant collagen distribution in an L450W mutant of the COL8A2 gene. Inv Ophthalmol Vis Sci 2005;46:4504-4511.

Gotzos V, Schwaller B, Hetzel N, et al. Expression of the calcium binding protein calretinin in WiDr cells and its correlation to their cell cycle. Exp Cell Res 1992;202:292-302.

Grayson M. The nature of hereditary deep polymorphous dystrophy of the cornea: Its association with iris and anterior chamber dysgenesis. Trans Am Ophthalmol Soc 1974;72:516-559.

Greenhill NS, Rüger BM, Hasan Q, Davis PF. The alpha1(VIII) and alpha2(VIII) collagen chains form two distinct homotrimeric proteins in vivo. Matrix Biol 2000;19:19-28.

Gwilliam R, Liskova P, Filipec M, et al. Posterior polymorphous corneal dystrophy in Czech families maps to chromosome 20 and excludes the VSX1 gene. Invest Ophthalmol Vis Sci 2005;46:4480-4484.

Hall PA, Watt FM. Stem cells: the regeneration and maintenance of cellular diversity. Development 1989;106:619-633.

Hamada R, Giraud JP, Graf B, Pouliquen Y. Analytical and statistical study of the lamellae, keratocytes and collagen fibrils of the central region of the normal human cornea (light and electron microscopy). Arch Ophthalmol Rev Gen Ophthalmol 1972;32:563-570.

Hand CK, Harmon DL, Kennedy SM, et al. Localization of the gene for autosomal recessive congenital hereditary endothelial dystrophy (CHED2) to chromosome 20 by homozygosity mapping, Genomics 1999;61:1-4.

Hanna C, Bicknell DS, O'Brien JE. Cell turnover in the adult human eye. Arch Ophthalmol 1961;65:695-698.

Hayashi K, Sueishi K, Tanaka K, Inomata H. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefes Arch Clin Exp Ophthalmol 1986;224:452-456.

Hellstrom I, Raycraft J, Kanan S, et al. Mesothelin variant 1 is released from tumor cells as a diagnostic marker. Cancer Epidemiol Biomarkers Prev 2006;15:1014-1020.

Héon E, Greenberg A, Kopp KK, et al. VSX1: A gene for Posterior polymorphous dystrophy and keratoconus. Hum Mol Genetics 2002;11:1029-1036.

Héon E, Mathers WD, Alward WL, et al. Linkage of Posterior polymorphous corneal dystrophy to 20q11. Hum Mol Genetics 1995;3:485-488.

Hidayat AA, Cockerham GC. Epithelial metaplasia of the corneal endothelium in Fuchs endothelial dystrophy. Cornea 2006;25:956-959.

Hesse M, Zimek A, Weber K, Magin TM. Comprehensive analysis of keratin gene clusters in humans and rodents. Eur J Cell Biol 2004;83:19-26.

Hirst LW, **Waring GO.** Clinical specular microscopy of Posterior polymorphous endothelial dystrophy. Am J Ophthalmol 1983;2:143-155.

Hogan MJ, Alvarado JA, Weddel JE. In Histology of the Human Eye. An Atlas and Textbook. W.B. Saunders, Philadelphia, PA, USA. 1971.

Hogan MJ, Bietti G. Hereditary deep dystrophy of the cornea (Polymorphous). Am J Ophthalmol 1969;68:777-788.

Hopfer U, Fukai N, Hopfer H, et al. Targeted disruption of Col8a1 and Col8a2 genes in mice leads to anterior segment abnormalities in the eye. FASEB J. 2005;19:1232-1244.

Hsu HJ, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NFκB activation. Cell 1995;81:495-504.

Hudson BG, Reeders ST, Tryggvason K. Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J Biol Chem 1993;268:26033-26036.

Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc Natl Acad Sci U S A 1996;93:136-140.

Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. Int J Cancer 1992;50:373-381.

Chang CY, Green CR, McGhee CN, Sherwin T. Acute wound healing in the human central corneal epithelium appears to be independent of limbal stem cell influence. Invest Ophthalmol Vis Sci 2008;49:5279-5286.

Chen Z, de Paiva CS, Luo L, et al. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells. 2004;22:355-366.

Chipev CC, Korge BP, Markova N, et al. A leucine---proline mutation in the H1 subdomain of keratin 1 causes epidermolytic hyperkeratosis. Cell 1992;70:821-828.

Ihanamäki T, Pelliniemi LJ, Vuorio E. Collagens and collagen-related matrix components in the human and mouse eye. Ret Eye Res 2004;23:403-434.

Illidge Ch, Kielty C, Shuttleworth A. Type VIII collagen: heterotrimeric chain association. Int J Biochem Cell Biol 2001;33:521-529.

Inada H, Izawa I, Nishizawa M, et al. Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. J Cell Biol 2001;155:415-426.

Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem 1998;67:609-652.

Irvine AD, Corden LD, Swensson O, et al. Mutations in cornea-specific keratin K3 or K12 cause Meesmann's corneal dystrophy. Nat Genet 1997;16:184-187.

Jackson BW, Grund C, Schmid E, et al. Formation of cytoskeletal elements during mouse embryogenesis, I. Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. Differentiation 1980;17:161-12179.

Jirsova K, Merjava S, Liskova P. Collagen in the human cornea – types, location and role. Cesk Slov Oftalmol 2008;64:167-170. Czech.

Johnson BL, **Brown SI.** Posterior polymorphous dystrophy: a light and electron microscopic study. Brit J Ophthalmol 1978;62:89-96.

Johnson DH, Bourne WM, Campbell RJ. The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas. Arch Ophthalmol 1982;100:1942-1947.

Johnston MC, Noden DM, Hazelton RD, et al. Origins of avian ocular and periocular tissues. Exp Eye Res 1979;29:27-43.

Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. Inv Ophthalmol Vis Sci 1996;37:645-655.

Jurkunas UV, Bitar MS, Funaki T, Azizi B. Evidence of oxidative stress in the pathogenesis of fuchs endothelial corneal dystrophy. Am J Pathol. 2010;177:2278-2289.

Kabosova A, Azar DT, Bannikov GA, et al. Compositional differences between infant and adult human corneal basement membranes. Invest Ophthalmol Vis Sci 2007;48:4989-4999.

Kadler KE, Holmes DF, Trotter JA, Chapman JA. Collagen fibril formation. Biochem J 1996;316:1-11.

Kasper M, Moll R, Stosiek P, Karsten U. Patterns of cytokeratin and vimentin expression in the human eye. Histochemistry 1988;89:369-377.

Kasper M, Stosiek P, Lane B. Cytokeratin and vimentin heterogeneity in human cornea. Acta Histochem 1992;93:371-381.

Kefalides NA. Structure and biosynthesis of basement membranes. Int Rev Connect Tissue Res 1973;6:63-104.

Kenney MC, Nesburn AB, Burgeson RE, et al. Abnormalities of the extracellular matrix in keratoconus corneas. Cornea 1997;16:345-351.

Kidson SH, Kume T, Deng K, et al. The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. Dev Biol 1999;211:306-322.

Kirkness CM, McCartney A, Rice NS, et al. Congenital hereditary corneal oedema of Maumenee: its clinical features, management, and pathology. Br J Ophthalmol 1987;71:130-144.

Ko EC, Jhala NC, Shultz JJ, Chhieng DC. Use of a panel of markers in the differential diagnosis of adenocarcinoma and reactive mesothelial cells in fluid cytology. Am J Clin Pathol 2001;116:709-715.

Koeppe L. (1916). Cited by Weisenthal RW, Krachmer JH. Posterior polymorphous corneal dystrophy: ten years of progress. Raven Press, New York 1988;82:457-466.

Krafchak CM, Pawar H, Moroi SE, et al. Mutations in TCF8 cause Posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells. Am J Hum Genet 2005;77:694-708.

Krachmer JH. Posterior polymorphous corneal dystrophy: a disease characterized by epithelial - like endothelial cells which influence management and prognosis. Trans Am Ophthalmol Soc 1985;83:413-475.

Kramer TR, Grossniklaus HE, Vigneswaran N, et al. Cytokeratin expression in corneal endothelium in the iridocorneal endothelial syndrome. Invest Ophthalmol Vis Sci 1992;33:3581-3585.

Ku NO, Liao J, Omary MB. Phosphorylation of human keratin 18 serine 33 regulates binding to 14-3-3 proteins. Embo J 1998;17:1892-1906.

Ku NO, Michie S, Resurreccion EZ, et al. Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression. Proc Natl Acad Sci USA 2002;99:4373-4378.

Kurpakus MA, Stock EL, Jones JC. The role of the basement membrane in differential expression of keratin proteins in epithelial cells. Dev Biol 1992;150:243-255.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.

Lane EB, Rugg EL, Navsaria H, et al. A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. Nature 1992;356:244-246.

Laganowski HC, Sherrard ES, Kerr Muir MG, Buckley RJ. Distinguishing features of the Iridocorneal endotelial syndrome and Posterior polymorphous dystrophy: value of endothelial specular microscopy. Brit J Ophthalmol 1991;75:212-216.

Laule A, Cable MK, Hoffman CE, et al. Endothelial cell population changes of human cornea during life. Arch Ophthalmol 1978;96:2031-2035.

Lavker RM, Dong G, Cheng SZ, et al. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. Invest Ophthalmol Vis Sci 1991;32:1864-1875.

Lazarides E, **Hubbard BD**. Immunological characterization of the subunit of the 100-A filaments from muscle cells. Proc Natl Acad Sci USA 1976;73:4344-4348.

Lauweryns B, van den Oord JJ, De Vos R, Missotten L. A new epithelial cell type in the human cornea. Invest Ophthalmol Vis Sci 1993a;34:1983-1990.

Lauweryns B, van den Oord JJ, Missotten L. The transitional zone between limbus and peripheral cornea. An immunohistochemical study. Invest Ophthalmol Vis Sci. 1993b;34:1991-1999.

Lee RE, Davison PF. The collagens of the developing bovine cornea. Exp Eye Res 1984;39:639-652.

Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J Cell Sci 1998;111:2867-2875.

Leinonen A, Mariyama M, Mochizuki T, et al. Complete primary structure of the human type IV collagen alpha4(IV) chain. J Biol Chem 1994;269:26172-26177.

Lemmink HH, Schroder CH, Monnens LA, Smeets HJ. The clinical spectrum of type IV collagen mutations. Hum Mutation 1997;9:477-499.

Leube RE, Bosch FX, Romano V, et al. Cytokeratin expression in simple epithelia. III. Detection of mRNAs encoding human cytokeratins nos. 8 and 18 in normal and tumor cells by hybridization with cDNA sequences in vitro and in situ. Differentiation 1986;33:69-85.

Levy SG, McCartney AC, Baghai MH, et al. Pathology of the iridocorneal - endothelial syndrome. The ICE cell. Invest Ophthalmol Vis Sci 1995a;36:2592-2601.

Levy SG, McCartney AC, Sawada H, et al. Descemet's membrane in the iridocorneal-endothelial syndrome: morphology and composition. Exp Eye Res 1995b;61:323-333.

Levy SG, Moss J, Noble BA, McCartney AC. Early-onset posterior polymorphous dystrophy. Arch Opthalmol 1996a;114:1265-1268.

Levy SG, Moss J, Sawada H, et al. The composition of wide-spaced collagen in normal and diseased Descemet's membrane. Curr Eye Res 1996b;15:45-52.

Liao J, Lowthert LA, Ghori N, Omary MB. The 70-kDa heat shock proteins associate with glandular intermediate filaments in an ATP-dependent manner. J Biol Chem 1995;270:915-922.

Liao J, Omary MB. 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. J Cell Biol 1996;133:345-357.

Liskova P, Filipec M, Merjava S, Jirsova K, Tuft SJ. Variable ocular phenotypes of posterior polymorphous corneal dystrophy caused by mutations in the ZEB1 gene. Ophthal Genet. 2010,31:230-234.

Liskova P, Prescott Q, Bhattacharya SS, Tuft SJ. British family with early-onset Fuchs' endothelial corneal dystrophy associated with p.L450W mutation in the COL8A2 gene. Br J Ophthalmol 2007a;91:1717-1718.

Liskova P, Tuft SJ, Gwilliam R, et al. Novel mutations in the ZEB1 gene identified in Czech and British patients with posterior polymorphous corneal dystrophy. Hum Mutat 2007b;28:638.

Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 1996;87:565-576.

Liu Y, Lyle S, Yang Z, Cotsarelis G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. J Invest Dermatol 2003;121:963-968.

Liu Y, Peng X, Tan J, et al. Zeb1 mutant mice as a model of posterior corneal dystrophy. Invest Ophthalmol Vis Sci 2008;49:1843-1849.

Ljubimov AV, Burgeson RE, Butkowski RJ, et al. Extracellular matrix alterations in human corneas with bullous keratopathy. Inv Ophthalmol Vis Sci 1996;37:997-1007.

Ljubimov AV, Burgeson RE, Butkowski RJ, et al. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab Invest 1995;72:461-473.

Lloyd C, Yu QC, Cheng J, et al. The basal keratin network of stratified squamous epithelia: defining K15 function in the absence of K14. J Cell Biol 1995;129:1329-1344.

Lukas W, Jones KA. Cortical neurons containing calretinin are selectively resistant to calcium overload and excitotoxicity in vitro. Neuroscience 1994;61:307-316.

Magin TM, Vijayaraj P, Leube RE. Structural and regulatory functions of keratins. Exp Cell Res 2007;313:2021-2032.

Marchevsky AM. Application of immunohistochemistry to the diagnosis of malignant mesothelioma. Arch Pathol Lab Med 2008;132:397-401.

Marshall GE, Konstas AG, Lee WR. Immunogold fine structural localization of extracellular matrix components in aged human cornea. Graefe's Arch Ophthalmol 1991;229:157-163.

Marshall GE, Konstas AG, Lee WR. Collagens in ocular tissues. Brit J Ophthalmol 1993;77:515-524.

Matsumoto K, Weber PA, Makley TA. Posterior polymorphous dystrophy – A histopathologic presentation. Ann Ophthalmol 1988;20:388-393.

Maurice DM. The structure and transparency of the cornea. J Physiol 1957;136:263-286.

McGowan SL, Edelhauser HF, Pfister RR, Whikehart DR. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. Mol Vis 2007;13:1984-2000.

McLean WH, Rugg EL, Lunny DP, et al. Keratin 16 and keratin 17 mutations cause pachyonychia congenita. Nat Genet 1995;9:273-278.

McLean WH, Eady RA, Dopping-Heppenstal PJ, et al. Mutations in the rod 1A domain of keratins 1 and 10 in bullous congenital ichthyosiform erythroderma (BCIE). J Invest Dermatol 1994;102:24-30.

Michelacci YM. Collagens and proteoglycans of the corneal extracellular matrix. Braz J Med Biol Res 2003;36:1037-1046.

Miettinen M, **Kovatich AJ.** HBME-1 a monoclonal antibody useful in the differential diagnosis of mesothelioma, adenocarcinoma and soft tissue and bone tumours. Appl Immunohistochem 1995;3:115-122.

Mishima S. Corneal thickness. Surv Ophthalmol 1968;13:57-96.

Mohamed MD, McKibbin M, Jafri H, et al. A new pedigree with recessive mapping to CHED2 locus on 20p13. Br J Ophthalmol 2001;85:758-9.

Moll I, Kuhn C, Moll R. Cytokeratin 20 is a general marker of cutaneous Merkel cells while certain neuronal proteins are absent. J Invest Dermatol 1995;104:910-915.

Moll I, Troyanovsky SM, Moll R. Special program of differentiytion expressed in keratinocytes of human haarscheiben: an analysis of individual cytokeratin polypeptides. J Invest Dermatol 1993;100:69-76.

Moll R, Divo M, Langbein L. The human keratins: biology and pathology. Histochem Cell Biolo 2008;129:705-733.

Moll R, Franke WW, Sciller DL, et al. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.

Moll R, Löwe A, Laufer J, Franke WW. Differential expression of cytokeratin 20 in human carcinomas: a new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol 1992;140:427-447.

Morgan G, Patterson A. Pathology of posterior polymorphous degeneration of the cornea. Brit J Ophthalmol 1967;51:433-437.

Morgan PR, Shirlaw PJ, Johnson NW, et al. Potential applications of anti-keratin antibodies in oral diagnosis. J Oral Pathol 1987;16:212-222.

Moroi SE, Gokhale PA, Schteingart MT, et al. Clinicopathologic correlation and genetic analysis in a case of Posterior polymorphous corneal dystrophy. Am J Ophthalmol 2003;4:461-470.

Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. Cell 1997;88:287-298.

Mullaney PB, Risco JM, Teichmann K, Millar L. Congenital hereditary endothelial dystrophy asociated with glaucoma. Ophthalmology 1995;2:186-192.

Myllyharju J, Kivirikko KI. Collagen and collagen-related diseases. Ann Med 2001;33:7-21.

Nelson JD, **Cameron JD**. Cornea. In: Krachmer JH, Mannis MJ, Holland EJ. (eds.): Cornea (2nd edition). Elsevier MOSBY Inc., 2005, pp 3-26.

Newsome DA, Foidart JM, Hassel JR, et al. Detection of specific collagen types in normal and keratoconus corneas. Inv Ophthalmol Vis Sci 1981;20:738-750.

Nishida T. Cornea. In: Krachmer JH, Mannis MJ, Holland EJ. (eds.): Cornea (2nd edition). Elsevier MOSBY Inc. 2005, pp 3-26.

Notara M, Alatza A, Gilfillan J, et al. In sickness and in health: Corneal epithelial stem cell biology, pathology and therapy. Exp Eye Res. 2010;90:188-195. Review.

Olsen T. On the calculation of power from curvature of the cornea. Br J Ophthalmol 1986;70:152-154.

Omary MB, Baxter GT, Chou CF, et al. PKC epsilon-related kinase associates with and phosphorylates cytokeratin 8 and 18. J Cell Biol 1992;117:583-593.

Omary MB, Ku NO, Liao J, Price D. Keratin modifications and solubility properties in epithelial cells and in vitro. Subcell Biochem 1998;31:105-140.

Ordonez NG. The value of antibodies 44-3A6, SM3, HBME-1, and thrombomodulin in differentiating epithelial pleural mesothelioma from lung adenocarcinoma: a comparative study with other commonly used antibodies. Am J Surg Pathol 1997;21:1399-1408.

Ordonez NG. The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. Am J Surg Pathol 2003a;27:1031-1051.

Ordonez NG. Application of mesothelin immunostaining in tumor diagnosis. Am J Surg Pathol 2003b;27:1418-1428.

Ortega N, Werb Z. New functional roles for non-collegenous domains of basement membrane collagens. J Cell Sci 2002;22:4201-4214.

Oshima RG, Baribault H, Caulin C. Oncogenic regulation and function of keratin 8 and 18. Cancer Metastasis Rev 1996;15:445-471.

Owens DW, Lane EB. Keratin mutations and intestinal pathology. J Pathol 2004;204:377-385.

Parry DA, Strelkov SV, Burkhard P, et al. Towards a molecular description of intermediate filament structure and assembly. Exp Cell Res 2007;313:2204-2216.

Patel DV, Grupcheva CN, McGhee CN. In vivo confocal microscopy of posterior polymorphous dystrophy. Cornea 2005;24:550-554.

Paulus W, Sage EH, Liszka U, Iruela-Arispe ML, Jellinger K. Increased levels of type VIII collagen in human brain tumours compared to normal brain tissue and non-neoplastic cerebral disorders. Br J Cancer 1991;63:367-371.

Pedersen OO, Rushood A, Olsen EG. Anterior mesenchymal dysgenesis of the eye. Congenital hereditary endothelial dystrophy and congenital glaucoma. Act Ophthalmol 1989;67:470-476.

Persechini A, Moncrief ND, Kretsinger RH. The EF-hand family of calcium-modulated proteins. Trends Neurosci 1989;12:462-467.

Pieramici SF, Afshari NA. Genetics of corneal dystrophies: the evolving landscape. Curr Ophthalmol 2006;17:361-366.

Pitz S, Moll R. Intermediate-filament expression in ocular tissue. Prog Ret Eye Res 2002;21:241-262.

Porter RM, Lunny DP, Ogden PH, et al. K15 expresson implies lateral differentiation within stratified epithelial basal cells. Lab Invest 2000;80:1701-1710.

Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 1995;64:403-434.

Prockop DJ, Sieron AL, Li SW. Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol 1998;16:399-408.

Pronk A, Leguit P, Hoynck van Papendrecht AA, et al. A cobblestone cell isolated from the human omentum: the mesothelial cell; isolation, identification, and growth characteristics. In Vitro Cell Dev Biol 1993;29A:127-134.

Ramaekers F, Huysmans A, Schaart G, et al Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp Cell Res 1987;170:235-249.

Raul U, Sawant S, Dange P, et al. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype. Int J Cancer 2004;111:662-668.

Reneker LW, Silversides DW, Xu L, Overbeek PA. Formation of corneal endothelium is essential for anterior segment development - a transgenic mouse model of anterior segment dysgenesis. Development 2000;127:533-542.

Riazuddin SA, Eghrari AO, Al-Saif A, et al. Linkage of a mild late-onset phenotype of Fuchs corneal dystrophy to a novel locus at 5q33.1-q35.2. Invest Ophthalmol Vis Sci. 2009;50:5667-5671.

Riazuddin SA, Zaghloul NA, Al-Saif A, et al. Missense mutations in TCF8 cause late-onset Fuchs corneal dystrophy and interact with FCD4 on chromosome 9p. Am J Hum Genet. 2010;86:45-53.

Richard G, DeLaurenzi V, Didona B, et al. Keratin 13 point mutation underlies the hereditary mucosal epithelial disorder white sponge nevus. Nat Genet 1995;11:453-455.

Richardson WP, Hettinger ME. Endothelial and epithelial-like cell formations in case of posterior polymorphous dystrophy. Arch Ophthalmol 1985;130:1520-1524.

Richardson MR, Segu ZM, Price MO, et al. Alterations in the aqueous humor proteome in patients with Fuchs endothelial corneal dystrophy. Mol Vis. 2010;16:2376-2383.

Risen LA, Binder PS, Nayak SK. Intermediate filaments and their organization in human corneal endothelium. Invest Ophthalmol Vis Sci 1987;28:1933-1938.

Rodrigues MM, Hidayat AA, Choe HS. Advances in corneal pathology. Ophthalmol Clin North Am 1995;8:83.

Rodrigues MM, Newsome DA, Krachmer JH, Sun TT. Posterior polymorphous dystrophy of the cornea: cell culture studies. Eye 1981;33:535-544.

Rodrigues MM, Sun TT, Krachmer JH, Newsome DA. Epithelization of the corneal endothelium in Posterior polymorphous dystrophy. Inv Ophthalmol Vis Sci 1980;19:832-835.

Rodrigues MM, Waring GO, Laibson PR, Weinreb S. Endothelial alterations in congenital corneal dystrophies. Am J Ophthalmol 1975;80:678-689.

Rogers J, Khan M, Ellis J. Calretinin and other CaBPs in the nervous system. Adv Exp Med Biol 1990;269:195-203.

Rogers JH. Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons. J Cell Biol 1987;105:1343-1353.

Rogers MA, Edler L, Winter H, et al. Characterization of new members of the human type II keratin gene family and general evaluation of the keratin gene domain on chromosome 12q13.13. Soc Invest Dermatol 2005;124:536-544.

Ross JR, Foulks GN, Sanfilippo FP, Howell DN. Immunohistochemical analysis of the pathogenesis of Posterior polymorphous dstrophy. Arch Ophthalmol 1995;113:340-345.

Rothnagel JA, Dominey AM, Dempsey LD, et al. Mutation in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. Science 1992;257:1128-1130.

Rozsa AJ, Beuerman RW. Density and organization of free nerve endings in the corneal epithelium of the rabbit. Pain 1982;14:105-120.

Rugg EL, McLean WH, Allison WE, et al. A mutation in the mucosal keratin K4 is associated with oral white sponge nevus. Nat Genet 1995;11:450-452.

Sack RA, Nunes I, Beaton A, Morris C. Host-defense mechanism of the ocular surfaces. Biosci Rep 2001;21:463-480.

Sage H. (1987). Cited by Marshall GE, Konstas AG, Lee WR. Collagens in ocular tissues. Brit J Ophthalmol 1993;77:515-524.

Sage H, Iruela-Arispe ML. Type VIII collagen in murine development. Association with capillary formation in vitro. Ann NY Acad Sci 1990;580:17-31.

Sandberg-Lall M. (2000). Cited by Michelacci YM. Collagens and proteoglycans of the corneal extracellular matrix. Braz J Med Biol Res 2003;36:1037-1046.

Sanes JR, Engvall E, Butkowski R, Hunter DD. Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J Cell Biol 1990;111:1685-1699.

Sawada H. The fine structure of bovine Descemet's membrane with special reference to biochemical nature. Cell Tissue Res 1982;226:241-255.

Sawada H, Konomi H, Hirosawa K. Characterization of the collagen in the hexagonal lattice of Descemet's membrane: its relation to type VIII collagen. J Cell Biol 1990;110:219-227.

Scott JE. How rational histochemistry produced order out of chaos in the "amorphous ground substance" (with a little help from biochemistry, biophysics etc.). Eur J Histochem 1998;42:29-34.

Sekundo W, Lee WR, Aitken DA, Kirkness CM. Multirecurrence of corneal posterior polymorphous dystrophy. An ultrastructural study. Cornea 1994;13:509-515.

Sevel D, Isaacs R. A re-evaluation of corneal development. Trans Am Ophthalmol Soc 1988;86:178-207.

Shamsuddin AK, Nirankari VS, Purnell DM, Chang SH. Is the corneal posterior cell layer truly endothelial? Ophthalmology 1986;93:1298-1303.

Shen AL, O'Leary KA, Dubielzig RR, et al. The PPCD1 mouse: characterization of a mouse model for posterior polymorphous corneal dystrophy and identification of a candidate gene. PLoS One. 2010;5:e12213.

Shuttleworth CA. Type VIII collagen. Int J Biochem Cell Biol 1997;29:1145-1148.

Scheef EA, Huang Q, Wang S, et al. Isolation and characterization of corneal endothelial cells from wild type and thrombospondin-1 deficient mice. Mol Vis 2007;13:1483-1495.

Schlötzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. Exp Eye Res 2005;81:247-264.

Schmidt E, Lisch W, Philips W, et al. A new, X-linked endothelial corneal dystrophy. Am J Ophthalmol 2006;141:478-487.

Schweizer J, Bowden PE, Coulombe PA, et al. New consensus nomenclature for mammalian keratins. J Cell Biol 2006;174:169-174.

Smith-Mungo LI, Kagan HM. Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 1998;16:387-398.

Sowden JC. Molecular and developmental mechanisms of anterior segment dysgenesis. Eye (Lond) 2007;21:1310-1318.

Steinert PM. The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. J Biol Chem 1990;265:8766-8774.

Stosiek P, Kasper M, Conrad K. Immunohistochemistry of cytokeratin expression in human blood vessel endothelia with special reference to synovial connective tissue. Acta Histochem 1990;89:61-66.

Studeny P, Farkas A, Vokrojova M, et al. Descemet's membrane endothelial keratoplasty with a stromal rim (DMEK-S). Br J Ophthalmol 2010;94:909-914.

Sundin OH, Broman KW, Chány HH, et al. A common locus for late-onset Fuchs corneal dystrophy maps to 18q21.2-q21.32. Incest Ophthalmol Vis Sci 2006a;47:3919-3926.

Sundin OH, Jun AS, Broman KW, et al. Linkage of late-onset Fuchs corneal dystrophy to a novel locus at 13pTel-13q12.13. Invest Ophthalmol Vis Sci 2006b;47:140-145.

Thoft RA, **Friend J.** The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 1983;24:1442-1443.

Timpl R. Structure and biochemical activity of basement membrane proteins. Eur J Biochem 1989;180:487-502.

Toivola DM, Nieminen MI, Hesse M, et al. Disturbances in hepatic cell-cycle regulation in mice with assembly-deficient keratins 8/18. Hepathology 2001;34:1174-1183.

Toma NM, Ebenezer ND, Inglehearn ChF, et al. Linkage of Congenital hereditary endothelial dystrophy to chromosome 20. Hum Mol Genetics 1995;12:2395-2398.

Trask DK, Band V, Zajchowski DA, et al. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc Natl Acad Sci USA 1990;87:2319-2323.

Traub P, Shoeman RL. Intermediate filament proteins: cytoskeletal elements with gene-regulatory function? Int Rev Cytol 1994;154:1-103.

Treseler PA, Foulks GN, Sanfilippo F. Expression of ABO blood group, hematopoietic, and other cell-specific antigens by cells in the human cornea. Cornea 1985;4:157-168.

Tseng SC. Concept and application of limbal stem cells. Eye 1989;3:141-157.

Tuori AJ, Virtanen I, Aine E, et al. The immunohistochemical composition of corneal basement membrane in keratoconus. Curr Eye Res 1997;16:792-801.

Upasani OS, Vaidya MM, Bhisey AN. Database on monoclonal antibodies to cytokeratins. Oral Oncology 2004;40:236-256.

Van der Velden LA, Manni JJ, Ramaekers FC, Kuijpers W. Expression of intermediate filament proteins in benign lesions of the oral mucosa. Eur Arch Otorhinolaryngol 1999;256:514-519.

Vincent AL, Niederer RL, Richards A, et al. Phenotypic characterisation and ZEB1 mutational analysis in posterior polymorphous corneal dystrophy in a New Zealand population. Mol Vis. 2009;15:2544-2553.

Vithana EN, Morgan P, Sundaresan P, et al. Mutations in sodium-borate cotransporter SLC4A11 cause recessive congenital hereditary endothelial dystrophy (CHED2), Nat Genet 2006;38:755-757.

Waring GO. Posterior collagenous layer of the cornea: ultrastructural classification of abnormal collagenous tissue posterior to Descemet´s membrane in 30 cases. Arch Ophthalmol 1982;100:122-134.

Waring GO, Bourne WM, Edelhauser HF, Kenyon KR. The corneal endothelium, normal and pathologic structure and function. Ophthalmology 1982;89:531-590.

Waring GO, Rodrigues MM, Laibson PR. Corneal dystrophies II. Endothelial dystrophies. Surv Ophthalmol 1978;23:147-168.

Waseem A, Dogan B, Tidman N, et al. Keratin 15 expression in stratified epithelia: downregulation in activated keratinocytes. J Invest Dermatol 1999;112:362-369.

Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett 2004;565:6-10.

Weber K, Shaw G, Osborn M, et al. Neurofilaments, a subclass of intermediate filaments: structure and expression. Cold Spring Harbor Symp. Quant Biol 1983;48:717-729.

Weston JA, Yoshida H, Robinson V, et al. Neural crest and the origin of ectomesenchyme: neural fold heterogeneity suggests an alternative hypothesis. Dev Dyn 2004;229:118-130.

Wilson SE, Hong JW. Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. Cornea. 2000;19:417-420.

Wollensak G, Witschel H. Vimentin and cytokeratin pattern in granular corneal dystrophy. Graefe's Arch Clin Exp Ophthalmol 1996;234:S110-S114.

Zatloukal K, Stumptner C, Lehner M, et al. Cytokeratin 8 protects from hepatotoxicity, and ist ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. Am J Pathol 2000;156:1263-1274.

Zimmermann DR, Trueb B, Winterhalter KH, et al. Type VI collagenis a major component of the human cornea. FEBS Letters 1986;197:55-58.

Yamaguchi N, Benya PD, van der Rest M, Ninomiya Y. The cloning and sequencing of alpha 1(VIII) collagen cDNAs demonstrate that type VIII collagen is a short chain collagen and contains triple-helical and carboxyl-terminal non-triple-helical domains similar to those of type X collagen. J Biol Chem. 1989;264:16022-16029.

9 LIST OF PUBLICATIONS AND SELECTED PRESENTATIONS

All of the author's related and unrelated publications are given in the list below, sorted in chronological order. Impact Factor (IF) values and citation reports correspond to the ISI Web of Knowledge.

9.1 Publications related to the thesis

[1] Jirsova K, **Merjava S,** Martincova R, Gwilliam R, Ebenezer ND, Liskova P, Filipec M. Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. *Exp Eye Res* 2007;84:680-686 (**IF = 2.651)**

cited by:

- **Eagle RC,** 2008. Immunohistochemistry in diagnostic ophthalmic pathology: a review. Clin. Exper. Ophthalmol. 36:675-688.
- **Klintworth GK,** 2009. Corneal dystrophies. Orphanet J. Rare Dis., 4:7.
- **Omary MB, Ku NO, Strnad P, et al**., 2009. Toward unraveling the complexity of simple epithelial keratins in human disease. J. Clin. Invest. 119:1794-1805.
- **Clausen I, Weidle E, Duncker G, et a**l., 2009. Mutational analysis of VSX-1 in one patient with posterior polymorphous corneal dystrophy and in three families with hereditary Fuchs endothelial dystrophy. Klinishe Monatsblatter fur Augenheilkunde. 226:466-469.
- **Vincent AL, Niederer RL, Richards A, et al**., 2009. Phenotypic characterisation and ZEB1 mutational analysis in posterior polymorphous corneal dystrophy in a New Zealand population. Molecular Vision, Volume: 15, Issue: 271-74, Pages: 2544-2553.
- **Kannabiran C,** 2009. Genetics of corneal endothelial dystrophies. J. Genetic., 88:487-494.
- **Pineda R, Jain V, Gupta P, et al.,** 2010. Descemet's Stripping Endothelial Keratoplasty: An Effective Treatment for Toxic Anterior Segment Syndrome with Histopathologic Findings. Cornea, 29:694-697.
- **Jakobiec FA, Bhat P,** 2010. Retrocorneal Membranes: A Comparative Immunohistochemical Analysis of Keratocytic, Endothelial, and Epithelial Origins. Am. J. Ophthalmol., 150:230-242.
- **Shen AL, O'Leary KA, Dubielzig RR, et al.** 2010. The PPCD1 Mouse: Characterization of a Mouse Model for Posterior Polymorphous Corneal Dystrophy and Identification of a Candidate Gene. PLOS ONE, Volume: 5, Issue: 8, Published: AUG 16 2010.
- **Hillenaar T, Mooy CM, Verjans GMGM, et al.** 2010. Zipper Cell Endotheliopathy A New Subset of Idiopathic Corneal Edema. Ophthalmology, 117:2255-2262.

[2] **Merjavá S,** Lisková P, Jirsová K. Immunohistochemical characterization of collagen IV in control corneas and in corneas obtained from patients suffering from posterior polymorphous corneal dystrophy. *Cesk Slov Oftalmol*. 2008;64:115-119. Slovak.

[3] Jirsová K, **Merjavá S,** Lisková P. Collagen in the human cornea – types, location and role. *Cesk Slov Oftalmol*. 2008;64:167-170. Czech.

[4] **Merjava S,** Liskova P, Sado Y, Davis PF, Greenhill NS, Jirsova K. Changes in the localization of collagens IV and VIII in corneas obtained from patients with posterior polymorphous corneal dystrophy. *Exp Eye Res.* 2009;88:945-952. (**IF = 2.538)**

cited by:

- **Jakobiec FA, Bhat P,** 2010. Retrocorneal Membranes: A Comparative Immunohistochemical Analysis of Keratocytic, Endothelial, and Epithelial Origins. Am. J. Ophthalmol., 150:230-242.
- **Saito K**, **Yonezawa T, Minaguchi J, et al.** 2011. Distribution of alpha(IV) collagen chains in the ocular anterior segments of adult mice. Connective Tissue Research, 52:147-156.
- **Herwig MC, Eter N, Holz FG, et al**. 2011. Corneal Clouding in Alport Syndrome. Cornea, 30:367-370.

[5] **Merjava S,** Neuwirth A, Mandys V, Jirsova K. Cytokeratins 8 and 18 in adult human corneal endothelium. *Exp Eye Res.* 2009;89:426-431. (**IF = 2.538)**

cited by:

- **Jakobiec FA, Bhat P,** 2010. Retrocorneal Membranes: A Comparative Immunohistochemical Analysis of Keratocytic, Endothelial, and Epithelial Origins. Am. J. Ophthalmol., 150:230-242.
- **Pai VC, Glasgow BJ**, 2010. MUC16 as a Sensitive and Specific Marker for Epithelial Downgrowth. Arch. Ophthalmol., 128:1407-1412.

[6] Jirsova K, **Merjava S,** Liskova P. 2009. Posterior polymorphous corneal dystrophy: A review of current knowledge. In: Korhonen L, Laine E. (eds.): New Topics in Eye Research. Nova Science Publishers, New York. 2009, 49-74.

[7] **Merjava S*,** Neuwirth A, Kalasova S, Vesela V, Jirsova K*. Mesothelial proteins are expressed in the human cornea. *Exp Eye Res*. 2010;91:623-629. * joint first authors. (**IF = 2.538)**

[8] Liskova P, Filipec M, **Merjava S**, Jirsova K, Tuft, S.J. Variable ocular phenotypes of posterior polymorphous corneal dystrophy caused by mutations in the ZEB1 gene. *Ophthal Genet.* 2010;31:230-234. (**IF = 1.406)**

[9] **Merjava S**, Neuwirth A, Tanzerova M, Jirsova K. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. *Histol. Histopathol*. 2011;26:323-331. (**IF = 2.404)**

[10] **Merjava S**, Brejchova K, Vernon A, Daniels JT, Jirsova K. Cytokeratin 8 is expressed in human corneo-conjunctival epithelium, particularly in limbal epithelial cells. *Invest Ophthalmol Vis Sci.* 2011;52:787-794. **(IF = 3.431)**

[11] **Merjava S**, Malinova E, Liskova P, Filipec M, Zemanova Z, Michalova K, Jirsova K. Combined indirect fluorescent immunohistochemistry with fluorescence in situ hybridization in a patient with posterior polymorphous corneal dystrophy. *Histochem Cell Biol.* 2011. Submitted.

9.2 Published abstracts

[12] Jirsova K, Vesela V, Martincova R, **Merjava S**, Liskova P, Filipec M. Proliferative capacity and cytokeratin expression in the abberant endothelium of posterior polymorphous corneal dystrophy. (Poster). Abstract from the Association for Research in Vision and Ophthalmology Conference. 2004. *Invest Ophthalmol Vis Sci* (**IF=3,577**) 2004, 45: E-Abstract 1527.

[13] **Merjava S,** Liskova P, Jirsova K. Changes in the α1 – α6 collagen IV chains in the corneas of posterior polymorphous corneal dystrophy patients. Poster presentation. Abstracts from the European Association for Vision and Eye Research Conference. October 3-6, 2007. Portoroz, Slovenia. *Acta Ophthalmologica Scandinavica* (**IF=1,848**), Published online 2 Oct 2007; Volume 85, Issue s240

[14] Jirsova K, **Merjava S,** Neuwirth A. Cytokeratin 8 and 18 expression in human corneal endothelium. Oral presentation. Abstracts from the European Association for Vision and Eye Research Conference. October 3-6, 2007. Portoroz, Slovenia. *Acta Ophthalmologica Scandinavica* (**IF=1,848**), Published online 2 Oct 2007; Volume 85, Issue s240

[15] **Merjava S,** Liskova P, Vesela V, Martincova R, Filipec M, Jirsova K. Changes in the corneas of posterior polymorphous corneal dystrophy patients. Poster presentation. 1st International Student Medical Congress, June 23-25. 2009. Košice, Slovakia. Abstract published in *Folia Medica Cassoviensia*; Tomus 64, No. 1, Suppl. 1, Issue s180-181.

[16] Noskova L, Liskova P, Stranecky V, Hartmannova H, Ivanek R, Jirsova K, **Merjava S**, Filipec M, Kmoch S. Posterior polymorphous corneal dystrophy – copy number, gene expression and candidate gene analyses within the PPCD1 candidate region on chromosome 20p11.2. Meeting Abstract. 34th Congress of the Federation-of-European-Biochemical-Societies. July 4-9. 2009. Prague, Czech Republic. *FEBS Journal* (**IF=3,139**). Published July 2009; Volume 276, Issue s1, 114 – 114.

[17] **Merjava S,** Kalasova S, Vesela V, Jirsova K. Cytokeratin 7 as a marker of corneal conjunctivalization in patients with limbal stem cell deficiency. Oral presentation. 2nd International Student Medical Congress, June 21-24. 2010. Košice, Slovakia. Abstract published in *Folia Medica Cassoviensia*; Tomus 65, No. 1, Suppl. 1, Issue s136-137.

Third best presentation award.

[18] **Merjava S,** Malinova E, Liskova P, Zemanova Z, Michalova K, Jirsova K. Recurrence of posterior polymorphous corneal dystrophy is caused by the overgrowth of the original diseased endothelium. Poster presentation. Abstracts from the European Association for Vision and Eye Research Conference. October 6-9, 2010. Hersonissos, Crete. *Acta Ophthalmologica Scandinavica* (**IF=2.441**). Published online 23 Sep. 2010; Volume 88, Issue s246

[19] Jirsova K, **Merjava S.** The presence and suggested role of mesothelial proteins in the human corneal endothelium. Oral presentation. Abstracts from the European Association for Vision and Eye Research Conference. October 6-9, 2010. Hersonissos, Crete. *Acta Ophthalmologica Scandinavica* (**IF=2.441**). Published online 23 Sep. 2010; Volume 88, Issue s246

9.3 Other selected presentations

[20] Jirsová K, **Merjavá S,** Martincová R, Lišková P, Filipec M. Immunohistochemical characterization of the changes in the endothelium with posterior polymorphous corneal dystrophy. Oral presentation. Book of abstract, page 94. *13th annual Meeting of the Czech ophthalmologic society.* Ústí nad Labem, Czech Republic, July 9-11. 2005.

[21] **Merjava S,** Jirsova K, Vesela V, Martincova R, Liskova P, Filipec M. Proliferative capacity and cytokeratin expression in the abberant endothelium of posterior polymorphous corneal dystrophy. Poster presentation. *The European Association for Vision and Eye Research Conference*, Vilamoura, Portugal, October 5-8, 2005.

[22] **Merjavá S,** Jirsová K. The changes in localization of α1 – α6 collagen IV chains in corneas obtained from patients with posterior polymorphous corneal dystrophy and in comparison with control corneas. Oral presentation. *16th annual Meeting of the Czech ophthalmologic society.* Špindlerov Mlýn, Czech Republic, September 24-27. 2008.

[23] **Merjava S,** Neuwirth A, Jirsova K. Cytokeratins 8 and 18 in normal human corneal endothelium. Poster presentation. *48th Annual Meeting of the Association for Cell Biology*, San Francisco, December 13-17. 2008.

[24] **Merjava S,** Jirsova K. Changes in the corneas from patients with posterior polymorphous corneal dystrophy. Oral presentation. *10th student's scientific conference 1.LF UK*, Prague, Czech Republic, May 2009.
Best paper award.

[25] Jirsova K, Kalasova S, **Merjava S.** Proteinase inhibitor PI-9 is expressed in human corneal endothelial cells. Oral presentation. *The 10th International Ocular Inflammation Society Congress*, Prague, Czech Republic, May 30 – June 2. 2009

[26] Jirsova K, **Merjava S.** Mesothelial cell markers are expressed in human corneal endothelial cells. Oral presentation. *The 9th Corneal conference*, Cardiff, United Kingdom, July 14-15. 2009.

[27] **Merjava S,** Kalasova S, Jirsova K. Characterization of the corneal endothelium. Poster presentation. *17th annual Meeting of the Czech ophthalmologic society.* Prague, Czech Republic, October 1-3. 2009.

[28] Jirsova K, **Merjava S.** The expression of mesothelin and other mesothelial proteins in the human cornea. *22nd Meeting of the European eye bank association*. Sitges, Barcelona, January 22 – 23. 2010.

[29] **Merjava S,** Jirsova K. Expression of cytokeratin 8 in human cornea. Oral presentation. *11th student's scientific conference 1.LF UK*, Prague, Czech Republic, May 2010.

[30] **Merjava S,** Malinova E, Zemanova Z, Michalova K, Jirsova K. A combination of indirect fluorescent immunohistochemistry with fluorescence in situ hybridization in patient with posterior polymorphous corneal dystrophy (Poster). *52nd Symposium of the Society for Histochemistry*, Prague, Czech Republic, September 1-4. 2010.
***** ***The best young investigator poster.***

[31] **Merjavá S,** Malinová E, Lišková P, Zemanová Z, Michalová K, Jirsová K. Recurrence of posterior polymorphous corneal dystrophy is caused by the overgrowth of the original diseased endothelium (E-Poster). *18th annual Meeting of the Czech ophthalmologic society*. Teplice, Czech Republic, September 23-25. 2010.

[32] Jirsova K, Dudakova L, Vesela V, Kalasova S, **Merjava S.** Cytokeratin 7 is a reliable marker of conjunctival overgrowth over corneas of patients suffering from limbal stem cell deficiency. *The 2nd Asia Cornea Society Biennial Scientific Meeting in Kyoto*, Japan, December 3-6. 2010.

9.4 Publications not related to the thesis

[33] Jirsova K, Dudakova L, Kalasova S, Vesela V, **Merjava S.** Cytokeratin 7 as a new marker of corneal conjunctivalization in patients with limbal stem cell deficiency. Invest Ophthalmol Vis Sci. 2010. Submitted.

10 APPENDIX

Appended publication 1

Merjava S, Neuwirth A, Tanzerova M, Jirsova K. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. *Histol. Histopathol*. 2011;26:323-331.

Appended publication 2

Merjava S, Brejchova K, Vernon A, Daniels J.T., Jirsova K. Cytokeratin 8 is expressed in human corneo-conjunctival epithelium, particularly in limbal epithelial cells. Invest Ophthalmol Vis Sci. 2011;52:787-94.

Appended publication 3

Merjava S, Neuwirth A, Mandys V, Jirsova K. Cytokeratins 8 and 18 in adult human corneal endothelium. *Exp Eye Res.* 2009;89:426-431.

Appended publication 4

Merjava S*, Neuwirth A, Kalasova S, Vesela V, Jirsova K*. Mesothelial proteins are expressed in the human cornea. *Exp Eye Res*. 2010;91:623-629. * joint first authors.

Appended publication 5

Jirsova K, **Merjava S**, Martincova R, Gwilliam R, Ebenezer ND, Liskova P, Filipec M. Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. *Exp Eye Res* 2007;84:680-686.

Appended publication 6

Merjava S, Liskova P, Sado Y, Davis PF, Greenhill NS, Jirsova K. Changes in the localization of collagens IV and VIII in corneas obtained from patients with posterior polymorphous corneal dystrophy. *Exp Eye Res.* 2009;88:945-952.

Appended publication 7

Merjava S, Malinova E, Liskova P, Filipec M, Zemanova Z, Michalova K, Jirsova K. Recurrence of posterior polymorphous corneal dystrophy is caused by the overgrowth of the original diseased host endothelium. *Histochem Cell Biol*. 2011. Submitted.

Histol Histopathol (2011) 26: 323-331

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva

Stanislava Merjava[1], Ales Neuwirth[2], Michaela Tanzerova[1] and Katerina Jirsova[1,3]

[1]Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and Charles University, Prague, Czech Republic, [2]Department of Immunobiology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic and [3]Ocular Tissue Bank, General Teaching Hospital and Charles University, Prague, Czech Republic

Summary. The aim of this study was to detect a spectrum of cytokeratins (CK) present in the adult human cornea, limbus and perilimbal conjunctiva.

Cryosections from seven corneo-scleral discs were fixed, and indirect immunofluorescent staining was performed using antibodies directed against CK1-CK10 and CK13-CK20. The percentage of positive cells was calculated in the epithelium of the cornea, limbus and perilimbal conjunctiva. Quantitative real time RT-PCR (qRT-PCR) was used to detect CK6 and CK18 expression in the corneal and conjunctival epithelium.

The most intense staining present throughout the cornea was observed for CK3, CK5 and CK14; CK19 was found at the corneal periphery only. CK4 and CK10/13 revealed mild to moderate positivity mostly in the superficial layers of the cornea. The suprabasal cell layers of all examined areas showed a strong positivity for CK16. A heterogeneous staining pattern with a centrifugal decrease in the signal was observed for CK8 and CK18. CK5/6, CK14 and CK19 were present in the limbus, where a positive signal for CK3 was observed in the suprabasal and superficial cells only. In contrast to the cornea, CK15 appeared in the basal and suprabasal layers of the limbus. The perilimbal conjunctiva showed strong immunostaining for CK10/13, CK14 and CK19. A moderate signal for CK7 was detected in the superficial layers of the conjunctiva. qRT-PCR confirmed CK6 and CK18 expression in the corneal and conjunctival epithelium.

The detailed characterization of the corneal, limbal and perilimbal conjunctival epithelium under normal circumstances may be useful for characterizing the changes occurring under pathological conditions.

Offprint requests to: Katerina Jirsova, PhD, Laboratory of the Biology and Pathology of the Eye and Ocular Tissue Bank, VFN, U nemocnice 2, 128 08, Prague, Czech Republic. e-mail: katerina.jirsova@vfn.cz

Key words: Cornea, Epithelium, Cytokeratins, Immunohistochemistry

Introduction

The cornea forms the outermost surface of the human eye, transmits light into the inner eye and has a refractive power of approximately 43 dioptres (Olsen, 1986). The cornea's outer surface is bordered by epithelium and its basal membrane, while its inner surface is bordered by the corneal endothelium with the underlying Descemet's membrane. The widest central part of the cornea is composed of the stroma (extracellular matrix with scattered keratocytes). The peripheral cornea continues through a transition zone - the limbus (border of the cornea and sclera) - to the conjunctiva and sclera. The conjunctiva consists of stratified non-keratinizing epithelium and varies in thickness and appearance from the eyelid margin to the limbus (Nelson and Cameron, 2005).

The approximately 50 μm thick corneal epithelium is composed of nonkeratinized, stratified cells. It consists of five to six layers: a monolayer of columnar basal cells, two or three layers of wing cells and two or three layers of superficial cells. The differentiation process requires about 7 to 14 days, and then the superficial cells are desquamated into the tear film (Hanna et al., 1961). The corneal epithelium is renewed throughout life from basal cells and from a population of limbal epithelial stem cells, which proliferate and migrate centripetally to the central epithelium (Davanger and Evenson, 1971; Thoft and Friend, 1983; Tseng, 1989; Chang et al., 2008). The epithelium of the eye contributes to the maintenance of the optically smooth eye surface, provides a barrier to external biological and chemical insults and protects the ocular surface from

microbial attack (Sack et al., 2001).

Cytokeratins (CKs) are epithelial-specific intermediate filaments, which are expressed in a tissue-specific and differentiation-dependent manner (Moll et al., 1982). CKs can be divided into two subfamilies: acidic type I CKs 9-20 (the genes of which are present on chromosome 17, except the gene for CK18, which is located on chromosome 12) and neutral to basic type II CKs 1-8 (whose genes are located on chromosome 12), (Moll et al., 2008). Each CK is a product of a distinct gene (Upasani et al., 2004). CKs are very stable, relatively resistant to degradation and very antigenic (Morgan et al., 1987). Their function is not only to maintain the shape of a cell and to protect the cell from mechanical stress, but they are also important for differentiation and specialized functions, including protection from apoptosis, stress and injury, the intracellular transduction of signals and malignant transformation (Oshima et al., 1996; Caulin et al., 2000; Zatloukal et al., 2000; Kim et al., 2006). Some CKs, particularly CKs 5, 7, 8/18, 19 and 20, exhibit characteristic expression patterns in human tumors; therefore, they have great importance in the immunohistochemical diagnosis of carcinomas (Moll et al., 2008).

Simple epithelium expresses predominantly CKs 8, 18, 7, 17 and 19 (Leube et al., 1986; Moll et al., 2008); additionally, CKs 7 and 19 are characteristic of glandular epithelium (Ramaekers et al., 1987). The basal cell layer of the stratified epithelium expresses CKs 5 and 14, while the cells of the superficial layers express CKs 1 and 10 or 4 and 13, depending on the state of epithelial keratinization and differentiation (Moll et al., 1982; Morgan et al., 1987). CK6 and CK16 are typical of hyperproliferative epithelium (van der Velden et al., 1999; Franssen et al., 2004). CK15 is a basal keratinocyte keratin and has been proposed as a potential marker of stem cells in the hair follicle bulge (Lloyd et al., 1995; Lyle et al., 1998; Liu et al., 2003) as well as in limbal stem cells (Yoshida et al., 2006).

Mutations in individual cytokeratin genes cause a variety of human autosomal-dominant familial diseases, in some of which ocular pathology has been described. In the hereditary blistering skin disease epidermolysis bullosa simplex, conjunctival and corneal blistering occurs due to various point mutations in the CK5 and CK14 genes (Coulombe et al., 1991; Lane et al., 1992; Lin et al., 1994). In Meesmann's corneal dystrophy, mutations in the CK3 and CK12 genes cause intraepithelial microcyst formation in the corneal epithelium (Irvine et al., 1997). Mutations in the genes for CKs 6, 16 and 17 or CKs 1 and 10 are associated with congenital pachyonychia types I and II or with bullous congenital ichthyosiform erythroderma, respectively, where no known eye pathology has been described (Chipev et al., 1992; Rothnagel et al., 1992; McLean et al., 1994; Bowden et al., 1995; McLean et al., 1995). Similarly, mutations in CK4 and CK13 are associated with white sponge nevus, without eye pathology (Richard et al., 1995; Rugg et al., 1995). No disease-causing mutations in the human CK7, CK15, CK19 or CK20 genes have yet been found (Owens and Lane, 2004; Moll et al., 2008).

The expression of CK3, which forms a dimmer with CK12, is well documented in the corneal epithelium, and both are believed to be cornea-specific (Moll et al., 1982; Lauweryns et al., 1993a). CK4 is expressed in the central cornea, CK13 in the peripheral cornea and CK19 in the peripheral cornea and in the basal cells of the limbus and conjunctiva (Lauweryns et al. 1993a,b). In the corneal epithelium CKs 7, 8, 11, 14, 16, 18 and 19/5 have been previously detected (Ross et al., 1995; Cockerham et al., 2002). In the adult human corneal endothelium, no CKs have been detected with the exception of the CK pair 8/18 (Kasper et al., 1992; Merjava et al., 2009).

The aim of this study was to determine, in detail, the presence of CK1-CK10 and CK13-CK20 in healthy human corneal epithelium, limbus and perilimbal conjunctiva, which can help the characterization of corneas under pathological conditions.

Materials and methods

Specimens

The study followed the tenets set out in the Declaration of Helsinki. Eleven corneo-scleral discs (11-17 mm in diameter, age from 38 to 74, mean age of 55.1±14.1 years) not acceptable for transplantation because of low endothelial density or positive serology results of the donor, obtained from the Ocular Tissue Bank Prague, were used. The time between death and storage in liquid nitrogen did not exceed 24 hours. First, seven corneo-scleral discs were dissected, snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at -70°C. Tissues were cryosectioned at a thickness of 7 µm, and four slices were mounted per slide. Finally, four corneal and conjunctival epithelial imprints on Biopore Millicell membranes (MILLICELL®- CM, PICM 01250, Millipore, Bedford MA) were used for the detection of CK6 and CK18 by quantitative real time RT-PCR (qRT-PCR) (total mRNA was isolated from cells on the Millicell membranes). For CKs which are not expressed in the cornea or conjunctiva, epithelia from breast skin (for CK2e), skin from the palm of the hand and the sole of the foot (for CK9, CK10), the mammary gland (for CK17) and the stomach mucosa (for CK20) were used as positive control tissues.

Indirect fluorescent immunohistochemistry

Seven different corneal samples were used to prepare cryosections for indirect immunofluorescence. Three cryosections on each slide were stained with a single antibody. The fourth section was used as a negative control (primary antibody omitted). Two

independent experiments were performed. Sections were fixed with cold acetone for 10 minutes, rinsed in phosphate buffered saline (PBS) and then incubated with the primary antibodies diluted in PBS containing 1 % bovine serum albumin (BSA) for 1 hour at room temperature. The following mouse monoclonal or goat (CK1) antibodies and dilutions were used: anticytokeratin 1 (1:250), anti-cytokeratin 3 (1:50), anti-cytokeratin 5 (1:400), anti-cytokeratin 6 (1:20), anticytokeratin 10 (1:50) (Santa Cruz Biotechnology, Santa Cruz, USA); anticytokeratin 2e (1:10), anticytokeratin 9 (1:15), anticytokeratin 16 (1:30), anticytokeratin 17 (1:25) and anticytokeratin 20 (1:15) (Acris Antibodies GmbH, Hiddenhausen, Germany); anticytokeratin 4 (1:200) (Sigma, St. Louis, USA); anticytokeratin 5/6 (1:40), anticytokeratin 7 (1:50), anticytokeratin 10/13 (1:25), anticytokeratin 18 (1:50) and anticytokeratin 19 (1:50) (DakoCytomation, Glostrup, Denmark); anticytokeratin 8 (1:400), anticytokeratin 14 (1:50) and anticytokeratin 15 (1:100) (Chemicon International Inc., Temecula, USA). The specimens were washed three times with PBS and incubated with the appropriate secondary antibodies (fluorescein isothiocyanateconjugated or rhodamine-conjugated antimouse or antigoat IgG, Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 hour at room temperature. After rinsing in PBS the slices were mounted with Vectashield - propidium iodide or 4,6diamidino2phenylindoldihydrochlorid (DAPI) (Vector Laboratories, Inc. Burlingame, USA) to counterstain the DNA within the nuclei.

Specimen assessment

Slices were examined by fluorescent microscopy using an Olympus BX51 (Olympus Co., Tokyo, Japan) at a magnification of 100-400x. Images were taken using a Vosskühler VDS CCD-1300 camera, (VDS Vosskühler GmbH, Germany), and NIS Elements software (Laboratory Imaging, Czech Republic) was used for cell analysis. The corneal, limbal and perilimbal conjunctival epithelia were evaluated separately. At least 300 epithelial and 100 endothelial cells directly connected to DM were examined, and the percentage of positive cells was calculated. To grade the intensity of cell staining, a scale was used: N: negative, 1: mild, 2: moderate, 3: intense, and 4: very intense staining. The mean range was calculated from three sections and two experiments.

Quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated from four corneal and conjunctival epithelial imprints on Millicell membranes using Rneasy Plus Microkit (Qiagen, Hilden, Germany). At least two corneal and conjunctival specimens were used per experiment. RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamers followed by PCR amplification with Sybr green master mix (Roche Diagnostics, Mannheim, Germany). The following specific oligonucleotides for CK6, CK18 and the housekeeping gene glyceraldehyde-3-phospate

Table 1. Fluorescent immunhistochemistry of different cytokeratins (CK) in individual layers (basal, suprabasal, superficial) of healthy corneal, limbal and perilimbal conjunctival epithelium.

CK type	Cornea			Limbus			Conjunctiva		
	basal	suprabas.	superfic.	basal	suprabas.	superfic.	basal	suprabas.	superfic.
average % of positive cells / intensity									
CK 1	21/1	26/1	26/1	N	10/1	16/1	N	15/1	15/1
CK 2e	N	N	N	N	N	N	N	N	N
CK 3	52/2	66/2	91/3	N	28/2	8/1	N	N	N
CK 4	19/1	46/1	70/2	N	37/1	63/2	N	54/2	77/3
CK 5	51/2	51/2	54/2	24/2	46/2	47/2	28/2	43/2	47/2
CK 6	N	14/1	11/1	N	7/1	9/1	N	N	9/1
CK 5/6	90/2	89/2	86/2	63/1	86/2	86/2	57/1	87/2	84/2
CK 7	N	N	N	N	N	N	N	N	31/2
CK 8	34/1	45/1	69/2	42/1	38/1	56/2	19/1	30/2	56/2
CK 9	N	N	N	N	N	N	N	N	N
CK 10	N	N	N	N	N	N	N	N	N
CK 10/13	10/1	29/1	45/2	N	39/2	62/2	10/1	70/2	82/3
CK 14	70/2	69/2	52/2	87/2	63/2	46/2	83/3	66/2	56/2
CK 15	N	N	N	46/2	21/1	N	67/2	23/1	17/1
CK 16	33/1	78/2	61/2	N	12/1	N	N	10/2	N
CK 17	N	N	N	N	N	N	N	N	N
CK 18	62/1	43/1	42/1	16/1	21/1	32/1	N	N	26/1
CK 19	54/2*	55/1*	65/2*	63/2	59/2	83/3	71/2	59/2	88/3
CK 20	N	N	N	N	N	N	N	N	N

Number of cells stained: % (average percentage) / N: negative, 1: mild, 2: moderate, 3: intense, and 4: very intense staining. *: assessed from the peripheral parts of the corneal epithelium.

dehydrogenase (GAPDH) were used: human CK6, sense primer 5' -agtttgcctccttcatcgac- 3', anti-sense primer 5' -cagcagggtccactttgttt- 3'; human CK18, sense primer 5' -cctgctgtccgtgtccat- 3', anti-sense primer 5' -ggaccggtagttggtgga- 3'; human GAPDH, sense primer 5' -agc cac atc gct cag acac- 3', anti-sense primer 5' -gcc caa tac gac caa atcc- 3'. The reactions were run in triplicate on a LC480 thermocycler (Roche Diagnostics).

The gene expression for CK6 and CK18 was quantified using a relative quantification model with efficiency correction using the following formula: ratio = $(E_{target})^{Ct\ target\ (control\text{-}treated)} / (E_{ref})^{Ct\ ref\ (control\text{-}treated)}$ according to Pfaffl (2001). The efficiency for each gene was estimated by the dilution calibration method. The sample with the lowest cytokeratin expression was taken as the calibrator (expression = 1), and the experiment was done

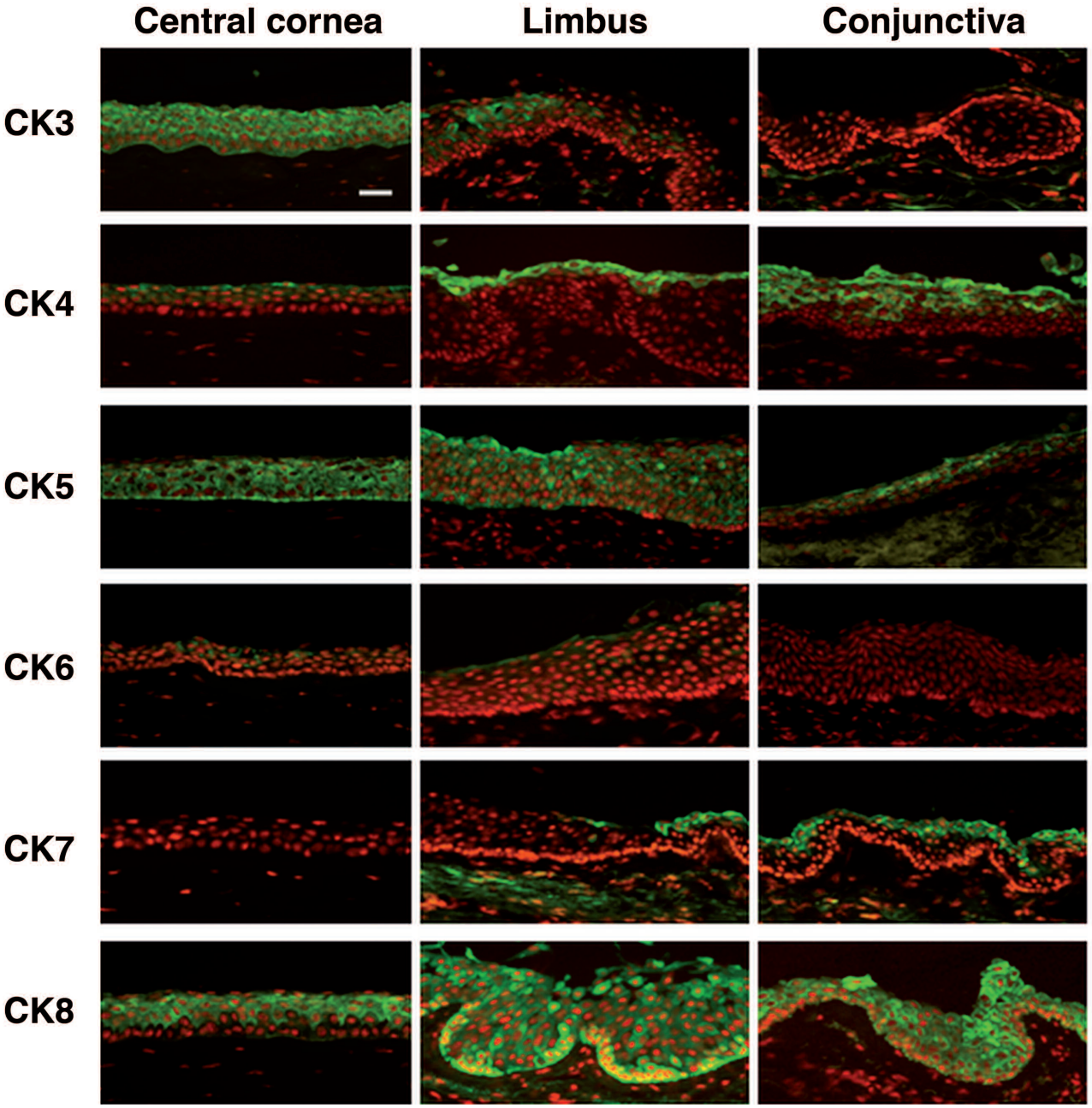

Fig. 1. The presence of neutral to basic cytokeratins (CK1 - CK8) in the epithelium of the adult human central cornea, limbus and conjunctiva. Indirect immunofluorescent staining was used. Scale bar: 50 μm.

in duplicate.

Results

The results of the immunohistochemical analysis of normal tissue stained with antibodies to various CKs are shown in Table 1 and representative figures are presented in Figs. 1, 2.

In the cornea moderate to intense positivity for CK3 was observed in most cells (70%; mean percentage from the values obtained in the basal, suprabasal and superficial layers). CK5, 5/6, 14 and 19 revealed moderate positivity dispersed throughout the corneal epithelium; CK5 was present in 52% of all detected

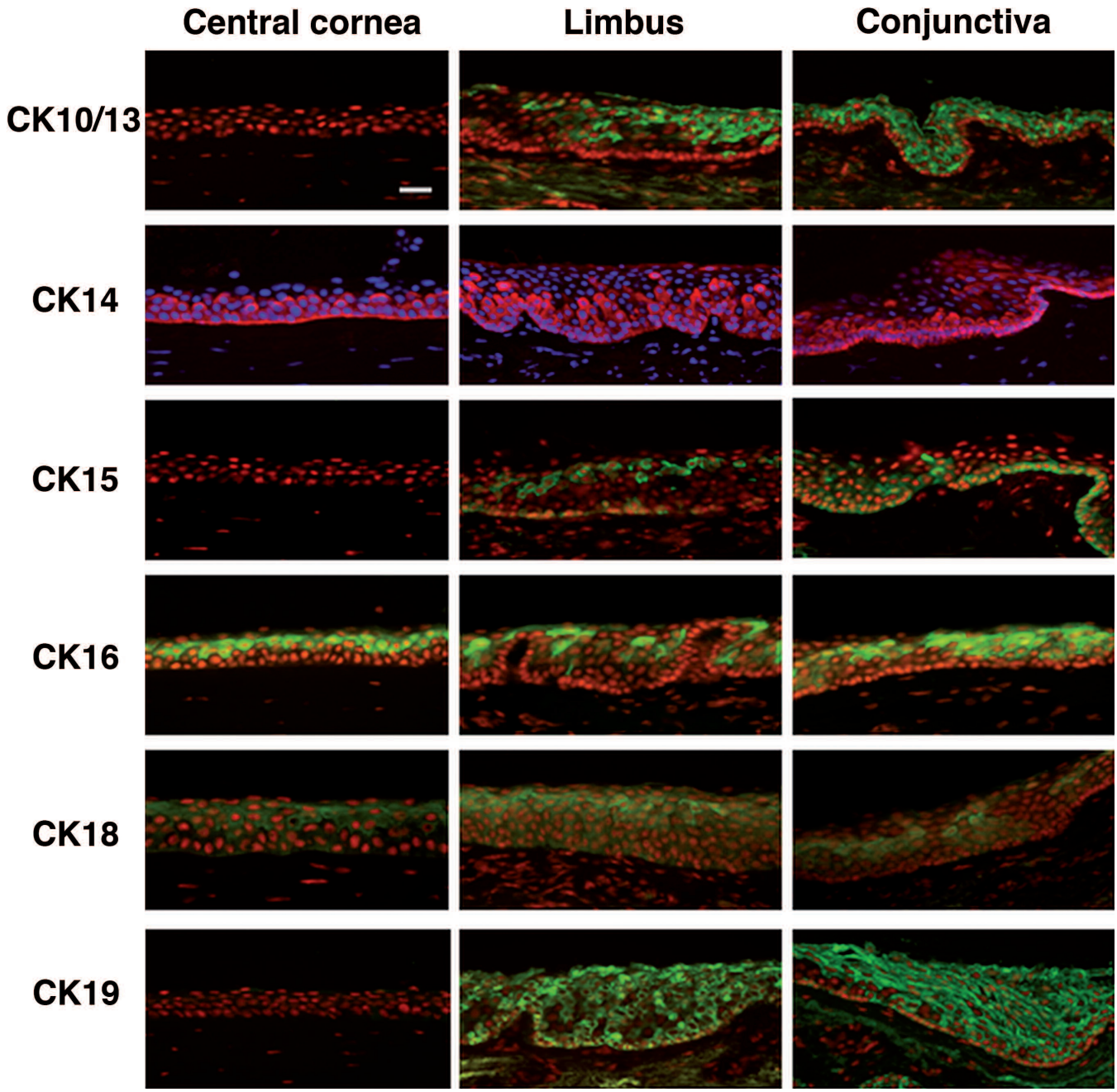

Fig. 2. The presence of acidic cytokeratins (CK9 - CK20) in the epithelium of the adult human central cornea, limbus and conjunctiva. Indirect immunofluorescent staining was used. Scale bar: 50 μm.

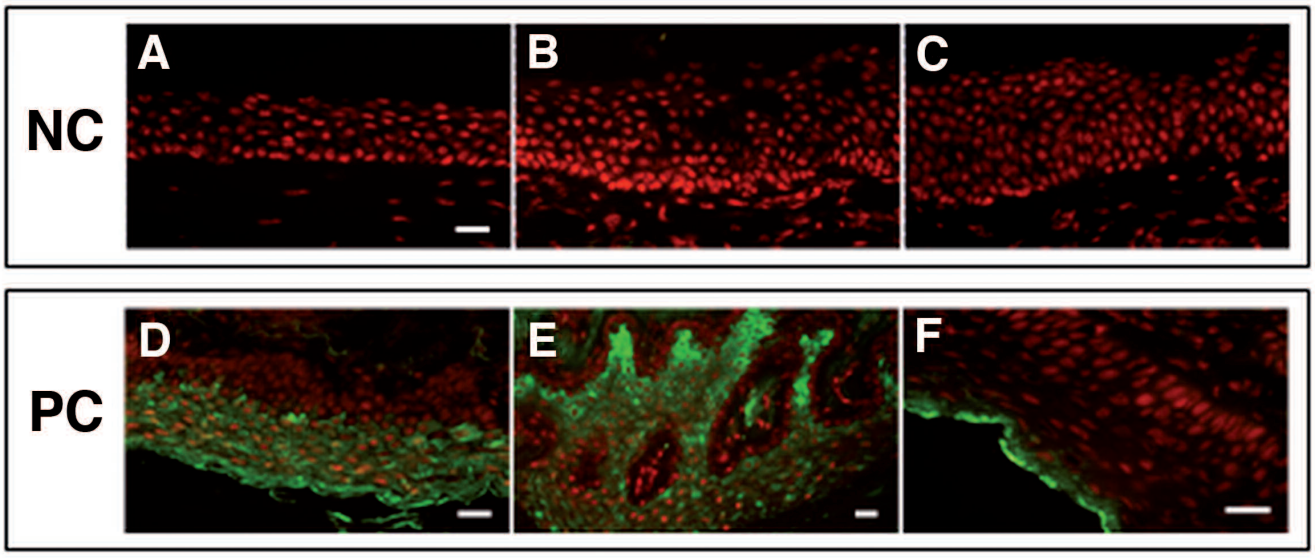

Fig. 3. Negative control (NC) of the corneal **(A),** limbal **(B)** and conjunctival **(C)** epithelium. Epithelia from breast skin for CK2e **(D)**, skin from the palm of the hand and the sole of the foot for CK9 **(E)** and CK10 **(F)** were used as positive control tissues (PC). Scale bar: 50 μm.

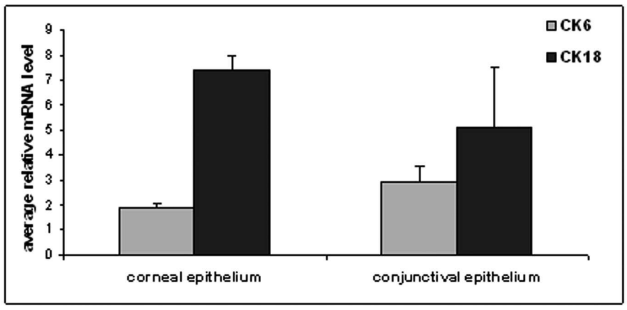

Fig. 4. Average relative expression of mRNA encoding cytokeratin 6 (CK6) and cytokeratin 18 (CK18) in four corneal and conjunctival epithelium samples. Relative CK6 and CK18 expression was normalized to GAPDH expression, and the sample with the lowest cytokeratin expression was taken as the calibrator (expression = 1).

cells; CK5/6 was present in 88% of all detected cells. CK6 revealed only mild positivity in 12% of the suprabasal and superficial cells. CK14 was detected in 70% of cells located in the basal layer and in 52% of cells of the superficial corneal layer. CK19 was present in 58% of cells; however, the immunostaining occurred mostly at the periphery of the cornea, while almost no positivity was detected in the central part of the cornea. A signal for CK16 was observed in all corneal epithelial layers; the most intense staining and the highest percentage of positive cells was present in the suprabasal layer (78% of cells). CK4 and CK10/13 revealed mild to moderate positivity in all corneal layers, where the percentage of positive cells increased from the basal (19 and 10%, respectively) to the superficial layer (70 and 45%, respectively), but the immunostaining for CK10/13 was present only in the peripheral part of the corneal epithelium. CK8 and CK18 were detected throughout all the corneal epithelial layers, with the majority of CK8 positive cells in the superficial layer (69%) and the highest number of CK18 positive cells in the basal layer (62%). All corneal epithelial layers were mildly positive for CK1. CK15 was expressed in a few epithelial basal cells at the corneal periphery in two of seven specimens. No staining for CK2e, CK7, CK9, CK10, CK17 and CK20 was observed in any corneal specimen. No signal was present in any of the negative controls for any of the studied CKs (Fig. 3a, b, c). The staining for CK2e, CK9, CK10, CK17 and CK20 is shown in the appropriate positive controls (Fig. 3d-f).

The strongest positivity in the limbus was detected for CKs 5, 5/6 and 14 (where the staining decreased from the basal to the superficial layers) as well as CK19. Heterogenous staining in each limbal layer was observed for CKs 8 and 18 (mean 45% and 23%, respectively). CK3 was present in 8% of the superficial and 28% of the suprabasal cells, while the basal layer was negative. The immunostaining declined from the border between the cornea and limbus towards the conjunctiva, in which staining for CK3 was negative. A signal in the superficial and suprabasal layers was observed for CK4, CK6, CK10/13 and CK1. CK16 was present in 12% of the suprabasal cells. In contrast to its absence in the cornea, CK15 appeared in the basal and suprabasal cells of the limbus.

The strongest positivity in the perilimbal conjunctiva was detected for CK5, 5/6, 14 and CK19, for which the

staining decreased from the basal to the superficial layers, and for CK10/13, 4 and CK1, for which the staining decreased in the opposite direction. CK16 was present in 10% of the suprabasal cells. Heterogenous staining in each conjunctival layer was observed for CK8 (mean 35% of cells). In contrast to the cornea and limbus, CK18 was detected only in the superficial layer (26% of cells) of the conjunctiva. CK15 appeared in each conjunctival layer, with the strongest positivity seen in the basal cells. Unlike the cornea and limbus, CK3 was absent throughout the whole conjunctival epithelium.

Of all the tested CKs, only CK8 and CK18 were present in the corneal endothelium in 46 and 23% of the cells, respectively. None of the tested CKs was expressed by keratocytes in the stroma.

The expression of CK6 and CK18 was confirmed by qRT-PCR. Relative CK6 and CK18 expression was normalized to an endogenously expressed housekeeping gene (GAPDH). Our data clearly showed the lower expression of CK6 and the higher expression of CK18 in both corneal and conjunctival epithelium (Fig. 4).

Discussion

This study describes in detail the CK spectrum in the human cornea, limbus and perilimbal conjunctiva. We have found that the expression of CK3, a corneal epitheliumspecific protein (Moll et al., 1982), decreases centrifugally from the superficial to the basal corneal layers. Moreover, we have found it in the suprabasal cells and in a minority of the superficial limbal cells, compared to the completely negative basal limbal cells. The absence of CK3 from the basal limbal cells makes it possible not only to distinguish between corneal and limbal cells, as necessary for the diagnosis of limbal stem cell deficiency (Donisi et al., 2003), but also between basal cells and cells from the upper limbal layers. This can be helpful for the subsequent separation and identification of limbal epithelial stem cells.

CK19, as a minor cytoskeletal component of the corneal epithelium, has been described as one of the major components in the conjunctival epithelium (Kasper et al., 1988; Elder et al., 1997; Kivelä and Uusitalo, 1998; Pitz and Mol 2002; Schlotzer-Schrehardt and Kruse, 2005). It exhibits the opposite direction in its labeling gradient than does CK3. The CK19 expression pattern through the central epithelium of the cornea is still a matter of some controversy. Our and several other studies have reported that CK19 is located in all layers of the conjunctival and limbal epithelium and that its presence decreases in the peripheral part of the cornea, and finally disappears in the central corneal epithelium (Kasper et al., 1988; Lauweryns et al., 1993a; Pitz and Mol, 2002). On the other hand, a few studies have found various numbers of CK19-positive cells in the central cornea (Chen et al., 2004; Yoshida et al., 2006). These differing results show that besides the methodological approach used in tissue processing, different antibody sensitivities or different fixation methods may contribute to controversial results (Mygind et al., 1988). On the other hand, we have seen no differences in staining for CK4, 5, 6, 8, 14, 16 or 18 between non-blocked sections and sections blocked in 2.5% BSA. It was postulated that the condition of the tissue used, including the time between death and processing of the tissue, may affect cytokeratin expression (Di Iorio et al., 2005). In our experiments the time between death and storage in liquid nitrogen did not exceed 24 hours.

More pronounced differences between the superficial layer of the cornea and the conjunctiva were found with CK7. This protein was intensely present throughout the surface epithelial layer of the conjunctiva yet absent from the superficial layer of the limbus and cornea. This CK was previously described by Krenzer and Freddo, (1997) only in goblet cells and by Elder et al. (1997) in basal and suprabasal epithelial cells of the central cornea.

The simple epithelial cytokeratins CK8 and CK18 were detected in the central corneal epithelium as well as in the limbal and conjunctival epithelium (confirmed using qRT-PCR). From our broad CK spectrum, only these two cytokeratins were detected in endothelial cells, as was shown previously (Merjava et al., 2009). Lastly, the simple epithelial cytokeratin CK17 was completely negative in our experiments, in contrast to the results of Elder et al., (1997) who found CK17 throughout the whole corneal, limbal and conjunctival epithelium. This could be explained by the different sensitivities of the antibodies used in the two studies.

Stratified epithelia CKs revealed the same expression pattern as was described by other authors (Moll et al., 1982; Morgan et al., 1987; Kurpakus et al., 1990). CK4 was detected predominantly in the superficial cell layers and CK14, as a marker of undifferentiated cells in stratified epithelia, mostly in the basal cells of the central, limbal and conjunctival epithelium, respectively. CK15, a minor cytoskeletal component of stratified tissue (Moll et al., 1982), was detected in the basal cells of stratified squamous epithelia as well as in basal cells of the limbus and conjunctiva (Lloyd et al., 1995). Previously, its expression was described in hair follicle bulge cells in the human scalp (Lyle et al., 1998). Our results support the finding that CK15 is predominantly expressed in the basal layers of the conjunctiva and limbus (Yoshida et al., 2006; Figueira et al., 2007; Lyngholm et al., 2008), an area where limbal epithelial stem cells occur, and thus can be considered as a limbal epithelial stem cell marker. Herein we are reporting for the first time the presence of CK6 in the corneal epithelium. Mild positivity was found when a monospecific antibody against CK6 was used, compared to the intense signal obtained using a dual specific antibody against the CK pair 5/6. These results indicate that the positivity observed using the CK5/6 antibody was predominantly CK5 positivity. CK6 and CK16 were detected mainly in the suprabasal cells of the corneal, limbal and conjunctival epithelium.

Although CK6 and CK16 are among the cytokeratins typical of proliferating cells, and we expected their occurrence in the basal layer of the epithelium, their presence in suprabasal cells is not unique (Sun et al., 1984; Mansbridge and Knapp, 1987; van der Velden et al., 1999). The expression of CK6 in the corneal and conjunctival epithelium was confirmed using qRT-PCR.

From CK1 and CK10, which are typical of keratinizing stratified epithelia (Moll et al., 1982), only CK1 was detected in a low amount, predominantly in the superficial cell layers of the corneal, limbal and conjunctival epithelium, which confirms that corneal epithelial cells are primarily nonkeratinized, but they do differentiate and desquamate (Hanna et al., 1961). Using an antibody against CK10 only, surprisingly no positivity was detected, in contrast to the positivity obtained using an antibody which detected CK10 and CK13 together. It seems likely that this positivity was the result of CK13 staining only, in agreement with the nonkeratinized pattern of the corneal epithelium, because CK13, together with CKs 4, 5 and 14, is typical of nonkeratinising stratified epithelia such as the buccal mucosa and alveolar mucosa (Clausen et al., 1986; Sawaf et al., 1991).

Taken together, our results show that the corneal, limbal and conjunctival epithelium express a wide spectrum of cytokeratins and that the corneal epithelium can be characterized as primary nonkeratinizing stratified epithelium (no CK10 and weak CK1 positivity, strong CK3, 4, 5, 13 and 14 positivity), but with the expression of some simple epithelial CKs (CKs 8 and 18).

Acknowledgements. This work was supported by the research project of the Czech Ministry of Education, Youth and Sports 0021620806/20610011. We would like to thank Dr. V. Vesela for excellent technical assistance with the preparation of the specimens.

References

Bowden P.E., Haley J.L., Kansky A., Rothnagel J.A., Jones D.O. and Turner R.J. (1995). Mutation of a type II keratin gene (K6a) in pachyonychia congenita. Nat. Genet. 10, 363-365.

Caulin C., Ware C.F., Magin T.M. and Oshima R.G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. J. Cell Biol. 149, 17-22.

Chang C.Y., Green C.R., McGhee C.N. and Sherwin T. (2008). Acute wound healing in the human central corneal epithelium appears to be independent of limbal stem cell influence. Invest. Ophthalmol. Vis. Sci. 49, 5279-5286.

Chen Z., de Paiva C.S., Luo L., Kretzer F.L., Pflugfelder S.C. and Li D.Q. (2004). Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22, 355-366.

Chipev C.C., Korge B.P., Markova N., Bale S.J., DiGiovanna J.J., Compton J.G. and Steinert P.M. (1992). A leucine-proline mutation in the H1 subdomain of keratin 1 causes epidermolytic hyperkeratosis. Cell 70, 821-828.

Clausen H., Vedtofte P., Moe D., Dablesteen E., Sun T.T. and Dale B. (1986). Differentiation-dependent expression of keratins in human oral epithelia. J. Invest. Dermatol. 86, 249-254.

Cockerham G.C., Laver N.V., Hidayat A.A. and McCoy D.L. (2002). An immunohistochemical anylysis and comparison of posterior polymorphous dystrophy with congenital hereditary endothelial dystrophy. Cornea 21, 787-791.

Coulombe P.A., Hutton M.E., Letai A., Hebert A., Paller A.S. and Fuchs E. (1991). Point mutation in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. Cell 66, 1301-1311.

Davanger M. and Evensen A. (1971). Role of the pericorneal structure in renewal of corneal epithelium. Nature 229, 560-561.

Di Iorio E., Barbaro V., Ruzza A., Ponzin D., Pellegrini G. and De Luca M. (2005). Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc. Natl. Acad. Sci. USA 102, 9523-9528.

Donisi P.M., Rama P., Fasolo A. and Ponzin D. (2003). Analysis of limbal stem cell deficiency by corneal impression cytology. Cornea 22, 533-538.

Elder M.J., Hiscott P. and Dart J.K. (1997). Intermediate filament expression by normal and diseased human corneal epithelium. Hum. Pathol. 28, 1348-1354.

Figueira E.C., Di Girolamo N., Coroneo M.T. and Wakefield D. (2007). The phenotype of limbal epithelial stem cells. Invest. Ophthalmol. Vis. Sci. 48, 144-156.

Franssen M.E., Boezeman J.B., Van De Kerkhof P.C. and Van Erp P.E. (2004). Monitoring hyperproliferative disorders in human skin: flow cytometry of changing cytokeratin expression. Cytometry B. Clin. Cytom. 57, 32-39.

Hanna C., Bicknell D.S. and O'Brien J.E. (1961). Cell turnover in the adult human eye. Arch. Ophthalmol. 65, 695-698.

Irvine A.D., Corden L.D., Swensson O., Swensson B., Moore J.E., Frazer D.G., Smith F.J., Knowlton R.G., Christophers E., Rochels R., Uitto J. and McLean W.H. (1997). Mutations in cornea-specific keratin K3 or K12 genes cause Meesmann's corneal dystrophy. Nat. Genet. 16, 184-187.

Kasper M., Moll R., Stosiek P. and Karsten U. (1988). Patterns of cytokeratin and vimentin expression in the human eye. Histochemistry 89, 369-377.

Kasper M., Stosiek P. and Lane B. (1992). Cytokeratin and vimentin heterogeneity in human cornea. Acta Histochem. 93, 371-381.

Kim S., Wong P. and Coulombe P.A. (2006). A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. Nature 441, 362-365.

Kivelä T. and Uusitalo M. (1998). Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye. Prog. Retin. Eye Res. 17, 385-428.

Krenzer K.L. and Freddo T.F. (1997). Cytokeratin expression in normal human bulbar conjunctiva obtained by impression cytology. Invest. Ophthalmol. Vis. Sci. 38, 142-152.

Kurpakus M.A., Stock E.L. and Jones J.C. (1990). Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. Invest. Ophthalmol. Vis. Sci. 31, 448-456.

Lane E.B., Rugg E.L., Navsaria H., Leigh I.M., Heagerty A.H., Ishida-Yamamoto A. and Eady R.A. (1992). A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. Nature 356, 244-246.

Lauweryns B., van den Oord J.J., De Vos R. and Missotten L. (1993a). A new epithelial cell type in the human cornea. Invest. Ophthalmol.

Vis. Sci. 34, 1983-1990.

Lauweryns B., van den Oord J.J. and Missotten L. (1993b). The transitional zone between limbus and peripheral cornea. An immunohistochemical study. Invest. Ophthalmol. Vis. Sci. 34, 1991-1999.

Leube R.E., Bosch F.X., Romano V., Zimbelmann R., Höfler H. and Franke W.W. (1986). Cytokeratin expression in simple epithelia. III. Detection of mRNAs encoding human cytokeratins nos. 8 and 18 in normal and tumor cells by hybridization with cDNA sequences *in vitro* and *in situ*. Differentiation 33, 69-85.

Lin A.N., Murphy F., Brodie S.E. and Carter D.M. (1994). Review of ophthalmic findings in 204 patients with epidermolysis bullosa. Am. J. Ophthalmol. 118, 384-390.

Liu Y., Lyle S., Yang Z. and Cotsarelis G. (2003). Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. J. Invest. Dermatol. 121, 963-968.

Lloyd C., Yu Q.C., Cheng J., Turksen K., Degenstein L., Hutton E. and Fuchs E. (1995). The basal keratin network of stratified squamous epithelia: defining K15 function in the absence of K14. J. Cell Biol. 129, 1329-1344.

Lyle S., Christofidou-Solomidou M., Liu Y., Elder D.E., Albelda S. and Cotsarelis G. (1998). The C8/144b monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. J. Cell Sci. 111, 3179-3188.

Lyngholm M., Hoyer P.E., Vorum H., Nielsen K., Ehlers N. and Mollgard K. (2008). Immunohistochemical markers for corneal stem cells in the early developing human eye. Exp. Eye Res. 87, 115-121.

Mansbridge J.N. and Knapp A.M. (1987). Changes in keratinocyte maturation during wound healing. J. Invest. Dermatol. 89, 253-263.

McLean W.H., Eady R.A., Dopping-Hepenstal P.J., McMillan J.R., Leigh I.M., Navsaria H.A., Higgins C., Harper J.I., Paige D.G., Morley S.M. and Lane E.B. (1994). Mutations in the rod 1A domain of keratins 1 and 10 in bullous congenital ichthyosiform erythroderma (BCIE). J. Invest. Dermatol. 102, 24-30.

McLean W.H., Rugg E.L., Lunny D.P., Morley S.M., Lane E.B., Swensson O., Dopping-Hepenstal P.J., Griffiths W.A., Eady R.A., Higgins C., Navsaria H.A., Leigh I.M., Strachen T., Kunkeler I. and Munro C.S. (1995). Keratin 16 and keratin 17 mutations cause pachyonychia congenita. Nat. Genet. 9, 273-278.

Merjava S., Neuwirth A., Mandys V. and Jirsova K. (2009). Cytokeratins 8 and 18 in adult human corneal endothelium. Exp. Eye Res. 89, 426-431.

Moll R., Franke W.W., Schiller D.L., Geiger B. and Krepler R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11-24.

Moll R., Divo M. and Langbein L. (2008). The human keratins: biology and pathology. Histochem. Cell Biol. 129, 705-733.

Morgan P.R., Shirlaw P.J., Johnson N.W., Leigh I.M. and Lane E.B. (1987). Potential applications of anti-keratin antibodies in oral diagnosis. J. Oral Pathol. 16, 212-222.

Mygind H., Nielsen B., Moe D., Clausen H., Dabelsteen E. and Clausen P.P. (1988). Antikeratin antibodies in routine diagnostic pathology. A comparison of 10 different commercial antikeratins. APMIS 96, 1009-1022.

Nelson J.D. and Cameron J.D. (2005). Cornea. In: Cornea. 2nd edition. Krachmer J.H., Mannis M.J. and Holland E.J. (eds). Elsevier MOSBY Inc. pp 3-26.

Olsen T. (1986). On the calculation of power from curvature of the cornea. Br. J. Ophthalmol. 70, 152-154.

Owens D.W. and Lane E.B. (2004). Keratin mutations and intestinal pathology. J. Pathol. 204, 377-385.

Oshima R.G., Baribault H. and Caulin C. (1996). Oncogenic regulation and function of keratins 8 and 18. Cancer Metastasis Rev. 15, 445-471.

Pfaffl M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pitz S. and Moll R. (2002). Intermediate-filament expression in ocular tissue. Prog. Retin. Eye Res. 21, 241-262.

Ramaekers F., Huysmans A., Schaart G., Moesker O. and Vooijs P. (1987). Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp. Cell. Res. 170, 235-249.

Richard G., De Laurenzi V., Didona B., Bale S.J. and Compton J.G. (1995). Keratin 13 point mutation underlies the hereditary mucosal epithelial disorder white sponge nevus. Nat. Genet. 11, 453-455.

Ross J.R., Foulks G.N., Sanfilippo F.P. and Howell D.N. (1995). Immunohistochemical analysis of the pathogenesis of posterior polymorphous dstrophy. Arch. Ophthalmol. 113, 340-345.

Rothnagel J.A., Dominey A.M., Dempsey L.D., Longley M.A., Greenhalgh D.A., Gagne T.A., Huber M., Frenk E., Hohl D. and Roop D.R. (1992). Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. Science 257, 1128-1130.

Rugg E.L., McLean W.H., Allison W.E., Lunny D.P., Macleod R.I., Felix D.H., Lane E.B. and Munro C.S. (1995). A mutation in the mucosal keratin K4 is associated with oral white sponge nevus. Nat. Genet. 11, 450-452.

Sack R.A., Nunes I., Beaton A. and Morris C. (2001). Host-defense mechanism of the ocular surfaces. Biosci. Rep. 21, 463-480.

Sawaf M.H., Ouhayoun J.P. and Forest N. (1991). Cytokeratin profiles in oral epithelia: a review and new classification. J. Biol. Buccale 19, 187-198.

Schlötzer-Schrehardt U. and Kruse F.E. (2005). Identification and characterization of limbal stem cells. Exp. Eye Res. 81, 247-264.

Sun T.T., Eichner R., Schermer A., Cooper D., Nelson W.G. and Weiss R.A. (1984). The transformed phenotype. In: The cancer cell. Vol. 1. Levine A., Topp W., van de Woude G. and Watson J.D. (eds). Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. pp 169-176.

Thoft R.A. and Friend J. (1983). The X, Y, Z hypothesis of corneal epithelial maintenance. Invest. Ophthalmol. Vis. Sci. 24, 1442-1443.

Tseng S.C. (1989). Concept and application of limbal stem cells. Eye 3, 141-157.

Upasani O.S., Vaidya M.M. and Bhisey A.N. (2004). Database on monoclonal antibodies to cytokeratins. Oral Oncol. 40, 236-256.

van der Velden L.A., Manni J.J., Ramaekers F.C. and Kuijpers W. (1999). Expression of intermediate filament proteins in benign lesions of the oral mucosa. Eur. Arch. Otorhinolaryngol. 256, 514-519.

Yoshida S., Shimmura S., Kawakita T., Miyashita H., Den S., Shimazaki J. and Tsubota K. (2006). Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. Invest. Ophthalmol. Vis. Sci. 47, 4780-4786.

Zatloukal K., Stumptner C., Lehner M., Denk H., Baribault H., Eshkind L.G. and Franke W.W. (2000). Cytokeratin 8 protects from hepatotoxicity, and ist ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. Am. J. Pathol. 156, 1263-1274.

Accepted September 27, 2010

Cytokeratin 8 Is Expressed in Human Corneoconjunctival Epithelium, Particularly in Limbal Epithelial Cells

Stanislava Merjava,[1] *Kristyna Brejchova,*[1] *Amanda Vernon,*[2] *Julie T. Daniels,*[2] *and Katerina Jirsova*[1,3]

Purpose. The purpose of this study was to investigate the expression of cytokeratin (CK) 8 in the corneoconjunctival epithelium.

Methods. In 17 cadaveric corneoscleral discs and 3 other discs, the presence of CK8 alone or CK8, together with CK3, CK15, vimentin, and integrin α6, was investigated by using indirect immunohistochemistry on radial cryosections. Four corneoscleral discs stored in organ culture were used for the preparation of tangential sections of the limbus and for the isolation of limbal epithelial cells and their subsequent cultivation. CK8 expression was examined by RT-PCR in the corneal, limbal, and conjunctival epithelium.

Results. Sixty percent of the cadaveric corneoscleral samples and all samples stored in organ culture revealed positivity for CK8 in the basal epithelial layer of the limbus. Positive basal cells formed a single line or separated clusters. The signal for CK8 became weaker toward the surface of the limbal epithelium. The colocalization of CK8 with vimentin and CK15 in the limbus was also found. CK3 showed only occasional positivity in some of the surface limbal cells. The expression of integrin α6 in the basal membrane was absent or decreased under the CK8-positive clusters. Cell cultures revealed strong positivity for CK8 in approximately 80% of the cultured cells, and CK8 expression in the cornea, limbus, and conjunctiva was determined by RT-PCR.

Conclusions. The study demonstrates the strong expression of CK8 in limbal epithelial basal cells, which is maintained during the differentiation and migration of the limbal cells toward the central corneal epithelium. (*Invest Ophthalmol Vis Sci.* 2011; 52:787–794) DOI:10.1167/iovs.10-5489

The corneal epithelium is a rapidly regenerating, nonkeratinized, stratified squamous epithelium that is continuously renewed throughout life from basal cells of the epithelium and from the population of limbal epithelial stem cells (LESCs), which proliferate and migrate centripetally to the corneal epithelium.[1–5] LESCs exhibiting high proliferative capacity are located in the basal layer of the limbus, a highly vascularized and innervated transition zone between the cornea and conjunctiva. Unipotent LESCs undergo asymmetric self-renewal cell division, in which one cell remains undifferentiated as a stem cell, while the fast-dividing progenitor cell, referred to as the transit amplifying cell (TAC), begins to divide and differentiates into the suprabasal and superficial cells of the corneal epithelium.[6–9]

LESCs are involved in the renewal of the corneal epithelium, which is important, not only for sustaining the integrity of the ocular surface, but also for the maintenance of visual function.[2,5] Despite extensive investigative effort, no definitive marker that may be helpful in identifying and isolating LESCs is known with certainty. Although cytokeratin (CK) expression alone is not sufficient to identify stem cells or progenitor TACs, the expression profile of several key cytokeratins (CK19, CK15), together with other known potential markers (ABCG2, p63), can be used to identify LESCs.[9–12] Adhesion molecules such as integrins can also play a role in the identification of LESCs and TACs (especially a lack of α6 and β4 integrins in the limbal area).[13]

CKs are intermediate filaments typical of epithelial cells, which are expressed in a tissue-specific, differentiation-dependent manner.[14] A broad spectrum of CKs has been detected in the corneal and limbal epithelium.[15–17] Limbal basal cells do not express CK3/12, which is a typical marker for advanced corneal epithelial differentiation,[14,18] although CK5/14, CK19, and vimentin have been detected in such cells.[13,16,18–21] CK15 was proposed to be a putative marker of stem cells in the hair follicle bulge[22] and a potential marker for LESCs and early TACs.[23,24]

CK8 (together with CK18) is the major component of the intermediate filaments of the simple and single-layered epithelia found in the liver and mammary gland, among other tissues.[25,26] CK8 and CK18 are the first cytokeratins expressed during embryogenesis, and the absence of CK8 causes midgestational lethality or colorectal hyperplasia and inflammation in mice.[27–29] The total amount of cellular CK8 and CK18 is kept at a stable level under physiological conditions,[14] whereas the expression of both often increases during carcinogenesis.[26,30] CK8 and -18 have a cytoprotective role against chemical insults[31] and modulate the cellular response to proapoptotic signals.[32–36] Their role in the regulation of the cell cycle has already been described.[37–40]

Recently, it was shown that besides the presence of CK8 and -18 in the corneal epithelium,[16] both CKs are expressed in the corneal endothelium.[41] Moreover, CK8 has been found in human superficial conjunctival cells as well as scattered throughout the mammalian conjunctival epithelium.[42,43] In this study, we report the strong expression of CK8 in the basal cells of the limbus, which is also retained during the differen-

From the [1]Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders and the [3]Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Czech Republic; the [2]Cells for Sight Transplantation and Research Programme, Department of Ocular Biology and Therapeutics, University College London (UCL) Institute of Ophthalmology, London, United Kingdom.

Supported by the research project of the Ministry of Education, Youth, and Sports of the Czech Republic MSM0021620806 and by Project 260501 from Charles University in Prague.

Submitted for publication March 8, 2010; revised June 14 and August 31, 2010; accepted September 14, 2010.

Disclosure: **S. Merjava**, None; **K. Brejchova**, None; **A. Vernon**, None; **J.T. Daniels**, None; **K. Jirsova**, None

Corresponding author: Stanislava Merjava, Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University, Unemocnice 2, 128 08, Prague, Czech Republic; merjava@centrum.cz.

tiation and migration of the limbal cells toward the central cornea. The possible function of CK8 in the activation, proliferation, and/or migration of limbal cells is not clear and requires further investigation.

Materials and Methods

Samples

The study was conducted according to the standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set forth in the Declaration of Helsinki. In total, 29 cadaveric corneoscleral discs (11–17 mm in diameter; mean age, 57.5 ± 17.4 years; range, 16–83) that were not acceptable for transplantation because of low endothelial cell density or a positive serology of the donor obtained from the Ocular Tissue Bank, Prague, were used.

Twenty corneoscleral discs (mean age, 56.8 ± 19.3 years) were dissected, snap frozen in liquid nitrogen, embedded in OCT, and stored at −70°C. The time between death and storage in liquid nitrogen did not exceed 24 hours. Tissues were cryosectioned radially at a thickness of 7 μm, to evaluate the whole structure of the cornea, and four sections were mounted per slide.

Four corneoscleral discs (mean age, 51.3 ± 7.3 years) were stored in organ culture in minimum essential medium with 2% fetal calf serum, as described elsewhere,[44] at 31°C for 8 to 9 days. After storage, the corneas were divided, and half of each cornea was used for the isolation of limbal epithelial cells and their subsequent cultivation. The second half was divided into two quarters, which were frozen (−70°C) and sectioned (7 μm) as tangential sections of the limbus, allowing evaluation of the complex architecture of the limbal crypts.[45]

Five corneoscleral discs (mean age, 65.4 ± 11.4 years) were used for the preparation of corneal epithelial, limbal epithelial, and perilimbal conjunctival samples. Lamellar rectangular dissections of the limbal and central corneal epithelial tissue (2 mm in diameter), both containing a minimum of underlying stromal tissue, were prepared with the use of a diamond knife (depth, ~100 μm). The samples of perilimbal conjunctiva were cut using corneal scissors. Immediately after preparation, the samples were placed in RNA stabilizer (RNAlater; Qiagen GmbH, Hilden, Germany); mRNA was isolated and subsequently used for semiquantitative reverse transcription polymerase chain reaction (RT-PCR).

Primary Limbal Epithelial Cell Isolation and Culture: Single-Cell Suspension Culture

Limbal epithelial cells (LECs) were cultured in corneal epithelial culture medium (CECM) consisting of DMEM F12 (1:1) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (Invitrogen), 0.1 nM cholera toxin (Sigma-Aldrich Corp., St. Louis, MO), 5 μg/mL human recombinant insulin (Sigma-Aldrich) and 10 ng/mL epidermal growth factor (Invitrogen).

LECs were isolated from one-half of each corneal button or rim after incubation in a 1.2 U/mL Dispase II (Stem Cell Technologies, Inc., Vancouver, BC, Canada) solution at 37°C for 30 minutes. After Dispase treatment, the tissue was transferred into a Petri dish on a drop (200 μL) of nonamimal trypsin enzyme (Tryple Select; Invitrogen-Gibco), epithelial side down, and then immediately inverted so that the epithelial side was uppermost. The epithelial cells were gently scraped from the limbal area with fine-pointed forceps. The cells were collected in 1 mL CECM. The cell suspension was pipetted up and down on the tissue segment to disperse the cells and then transferred into a T-25 tissue culture flask (Fisher Scientific, Loughborough, UK) containing growth-arrested 3T3 mouse fibroblasts plated at a cell density of 7×10^5 cells/mL. The fibroblasts had been growth arrested by treatment for 2 hours in Dulbecco's modified Eagles' medium (DMEM; Invitrogen) supplemented with 10% newborn calf serum (Invitrogen), 1% antibiotic-antimycotic solution (Invitrogen), and 4 μg/mL mitomycin C (Sigma-Aldrich). The cultures were incubated at 37°C and 5% CO_2 in air. The culture medium was changed three times a week, and the cultures were passaged on reaching 80% to 90% confluence. At each passage six-well plates and eight-chamber slides containing growth-arrested 3T3 mouse fibroblasts (2.6×10^5 cells/mL per well or 2.8×10^4 cells/mL per chamber) were seeded by LECs (700 LECs/well and 500 LECs/chamber, respectively).

Indirect Immunofluorescence on Radial Sections

Slices obtained from all corneoscleral specimens were stained with anti-CK8 antibody, and each staining was performed in duplicate. A negative control (primary antibody omitted) was included in every slide. Immunohistochemistry on cryosections was performed, as described previously.[41] The mouse monoclonal antibody anti-CK8 (clone 4.1.18, 1:400; Millipore, Bedford MA) was used, and subsequently the sections were incubated with the appropriate secondary antibody (fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA).

Double-Staining on Radial and Tangential Sections

Double-staining was performed on six different corneoscleral samples (three were used for radial and three for tangential sections). After the samples were fixed in cold acetone (10 minutes) and rinsed in phosphate-buffered saline (PBS), 5% donkey serum was applied. A mixture of mouse anti-CK8 antibody (Millipore) and goat anti-CK3 (clone C-14, 1:50), anti-CK15 (clone A-13, 1:400), anti-integrin α6 (clone C-18, 1:40), or anti-vimentin (clone C-20, 1:100; all from Santa Cruz Biotechnology, Santa Cruz, CA) was applied to the sections in one step, followed by a mixture of FITC-conjugated donkey anti-mouse IgG and TRITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories). All antibodies were diluted in PBS as described by the manufacturers.

Indirect Immunofluorescence on Cultivated Cells

Immunocytochemical staining was performed to localize CK8. In brief, confluent corneal epithelial cultures were fixed in 4% paraformaldehyde in PBS at room temperature for 10 minutes and treated in blocking solution (5% normal goat serum, Jackson ImmunoResearch Laboratories). The blocking solution used for immunocytochemistry was supplemented with 0.33% Triton X100 (Sigma-Aldrich) to permeabilize the cell membranes. The primary antibody (monoclonal mouse anti-human CK8; Millipore) was applied for 1 hour at room temperature, followed by incubation with a secondary antibody conjugated with fluorescent dye (Alexa Fluor 488 conjugated goat anti-mouse IgG, Invitrogen) and mounted with antifade medium (Vectastain; Vector Laboratories, Inc., Burlingame, CA).

Immunohistochemistry Assessment

Sections were examined by fluorescence microscopy (model BX51; Olympus Co., Tokyo, Japan) and by an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at a magnification of 100× to 400×. The images were obtained with one of two cameras (VDS CCD-1300QF; VDS Vosskühler GmbH, Osnabrück, Germany, or Jenoptik ProgRes C12plus; Jenoptik; Laser.Optik.Systeme GmbH, Jena, Germany) and produced with image-management software (NIS Elements; Laboratory Imaging, Prague, Czech Republic). At least 300 cells of the central corneal, limbal, and perilimbal conjunctival epithelium were examined, and the percentage of positive cells was calculated. The following scale was used to grade the intensity of cell staining: N, negative; 1, weak; 2, moderate; 3, intense; and 4, very intense. The mean range was calculated from three sections and two experiments.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from the corneal, limbal, and perilimbal conjunctival epithelium of five individual corneoscleral discs

TABLE 1. The Average Percentage of CK8-Positive Cells and the Intensity of the Signal in the Corneal, Limbal, and Perilimbal Conjunctival Epithelium of 20 Corneoscleral Discs

Sample	Age (y)	Basal Cells of the Limbus*	Suprabasal and Superficial Cells of the Limbus*	Corneal Epithelium*	Perilimbal Conjunctiva*
1	16	N	50/2	50/2	50/1
2	26	N	N	N	N
3	29	N	N	N	N
4	30	N	30/1 s	55/1 s	25/1 s
5	38	Line/3	60/2	80/3†	80/3
6	49	Line/3	50/2	85/3 s	50/2
7	49	Clusters/1	5/1	40/1 s	20/1 s
8	51	N	N	N	N
9	59	N	5/3 s	40/1	N
10	61	Line/3	30/1	50/2†	80/2
11	66	Line/1	65/1	25/1†	25/1†
12	67	Line/1	50/1	35/1†	30/1†
13	68	N	N	N	N
14	70	N	N	N	N
15	73	Clusters/3	30/1	30/1	75/2
16	74	Line + clusters/3	20/2	10/2 s	N
17	74	Line + clusters/4	50/2	d	80/3
18	77	Line/1	35/1	40/1 s	30/1
19	77	Clusters/3	60/2	70/2	80/3
20	82	Clusters/4	35/1	60/2†	80/3

N, negative; 1, mild; 2, moderate; 3, intense; and 4, very intense staining.
* Data shown are the average percent of positive cells/intensity.
† No or weak positivity in the basal layer; d, damaged; s, positivity in the superficial cells only.

(RNeasy Plus Microkit; Qiagen). Six microliters of total RNA were reverse transcribed into cDNA in a 20-μL reaction mixture (SuperScript III/RNase OUT Enzyme Mix; Invitrogen), according to the manufacturer's instructions. Subsequently, equal amounts of cDNA from five individual samples were amplified with the following specific oligonucleotides for CK8 and the housekeeping gene glyceraldehyde-3-phospate dehydrogenase (GAPDH): human CK8, sense primer 5′-ATCAGCTCCTCGAGCTTCTC-3′, anti-sense primer 5′-TC-

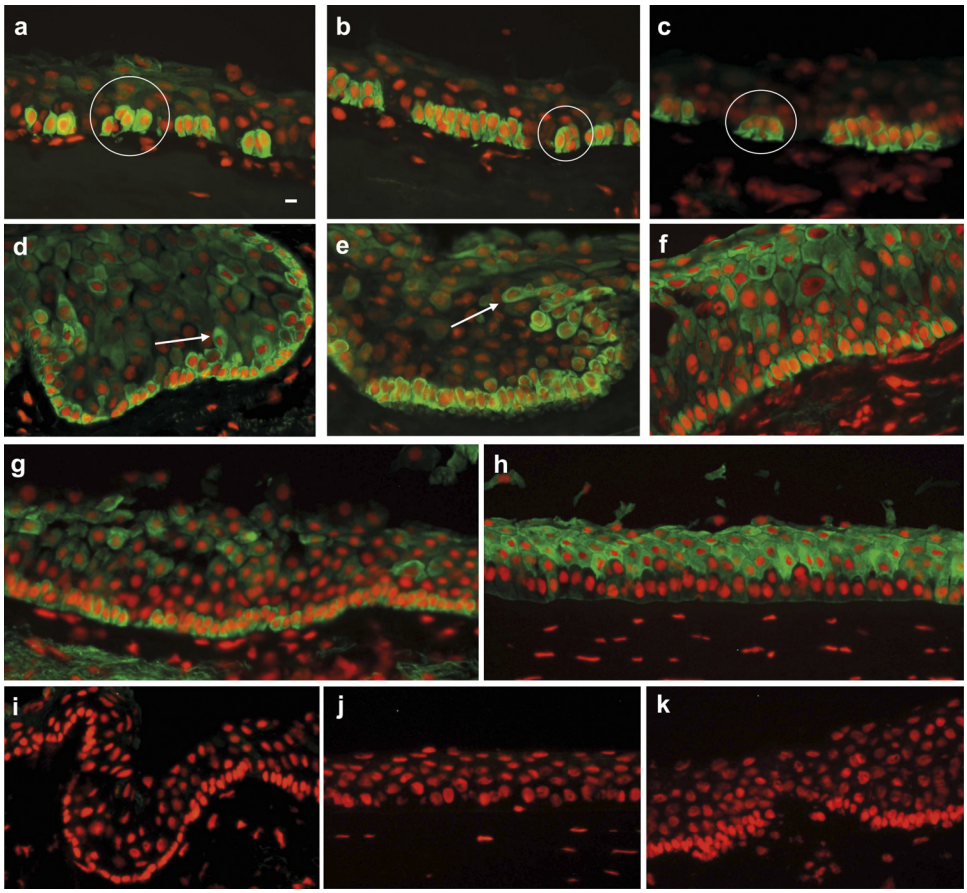

FIGURE 1. Immunolocalization of CK8 in radial sections of the limbus and cornea. CK8 expression in the limbus: positive limbal basal cells forming clusters (*circles*; **a–c**) or lines with clearly visible elongated CK8-positive cells projecting from the basal layer (*arrows*; **d**, **e**); limbal specimen in which, besides a basal line, CK8 is abundantly present throughout the suprabasal and superficial limbal layers (**f**). If the limbal basal cells were positive for CK8 (**g**), the central corneal epithelium of the same specimen was positive as well (**h**). If CK8 staining was absent from the limbal epithelium (**i**), the central corneal epithelium of the same specimen was negative also (**j**). Negative control of the limbus (**k**). Scale bar, 10 μm.

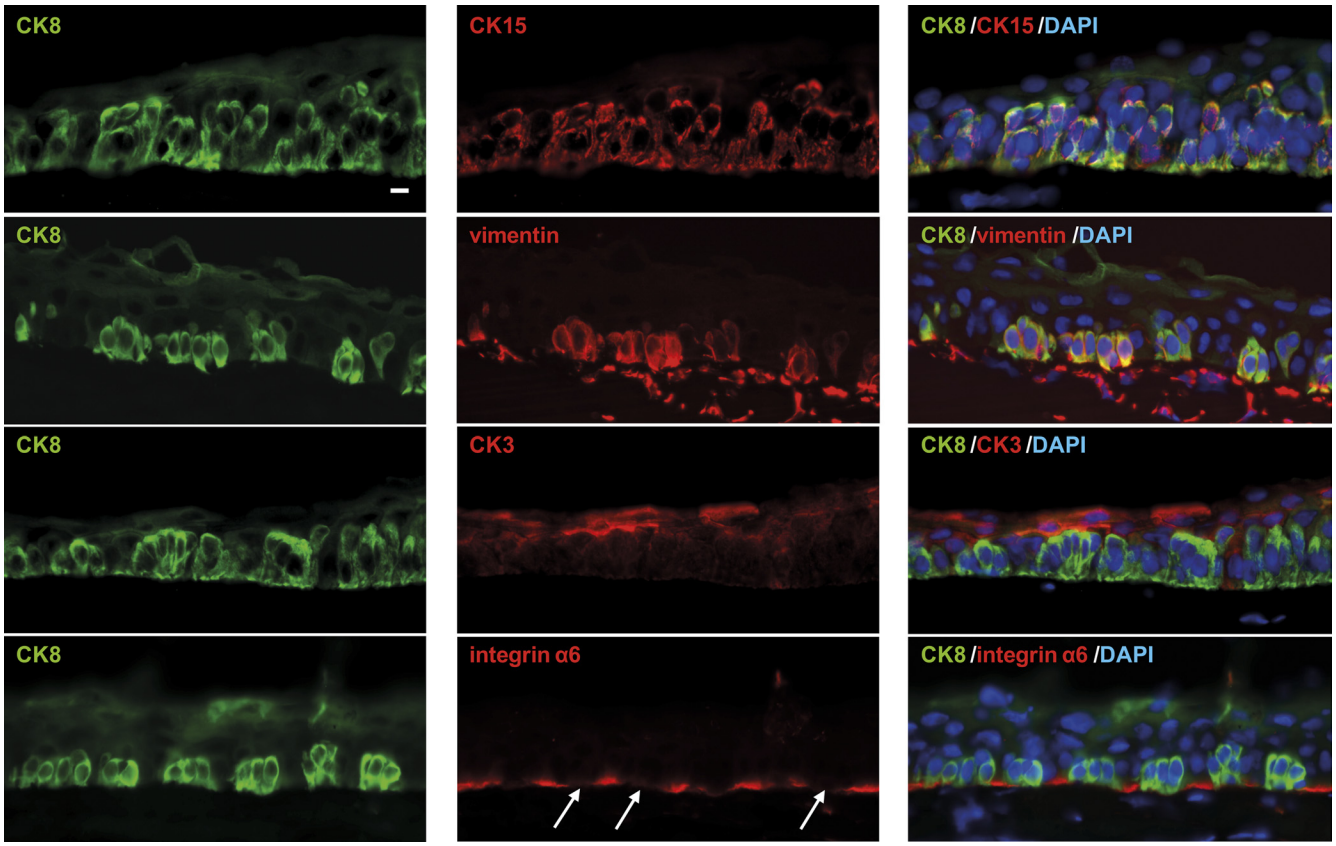

Figure 2. Immunolocalization of CK8 with CK15, vimentin, CK3 or integrin α6 in the basal layer of the limbus on radial sections. CK8 (*green,* FITC) colocalized with CK15 (*red,* TRITC) and with vimentin (*red*, TRITC). CK3 (*red,* TRITC), in contrast to CK8, was completely absent from the basal cells of the limbus, whereas a few suprabasal and superficial cells were CK3-positive. The expression of integrin α6 (*red,* TRITC) decreased in the areas where CK8-positive clusters occurred (*arrows*). Nuclei were counterstained with DAPI (*blue*). Scale bar, 10 μm.

CAGGAACCGTACCTTGTC-3′; and human GAPDH, sense primer 5′-AGCCACATCGCTCAGACAC-3′, anti-sense primer 5′-GCCCAATACGACCAAATCC-3′. The number of PCR cycles for the different primer pairs was 35 cycles for both CK 8 and GAPDH. Each cycle consisted of denaturation for 25 seconds at 95°C, annealing for 30 seconds at 65°C for CK 8, 60°C for GAPDH and elongation for 45 seconds at 72°C. PCR products were analyzed by ethidium bromide–stained 2% agarose gel electrophoresis.

Results

Indirect Immunofluorescence on Cadaveric Corneoscleral Discs

The presence of CK8-positive cells in various locations of the corneoscleral discs assessed using indirect immunohistochemical fluorescence is shown in Table 1.

Sixty percent of the samples revealed weak to strong positivity for CK8 in the cytoplasm of cells of the limbal basal layer. Most of the positive basal cells were small, with a high nuclear–cytoplasmic ratio, and formed clusters (Figs. 1a–c) or showed continuous staining (positivity in a single line; Figs. 1d–f). In addition, positive elongated cells in the near vicinity of CK8-positive clusters were detected in the suprabasal layers of the limbal epithelium (Figs. 1d, 1e). Weak to moderate positivity was detected in approximately 40% of the surface cells of the limbus. In most specimens, the intensity of the signal in the superficial cells was weaker than the signal in the basal limbal layer. The central corneal epithelium was positive for CK8, predominantly in the superficial and suprabasal layers (Fig. 1h), but some heterogeneous positivity was detected in the basal layer of several samples as well (Table 1). In each specimen that contained positive limbal basal cells, the epithelium of the cornea was positive as well (Figs. 1g, 1h; Table 1). Similarly, in most specimens in which CK8 was absent from the limbal basal cells, the epithelium of the central cornea was negative (Figs. 1i, 1j). No signal was seen in any of the negative controls (Fig. 1k).

Cells in the basal layer of the limbus showed positive staining for CK8/CK15 and CK8/vimentin in double-immunostained radial sections (Fig. 2). On the other hand, only a few CK3-positive cells were scattered in the superficial layer of the

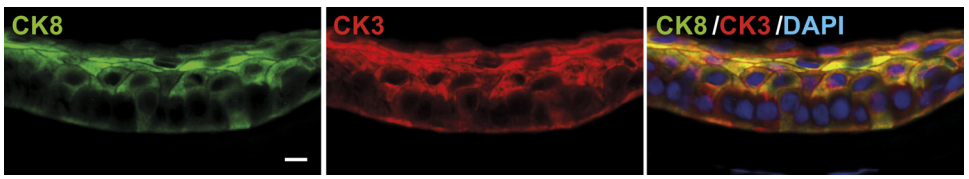

Figure 3. Immunolocalization of CK8 (*green*, FITC) and CK3 (*red*, TRITC) in the central corneal epithelium. Nuclei were counterstained with DAPI (*blue*). Scale bar, 10 μm.

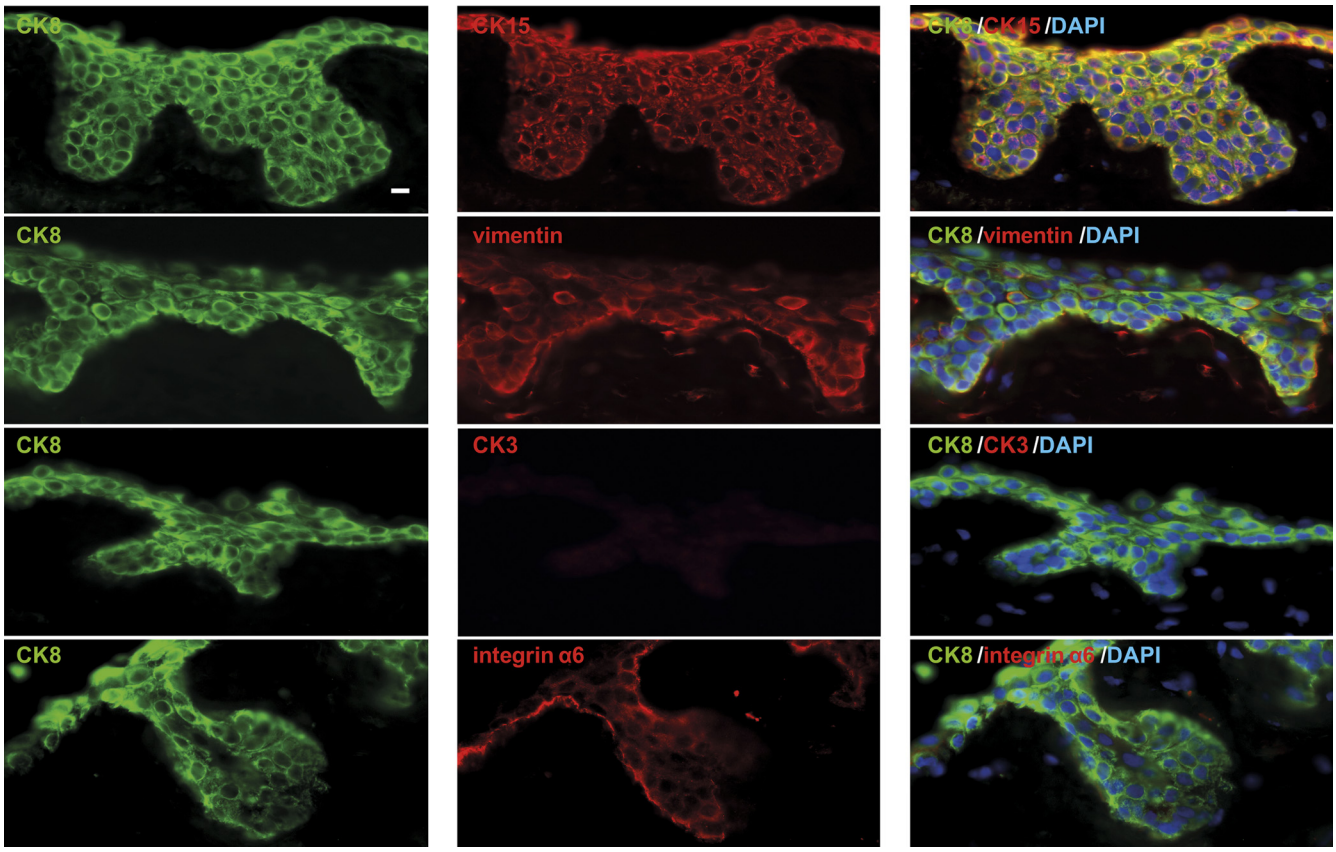

Figure 4. The immunohistochemical localization of CK8 in tangential sections of corneas after storage in organ culture. CK8 (*green,* FITC) colocalized with CK15 (*red,* TRITC) and with vimentin (*red*, TRITC) in the limbal crypts. The limbal epithelial cells were CK8 positive, whereas no CK3 (*red,* TRITC) signal was detected in these cells. The difference between CK8-positive and integrin α6 (*red,* TRITC)-negative areas was less apparent than in radial sections. Nuclei were counterstained with DAPI (*blue*). Scale bar, 10 μm.

limbus, and no CK3-positive cells were present in the basal layer of the limbus. In the CK3-positive central corneal epithelium, CK8-positive cells were found as well (Fig. 3). Positive staining for integrin α6 in the basal membrane decreased or was absent in the area of the clusters of CK8-positive cells (Fig. 2).

Indirect Immunofluorescence Staining of Corneas Stored in Organ Culture

Strong positivity for CK8 in the basal cells of the limbus located in the limbal crypts was detected in all tangential sections of all corneoscleral samples. Elongated CK8-positive cells were detected in the suprabasal layer of the limbus, and a strong signal was observed in some superficial cells. Storage in organ culture for 8 to 9 days led to a decrease in the number of epithelial layers to one to three.

Double immunofluorescence staining of tangential sections showed positive staining for CK8/CK15 and CK8/vimentin of cells in the basal layer of the limbal crypts. No immunostaining for CK3 was present in the limbal crypts. The most intensive staining for integrin α6 was observed in the basal membrane of the limbus. The difference between CK8-positive and integrin α6-negative areas was less apparent than in radial sections (Fig. 4).

A strong signal for CK8 was detected in approximately 80% of the cultured LECs. Most of these positive cells were small, with a high nuclear–cytoplasmic ratio, but the signal was retained in elongated cells as well (Fig. 5).

CK8 mRNA was found in the corneal, limbal, and perilimbal conjunctival epithelium of all five corneoscleral discs, using semiquantitative RT-PCR, as was GAPDH mRNA, which served as an internal control. Representative results are shown in Figure 6.

Discussion

Our results clearly showed CK8 expression in the corneal, limbal, and conjunctival epithelium. A strong signal for CK8 was detected, especially in the basal layer of the limbal epithelium. Similar staining with respect to this location had been shown for CK15 and -19.[13,24] Most of our radial and all our tangential sections revealed an intense signal for CK8 located in the basal layer of the limbal crypts, which extended into the suprabasal and superficial epithelial layers of the limbus and cornea, where the signal intensity gradually decreased. The location of the CK8-positive cells, which is in accordance with the X, Y, Z hypothesis,[4] indicates that the expression of CK8

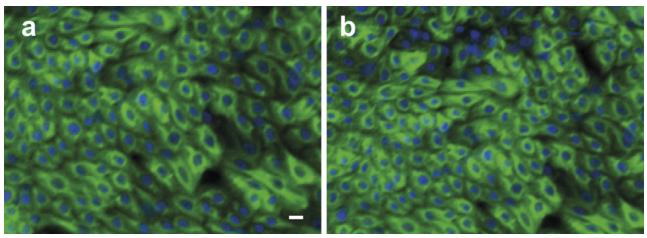

Figure 5. The immunohistochemical localization of CK8 in cell cultures obtained from limbal epithelial cells (**a**, **b**). Scale bar, 10 μm.

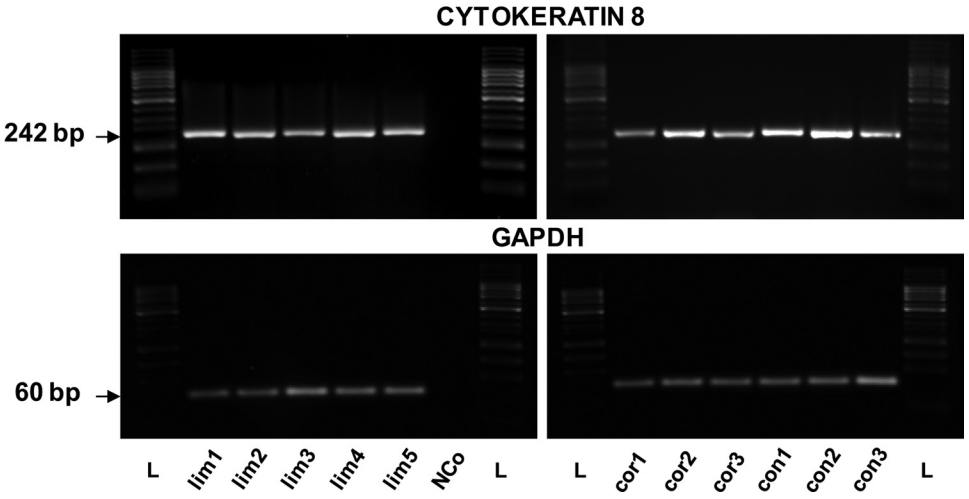

FIGURE 6. Expression of cytokeratin 8 in five samples of limbal epithelium (lim) and in three representative samples of corneal (cor) and perilimbal conjunctival epithelium (con) determined by RT-PCR. GAPDH was used as an internal control. NCo-negative control (reaction without sample cDNA), a marker for internal contamination, L-50-bp DNA ladder (25–1000 bp).

probably persists during the differentiation process from LESCs through TACs up to terminally differentiated corneal epithelial cells. The explanation for the absence of CK8 in some specimens could lie in the fact that radial sections from cadaveric corneas were not obtained solely from the superior or inferior areas (areas rich in limbal crypts)[46] or that its absence reflects a situation in which the basal limbal cells are in a quiescent state and thus do not proliferate.

In the central cornea, CK8 immunostaining was located predominantly in the superficial and suprabasal layers, where it partially colocalized with the corneal differentiation marker CK3. Occasional positivity in the basal layer of the central cornea may be explained by the fact that these basal cells proliferate, differentiate, and, in this way, participate in the renewal of the central corneal epithelium.[1,3] Moreover, we have found CK8 in all layers of the perilimbal conjunctiva (Table 1) and bulbar conjunctiva (data not shown). All the obtained data support our idea that CK8 may be expressed in activated basal cells that are ready to divide and differentiate.

Our hypothesis that CK8 expression persists during differentiation was confirmed by its detection in cultured LECs obtained from corneoscleral buttons after long-term storage. It was found that most cells in LEC cultures are limbal rather then corneal in phenotype (expressing CK19, β1 integrin, and p63).[47] This finding does not, however, prove that any of these cultured cells are stem cells. It is likely that LEC cultures consist of a heterogeneous population of LESCs and a gradient of differentiated cells. Our results showed that both cells with a high nucleocytoplasmic ratio as well as more differentiated elongated cells with a larger cytoplasm in LEC sheets were positively stained for CK8.

Regarding the localization of CK8 in the basal layers of the limbus, we wanted to determine the phenotype of the CK8-positive basal cells of the limbal epithelium using double-staining immunohistochemistry. CK8 in the limbus was clearly localized in the same cells as CK15 and vimentin, which are accepted as potential stem cell markers.[19,24] An inverse staining gradient was seen between CK8 and cornea-specific CK3 protein as well as between CK8 and integrin α6 in the limbus. Integrin α6 (together with integrin β4) is a component of hemidesmosomes, localized specifically to the basal membrane of basal cells.[48] It has been suggested that the lack of its expression is an inherent feature of LESCs, reflecting their need for independence, and could facilitate the migration of cells derived from LESCs.[9] Although we agree with this interpretation, it is at odds with the human study by Kim et al.,[49] and with the study by Pajoohesh-Ganji et al.[50] in the mouse, which showed that more adherent cells expressing elevated integrin levels are more stemlike than cells expressing lower integrin levels.

On the basis of all these facts, the possibility that CK8 is a newly found marker for LESCs could be considered, but CK8 is present in abundance in half of the basal limbal epithelial cells; moreover, it is still present in elongated cells projecting from CK8-positive clusters up to the cells in the central corneal epithelium. As stem cells represent less than 10% of the total limbal basal cell population,[51] it is clear that CK8 cannot be considered as a potential marker specific only for LESCs.

Much more interesting would be to elucidate the exact function of CK8 in the human cornea, especially in the limbal epithelium. Providing mechanical strength in the single layered epithelia and interacting with desmosomes are the basic, but not the only, functions of CK8 and -18. The expression of both CKs increases the migratory and invasive ability of transfected cells.[52] They bind DNA, RNA as well as molecules that are important in signal transduction.[53–56] The CK8/18 pair plays an important role in cell-cycle regulation through phosphoserine-binding protein 14-3-3, which is a key regulator in signal-transduction/cell-cycle checkpoint control.[57] During the S/G_2/M phases, when cytokeratins become hyperphosphorylated, 14-3-3 binds to CK8/18 and, due to cdc25 (dual-specificity phosphatase), remains free to dephosphorylate the cdc2/cyclin B complex, thus cells come through the M-phase checkpoint.[54,58–60] The key role of CK8 in cell-cycle regulation was demonstrated and confirmed in CK8-null mice, where hepatic cells exhibited an altered cellular redistribution of 14-3-3 protein into the nuclei and its binding to cdc25. All this leads to cell-cycle deregulation and finally, in the G_2-phase, to cell-cycle arrest.[38,40]

Although the expression of CK8 is very important for normal cell signaling and cell-cycle regulation,[38,40] as well as for the migratory and invasive ability of cells,[52] its exact function in the cells of the corneoscleral discs is not known yet. The obtained data support our hypothesis that CK8 could play some still unidentified role in the activation of cells and their proliferation and migration. The signal for CK8 is retained in cells during their differentiation, but the exact relation between CK8 expression and the renewal of cells in the corneoconjunctival area remains to be elucidated.

Acknowledgments

The authors thank Viera Vesela and Sarka Kalasova for excellent technical assistance with the preparation of the specimens.

References

1. Chang CY, Green CR, McGhee CN, Sherwin T. Acute wound healing in the human central corneal epithelium appears to be independent of limbal stem cell influence. *Invest Ophthalmol Vis Sci.* 2008;49:5279–5286.
2. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature.* 1971;229:560–561.
3. Dua HS, Miri A, Alomar T, Yeung AM, Said DG. The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. *Ophthalmology.* 2009;116:856–863.
4. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci.* 1983;24:1442–1443.
5. Tseng SC. Concept and application of limbal stem cells. *Eye (Lond).* 1989;3:141–157.
6. Hall PA, Watt FM. Stem cells: the generation and maintenance of cellular diversity. *Development.* 1989;106:619–633.
7. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci.* 1998;111:2867–2875.
8. Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell.* 1997;88:287–298.
9. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res.* 2005;81:247–264.
10. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci.* 2005;118:1715–1724.
11. de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells.* 2005;23:63–73.
12. Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett.* 2004;565:6–10.
13. Lauweryns B, van den Oord JJ, Missotten L. The transitional zone between limbus and peripheral cornea: an immunohistochemical study. *Invest Ophthalmol Vis Sci.* 1993;34:1991–1999.
14. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell.* 1982;31:11–24.
15. Cockerham GC, Laver NV, Hidayat AA, McCoy DL. An immunohistochemical analysis and comparison of posterior polymorphous dystrophy with congenital hereditary endothelial dystrophy. *Cornea.* 2002;21:787–791.
16. Kasper M, Stosiek P, Lane B. Cytokeratin and vimentin heterogeneity in human cornea. *Acta Histochem.* 1992;93:371–381.
17. Ross JR, Foulks GN, Sanfilippo FP, Howell DN. Immunohistochemical analysis of the pathogenesis of posterior polymorphous dystrophy. *Arch Ophthalmol.* 1995;113:340–345.
18. Lauweryns B, van den Oord JJ, De Vos R, Missotten L. A new epithelial cell type in the human cornea. *Invest Ophthalmol Vis Sci.* 1993;34:1983–1990.
19. Kasper M, Moll R, Stosiek P, Karsten U. Patterns of cytokeratin and vimentin expression in the human eye. *Histochemistry.* 1988;89:369–377.
20. Kurpakus MA, Stock EL, Jones JC. Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. *Invest Ophthalmol Vis Sci.* 1990;31:448–456.
21. Morgan PR, Shirlaw PJ, Johnson NW, Leigh IM, Lane EB. Potential applications of anti-keratin antibodies in oral diagnosis. *J Oral Pathol.* 1987;16:212–222.
22. Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *J Cell Sci.* 1998;111:3179–3188.
23. Lyngholm M, Hoyer PE, Vorum H, Nielsen K, Ehlers N, Mollgard K. Immunohistochemical markers for corneal stem cells in the early developing human eye. *Exp Eye Res.* 2008;87:115–121.
24. Yoshida S, Shimmura S, Kawakita T, et al. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci.* 2006;47:4780–4786.
25. Franke WW, Schiller DL, Moll R, et al. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J Mol Biol.* 1981;153:933–959.
26. Oshima RG, Baribault H, Caulin C. Oncogenic regulation and function of keratins 8 and 18. *Cancer Metastasis Rev.* 1996;15:445–471.
27. Baribault H, Penner J, Iozzo RV, Wilson-Heiner M. Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes Dev.* 1994;8:2964–2973.
28. Baribault H, Price J, Miyai K, Oshima RG. Mid-gestational lethality in mice lacking keratin 8. *Genes Dev.* 1993;7:1191–1202.
29. Jackson BW, Grund C, Schmid E, Burki K, Franke WW, Illmensee K. Formation of cytoskeletal elements during mouse embryogenesis: intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. *Differentiation.* 1980;17:161–179.
30. Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. *Proc Natl Acad Sci U S A.* 1990;87:2319–2323.
31. Bauman PA, Dalton WS, Anderson JM, Cress AE. Expression of cytokeratin confers multiple drug resistance. *Proc Natl Acad Sci U S A.* 1994;91:5311–5314.
32. Caulin C, Salvesen GS, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol.* 1997;138:1379–1394.
33. Caulin C, Ware CF, Magin TM, Oshima RG. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J Cell Biol.* 2000;149:17–22.
34. Gilbert S, Loranger A, Daigle N, Marceau N. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis: the protection occurs through a receptor-targeting modulation. *J Cell Biol.* 2001;154:763–773.
35. Gilbert S, Ruel A, Loranger A, Marceau N. Switch in Fas-activated death signaling pathway as result of keratin 8/18-intermediate filament loss. *Apoptosis.* 2008;13:1479–1493.
36. Inada H, Izawa I, Nishizawa M, et al. Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J Cell Biol.* 2001;155:415–426.
37. Galarneau L, Loranger A, Gilbert S, Marceau N. Keratins modulate hepatic cell adhesion, size and G1/S transition. *Exp Cell Res.* 2007;313:179–194.
38. Ku NO, Michie S, Resurreccion EZ, Broome RL, Omary MB. Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression. *Proc Natl Acad Sci U S A.* 2002;99:4373–4378.
39. Magin TM, Vijayaraj P, Leube RE. Structural and regulatory functions of keratins. *Exp Cell Res.* 2007;313:2021–2032.
40. Toivola DM, Nieminen MI, Hesse M, et al. Disturbances in hepatic cell-cycle regulation in mice with assembly-deficient keratins 8/18. *Hepatology.* 2001;34:1174–1183.
41. Merjava S, Neuwirth A, Mandys V, Jirsova K. Cytokeratins 8 and 18 in adult human corneal endothelium. *Exp Eye Res.* 2009;89:426–431.
42. Krenzer KL, Freddo TF. Cytokeratin expression in normal human bulbar conjunctiva obtained by impression cytology. *Invest Ophthalmol Vis Sci.* 1997;38:142–152.
43. Zhang W, Zhao J, Chen L, Urbanowicz MM, Nagasaki T. Abnormal epithelial homeostasis in the cornea of mice with a destrin deletion. *Mol Vis.* 2008;14:1929–1939.
44. Nejepinska J, Juklova K, Jirsova K. Organ culture, but not hypothermic storage, facilitates the repair of the corneal endothelium following mechanical damage. *Acta Ophthalmol.* 2010;88:413–419.
45. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells.* 2004;22:355–366.
46. Shortt AJ, Secker GA, Notara MD, et al. Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. *Surv Ophthalmol.* 2007;52:483–502.
47. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. *Exp Eye Res.* 2004;79:41–49.

48. Tervo K, Tervo T, van Setten GB, Virtanen I. Integrins in human corneal epithelium. *Cornea.* 1991;10:461–465.
49. Kim HS, Luo L, Pflugfelder SC, Li DQ. Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2005;46:840–848.
50. Pajoohesh-Ganji A, Pal-Ghosh S, Simmens SJ, Stepp MA. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. *Stem Cells.* 2006;24:1075–1086.
51. Lavker RM, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia: implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. *Invest Ophthalmol Vis Sci.* 1991;32:1864–1875.
52. Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype. *Int J Cancer.* 2004;111:662–668.
53. Liao J, Lowthert LA, Ghori N, Omary MB. The 70-kDa heat shock proteins associate with glandular intermediate filaments in an ATP-dependent manner. *J Biol Chem.* 1995;270:915–922.
54. Liao J, Omary MB. 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. *J Cell Biol.* 1996;133:345–357.
55. Omary MB, Baxter GT, Chou CF, Riopel CL, Lin WY, Strulovici B. PKC epsilon-related kinase associates with and phosphorylates cytokeratin 8 and 18. *J Cell Biol.* 1992;117:583–593.
56. Traub P, Shoeman RL. Intermediate filament proteins: cytoskeletal elements with gene-regulatory function? *Int Rev Cytol.* 1994;154:1–103.
57. Aitken A, Jones D, Soneji Y, Howell S. 14-3-3 proteins: biological function and domain structure. *Biochem Soc Trans.* 1995;23:605–611.
58. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol.* 2000;40:617–647.
59. Ku NO, Liao J, Omary MB. Phosphorylation of human keratin 18 serine 33 regulates binding to 14-3-3 proteins. *EMBO J.* 1998;17:1892–1906.
60. Omary MB, Ku NO, Liao J, Price D. Keratin modifications and solubility properties in epithelial cells and in vitro. *Subcell Biochem.* 1998;31:105–140.

Experimental Eye Research 89 (2009) 426–431

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Cytokeratins 8 and 18 in adult human corneal endothelium

Stanislava Merjava [a], Ales Neuwirth [a], Vaclav Mandys [c], Katerina Jirsova [a,b,*]

[a] Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[b] Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[c] Department of Pathology, Third Faculty of Medicine, Charles University, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 9 January 2009
Accepted in revised form 20 April 2009
Available online 3 May 2009

Keywords:
cornea
endothelium
cytokeratin 8
cytokeratin 18

ABSTRACT

The aim of this study was to determine if cytokeratins (CKs) 8 and 18 – typical epithelial cell markers – are constitutively expressed in adult human corneal endothelium. Cryosections, paraffin-embedded sections and corneal endothelial imprints obtained from eleven adult human corneal discs not suitable for transplantation were used. Different fixative solutions were applied before indirect immunofluorescent or enzymatic staining was performed with antibodies against CK8 (Chemicon), CK18 (Dako and Sigma) and CK8/18 (Novocastra). Semi-quantitative RT-PCR and Western blotting (mRNA or proteins were isolated from Millicell membranes) were used to determine cytokeratin mRNA and protein levels. Approximately 50% of the corneal endothelial cells were positive for CK8 (Chemicon), CK18 (Sigma) and the CK pair 8/18 (Novocastra) in the endothelium when acetone was used for fixation. Four and 52% CK18-positive cells were observed using immunofluorescent and enzymatic immunohistochemistry, respectively, when the CK18 antibody from Dako was used. No signal was detected when 4% formalin or 10% paraformaldehyde was used as a fixative, irrespective of the antibody used. CK8 and CK18 proteins and mRNAs were detected in the endothelium of all tested corneas by Western blotting or semi-quantitative RT-PCR, respectively. We detected both CK8 and CK18 in the endothelium of all specimens at both the protein and mRNA levels. These results clearly demonstrate that cells of the corneal endothelium express CKs 8 and 18 and share some features with simple epithelia.

1. Introduction

The endothelium, the innermost part of the cornea, is a monolayer of flat hexagonal cells which are essential for maintaining corneal transparency through its dehydration (Waring et al., 1982). Studies on fetal avian corneas showed that the corneal endothelium is derived from neural crest cells that migrate during embryogenesis into the primary mesenchyme, lining the optic cup to form a more complex cell population – the secondary mesenchyme. The crest cells of the secondary mesenchyme migrate into the eye in three waves and differentiate into the endothelium, trabecular meshwork, and corneal keratocytes and contribute to the development of the iris (Bahn et al., 1984; Johnston et al., 1979).

Since the development of the avian corneal endothelium is well established, compared to the development of the corneal endothelium in humans (Johnston et al., 1979), many investigators accept a neural crest origin of the corneal endothelial cells in man as well (Bahn et al., 1984; Hayashi et al., 1986). Sevel and Isaacs (1988) found that the endothelium and Descemet's membrane develop from the posterior accumulation of stromal mesenchymal cells. The term "mesenchymal cells" is used in their study only due to its being commonly accepted terminology in embryology texts, and in fact it is the mesectoderm (Sevel and Isaacs, 1988), the newly named ectomesenchyme, whose exact origin is discussed (Weston et al., 2004). Other investigators have postulated that human corneal endothelium is derived from mesenchymal tissues originating from the mesoderm (Risen et al., 1987).

Although the posterior cell layer of the cornea is commonly named "endothelium", this is a misnomer. The corneal endothelium differs from true vascular endothelium not only by its origin, but also by the absence of typical vascular endothelium markers such as Weibel-Palade bodies and factor VIII, or vascular cell adhesion molecule-1 (VCAM-1) (Foets et al., 1992a,b; Shamsuddin et al., 1986).

Human corneal endothelial cells display an unusual combination of neurofilaments, neuronal markers (neural cell adhesion molecule, neuron specific enolase and S-100 protein), the

* Corresponding author at: Laboratory of the Biology and Pathology of the Eye and Ocular Tissue Bank, VFN, U nemocnice 2, 128 08 Prague, Czech Republic. Tel.: +420 224 963 008; fax: +420 224 962 338.
E-mail address: katerina.jirsova@vfn.cz (K. Jirsova).

0014-4835/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2009.04.009

mesenchymal cell marker vimentin and probably epithelial markers as well – cytokeratins (Foets et al., 1990, 1992a,b; Hayashi et al., 1986; Risen et al., 1987; Shamsuddin et al., 1986).

Cytokeratins (CKs) are expressed in a cell type- and differentiation-dependent manner (Moll et al., 1982). CK8 and CK18 are the first cytokeratins expressed during embryogenesis (Jackson et al., 1980). The absence of CK18 leads to a disruption of the keratin filament network in the liver and pancreas, while the absence of CK8 is incompatible with life (Baribault et al., 1993; Ku et al., 1995). Both CKs have been found in simple epithelium, including corneal epithelial cells, vascular endothelium and in the pleural and peritoneal mesothelium (Kasper et al., 1992; Pronk et al., 1993; Stosiek et al., 1990). The total amount of cellular CK8 and CK18 is kept at a stable level under physiological conditions (Moll et al., 1982), while the expression of both often increases during carcinogenesis (Oshima et al., 1996; Trask et al., 1990). Moreover, the filament formation of CK8 and CK18 may have implications for the induction of a transformed phenotype in stratified epithelium, which may result in increased invasive potential and tumorigenicity (Raul et al., 2004).

In human corneal endothelial cells, homogeneous immunostaining for CK18 and 19 and heterogeneous immunostaining for CK7 and 8 was also shown previously by Foets et al. (1990); however, the expression of CK7 and CK19 in healthy human corneal endothelium was not confirmed in numerous subsequent studies (Cockerham et al., 2002; Jirsova et al., 2007; Levy et al., 1995). The expression of CK8 and CK18 in normal adult and fetal endothelium is a matter of some controversy. In a study by Kasper et al. (1992), Wollensak and Witschel (1996) as well as in our previous study, which was focused primarily on the expression of CKs in the corneas of patients suffering from posterior polymorphous corneal dystrophy, an occasional positivity for CKs 8 and 18 in the control endothelium was observed (Jirsova et al., 2007). In contrast, no CK8 or CK18 expression was detected in other studies (Cockerham et al., 2002; Kramer et al., 1992; Levy et al., 1995). The heterogeneous presence of CKs 8 and 18 was found in human corneal epithelium (Kasper et al., 1992).

The phenotypic diversity of the corneal endothelium is manifested by its instability in some endothelial pathologies, including posterior polymorphous corneal dystrophy or Fuchs endothelial corneal dystrophy, in which abnormal endothelial cells acquire characteristics of "fibroblast-like" or mostly "epithelial-like" cells (Boruchoff and Kuwabara, 1971; Hidayat and Cockerham, 2006; Johnson and Brown, 1978). The condition characterized by a slow, continuous loss of morphologically and physiologically altered endothelial cells may eventually lead to corneal edema, as was found in Fuchs endothelial corneal dystrophy (Adamis et al., 1993).

As the presence of CKs 8 and 18 in healthy corneal endothelium is a matter of controversy, the aim of this study was to definitively determine the pattern of their expression in adult human corneas, which is important for understanding the behavior of healthy as well as pathological endothelial cells. Further, such knowledge may lead to a better understanding of the development and differentiation processes in the posterior corneal layers, including the type of progenitor cells involved.

2. Materials and methods

2.1. Specimens

The study followed the tenets set out in the Declaration of Helsinki. Eleven adult corneal discs (mean age 65.0 ± 12.7 years) not acceptable for transplantation because of low endothelial cell count or a positive serology of the donor were obtained from the Ocular Tissue Bank, Prague. The time between death and storage in liquid nitrogen did not exceed 24 h.

Six corneal buttons were dissected into three parts: the first third was snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at −70 °C; the second third was fixed in 10% paraformaldehyde in phosphate buffered saline (PBS), dehydrated in alcohol and xylol and embedded in paraffin. Tissue slices 7 μm thick were cut as cryosections and 5 μm thick as paraffin sections. Slides containing four slices were used for indirect fluorescent or enzymatic immunohistochemistry. The last third and an additional five corneas were used for impression cytology of the endothelium (six imprints per cornea) on Biopore Millicell membranes (MILLICELL®-CM, PICM 01250, Millipore, Bedford, MA). The membranes were then used for indirect fluorescent immunohistochemistry, RT-PCR and Western blotting.

2.2. Indirect immunofluorescence

2.2.1. Cryosections

Six different corneal samples were used to prepare cryosections for indirect immunofluorescence. Three cryosections on each slide were stained with a single antibody. The fourth slice was used as a negative control (primary antibody omitted). Two independent experiments were performed. The tissue was fixed with cold acetone for 10 min, rinsed in PBS and incubated with the primary antibody diluted in 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The following mouse monoclonal antibodies and dilutions were used: anti-cytokeratin 8 (clone 4.1.18, 1:400, Chemicon International Inc., Temecula, USA), anti-cytokeratin 18 (clone DC 10, 1:50, DakoCytomation, Glostrup, Denmark), anti-cytokeratin 18 (clone CY-90, 1:800, Sigma, St. Louis, USA) and anti-cytokeratin 8/18 (clone 5D3, 1:75, Novocastra Laboratories Ltd., Vision BioSystems, Newcastle, UK). Then the specimens were washed three times in PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated anti-mouse IgG, 1:500, Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 h at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide (Vector Laboratories, Inc., Burlingame, USA) to counterstain nuclear DNA.

2.2.2. Endothelial imprints

Two endothelial imprints of each of six different corneas were used for indirect immunofluorescence on Millicell membranes. The Millicell membranes with endothelial imprints were released from plastic holders (by treatment with acetone for 1 min) and placed cell side up on round 12 mm coverslips. Then the cells were rinsed in PBS and permeabilised in 0.2% Triton X-100. After washing, the membranes were exposed to a blocking solution (2.5% BSA in PBS), then incubated with the primary antibodies (as described above) diluted in 0.1% BSA in PBS for 1 h at room temperature. The membranes were washed, incubated with the secondary antibody, mounted and counterstained as described above.

2.3. Enzyme immunohistochemistry

Paraffin-embedded specimens from six corneas were mounted on slides (Superfrost Plus, Menzel GmbH & Co KG, Germany), deparaffinized in xylene, rehydrated through a decreasing ethanol gradient, and rinsed in distilled water. Antigen unmasking was performed with 0.1% trypsin for CK8, CK18 (Sigma) and the CK pair 8/18 or with high pH Target Retrival Solution (DakoCytomation, Glostrup, Denmark) for CK18 (Dako). After rinsing, the endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min at room temperature. The slides were washed and pretreated with

protein blocking antigen (UltraTech HRB AEC Kit, Immunotech, France) for 5 min. The same primary antibodies as described above were used, then the slides were rinsed and incubated with a biotinylated secondary antibody for 30 min at room temperature. After washing in PBS the tertiary complex (streptavidin/horseradish peroxidase) was applied for 30 min at room temperature. Cytokeratin staining was visualized by 3-amino-9-ethylcarbazole (Immunotech) for 1 min. The slides were rinsed in distilled water, counterstained with Harris hematoxilin and mounted in Aquatex medium (Merck KGaA, Germany). The same procedure was used for the detection of CKs 8 and 18 in cryosections.

2.4. Immunohistochemistry assessment

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200–400×. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany). At least one hundred endothelial cells per section (300/slide), directly connected to Descemet's membrane, were examined, and the percentage of positive cells was calculated. The intensity of cell staining was graded using a scale according to Diebold et al. (1997): 0: no discernible staining, 1: mild, 2: moderate, 3: intense, and 4: very intense staining.

2.5. Semi-quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from five corneas (two endothelial imprints from each cornea on Millicell membranes) by means of TRI Reagent (Molecular Research Center, Cincinnati, OH). Nine μl of total RNA were reverse transcribed into cDNA in a 20 μl reaction mixture using SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The cDNA samples of each individual RNA extraction were first normalized to yield equal amounts of β-actin. Subsequently, equal amounts of cDNA from five individual samples were amplified with the following specific primers: human CK8, sense primer 5′ – ATC AGC TCC TCG AGC TTC TC – 3′, anti-sense primer 5′ – TCC AGG AAC CGT ACC TTG TC – 3′; human CK18, sense primer 5′ – TCA GCA GAT TGA GGA GAG CA – 3′, anti-sense primer 5′ – TCT GAC TCA AGG TGC AGC AG – 3′; and β-actin, sense primer 5′ – GGC ATC CTC ACC CTG AAG TA – 3′, anti-sense primer 5′ – AAG GTC TCA AAC ATG ATC TGG GT – 3′. The number of PCR cycles for the different primer pairs were 32 cycles for β-actin and 35 cycles for CKs 8 and 18. Each cycle consisted of denaturation for 25 s at 95 °C, annealing for 30 s at 56 °C for β-actin and 65 °C for CKs 8 and 18 and elongation for 45 s at 72 °C. PCR products were analyzed by ethidium bromide stained 2% agarose gel electrophoresis.

2.6. Western blot analysis

Five corneas were used for analysis. To prepare whole-cell extracts, endothelial cells on Millicell membranes were treated in lysis buffer, containing 0.2% Triton X-100, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and protease inhibitors in PBS, followed by centrifugation for 15 min at 14 000 g. The protein concentration was determined using a commercial kit (BCA Protein Assay Kit, Pierce, Rockford, USA). Equal volumes of protein extract and sample buffer were mixed, reduced by 5% beta-mercaptoethanol, and fractionated on 5–10% SDS-poly-acrylamide gels (Laemmli, 1970). After electrophoresis was complete, the proteins were transferred to nitrocellulose membranes (Serva Electroforesis GmbH, Heidelberg, Germany) and blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) at 4 °C overnight. After washing in PBS-T, the membranes were probed with mouse and rabbit antibodies against CK8 (1:1000, Chemicon), CK18 (1:1000) and β-actin (1:2000, Abcam, Cambridge, UK) for 2 h at a room temperature. After washing in PBS-T, the membranes were incubated with ImmunoPure® Peroxidase conjugated goat anti-mouse or anti-rabbit IgG antibody (1:12 000) (Pierce Biotechnology, Rockford, USA) with 1% BSA for 40 min at room temperature and washed with PBS-T. Positive reactions were visualized using an enhanced chemiluminescent technique with SuperSignal® West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Rockford, USA) for 5 min and examined using a Syngene membrane documentation system Chemigeniius-Q and GeneSnap program (Synoptics Ltd., Cambridge, UK).

3. Results

3.1. Indirect immunofluorescence

The endothelium of all corneal cryospecimens formed a monolayer of flat cells attached to Descemet's membrane. CK-positive endothelial cells were scattered around the endothelial layer with an irregular staining intensity. The mean percentage of endothelial cells positive for CK8 was 54% with moderate intensity. Mild staining for CK18 was observed in 4% and 66% of all endothelial cells using antibodies from Dako or Sigma, respectively. Moderate staining was observed for the CK pair 8/18 in 54% of endothelial cells (Table 1), (Fig. 1A–D). Heterogeneous positive staining for both CKs after acetone fixation was detected in the corneal epithelium (data not shown), which served as a positive control. No signal was detected in either the endothelium or the epithelium when 4% formalin or 10% paraformaldehyde was used for fixation (data not shown). No correlation between expression patterns and post-mortem time was detected.

Table 1
Fluorescent immunhistochemistry of the expression of different CKs on cryosections and endothelial imprints of the corneal endothelium (CE).

CK type	CE1	CE2	CE3	CE4	CE5	CE6	% of positive specimens (average percentage of positive cells/average intensity)
Cryosections	Percentage of positive cells/intensity						
CK8 (Chemicon)	35/1	30/2	80/2	55/2	68/2	55/1	100 (54/2)
CK18 (Dako)	1/1	2/1	6/1	1/1	12/1	1/1	100 (4/1)
CK18 (Sigma)	70/1	70/1	60/2	40/1	80/2	75/1	100 (66/1)
CK8/18 (Novocastra)	70/2	55/2	50/2	30/2	55/3	65/2	100 (54/2)
Endothelial imprints							
CK8 (Chemicon)	75/1	80/1	50/2	50/2	47/3	20/2	100 (54/2)
CK18 (Dako)	20/1	1/1	10/1	10/1	7/1	2/1	100 (8/1)
CK18 (Sigma)	20/1	30/1	25/2	60/1	75/2	20/1	100 (38/1)
CK8/18 (Novocastra)	70/2	40/2	25/2	68/2	38/2	40/2	100 (47/2)

Number of cells stained: % (actual percentage)/0: no discernible staining, 1: mild, 2: moderate, 3: intense, and 4: very intense staining.

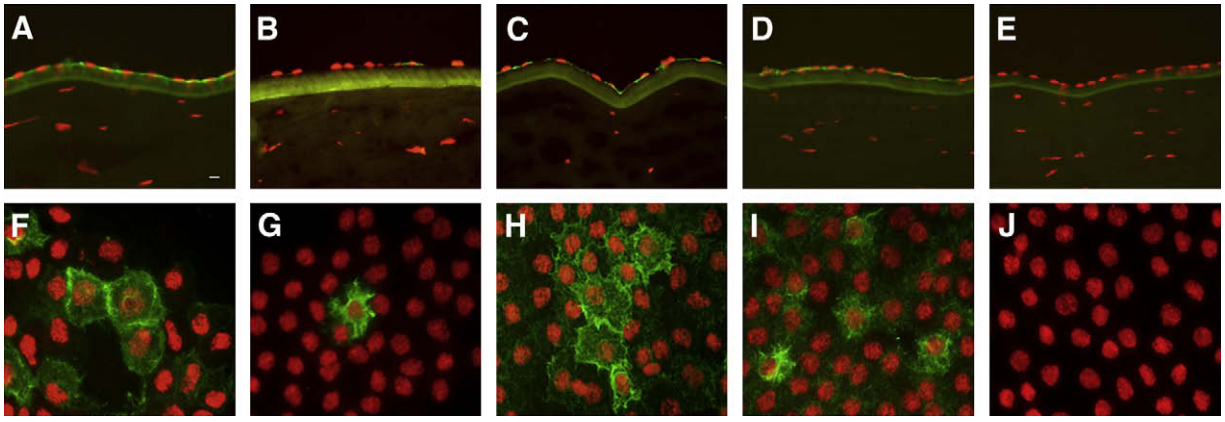

Fig. 1. Indirect immunofluorescent staining of the adult human corneal endothelium. Expression of cytokeratins 8 and 18 on cryosections (A–E) and on endothelial imprints (F–J). Immunostaining for CK8 (A, F), CK18 – Dako (B, G), CK18 – Sigma (C, H), the CK pair 8/18 (D, I), and the negative control (primary antibody omitted) (E, J). Scale bar represents 10 μm.

Endothelium harvested on Millicell membranes formed a monolayer of flat, mostly hexagonal cells with moderate positivity for CK8 in 54% and mild positivity for CK18 (Dako) in 8% of all examined cells. The mean percentage of mild CK18-positive (Sigma) cells reached 38%. The CK pair 8/18 showed moderate positivity in 47% of endothelial cells (Table 1), (Fig. 1F–I). The staining intensity and the distribution of individual CKs in the endothelial cells were irregular. The majority of positive cells accumulated in clusters in the endothelial monolayer without any apparent preference for the central or peripheral zones of the cornea. CK8 formed fibrils, especially under the cell membrane, and copied the cell shape (Fig. 1F). CK18 formed a fibrillar network between the nuclear and inner cell membranes (Fig. 1G).

3.2. Enzymatic immunohistochemistry

Moderate immunoreactivity for CK8 was detected in 71% of endothelial cells. Anti-CK18 antibodies from both Dako and Sigma showed moderate positivity in 52 and 70% of cells, respectively. The CK pair 8/18 revealed moderate positivity in 49% of cells on cryosections fixed in cold acetone (Table 2), (Fig. 2A–D).

We did not detect any immunoreactivity in the endothelium or epithelium of paraffin-embedded specimens fixed with 10% paraformaldehyde (Fig. 2E–H), acetone or 4% formalin (data not shown).

3.3. Semi-quantitative reverse transcription polymerase chain reaction

CK8 and CK18 mRNAs were found in the endothelium of all five corneas using semi-quantitative RT-PCR as was β-actin mRNA, which served as an internal control. Representative results are shown in Fig. 3.

3.4. Western blot analysis

We detected CK8, CK18 (Abcam) and β-actin proteins in the endothelial cells of all the examined specimens (Fig. 4).

4. Discussion

To provide evidence that CK8 and CK18 are expressed in the adult human corneal endothelium, we have used different fixation and processing methods prior to immunohistochemical analysis. In addition, we have confirmed the results at the mRNA level by RT-PCR and at the protein level by Western blotting. We have clearly shown that CK8 and CK18 are expressed constitutively in the adult human corneal endothelium. Approximately 50% of endothelial cells revealed positivity for CK8 and CK18 using immunohistochemical approaches. The endothelial imprints allowed transversal analysis of the endothelial cells and due to it was showed that both CKs formed fibrils. This is in contrary to results published by Raul et al. (2004), who described that CK8 is diffusely localized within the cytoplasm, while the expression of CK18 is much more extensive and it forms fibrils.

It was postulated previously that commercially available anti-keratin antisera can show great variability with respect to reactivity, quality and the methodological approach used (Mygind et al., 1988). Our immunohistochemical experiments clearly confirm these suggestions. Cryosectioned specimens, when acetone was used as the fixative, were positive, but no immunostaining was detected after 4% formalin or 10% paraformaldehyde fixation. Similarly no positive immunostaining in the endothelium and epithelium was found on paraffin-embedded sections with any of the fixative solutions used, including acetone. Our results demonstrate the widely divergent ability of two different antibodies to detect CK18. Four to 8% positive cells were detected using anti-CK18 from Dako on cryosections and imprints, compared to 38–66%

Table 2
Enzymatic immunohistochemistry of the expression of different CKs on cryosections of the corneal endothelium (CE).

CK type	CE1	CE2	CE3	CE4	CE5	CE6	% of positive specimens (average percentage of positive cells/average intensity)
	Percentage of positive cells/intensity						
CK8 (Chemicon)	75/1	70/2	70/2	50/3	90/3	70/2	100 (71/2)
CK18 (Dako)	50/2	80/2	50/2	20/2	90/3	20/2	100 (52/2)
CK18 (Sigma)	60/2	95/2	75/3	20/2	90/3	80/2	100 (70/2)
CK8/18 (Novocastra)	50/2	70/2	50/2	5/1	50/2	70/2	100 (49/2)

Number of cells stained: % (actual percentage)/0: no discernible staining, 1: mild, 2: moderate, 3: intense, and 4: very intense staining.

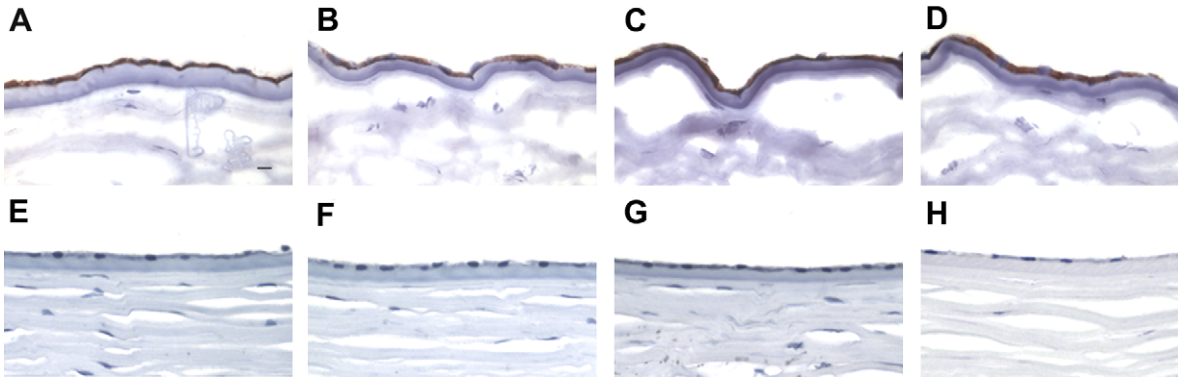

Fig. 2. Enzymatic immunohistochemical staining of the adult human corneal endothelium for CK8 (A, E), CK18 – Dako (B, F), CK18 – Sigma (C, G) and CK pair 8/18 (D, H). Immunostaining is present in the corneal endothelium when cryosections are used (A–D). No CKs are detected when paraffin-embedded sections are used (E–H). Scale bar represents 10 μm.

positive endothelial cells using anti-CK18 from Sigma. A similar percentage (52–70%) was seen when enzymatic immunohistochemistry was used; these results suggest that the Sigma CK18 antibody is better suited for detecting CK18 in the corneal endothelium than the one from Dako. Our observation shows that besides the methodological approach used in tissue processing, different antibody sensitivities may have contributed to the controversial results obtained in earlier studies. Different CK8 or CK18 antibody binding behaviors have been found in various cell types (Bosch et al., 1988; Kasper, 1991; Kasper et al., 1991). These differences may be explained by the existence of conformation-dependent epitopes or the existence of isoforms for individual cytokeratin polypeptides (Kasper et al., 1992). As a result, it is necessary to work with an enlarged antibody panel to avoid misleading results in the immunolocalization of cytokeratin proteins (Kasper, 1991).

In most of the experiments in which paraffin sections were used for immunohistochemistry, no CK8 or CK18 was detected (Cockerham et al., 2002; Kramer et al., 1992; Levy et al., 1995). On the other hand, Kasper et al. who stained for CKs 8 and 18 in both cryosections and paraffin sections, localized these intermediate filaments in the endothelium of specimens obtained from healthy adult and fetal human corneas using formaldehyde-resistant antibodies (Kasper et al., 1992). Similarly in the study performed by Wollensak and Witschel (1996), CK18 was found in the adult corneal endothelium but only in two of four specimens on paraffin sections after formaldehyde fixation using the monoclonal antibody from Sigma. It seems likely that paraformaldehyde or formalin fixation or the process of preparing paraffin-embedded sections causes alterations in the antigen epitopes of CKs 8 and 18. In contrast to our previous study (Jirsova et al., 2007) as well as to studies performed by other authors (Cockerham et al., 2002; Levy et al., 1995), in which no CKs besides CK8 and 18 were found in control endothelium, Foets et al. (1990) detected a uniform or occasional positivity for CKs 7, 8, 18 and 19.

The expression of CKs is dramatically changed in patients suffering from corneal endothelial dystrophies, where the endothelium displays signs of epithelialization (Boruchoff and Kuwabara, 1971; Hidayat and Cockerham, 2006). Abnormal cells gain

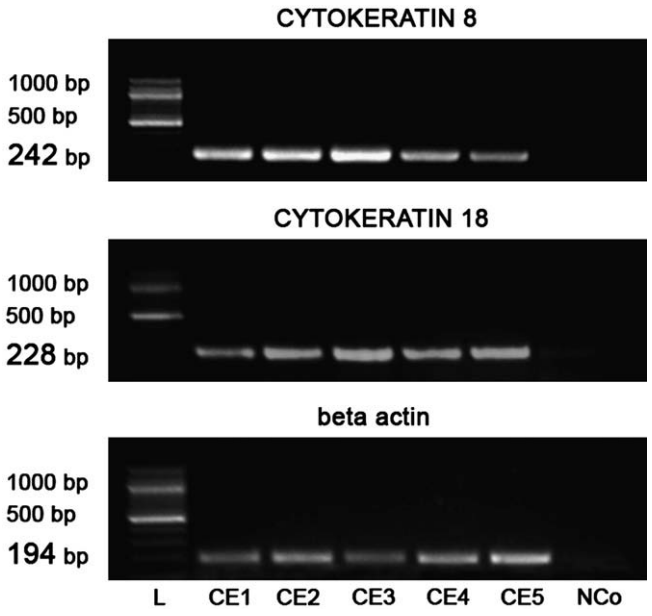

Fig. 3. Expression of the cytokeratin 8 and 18 genes in the corneal endothelium (CE) determined by RT-PCR. Beta actin was used as an internal control. NCo – negative control (reaction without sample cDNA) a marker for internal contamination, L – ladder.

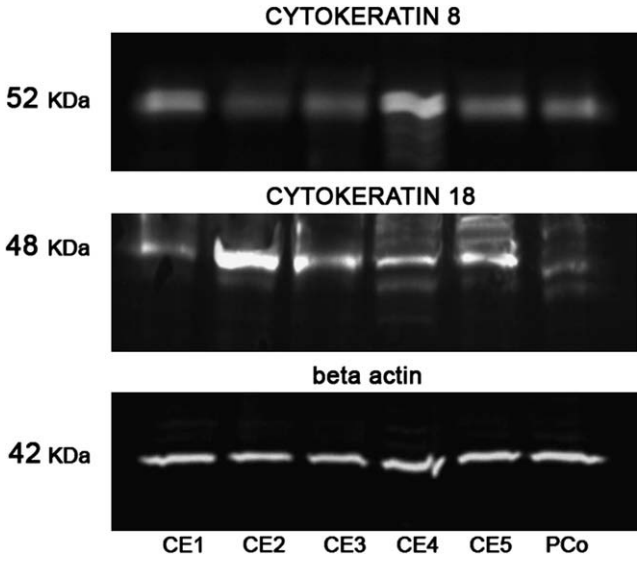

Fig. 4. Expression of cytokeratin 8 and 18 proteins in adult corneal endothelium (CE) cells as determined by Western blotting. Beta actin was used as an internal control. PCo – positive control (corneal epithelium).

proliferative activity and express various CKs, including CK7, 19 and the CK pair 8/18 (Cockerham et al., 2002; Hidayat and Cockerham, 2006; Jirsova et al., 2007; Rodrigues et al., 1980). The mechanisms that underlie the activation of diverse CK expression under these pathological conditions have not yet been elucidated, but it seems likely that it can be linked to the altered differentiation of endothelial cell progenitors.

The function of CKs 8 and 18 in the corneal endothelium may be connected with their cytoprotective role against chemical insults and the modulation of the cellular response to proapoptotic signals. Both CKs are dramatically reorganized during apoptosis; CK18 is a target for caspase-mediated proteolysis. The occurrence of cytokeratins 8 and 18 in different cell types leads to resistance to TNF-α- and Fas-mediated apoptosis; conversely, their loss causes a switch in the Fas-activated death signaling pathway (Caulin et al., 1997, 2000; Gilbert et al., 2001, 2008; Inada et al., 2001). The occurrence of these cytokeratins in the adult corneal endothelium may support the immune privilege of the cornea and augment protection against cytotoxic agents.

Whether the corneal endothelium is derived only from mesenchymal tissue (Risen et al., 1987) or whether neural crest cells participate on its development is a question open to discussion. In any case, our study clearly demonstrates that the corneal endothelium expresses, in addition to mesenchymal and neuronal proteins (Hayashi et al., 1986; Shamsuddin et al., 1986), epithelial cell markers as well.

Acknowledgements

This work was supported by the research project of the Ministry of Education, Youth and Sports of the Czech Republic 0021620806/20610011. We would like to thank Dr. V. Vesela for excellent technical assistance with the preparation of the specimens.

References

Adamis, A.P., Filatov, V., Tripathi, B.J., Tripathi, R.C., 1993. Fuchs' endothelial dystrophy of the cornea. Surv. Ophthalmol. 38, 149–168.
Bahn, C.F., Falls, H.F., Varley, G.A., Meyer, R.F., Edelhauser, H.F., Bourne, W.M., 1984. Classification of corneal endothelial disorders based on neural crest origin. Ophthalmology 91, 558–563.
Baribault, H., Price, J., Miyai, K., Oshima, R.G., 1993. Mid-gestational lethality in mice lacking keratin 8. Genes. Dev. 7, 1191–1202.
Boruchoff, S.A., Kuwabara, T., 1971. Electron microscopy of posterior polymorphous degeneration. Am. J. Ophthalmol. 72, 879–887.
Bosch, F.X., Leube, R.E., Achtstätter, T., Moll, R., Franke, W.W., 1988. Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. J. Cell Biol. 106, 1635–1648.
Caulin, C., Salvesen, G.S., Oshima, R.G., 1997. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J. Cell Biol. 138, 1379–1394.
Caulin, C., Ware, C.F., Magin, T.M., Oshima, R.G., 2000. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. J. Cell Biol. 149, 17–22.
Cockerham, G.C., Laver, N.V., Hidayat, A.A., McCoy, D.L., 2002. An immunohistochemical analysis and comparison of posterior polymorphous dystrophy with congenital hereditary endothelial dystrophy. Cornea 21, 787–791.
Diebold, Y., Calonge, M., Fernández, N., Lázaro, M.C., Callejo, S., Herreras, J.M., Pastor, J.C., 1997. Characterization of epithelial primary cultures from human conjunctiva. Graefe's Arch. Clin. Exp. Ophthalmol. 235, 268–276.
Foets, B.J., van den Oord, J.J., Desmet, V.J., Missotten, L., 1990. Cytoskeletal filament typing of human corneal endothelial cells. Cornea 9, 312–317.
Foets, B., van den Oord, J., Engelmann, K., Missotten, L., 1992a. A comparative immunohistochemical study of human corneotrabecular tissue. Graefe's Arch. Clin. Exp. Ophthalmol. 230, 269–274.
Foets, B.J., van den Oord, J.J., Volpes, R., Missotten, L., 1992b. In situ immunohistochemical analysis of cell adhesion molecules on human corneal endothelial cells. Br. J. Ophthalmol. 76, 205–209.
Gilbert, S., Loranger, A., Daigle, N., Marceau, N., 2001. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. J. Cell Biol. 154, 763–773.
Gilbert, S., Ruel, A., Loranger, A., Marceau, N., 2008. Switch in Fas-activated death signaling pathway as result of keratin 8/18-intermediate filament loss. Apoptosis 13, 1479–1493.
Hayashi, K., Sueishi, K., Tanaka, K., Inomata, H., 1986. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefe's Arch. Clin. Exp. Ophthalmol. 224, 452–456.
Hidayat, A.A., Cockerham, G.C., 2006. Epithelial metaplasia of the corneal endothelium in Fuchs endothelial dystrophy. Cornea 25, 956–959.
Inada, H., Izawa, I., Nishizawa, M., Fujita, E., Kiyono, T., Takahashi, T., Momoi, T., Inagaki, M., 2001. Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. J. Cell Biol. 155, 415–426.
Jackson, B.W., Grund, C., Schmid, E., Burki, K., Franke, W.W., Illmensee, K., 1980. Formation of cytoskeletal elements during mouse embryogenesis. Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. Differentiation 17, 161–179.
Jirsova, K., Merjava, S., Martincova, R., Gwilliam, R., Ebenezer, N.D., Liskova, P., Filipec, M., 2007. Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. Exp. Eye Res. 84, 680–686.
Johnson, B.L., Brown, S.I., 1978. Posterior polymorphous dystrophy: a light and electron microscopic study. Br. J. Ophthalmol. 62, 89–96.
Johnston, M.C., Noden, D.M., Hazelton, R.D., Coulombre, J.L., Coulombre, A.J., 1979. Origins of avian ocular and periocular tissues. Exp. Eye Res. 29, 27–43.
Kasper, M., 1991. Heterogeneity in the immunolocalization of cytokeratin specific monoclonal antibodies in the rat eye: evaluation of unusual epithelial tissue entities. Histochemistry 95, 613–620.
Kasper, M., Perry, G., Stosiek, P., 1991. Cytokeratin expression in human spinal meninges and ependymal cells. J. Hirnforsch. 32, 19–25.
Kasper, M., Stosiek, P., Lane, B., 1992. Cytokeratin and vimentin heterogeneity in human cornea. Acta Histochem. 93, 371–381.
Kramer, T.R., Grossniklaus, H.E., Vigneswaran, N., Waring, G.O., Kozarsky, A., 1992. Cytokeratin expression in corneal endothelium in the iridocorneal endothelial syndrome. Invest. Ophthalmol. Vis. Sci. 33, 3581–3585.
Ku, N.O., Michie, S., Oshima, R.G., Omary, M.B., 1995. Chronic hepatitis, hepatocyte fragility, and increased soluble phosphoglycokeratins in transgenic mice expressing a keratin 18 conserved arginine mutant. J. Cell Biol. 131, 1303–1314.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Levy, S.G., McCartney, A.C., Baghai, M.H., Barrett, M.C., Moss, J., 1995. Pathology of the iridocorneal - endothelial syndrome. The ICE cell. Invest. Ophthalmol. Vis. Sci. 36, 2592–2601.
Moll, R., Franke, W.W., Schiller, D.L., Geiger, B., Krepler, R., 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11–24.
Mygind, H., Nielsen, B., Moe, D., Clausen, H., Dabelsteen, E., Clausen, P.P., 1988. Antikeratin antibodies in routine diagnostic pathology. A comparison of 10 different commercial antikeratins. APMIS 96, 1009–1022.
Oshima, R.G., Baribault, H., Caulin, C., 1996. Oncogenic regulation and function of keratins 8 and 18. Cancer Metastasis Rev. 15, 445–471.
Pronk, A., Leguit, P., Hoynck van Papendrecht, A.A., Hagelen, E., van Vroonhoven, T.J., Verbrugh, H.A., 1993. A cobblestone cell isolated from the human omentum: the mesothelial cell; isolation, identification, and growth characteristics. In Vitro Cell. Dev. Biol. 29A, 127–134.
Raul, U., Sawant, S., Dange, P., Kalraiya, R., Ingle, A., Vaidya, M., 2004. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype. Int. J. Cancer 111, 662–668.
Risen, L.A., Binder, P.S., Nayak, S.K., 1987. Intermediate filaments and their organization in human corneal endothelium. Invest. Ophthalmol. Vis. Sci. 28, 1933–1938.
Rodrigues, M.M., Sun, T.T., Krachmer, J., Newsome, D., 1980. Epithelization of the corneal endothelium in posterior polymorphous dystrophy. Invest. Ophthalmol. Vis. Sci. 19, 832–835.
Sevel, D., Isaacs, R., 1988. A re-evaluation of corneal development. Trans. Am. Ophthalmol. Soc. 86, 178–207.
Shamsuddin, A.K., Nirankari, V.S., Purnell, D.M., Chang, S.H., 1986. Is the corneal posterior cell layer truly endothelial? Ophthalmology 93, 1298–1303.
Stosiek, P., Kasper, M., Conrad, K., 1990. Immunohistochemistry of cytokeratin expression in human blood vessel endothelia with special reference to synovial connective tissue. Acta Histochem. 89, 61–66.
Trask, D.K., Band, V., Zajchowski, D.A., Yaswen, P., Suh, T., Sager, R., 1990. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 87, 2319–2323.
Waring, G.O., Bourne, W.M., Edelhauser, H.F., Kenyon, K.R., 1982. The corneal endothelium. Normal and pathologic structure and function. Ophthalmology 89, 531–590.
Weston, J.A., Yoshida, H., Robinson, V., Nishikawa, S., Fraser, S.T., Nishikawa, S., 2004. Neural crest and the origin of ectomesenchyme: neural fold heterogeneity suggests an alternative hypothesis. Dev. Dyn. 229, 118–130.
Wollensak, G., Witschel, H., 1996. Vimentin and cytokeratin pattern in granular corneal dystrophy. Graefe's Arch. Clin. Exp. Ophthalmol. 234 (Suppl. 1), S110–S114.

Experimental Eye Research 91 (2010) 623–629

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Mesothelial proteins are expressed in the human cornea

Katerina Jirsova [a,b,1,*], Ales Neuwirth [c], Sarka Kalasova [a], Viera Vesela [a], Stanislava Merjava [a,1]

[a] Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[b] Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic
[c] Department of Immunobiology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 23 February 2010
Accepted in revised form 1 August 2010
Available online 13 August 2010

Keywords:
cornea
mesothelin
HBME-1 (Hector Battifora mesothelial cell-1) protein
calbindin 2

ABSTRACT

The goal of our study was to determine whether proteins typical of the human mesothelial cell phenotype, such as mesothelin, HBME-1 (Hector Battifora mesothelial cell-1) protein and calbindin 2, are expressed in the human cornea, especially in endothelial cells.

Cryosections and endothelial and epithelial imprints of sixteen human cadaverous corneoscleral discs were used. The presence of proteins was examined using immunohistochemistry and Western blotting, while mRNA levels were determined by qRT-PCR.

A strong signal for mesothelin was present in the corneal epithelium, while less intense staining was visible in the endothelium. Similarly, higher and lower mRNA levels were detected using qRT-PCR in the corneal epithelium and endothelium, respectively. HBME-1 antibody strongly stained the corneal endothelium and stromal keratocytes. Marked positivity was present in the corneal stromal extracellular matrix, while no staining was present in the sclera. Calbindin 2 was detected using immunohistochemistry and Western blotting in the corneal epithelium, endothelium and stroma. qRT-PCR confirmed its expression in epithelial and endothelial cells.

Three proteins expressed constitutively in mesothelial cells were detected in the human cornea. The possible function of mesothelin in cell–cell contact on the ocular surface is discussed. The presence of HBME-1 protein in the endothelial layer may indicate a still unknown function that could be shared with mesothelial cells of the pleura and peritoneum. The much more pronounced occurrence of calbindin 2 in the corneal epithelium compared to fewer positive endothelial cells explains the higher turnover of epithelial cells compared to the proliferatively inactive endothelium.

1. Introduction

The vertebrate corneal epithelium develops from the surface ectoderm, while the corneal endothelium and keratocytes originate from the lateral plate mesoderm and the neural crest, which together form the periocular mesenchyme. A broad spectrum of transcriptional factors is required for the appropriate differentiation of the periocular mesenchyme to keratocytes and corneal endothelial cells (Cvekl and Tamm, 2004; Gage et al., 2005; Kidson et al., 1999; Reneker et al., 2000; Sowden, 2007).

The distinctive phenotypical heterogeneity of the human corneal endothelium (Foets et al., 1990, 1992a; Hayashi et al., 1986) may reflect the fact that the mammalian endothelium originates from both neural and mesodermal cells, compared to the avian endothelium, which is formed only by cells of neural crest cell origin (Gage et al., 2005; Johnston et al., 1979; Sowden, 2007). Human corneal endothelial cells express neural cell markers such as neuron-specific enolase, S-100 protein, neuron cell adhesion molecule and neurofilaments, in accordance with the crucial participation of the neural crest in endothelial cell development (Foets et al., 1992a, 1992b; Hayashi et al., 1986; Shamsuddin et al., 1986). In addition, epithelial cell markers – cytokeratins 8 and 18 – have been detected immunohistochemically in normal endothelium, and subsequently the constitutive expression of both cytokeratins was confirmed using a molecular biology approach (Foets et al., 1990; Merjava et al., 2009). On the other hand, the absence of markers typical of the vascular endothelium such as factor VIII, VCAM and PECAM (CD 31) in human corneal endothelial

* Corresponding author. Laboratory of the Biology and Pathology of the Eye, VFN, U nemocnice 2, 128 08 Prague, Czech Republic. Tel.: +420 224 963 008; fax: +420 224 962 338.
E-mail address: katerina.jirsova@vfn.cz (K. Jirsova).
[1] These contributed equally to this work and therefore should be considered as co-first authors.

0014-4835/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2010.08.002

cells (Foets et al., 1992b; Scheef et al., 2007; Shamsuddin et al., 1986; Treseler et al., 1985–1986) clearly demonstrates that the term endothelium, although widely used for the posterior cells of the cornea, is a misnomer (Shamsuddin et al., 1986).

Of the mesothelial cell markers, vimentin is present in human corneal endothelium (Foets et al., 1990; Hayashi et al., 1986; Risen et al., 1987), but this protein is observed in a number of various cell types of mesodermal origin, including different epithelia as well as the vascular endothelium, and thus cannot be considered as a protein specific only for mesothelial cells (Blose and Meltzer, 1981; Celis et al., 1983).

Because of the partially shared origin of mesothelial cells and human corneal endothelial cells, we expected that some of the markers typical of mesothelial cells may be expressed by the corneal endothelium. Moreover, endothelial cells are located on the basal membrane on one side and are in contact with the aqueous humor of the anterior chamber on the other, thus they occupy a similar location as do typical mesothelial cells in the pleura or peritoneum; one may expect a sharing of some phenotypical markers between the two cell types due to their similar roles, at least in terms of fluid movement between a tissue and a cavity.

Little is known about mesothelial cell markers and their presence in the adult human cornea. We have therefore chosen mesothelin, HBME-1 (Hector Battifora mesothelial cell-1) protein and calbindin 2, proteins typically expressed in the mesothelium (Ko et al., 2001; Miettinen and Kovatich, 1995; Ordonez, 2003a), and studied their presence and expression in different corneal layers, focusing on endothelial cells.

Mesothelin (MSLN) is a membrane-bound peptide, which is physiologically expressed in mesothelial cells lining the pleura, pericardium and peritoneum; limited immunoreactivity is manifested in various epithelial cells. Its biological function is not known (Chang and Pastan, 1996; Chang et al., 1992; Ordonez, 2003a). However, mesothelin is strongly expressed in malignant mesotheliomas as well as in various carcinomas and adenocarcinomas; it may be released from cell membranes, and a soluble form has been detected in the serum of mesothelioma patients (Hellstrom et al., 2006; Ordonez, 2003a, 2003b).

The protein to which the HBME-1 antibody reacts is still unidentified. HBME-1 antibody reacts strongly to mesothelial cells, especially in the pleura and peritoneum. The antibody was raised against cultured mesothelioma cells, and because it neither immunoblots nor immunoprecipitatates, the antigen has remained unidentified (personal communication H. Battifora in Miettinen and Kovatich, 1995). Due to its overexpression in various neoplastic epithelial cells, this unknown protein nonetheless serves as a useful marker in the differential diagnosis of epithelial mesothelioma on one hand and sarcoid mesothelioma or adenocarcinoma on the other (Dahlstrom et al., 2001; Miettinen and Kovatich, 1995). It is located both in the cytoplasm and plasmatic membrane of mesothelioma cells, where a thick brush pattern indicates the presence of this protein in some of the microvilli (Battifora and McCaughey, 1995; Miettinen and Kovatich, 1995; Ordonez, 1997).

The third protein that we studied is a 29-kDa calcium-binding protein, calbindin 2 (CALB-2), known as calretinin as well. Together with calbindin 1, calmodulin, parvalbumin, S-100 protein and troponin C, calbindin 2 is a member of the troponin C large superfamily characterised by a structural motif described as the EF-hand (Baimbridge et al., 1992; Persechini et al., 1989). This protein was originally cloned from the chick retina, and it is abundantly expressed in the neurons of the central and peripheral nervous systems (Rogers, 1987; Rogers et al., 1990), including murine corneal sensory neurons (Felipe et al., 1999). Outside of neurons, it is typically found in mesothelial and mesothelioma cells as well as in various other cell types (Al Moghrabi et al., 2007; Doglioni et al., 1996; Ko et al., 2001; Marchevsky, 2008). The function of calbindin 2 is not fully understood, but it is thought to act as a buffer to prevent abnormal intracellular calcium increases (Rogers, 1987; Rogers et al., 1990). Its presence protects neuronal cells against various types of injury (Lukas and Jones, 1994). In addition, this protein was localized by Gotzos et al. (1992) in both the cytoplasm and the cell nuclei in phase G1 and during mitosis in human adenocarcinoma cells, where it associates with the kinetochor and polar microtubules. This localization indicates a role in spindle microtubule stability and chromosome separation (Gotzos et al., 1992).

None of the above mentioned proteins has yet been described in the cornea, other than the presence of calbindin 2 in mouse neurons (Felipe et al., 1999). In the present study, we determined the presence and expression of these proteins throughout the cornea as well as in the limbus and adjacent conjunctiva.

2. Methods

2.1. Specimens

The study followed the ethical standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki. In total, sixteen adult corneoscleral discs (9 men and 7 women; mean age 62.3 ± 13.9 years) not acceptable for transplantation because of low endothelial cell density or a positive serology of the donor were obtained from the Ocular Tissue Bank Prague. One cadaverous peritoneum, which was used as a positive control, was retrieved from the Institute of Forensic Medicine and Toxicology of the First Faculty of Medicine and General University Hospital, Prague.

Five corneal buttons were dissected and snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at −70 °C. Four 7 μm thick cryosections were placed per slide and used for indirect fluorescent or enzymatic immunohistochemistry. Three corneas were used for impression cytology of the endothelium and epithelium (two imprints of each per cornea) on Suppor®-200 Membrane Disc Filters (Suppor®-200, P/N 60298, Pall Corporation, Michigan) and subsequently used for indirect fluorescent or enzymatic immunohistochemistry.

Six corneas were used for impression cytology of the endothelium and corneal epithelium on Biopore Millicell membranes (MILLICELL® – CM, PICM 01250, Millipore, Bedford MA). After the rest of the endothelium, epithelium and limbus were removed, the native stromal tissue was processed. All these samples were frozen and subsequently used for the detection of mesothelin and calbindin 2 by Western blotting and qRT-PCR (proteins and mRNAs were isolated from cells on the Millicell membranes and from stromal tissue). Additionally, two Descemet's membrane–endothelium lamellae with supporting stromal tissue (Studeny et al., 2009), not acceptable for grafting, were used for immunohistochemistry to detect HBME-1 protein and mesothelin in a fully confluent endothelial mosaic. The time between death and storage in liquid nitrogen for all these samples did not exceed 24 h.

2.2. Indirect immunofluorescence

2.2.1. Cryosections

Three sections on each slide were stained with a single antibody, and the fourth section was used as a negative control (primary antibody omitted); this negative control was included on every slide. Immunohistochemistry on cryosections was performed as described previously (Merjava et al., 2009). The following mouse or goat monoclonal antibodies and dilutions were used: anti-mesothelin

(clone K1, 1:50), anti-calretinin (calbindin 2, clone N-18, 1:75, Santa Cruz Biotechnology, Santa Cruz, USA) and anti-human mesothelial cell protein (clone HBME-1, 1:50, DakoCytomation, Glostrup, Denmark). Subsequently, the sections were incubated with the appropriate secondary antibody (fluorescein isothiocyanate-conjugated or rhodamine-conjugated anti-mouse or anti-goat IgG, Jackson ImmunoResearch Laboratories, West Grove, USA).

2.2.2. Endothelial and epithelial imprints

Suppor®-200 membranes were first moistened with 10 μl of distilled water and placed on the endothelial or epithelial surface of the cornea for 5 s. The primary imprints were reprinted onto slides (Superfrost Plus, Menzel GmbH & Co KG, Germany). After fixation in cold acetone for 10 min, the cells were rinsed in PBS and permeabilized in 0.2% TRITON X-100 and rinsed. Subsequently, the immunostaining was performed as described above.

2.3. Enzyme immunohistochemistry

2.3.1. Cryosections

The tissue was fixed with cold acetone for 10 min, rinsed with PBS and the endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature. The slides were pretreated with protein blocking antigen (UltraTech HRB AEC kit, Immunotech, France) for 5 min and then incubated with the primary antibody (as listed above) diluted in PBS containing 1% BSA for 1 h. The biotinylated secondary antibody and subsequently the tertiary complex (streptavidin/horseradish peroxidase) were both applied for 30 min. The immunostaining reaction was detected using 3-amino-9-ethylcarbazol for 1 min (Immunotech). The slides were rinsed in distilled water, counterstained with Harris hematoxilin and mounted in Aquatex medium (Merck KGaA, Germany).

2.3.2. Endothelial and epithelial imprints

The immunohistological protocol for endothelial and epithelial imprints (cells from Suppor®-200 membranes reprinted on Superfrost slides) was the same as for enzymatic immunohistochemistry on cryosections, with the addition of a permeabilization step in 0.2% TRITON X-100 between the first rinsing in PBS and blocking with 3% hydrogen peroxide.

2.4. Immunohistochemistry assessment

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200–1000×. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany) and JENOPTIK ProgRes C12plus (Jenoptik, Laser. Optik. Systeme GmbH, Jena, Germany). At least three hundred epithelial cells per section (900/slide) and one hundred endothelial cells per section (300/slide), directly connected to Descemet's membrane, were examined, and the percentage of positive cells was calculated. The intensity of cell staining was graded using a scale: 0: no discernible staining, 1: weak, 2: moderate, 3: intense, and 4: very intense staining. The presence of each protein was determined as well in the limbus and adjacent perilimbal conjunctiva.

2.5. Western blot analysis

Six corneas were used for analysis. To prepare whole-cell extracts, the stroma, epithelial and endothelial cells on Millicell membranes were treated in lysis buffer, containing 0.2% Triton X-100, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and protease inhibitors in PBS, followed by centrifugation for 15 min at 14 000g. The protein concentration was determined using a commercial kit (BCA Protein Assay Kit, Pierce, Rockford, USA). Equal volumes of protein extract and sample buffer were mixed, reduced by 5% beta-mercaptoethanol, and fractionated on 5–10% SDS-poly-acrylamide gels (Laemmli, 1970). After electrophoresis was complete, the proteins were transferred to nitrocellulose membranes (Serva Electroforesis GmbH, Heidelberg, Germany) and blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) at 4 °C overnight. After washing in PBS-T, the membranes were probed with mouse and goat antibodies against mesothelin (clone K1, 1:300), calretinin (calbindin 2, clone N-18, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA) and β-actin (1:2 000, Abcam, Cambridge, UK) for 2 h at room temperature. After washing in PBS-T, the membranes were incubated with ImmunoPure® peroxidase conjugated goat anti-mouse antibody (1:12 000), (Pierce Biotechnology, Rockford, USA) or peroxidase conjugated rabbit anti-goat antibody (1:8 000, Invitrogene Corporation, Camarillo, USA) with 1% BSA for 40 min at room temperature and washed with PBS-T. Positive reactions were visualized using an enhanced chemiluminescent technique with SuperSignal® West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Rockford, USA) for 5 min and examined using a Syngene membrane documentation system Chemigeniius-Q and GeneSnap program (Synoptics Ltd., Cambridge, UK).

2.6. Quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated from imprints of the corneal epithelium and endothelium on Millicell membranes and from stromal samples using an Rneasy Plus Microkit (Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamers followed by PCR amplification with Sybr green master mix (Roche Diagnostics, Mannheim, Germany). The following specific oligonucleotides for mesothelin, calbindin 2 and a houskeeping gene glyceraldehyde-3-phospate dehydrogenase (GAPDH) were used: human mesothelin, sense primer 5′-gaa tgt gag cat gga ctt gg-3′, anti-sense primer 5′-acg tgg ggt ccc aga agt-3′; human calbindin 2, sense primer 5′-ttc agg gca tga agc tgac-3′, anti-sense primer 5′-tca tgc tcg tca atg tag cc-3′; human GAPDH, sense primer 5′-agc cac atc gct cag acac-3′, anti-sense primer 5′-gcc caa tac gac caa atcc-3′. The reactions were run in triplicates on a LC480 thermocycler (Roche Diagnostics). The gene expression for mesothelin and calbindin 2 was quantified using a relative quantification model with efficiency correction using the following formula: $\text{ratio} = (E_{\text{target}})^{\text{Ct target(control-treated)}} / (E_{\text{ref}})^{\text{Ct ref(control-treated)}}$ according to Pfaffl (2001). The efficiency for each gene was estimated by the dilution calibration method. The tissue expression in individuals was quantified separately, where the sample with the highest mesothelin or calbindin 2 expression from a given individual was set to a relative expression value of 100%. The data were analyzed by GraphPad Prism software.

3. Results

The results of the immunohistochemical analysis of cryospecimens stained with antibodies to mesothelin, HBME-1 protein and calbindin 2 are shown in Table 1.

3.1. Mesothelin

We found a weak to strong signal for mesothelin in each of the 5 tested cryospecimens in all corneal epithelial layers, with the most intense signal in the superficial epithelium (Table 1; Fig. 1a). A similar staining pattern was observed in the limbal and conjunctival epithelium (data not shown). The membrane-bound

Table 1
The mean (average) percentage of positive cells and staining intensity of mesothelin, HBME-1 protein and calbindin 2 in different locations of the human cornea.

protein	epithelium			stroma	endothelium
	superficial	suprabasal	basal		
	average % of positive cells/intensity				
mesothelin	75/4	70/3	75/3	N	60/2
HBME-1	N	N	N	100/4	86/2
calbindin 2	25/1	77/2	77/2	100/1	66/2

Number of cells stained: % (average percentage)/N: negative, 1: mild, 2: moderate, 3: intense, and 4: very intense staining.

localization of mesothelin was readily apparent on cryosections, clearly showing the borders between adjacent epithelial cells. This staining pattern was found as well on the corneal epithelial imprints (Fig. 1b). The immunostaining of the corneal endothelium was dependent upon the concentration of the primary antibody. Very weak staining of the cells was obtained using a dilution of 1:50, while a higher concentration (1:25) led to moderate–intense positivity (Fig. 1a). A pronounced cytoplasmic staining was manifested in almost 100% of the cells on the endothelium–Descemet's membrane lamella (Fig. 1c). The stroma, the basal membrane of the epithelium, Descemet's membrane and Bowman's layer were completely negative. An intense signal was detected in the surface cells of the peritoneum (Fig. 1d), and staining was absent in all negative control specimens (Fig. 1e).

We detected two isoforms (40 and 69 KDa) of mesothelin in all layers of the cornea, in the endothelium, stroma and epithelium, using Western blot analysis. Moreover, the expression of mesothelin was determined by qRT-PCR in all three corneal layers. The strongest expression was found in the epithelium, less in the stroma and the endothelium (Fig. 2). Relative mesothelin expression was normalized to an endogenously expressed housekeeping gene (GAPDH).

3.2. HBME-1

Homogenous staining using the HBME-1 antibody was observed in each of the tested corneal specimens. HBME-1 protein was expressed by keratocytes and appeared throughout the whole stroma, including Bowman's layer (Fig. 3a). A prominent sharp border of the staining was noted in all specimens between the positive corneal stroma and the negative stroma of the sclera (Fig. 3b). Human corneal endothelial cells revealed moderate to intense positivity. Flat specimens (endothelial imprints and lamellae) enabled a detailed visualization of the signal and revealed almost 100% positivity. While a minority of the cells revealed membrane staining in a brush border pattern, strong cytoplasmic staining was found in most of the positive cells (Fig. 3c and d).

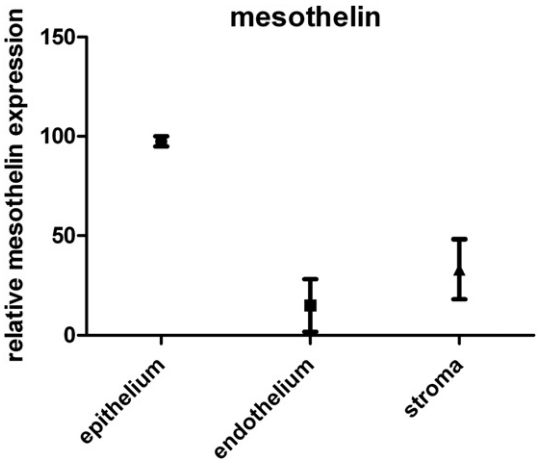

Fig. 2. Relative expression of mesothelin in corneal samples quantified by qRT-PCR. The majority of mesothelin expression was detected in the epithelium, but pronounced expression was detected in stromal and endothelial samples as well. Data were normalized to a housekeeping gene (GAPDH). Each bar represents mean ± SEM of 6 experimental samples.

HBME-1 staining was not detected in the corneal epithelium or in either basal membrane. The limbus, conjunctiva and sclera were completely negative as well as all negative controls. Superficial mesothelial cells of the peritoneum revealed strong intracellular positivity (Fig. 3e).

3.3. Calbindin 2

Calbindin 2 was detected in the corneal, limbal and conjunctival epithelia as well as in endothelial cells using immunohistochemical methods (Table 1; Fig. 4a). Both cytoplasmic and nuclear staining were clearly visible on the epithelial and endothelial imprints. Intranuclear dots, probably representing an association with kinetochor and polar microtubules, were more readily detectable in superficial epithelial cells compared to endothelial cells. The nuclei of both the endothelium and epithelium revealed mostly one or two, less often three or more, dots (Fig. 4b and c). Moreover, a strong signal was found at the plasmatic membrane of epithelial as well as endothelial cells. Weak immunostaining without any gradient was observed throughout the whole corneal stroma and in the surface cells of the peritoneum (Fig. 4d). No positive staining was observed in any negative control (Fig. 4e).

Using Western blot analysis, calbindin 2 was detected in the stroma, epithelium and endothelium. qRT-PCR revealed that calbindin 2 is expressed in the epithelial and to a lesser extent in the endothelial cells of the cornea, while no expression was detected in the stroma (Fig. 5). Relative calbindin 2 expression was normalized to an endogenously expressed housekeeping gene (GAPDH).

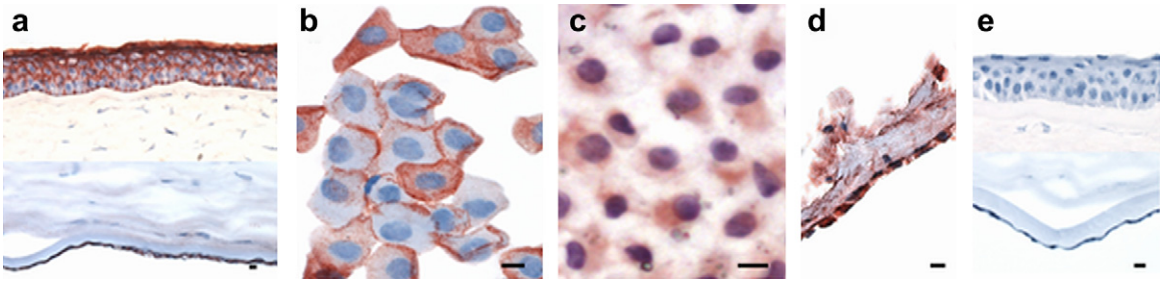

Fig. 1. Enzymatic immunohistochemical detection of mesothelin in the human cornea. Positivity in the epithelium and endothelium on cryosections (a). Detail of the membrane-bound signal in the epithelial imprints (b) and the cytoplasmic signal in the endothelial cells of lamella (c). Surface cells of the peritoneum as a positive control (d) and corneal negative control (e). Scale bar represents 10 μm.

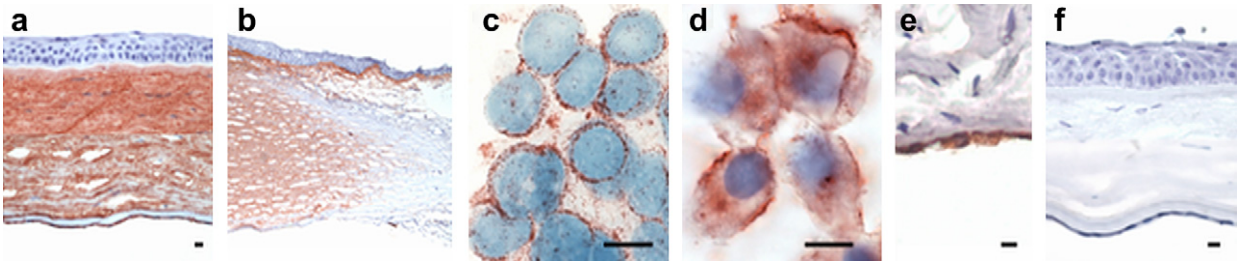

Fig. 3. Enzymatic immunohistochemical detection of HBME-1 protein. A strong signal in the stroma and endothelium of corneal cryosections (a). A sharp border between the corneal (positive) and scleral (negative) stroma (b). A clear brush border pattern in the endothelial imprints (c) and strong cytoplasmic signal in the endothelial cells of endothelial lamella (d). Superficial cells of the peritoneum as a positive control (e) and corneal negative control (f). Scale bar represents 10 μm.

4. Discussion

In this study we clearly demonstrate that mesothelin, HBME-1 protein and calbindin 2, which are considered to be reliable markers of healthy and neoplastic mesothelium (King et al., 2006; Marchevsky, 2008; Miettinen and Kovatich, 1995; Yaziji et al., 2006), are abundantly expressed in the human cornea.

Mesothelin has not yet been described in the eye, excluding its expression in the rat limbus (Adachi et al., 2006). Surprisingly, we have found this protein in the endothelium, stroma, and more intensely in the epithelium of the cornea, limbus and conjunctiva. Although the immunostaining of the endothelial cells depended on the concentration of the primary antibody, Western blot analysis as well as qRT-PCR clearly confirmed its presence in this cell layer.

The abundant expression of mesothelin in healthy mesothelial cells of the pleura, peritoneum and pericardium is well documented (Chang et al., 1992; Ordonez, 2003a), but its role under physiological conditions is still unknown, and no histological differences were found in various mesothelin-expressing tissues between mesothelin knockout and wild-type mice (Bera and Pastan, 2000). Under pathological conditions, the non-covalent bond between membrane-bound mesothelin and the N-linked glycans of mucin 16 (MUC16, formerly CA125) mediates the heterotypic cell adhesion of MUC16 expressed in the OVCAR-3 ovarian tumor cell line to an endothelial-like cell line expressing mesothelin, which may be inhibited by anti-mesothelin antibody (Gubbels et al., 2006; Rump et al., 2004). Because of the abundant occurrence of MUC16 (Argueso et al., 2003; Blalock et al., 2008) and mesothelin in corneal and conjunctival epithelium, a comparable bond between these two proteins on the ocular surface may be expected. Additionally, the immunostaining of the conjunctival surface using H185 antibody, which reacts with MUC16, was altered in patients suffering from non-Sjögren's dry eye compared to the normal ocular surface epithelium (Argueso et al., 2003; Danjo et al., 1998). Nevertheless, a possible role for mesothelin and MUC16 on the ocular surface under normal conditions, and potential alterations in the interaction between mesothelin and MUC16 in terms of the ocular surface changes seen in dry eye patients, still remain to be elucidated.

The HBME-1 antibody clearly stains corneal endothelial cells, which display an abundant cytoplasmic signal. In the corneal endothelium, the cytoplasmic staining is more distinct than the signal present in the cell membrane, which is different from mesothelial and most neoplastic cells, in which microvillous staining predominates over cytoplasmic staining (Dahlstrom et al., 2001). We did not find any difference in the intensity of the staining between the central and peripheral parts of the endothelium, as was expected due to the increased centrifugal presence of microvili in the human corneal endothelial cells (Svedbergh and Bill, 1972). The presence of a signal for HBME-1 protein seen in the microvilli of the endothelium, but not in the corneal epithelium, may reflect the different function of the corneal epithelium and endothelium, which is in contact with the aqueous humor and may help to maintain its fluidity.

Interestingly, HBME-1 staining was found throughout the extracellular space of all stromal layers. The very intense staining in the central and peripheral cornea sharply diminished in the limbal area and was missing from the scleral stroma. This localization indicates that the antibody reacts with some protein abundantly present in the extracellular matrix of the corneal stroma but missing from the sclera. Unfortunately, HBME-1 antibody is not suitable for immunoblots or immunoprecipitates (personal communication Hector Battifora in Miettinen and Kovatich, 1995), as we have confirmed using Western blot analysis (data not shown). Moreover, the exact antigen with which the antibody reacts is still unknown, so we were unable to prepare primers for qRT-PCR evaluation. Thus, the function and importance of this protein in the cornea remain to be elucidated.

The intracellular localization of the third detected protein – calbindin 2 – is already well established. We detected calbindin 2 in all corneal layers using immunohistochemistry and Western blotting. Several intranuclear spots were clearly visible on our specimens of epithelial and endothelial imprints, but in contrast we were unable to detect calbindin 2 expression in the stroma using qRT-PCR.

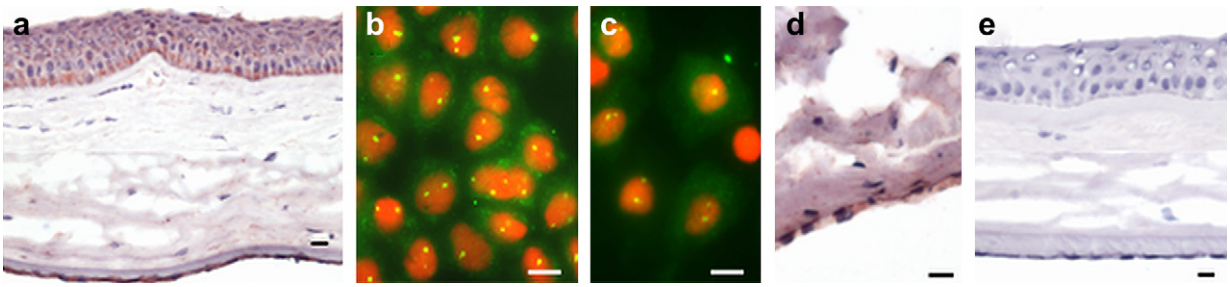

Fig. 4. Immunohistochemical detection of calbindin 2. Positivity in the epithelium and endothelium of corneal cryosections (a). Calbindin 2-positive intranuclear dots in the epithelial (b) and endothelial (c) imprints. Surface cells of the peritoneum as a positive control (d) and corneal negative control (e). Scale bar represents 10 μm.

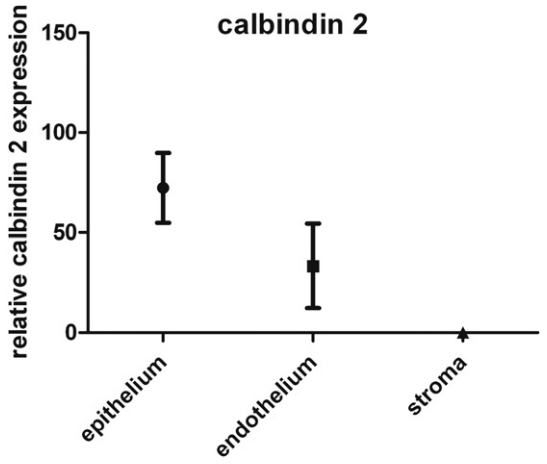

Fig. 5. Relative expression of calretinin in corneal samples quantified by qRT-PCR. Calretinin is expressed in epithelial and in some endothelial samples, while the stroma is negative. Data were normalized to a housekeeping gene (GAPDH). Each bar represents mean ± SEM of 6 experimental samples.

Nonetheless, because of the characteristic specific staining (intranuclear dots) of this protein we expected its presence in keratocytes as well. The intracellular localization of calbindin 2 (calretinin) is consistent with the previous findings of Gotzos et al., who demonstrated that this protein is associated with kinetochor and polar microtubules in phase G1 and mitosis (Gotzos et al., 1992). The more intense staining of the dots in epithelial cells compared to the less positive endothelium may be connected to the high turnover of epithelial cells compared to the proliferatively inactive endothelial cells, which are inhibited in the G1 phase (Joyce et al., 1996).

In addition to mesothelin, HBME-1 protein and calbindin 2, thrombomodulin is considered to be a reliable marker of mesothelial cell origin (King et al., 2006; Marchevsky, 2008; Ordonez, 2003b; Yaziji et al., 2006). Thrombomodulin is an integral membrane protein expressed on the surface of the vascular endothelium and functions as a cofactor in the thrombin-induced activation of protein C in the anticoagulant pathway (Esmon and Owen, 1981, 2004). Its presence in the human cornea, including in the endothelial cells where it appears to be involved in maintaining the fluidity of the aqueous humor, has already been demonstrated (Ikeda et al., 2000). The expression of mesothelial markers extends the phenotypical heterogeneity of human corneal endothelial cells. The phenotypical uniqueness of corneal endothelial cells consists in the presence of a combination of neural, epithelial, and mesothelial cell line markers in this cell type (Foets et al., 1990; Foets et al., 1992b; Merjava et al., 2009; Risen et al., 1987).

In summary, we report here the presence of the mesothelial proteins mesothelin, HBME-1 protein and calbindin 2 in the normal adult human cornea and hypothesize about the potential roles of these proteins in ocular tissue.

Acknowledgements

This work was supported by the research project of the Ministry of Education, Youth and Sports of the Czech Republic MSM0021620806 and by the Project 260501 from Charles University in Prague.

References

Adachi, W., Ulanovsky, H., Li, Y., Norman, B., Davis, J., Piatigorsky, J., 2006. Serial analysis of gene expression (SAGE) in the rat limbal and central corneal epithelium. Invest. Ophthalmol. Vis. Sci. 47, 3801–3810.

Al Moghrabi, H., Elkeilani, A., Thomas, J.M., Mai, K.T., 2007. Calretinin: an immunohistochemical marker for the normal functional endometrial stroma and alterations of the immunoreactivity in dysfunctional uterine bleeding. Pathol. Res. Pract. 203, 79–83.

Argueso, P., Spurr-Michaud, S., Russo, C.L., Tisdale, A., Gipson, I.K., 2003. MUC16 mucin is expressed by the human ocular surface epithelia and carries the H185 carbohydrate epitope. Invest. Ophthalmol. Vis. Sci. 44, 2487–2495.

Baimbridge, K.G., Celio, M.R., Rogers, J.H., 1992. Calcium-binding proteins in the nervous system. Trends Neurosci. 15, 303–308.

Battifora, H., McCaughey, W.T.E., 1995. Tumors of the Serous Membranes, third ed. American Registry of Pathology, Washington D.C. 72.

Bera, T.K., Pastan, I., 2000. Mesothelin is not required for normal mouse development or reproduction. Mol. Cell. Biol. 20, 2902–2906.

Blalock, T.D., Spurr-Michaud, S.J., Tisdale, A.S., Gipson, I.K., 2008. Release of membrane-associated mucins from ocular surface epithelia. Invest. Ophthalmol. Vis. Sci. 49, 1864–1871.

Blose, S.H., Meltzer, D.I., 1981. Visualization of the 10-NM filament vimentin rings in vascular endothelial cells in situ: close resemblance to vimentin cytoskeletons found in monolayers in vitro. Exp. Cell. Res. 135, 299–309.

Celis, J.E., Larsen, P.M., Fey, S.J., Celis, A., 1983. Phosphorylation of keratin and vimentin polypeptides in normal and transformed mitotic human epithelial amnion cells: behavior of keratin and vimentin filaments during mitosis. J. Cell Biol. 97, 1429–1434.

Chang, K., Pastan, I., 1996. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc. Natl. Acad. Sci. USA 93, 136–140.

Chang, K., Pastan, I., Willingham, M.C., 1992. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. Int. J. Cancer 50, 373–381.

Cvekl, A., Tamm, E.R., 2004. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. Bioessays 26, 374–386.

Dahlstrom, J.E., Maxwell, L.E., Brodie, N., Zardawi, I.M., Jain, S., 2001. Distinctive microvillous brush border staining with HBME-1 distinguishes pleural mesotheliomas from pulmonary adenocarcinomas. Pathology 33, 287–291.

Danjo, Y., Watanabe, H., Tisdale, A.S., George, M., Tsumura, T., Abelson, M.B., Gipson, I.K., 1998. Alteration of mucin in human conjunctival epithelia in dry eye. Invest. Ophthalmol. Vis. Sci. 39, 2602–2609.

Doglioni, C., Dei Tos, A.P., Laurino, L., Iuzzolino, P., Chiarelli, C., Celio, M.R., Viale, G., 1996. Calretinin: a novel immunocytochemical marker for mesothelioma. Am. J. Surg. Pathol. 20, 1037–1046.

Esmon, C.T., Owen, W.G., 1981. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc. Natl. Acad. Sci. USA 78, 2249–2252.

Esmon, C.T., Owen, W.G., 2004. The discovery of thrombomodulin. J. Thromb. Haemost. 2, 209–213.

Felipe, C.D., Gonzalez, G.G., Gallar, J., Belmonte, C., 1999. Quantification and immunocytochemical characteristics of trigeminal ganglion neurons projecting to the cornea: effect of corneal wounding. Eur. J. Pain 3, 31–39.

Foets, B.J., van den Oord, J.J., Desmet, V.J., Missotten, L., 1990. Cytoskeletal filament typing of human corneal endothelial cells. Cornea 9, 312–317.

Foets, B., van den Oord, J., Engelmann, K., Missotten, L., 1992a. A comparative immunohistochemical study of human corneotrabecular tissue. Graefes Arch. Clin. Exp. Ophthalmol. 230, 269–274.

Foets, B.J., van den Oord, J.J., Volpes, R., Missotten, L., 1992b. In situ immunohistochemical analysis of cell adhesion molecules on human corneal endothelial cells. Br. J. Ophthalmol. 76, 205–209.

Gage, P.J., Rhoades, W., Prucka, S.K., Hjalt, T., 2005. Fate maps of neural crest and mesoderm in the mammalian eye. Invest. Ophthalmol. Vis. Sci. 46, 4200–4208.

Gotzos, V., Schwaller, B., Hetzel, N., Bustos-Castillo, M., Celio, M.R., 1992. Expression of the calcium binding protein calretinin in WiDr cells and its correlation to their cell cycle. Exp. Cell Res. 202, 292–302.

Gubbels, J.A., Belisle, J., Onda, M., Rancourt, C., Migneault, M., Ho, M., Bera, T.K., Connor, J., Sathyanarayana, B.K., Lee, B., Pastan, I., Patankar, M.S., 2006. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. Mol. Cancer 5, 50.

Hayashi, K., Sueishi, K., Tanaka, K., Inomata, H., 1986. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefes Arch. Clin. Exp. Ophthalmol. 224, 452–456.

Hellstrom, I., Raycraft, J., Kanan, S., Sardesai, N.Y., Verch, T., Yang, Y., Hellstrom, K.E., 2006. Mesothelin variant 1 is released from tumor cells as a diagnostic marker. Cancer Epidemiol. Biomarkers Prev. 15, 1014–1020.

Ikeda, T., Ishii, H., Higuchi, T., Sato, K., Hayashi, Y., Ikeda, K., Hirabayashi, Y., 2000. Localization of thrombomodulin in the anterior segment of the human eye. Invest. Ophthalmol. Vis. Sci. 41, 3383–3390.

Johnston, M.C., Noden, D.M., Hazelton, R.D., Coulombre, J.L., Coulombre, A.J., 1979. Origins of avian ocular and periocular tissues. Exp. Eye Res. 29, 27–43.

Joyce, N.C., Meklir, B., Joyce, S.J., Zieske, J.D., 1996. Cell cycle protein expression and proliferative status in human corneal cells. Invest. Ophthalmol. Vis. Sci. 37, 645–655.

Kidson, S.H., Kume, T., Deng, K., Winfrey, V., Hogan, B.L., 1999. The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. Dev. Biol. 211, 306–322.

King, J., Thatcher, N., Pickering, C., Hasleton, P., 2006. Sensitivity and specificity of immunohistochemical antibodies used to distinguish between benign and malignant pleural disease: a systematic review of published reports. Histopathology 49, 561–568.

Ko, E.C., Jhala, N.C., Shultz, J.J., Chhieng, D.C., 2001. Use of a panel of markers in the differential diagnosis of adenocarcinoma and reactive mesothelial cells in fluid cytology. Am. J. Clin. Pathol. 116, 709–715.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lukas, W., Jones, K.A., 1994. Cortical neurons containing calretinin are selectively resistant to calcium overload and excitotoxicity in vitro. Neuroscience 61, 307–316.

Marchevsky, A.M., 2008. Application of immunohistochemistry to the diagnosis of malignant mesothelioma. Arch. Pathol. Lab. Med. 132, 397–401.

Merjava, S., Neuwirth, A., Mandys, V., Jirsova, K., 2009. Cytokeratins 8 and 18 in adult human corneal endothelium. Exp. Eye Res. 89, 426–431.

Miettinen, M., Kovatich, A.J., 1995. HBME-1 a monoclonal antibody useful in the differential diagnosis of mesothelioma, adenocarcinoma and soft tissue and bone tumours. Appl. Immunohistochem. 3, 115–122.

Ordonez, N.G., 1997. The value of antibodies 44-3A6, SM3, HBME-1, and thrombomodulin in differentiating epithelial pleural mesothelioma from lung adenocarcinoma: a comparative study with other commonly used antibodies. Am. J. Surg. Pathol. 21, 1399–1408.

Ordonez, N.G., 2003a. The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. Am. J. Surg. Pathol. 27, 1031–1051.

Ordonez, N.G., 2003b. Application of mesothelin immunostaining in tumor diagnosis. Am. J. Surg. Pathol. 27, 1418–1428.

Persechini, A., Moncrief, N.D., Kretsinger, R.H., 1989. The EF-hand family of calcium-modulated proteins. Trends Neurosci. 12, 462–467.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Reneker, L.W., Silversides, D.W., Xu, L., Overbeek, P.A., 2000. Formation of corneal endothelium is essential for anterior segment development – a transgenic mouse model of anterior segment dysgenesis. Development 127, 533–542.

Risen, L.A., Binder, P.S., Nayak, S.K., 1987. Intermediate filaments and their organization in human corneal endothelium. Invest. Ophthalmol. Vis. Sci. 28, 1933–1938.

Rogers, J., Khan, M., Ellis, J., 1990. Calretinin and other CaBPs in the nervous system. Adv. Exp. Med. Biol. 269, 195–203.

Rogers, J.H., 1987. Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons. J. Cell Biol. 105, 1343–1353.

Rump, A., Morikawa, Y., Tanaka, M., Minami, S., Umesaki, N., Takeuchi, M., Miyajima, A., 2004. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. J. Biol. Chem. 279, 9190–9198.

Scheef, E.A., Huang, Q., Wang, S., Sorenson, C.M., Sheibani, N., 2007. Isolation and characterization of corneal endothelial cells from wild type and thrombospondin-1 deficient mice. Mol. Vis. 13, 1483–1495.

Shamsuddin, A.K., Nirankari, V.S., Purnell, D.M., Chang, S.H., 1986. Is the corneal posterior cell layer truly endothelial? Ophthalmology 93, 1298–1303.

Sowden, J.C., 2007. Molecular and developmental mechanisms of anterior segment dysgenesis. Eye (Lond) 21, 1310–1318.

Studeny, P., Farkas, A., Vokrojova, M., Liskova, P., Jirsova, K., 2009. Descemet's membrane endothelial keratoplasty with a stromal rim (DMEK-S). Br. J. Ophthalmol. (Epub ahead of print).

Svedbergh, B., Bill, A., 1972. Scanning electron microscopic studies of the corneal endothelium in man and monkeys. Acta Ophthalmol. (Copenh) 50, 321–336.

Treseler, P.A., Foulks, G.N., Sanfilippo, F., 1985–1986. Expression of ABO blood group, hematopoietic, and other cell-specific antigens by cells in the human cornea. Cornea 4 (3), 157–168.

Yaziji, H., Battifora, H., Barry, T.S., Hwang, H.C., Bacchi, C.E., McIntosh, M.W., Kussick, S.J., Gown, A.M., 2006. Evaluation of 12 antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma: identification of a three-antibody immunohistochemical panel with maximal sensitivity and specificity. Mod. Pathol. 19, 514–523.

Available online at www.sciencedirect.com
ScienceDirect
Experimental Eye Research 84 (2007) 680–686
EXPERIMENTAL EYE RESEARCH
www.elsevier.com/locate/yexer

Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients

Katerina Jirsova [a,b,*], Stanislava Merjava [b], Radka Martincova [b], Rhian Gwilliam [c], Neil D. Ebenezer [d], Petra Liskova [b,d], Martin Filipec [a,b,e]

[a] *Ocular Tissue Bank, General Teaching Hospital and Charles University, U Nemocnice 2, Prague 128 08, Czech Republic*
[b] *Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and Charles University, U Nemocnice 2, Prague 128 08, Czech Republic*
[c] *The Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK*
[d] *Division of Molecular Genetics, Institute of Ophthalmology, UCL, London EC1V 9EL, UK*
[e] *Eye Clinic Lexum, U spolecenske zahrady 3, 140 00 Prague, Czech Republic*

Received 11 August 2006; accepted in revised form 9 December 2006
Available online 15 December 2006

Abstract

Posterior polymorphous corneal dystrophy (PPCD) is a hereditary bilateral disorder affecting Descemet's membrane and the endothelium. The aim of the present study was to determine the spectrum of cytokeratin (CK) expression in cells on the posterior surface of the cornea in PPCD patients. Ten corneal buttons and one specimen of the trabecular meshwork (TM) from PPCD patients who underwent graft or glaucoma surgery were used, as well as six corneal buttons and two TM specimens obtained from healthy donors as controls. Cryosections were fixed and indirect immunofluorescent staining was performed using antibodies directed against a wide spectrum of cytokeratins (CKs). The number of positive cells and the intensity of the staining were assessed using fluorescent microscopy. All 10 PPCD corneal specimens had areas of endothelium displaying typical endothelial morphology as well as areas consisting of layers two to six cells thick with both flat endothelial-like cells and polygonal cells with round nuclei and a large cytoplasm. Both of these morphologically distinct cell types showed strong immunostaining for CK7, CK19, CK8 and CK18, while weaker positive signals were observed for CK1, CK3/12, CK4, CK5/6, CK10, CK10/13, CK14, CK16 and CK17. PPCD endothelium was completely negative for CK2e, CK9, CK15, and CK20. Focal positivity was detected in PPCD TM for CK4, CK7 and CK19. CK8 and CK18 were the only CKs expressed in control endothelium. PPCD and control epithelium displayed similar staining patterns. The distinct positivity for CK3/12, CK4, CK5/6, CK10/13, CK14, CK16 and CK17 was observed in aberrant PPCD endothelium for the first time. We demonstrate that the abnormal endothelium of PPCD patients expresses a mixture of CKs, with CK7 and CK19 predominating. In terms of CK composition, the aberrant PPCD endothelium shares features of both simple and squamous stratified epithelium with a proliferative capacity. The wide spectrum of CK expression is most probably not indicative of the transformation of endothelial cells to a distinct epithelial phenotype, but more likely reflects the modified differentiation of metaplastic epithelium.
Published by Elsevier Ltd.

Keywords: posterior polymorphous corneal dystrophy; immunohistochemistry; cytokeratins; endothelium

1. Introduction

Posterior polymorphous corneal dystrophy (PPCD, OMIM #122000) is a bilateral disorder of the corneal endothelium and Descemet's membrane (DM), inherited in an autosomal dominant manner (Cibis et al., 1977; Hogan and Bietti, 1969).

* Corresponding author. Laboratory of the Biology and Pathology of the Eye and Ocular Tissue Bank, VFN, U Nemocnice 2, 128 08, Prague, Czech Republic. Tel.: +420 224 963 008; fax: +420 224 962 338.
E-mail address: katerina.jirsova@vfn.cz (K. Jirsova).

0014-4835/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.exer.2006.12.006

At least three different genes are implicated in PPCD: the gene encoding the α2 chain of type VIII collagen (COL8A2; OMIM *120252) on chromosome 1 (Biswas et al., 2001); the visual system homeobox gene 1 (VSX1; OMIM *605020) on chromosome 20 (Héon et al., 2002); and the human zinc finger transcription factor 8 gene (TCF8; OMIM *189909) on chromosome 10 (Krafchak et al., 2005). In addition, linkage analysis performed in two large Czech families delineated the PPCD locus on chromosome 20p.11.2 to a 2.7 cM interval between flanking markers D20S48 and D20S139; the PPCD gene for this locus has not yet been reported (Gwilliam et al., 2005).

PPCD is characterized biomicroscopically by vesicular lesions, bands and geographic opacities at the level of posterior DM and the endothelium. The disease is usually non-progressive in most affected subjects. However, in some patients secondary changes such as corneal edema or glaucoma may lead to visual impairment and necessitate surgical management in the form of keratoplasty or an anti-glaucomatous procedure (Cibis et al., 1977).

The corneal endothelium is a monolayer of flat hexagonal cells, which in humans are normally arrested in the G_1-phase of the cell cycle (Joyce et al., 1996). In PPCD patients, local foci of the original cells lose their endothelial characteristics and acquire an epithelial-like phenotype (Hirst, 1983). This epithelialization has been demonstrated using both light and electron microscopy (Krachmer, 1985; Matsumoto et al., 1988). It was demonstrated that aberrant cells are joined together by abundant desmosomes, that they exhibit numerous microvilli on their apical surface, and that there are abundant keratofibrils in a cytoplasm depleted of organelles (Boruchoff and Kuwabara, 1971; Levy et al., 1996; Rodrigues et al., 1980). Proliferation and the formation of multilayered areas extending outwards from the cornea over the trabecular meshwork (TM), leading to secondary glaucoma, have also been observed (Krachmer, 1985).

Cytokeratins (CKs) are typical epithelial cell markers expressed in a tissue-specific and differentiation-dependent manner (Moll et al., 1982). The epithelialization of the PPCD endothelium was confirmed immunohistochemically by Rodrigues et al. (1981), who detected the presence of CKs using non-specific antisera against total human epidermal CKs. Pan-cytokeratin expression in the abnormal PPCD endothelial layer was later verified using AE1/AE3 antibody (Molia et al., 1999; Ross et al., 1995). CK7 and the CK pair 8/18 (CAM 5.2 antibody) have also been detected in aberrant PPCD cells (Cockerham et al., 2002; Moroi et al., 2003).

The underlying cause of the endothelial changes in PPCD is currently unknown. Proliferation and epithelial characteristics of pathological endothelium have also been demonstrated immunohistochemically in other corneal diseases. Positive immunostaining using AE1, AE3, CK3, CK5 and CK19 antibodies was observed in iridocorneal-endothelial syndrome (Hirst et al., 1995; Levy et al., 1995), while the epithelialization of the endothelium in congenital hereditary endothelial dystrophy (CHED) was detected using antibodies against CK7, CK8 and CK18 (Cockerham et al., 2002).

In this study we used a wide spectrum of antibodies directed against CKs with the aim of characterizing the aberrant cells arising on the posterior corneal surface in patients diagnosed with PPCD. Antibodies to the typical corneal epithelium CK pair (CK3/12), CKs expressed predominantly in simple epithelia (CK7, CK8, CK17, CK18, CK19), CKs of stratified epithelia (CK4, CK13), CKs of basal epithelia (CK5, CK14, CK15), CKs typical of hyperproliferative epithelia (CK6, CK16) and CKs expressed in keratinized and terminally differentiated squamous epithelia (CK1, CK10) were used (Moll et al., 1982; van der Velden et al., 1999).

2. Materials and methods

2.1. Specimens

The study was approved by the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and followed the tenets set out in the Declaration of Helsinki. The diagnosis of PPCD was based on slit lamp examination, specular microscopy and family history.

Ten corneal buttons were obtained by keratoplasty surgery from nine PPCD patients (five males and four females) from eight families of Czech origin, ranging in age from 14 to 71 years, with a median age of 40 years. Each eye requiring corneal transplantation developed stromal and epithelial edema. None of the examined patients suffered from secondary glaucoma or any other ophthalmic disorder.

One cornea was obtained from a member of a family mapped to 20p11.2 (Gwilliam et al., 2005) (Table 1; P1), eight corneas from seven patients from six families originating in the same geographic area in the western part of the Czech Republic (Table 1; P2, P3, P5–P8) and one cornea from a patient with an identified change in the coding sequence of the TCF8 gene (unpublished) (Table 1; P4). One TM specimen was obtained during glaucoma surgery performed in an eye two years after keratoplasty in a patient from one of the families clustered in the western part of the Czech Republic (the cornea of this patient was not included in the study). Six healthy corneal buttons and two TM specimens from donors with no prior ocular history obtained from the Ocular Tissue Bank (Prague) were included as control tissue. For CKs that are not expressed in the cornea or conjunctiva, epithelia from breast skin (for CK2e), skin from the palm of the hand and the sole of the foot (for CK9) and stomach mucosa (for CK20) were used as positive control tissues.

Corneal buttons were dissected, embedded in optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen. Tissues were cryosectioned at a thickness of 7 μm and four slices were mounted per slide.

2.2. Histology and indirect immunofluorescent microscopy

Slides from each PPCD patient and controls were stained with haematoxilin and eosin for morphological assessment of the endothelial layer by light microscopy.

Slices obtained from all 10 corneal specimens were stained with each antibody, and each staining was performed in

Table 1
The morphological characterization of abnormal endothelial layers obtained from nine PPCD corneas

Patient no.	P1	P2	P3	P4	P5 LE	P5 RE	P6	P7	P8	P9
Number of endothelial layers (min./max.)	1/6	1/2	1/3	1/5	1/5	1/3	1/3	1/4	1/1	1/3
The number of layers prevalent in sections	2–3	1	1–2	1	1–2	1–2	1	2	1	2–3
Cell type characterization of abnormal endothelial layer	EP	EN/EP	EN/EP	EN/EP	EN/EP	EN	EN/EP	EN/EP	EN/EP	EP

PPCD corneas revealed a mixture of endothelial (EN) and epithelial (EP) cell types. Corneas from the left eye (LE) and right eye (RE) of patient 5 were studied.

duplicate. A negative control (primary antibody omitted) was included on every slide. The tissue was fixed with cold acetone for 10 min, rinsed in phosphate buffered saline (PBS) and incubated with primary antibodies diluted in PBS containing 1% bovine serum albumin for 1 h at room temperature. The following mouse or goat monoclonal antibodies and dilutions were used: cytokeratin 1 (1:250) and cytokeratin 10 (1:50) (Santa Cruz Biotechnology, Santa Cruz, USA); cytokeratin 2e (1:50), cytokeratin 9 (1:50), cytokeratin 16 (1:30), cytokeratin 17 (1:25) and cytokeratin 20 (1:50) (Acris Antibodies GmbH, Hiddenhausen, Germany); cytokeratin 3/12 (1:400) (Research Diagnostics Inc., Flanders, NJ, USA); cytokeratin 4 (1:200) (Sigma, St. Louis, USA); cytokeratin 5/6 (1:40), cytokeratin 7 (1:50), cytokeratin 10/13 (1:25), cytokeratin 18 (1:50) and cytokeratin 19 (1:50) (DakoCytomation, Glostrup, Denmark); cytokeratin 8 (1:400), cytokeratin 14 (1:50) and cytokeratin 15 (1:100) (Chemicon International Inc., Temecula, USA). The specimens were then washed three times with PBS and incubated with appropriate secondary antibodies [fluorescein isothiocyanate-conjugated or rhodamine-conjugated (for CK14 only) anti-mouse or anti-goat IgG, all from Jackson ImmunoResearch Laboratories, West Grove, USA] for 1 h at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide or DAPI (Vector Laboratories, Inc. Burlingame, USA) to counterstain the DNA within the nuclei. The slices prepared from TM specimens were stained for CK4, CK7 and CK19.

The slices were examined by fluorescent microscopy using an Olympus BX 51 at a magnification of 200–1000×. Images were taken using a Vosskühler VDS CCD-1300 camera, (VDS Vosskühler GmbH, Germany). The positivity was assessed separately in each corneal layer. At least 100 cells directly connected to DM or connected to DM through an intermediate cell were examined, and the percentage of positive cells was calculated. To grade the intensity of cell staining, a scale according to Diebold et al. (1997) was used: 0, no discernible staining; 1+, mild; 2+, moderate; 3+, intense; and 4+, very intense staining.

3. Results

3.1. Histology

The endothelium of all control specimens was formed of one layer of flat cells adjoined to DM (Fig. 1A). PPCD endothelium consisted predominantly of one or two cell layers (Table 1), but areas with up to six cell layers forming loop projections detached from DM were also observed. These multilayered foci were mostly composed of cells containing prominent round nuclei and more abundant cytoplasm (Fig. 1B). At the basal zone, adjacent to DM, most cells were flat with elongated nuclei. However, many areas of DM were free of any cells.

3.2. Indirect immunofluorescent microscopy

The results of the immunohistochemical analysis of PPCD and normal corneas stained with antibodies to various CKs are shown in Table 2.

The endothelium of all control corneal specimens was positive for CK8 (54%/2+) and CK18 (32%/2+); none of the

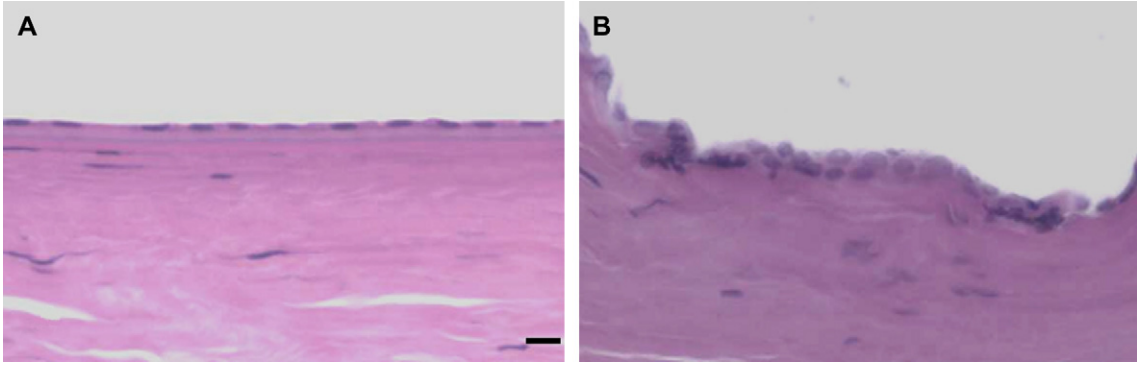

Fig. 1. Control endothelium is composed of one layer of regular flat cells (A) compared to PPCD multilayered endothelium displaying cells containing prominent round nuclei and more abundant cytoplasm (B). Hematoxilin and eosin staining, scale bar represents 10 μm.

Table 2
The immunostaining of different CKs in PPCD and control corneal endothelium

CK type	Patient no.									Control	PPCD
	P1	P2	P3	P4	P5 LE	P5 RE	P6	P7	P8	P9	
	% of positive cells/intensity										% positive specimens (average percentage of positive cells/average intensity)
CK1	N	N	N	N	N	5/1+	30/1+	25/1+	N	N	0	30 (20/1+)
CK3/12	76/2+	72/2+	62/2+	80/2+	50/1+	3/1+	40/1+	24/1+	43/1+	N	0	90 (51/1+)
CK4	28/4+	5/1+	41/1+	35/2+	17/1+	7/2+	33/1+	20/2+	5/1+	65/2+	0	100 (26/2+)
CK5/6	24/1+	10/1+	60/1+	30/1+	7/1+	11/1+	1/2+	60/1+	3/1+	85/2+	0	100 (29/1+)
CK7	100/4+	95/4+	100/4+	100/4+	80/2+	88/3+	95/3+	88/2+	88/3+	100/3+	0	100 (93/3+)
CK8	95/4+	97/4+	100/4+	100/4+	75/2+	85/2+	76/3+	80/3+	100/4+	80/2+	100 (54/2+)	100 (89/3+)
CK10	N	N	9/1+	N	N	N	N	N	N	15/2+	0	20 (12/1+)
CK10/13	N	N	N	8/1+	10/1+	50/1+	53/2+	10/1+	5/1+	45/1+	0	70 (26/1+)
CK14	N	21/1+	45/3+	90/3+	100/4+	100/3+	11/3+	100/4+	N	100/4+	0	80 (71/3+)
CK16	4/1+	1/1+	39/1+	42/2+	49/2+	60/2+	64/1+	37/2+	1/2+	20/2+	0	100 (32/2+)
CK17	9/1+	10/2+	60/3+	75/2+	50/2+	55/2+	18/2+	60/1+	8/2+	70/2+	0	100 (42/2+)
CK18	100/4+	95/3+	100/3+	47/2+	3/1+	10/1+	71/4+	13/1+	83/2+	72/2+	100 (32/2+)	100 (59/2+)
CK19	100/4+	100/3+	100/3+	100/4+	70/1+	98/3+	95/2+	90/1+	78/3+	100/3+	0	100 (93/3+)

Number of cells stained: actual percentage (%)/staining. Staining: 0, no discernible staining; 1+, mild; 2+, moderate; 3+, intense; and 4+, very intense staining. CK2e, CK9, CK15, and CK20 were negative in control as well as in PPCD corneas (not shown). LE, left eye; RE, right eye.

other tested CKs was expressed in control endothelium (Fig. 2A).

Each of the 10 PPCD specimens was positive for CK4, CK5/6, CK7, CK8, CK16, CK17, CK18 and CK19. Nine of 10 PPCD specimens were positive for CK3/12 and CK14, seven for CK10/13, three for CK1 and two for CK10 (Fig. 2B).

The intensity and distribution of CK staining were irregular and varied among PPCD corneas and also within individual specimens. Very intense staining was present in 93% of the cells of abnormal endothelium using antibodies to CK7 as well as to CK19. The number of cells showing positive staining for CK8 and CK18 in PPCD specimens was 1.9 and 1.8 times greater than the number seen in controls, respectively.

CK14, CK16 and CK17 were stained moderately to intensely, but in no more than 71% of abnormal endothelial layer cells (CK14). Mild positivity for CK3/12 was present in 51% of PPCD endothelial cells. Less than one-third positive cells were observed for CK1, CK4, CK5/6, CK10 and CK10/13, with mild or moderate staining intensity. CK1 and CK10 were detected only in surface cells. No immunoreactivity was found in the PPCD endothelial layer for CK2e, CK9, CK15 or CK20 (Fig. 2B).

Control and PPCD corneal epithelia showed a very similar CK staining pattern, both quantitatively and qualitatively. CK3/12 showed strong positivity in all corneal epithelial layers. CK4 was negative in the basal layer, while a positive signal increased from the suprabasal to the superficial layer. CK14 showed strong positivity in the basal layer, decreasing to the suprabasal layers. CK16 and CK5/6 were present in all epithelial layers, mostly in the suprabasal layer. CK7 and CK17 were not present in either control or PPCD epithelium. CK18 and CK19 positivity was found in about 50% of control as well as PPCD epithelial cells, with no difference between the basal, suprabasal and superficial layers. The epithelium was negative for CK1 and CK10.

The cytoplasm of the stromal cells was negative in all the examined corneas with all the antibodies used.

A few positive cells were found on the surface of the TM of PPCD specimens using CK4, CK7 and CK19 antibodies and in the same focus in several consecutive sections. The control specimens were completely negative.

4. Discussion

Despite extensive research into PPCD, the mechanism leading to the transformation of PPCD endothelium into cells with epithelial characteristics is still unknown. By investigating CK expression, we focused on characterizing these epithelial-like cells with the aim of improving our knowledge of PPCD pathogenesis. Besides immunostaining for CK7 and CK8/18, which has been previously observed in aberrant PPCD endothelium (Cockerham et al., 2002), we have found distinct positivity for CK3/12, CK4, CK5/6, CK10/13, CK14, CK16, CK17 and CK19.

We have observed that the pronounced expression of CK7 and CK19 is the most characteristic pattern in the CK composition of abnormal PPCD endothelium. Both CKs are usually

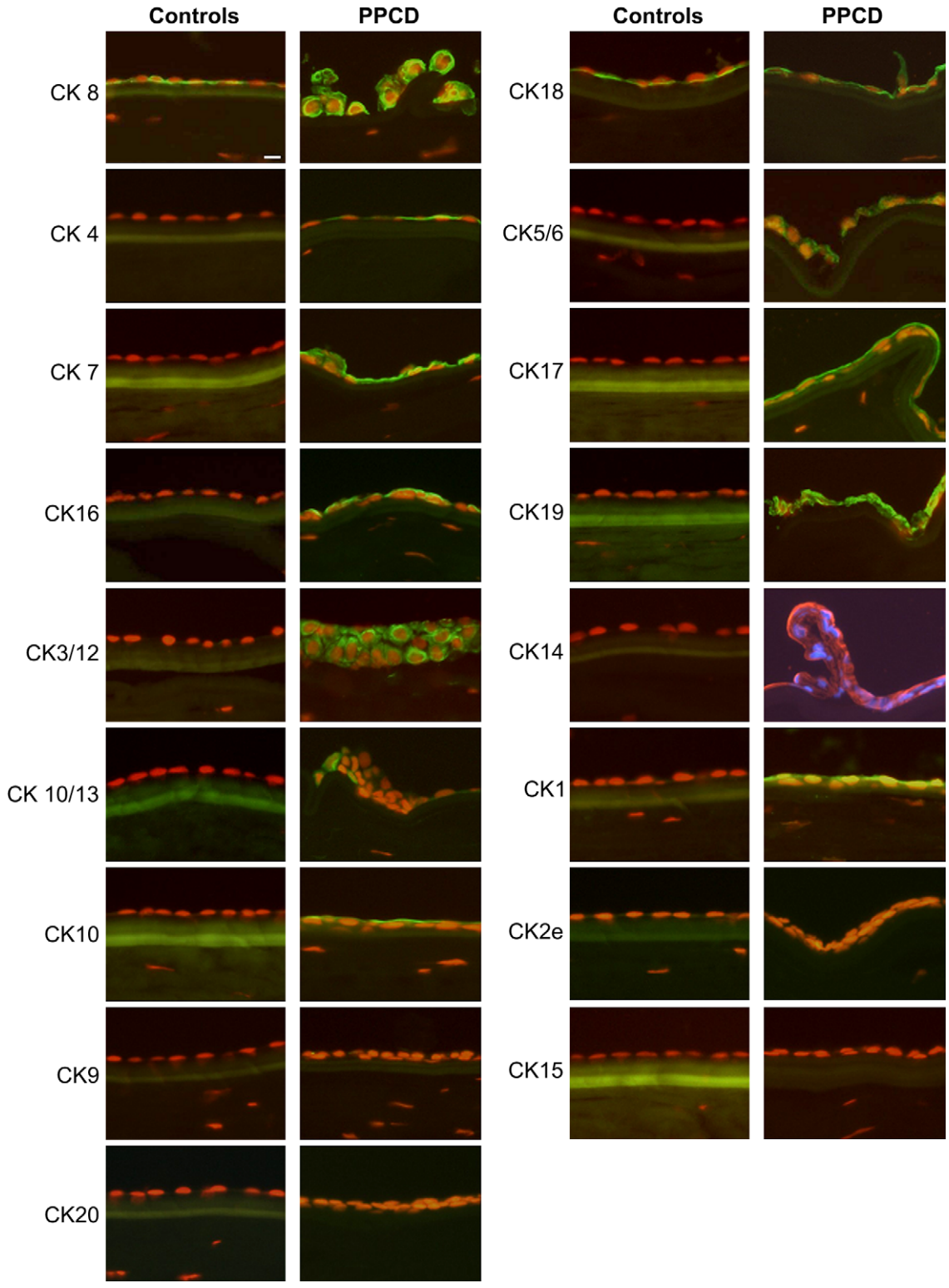

Fig. 2. Immunolocalisation of various CKs in control (A) and PPCD (B) corneal sections. Cell nuclei were stained with propidium iodide or DAPI. Scale bar represents 10 μm.

present in ductal and glandular epithelia (Ramaekers et al., 1987). CK7 was previously detected in the endothelium of all seven PPCD corneas studied (Cockerham et al., 2002), as well as in the endothelium of a proband from a PPCD pedigree with an identified disease-causing mutation in the TCF8 gene (Moroi et al., 2003). The question whether the expression of CK7 in PPCD may arise as a consequence of a less differentiated phenotype of pathological metaplastic epithelial cells remains to be elucidated. CK19 is a proposed limbal stem cell marker and can be characteristic of not fully differentiated

cells as well as of metaplastic epithelium (Lauweryns et al., 1993; Mandys et al., 2003).

The presence of the simple epithelial CK pair 8/18 in normal human endothelium is a matter of some controversy. While some studies found control endothelium to be free of CK8 and CK18 expression (Kramer et al., 1992; Levy et al., 1995), other authors detected both of these CKs in the corneal endothelium (Kasper et al., 1992; Wollensak and Witschel, 1996). We detected both in the endothelium of all control and PPCD specimens.

Interestingly, we also detected CK3/12, the expression of which is normally restricted to the corneal epithelium (Moll et al., 1982), in all of the examined PPCD patients, suggesting that the aberrant endothelium formed during PPCD shares features of the corneal epithelium. The expression of the basal cell marker CK14 and the stratification marker CK4 may correlate with the ability of the pathologically altered endothelium to form multilayered structures, while the expression of the hyperproliferation-associated markers CK6 and CK16 (van der Velden et al., 1999) may correlate with the proliferative capacity of these aberrant PPCD cells. Due to the weak positive signals for CK1 and CK10, markers for terminal differentiation and cornification (van der Velden et al., 1999), found in a few PPCD cells, we conclude that most of the abnormal endothelial PPCD cells are not terminally differentiated epithelium. The overall CK composition of aberrant PPCD endothelium shows features of both simple (CK7, CK8, CK18, CK17, CK19) and stratified (CK4, CK13) epithelium, along with a proliferative capacity (CK6 and CK16).

The broad CK spectrum expressed in our PPCD patients indicates that the altered cells are not already transformed into a distinct differentiated epithelial phenotype, but more likely it is a sign of the deranged maturation of an emerging metaplastic epithelium. In addition to the endothelium, we detected the epithelialization of superficial cells in PPCD TM, probably reflecting the capacity of the abnormal cells to migrate outwards from the cornea and to overgrow the surrounding tissues. The inter-individual heterogeneity in CK expression can arise from a situation in which foci of variously differentiated epithelium are irregularly scattered throughout the endothelium of corneal sections.

It is still unclear what causes a shift of endothelial cells to cells with epithelial characteristics appearing on the posterior corneal layer. It is not known if the alteration in CK expression is a more-or-less direct consequence of genetic changes or a secondary response to a more general dysregulation, independent of genetic mutations. One possible explanation could be that changes in CK expression are a result of an abnormal basement membrane composition. This mechanism of abnormal cytokeratin expression has been previously described in epithelial cells from bovine conjunctiva (Kurpakus et al., 1992). It was demonstrated that mutations in the TCF8 gene lead to the abnormal expression of COL4A3 (Krafchak et al., 2005) and that mutations in COL8A2 in PPCD corneas (Biswas et al., 2001) result in changes in basement membrane composition (aberrant formation of DM). A non-genetic explanation for the altered CK expression is suggested by the fact that, in addition to PPCD and CHED, epithelialization of the endothelium with CK expression also occurs in iridocorneal-endothelial syndrome corneas, which is not a hereditary disorder (Hirst and Waring, 1983). Moreover, similar changes were observed in corneas from patients with bullous keratopathy after ruptures in Descemet's membrane as a consequence of forceps injury at birth (Tetsumoto et al., 1993). Support for this theory comes from our observation that there is no difference in CK composition between patients with mutations in different genes.

We can conclude that the pattern of CK expression found in the cells on the posterior surface of PPCD corneas is most probably related to a metaplastic process during which endothelial cells are shifted to endo-epithelial and epithelial phenotypes. A considerable number of the expressed CKs are indicative of pathologically altered differentiation. The exact mechanisms mediating this metaplastic process remain to be elucidated.

Acknowledgements

This work was supported by the grant of the Grant Agency of the Czech Republic 201898301/031040 and the Czech Ministry of Education, Youth and Sports research project 0021620806/20610011. We would like to thank Dr. V. Vesela for excellent technical assistance with the preparation of the specimens. We thank Prof. K. Smetana and Dr. O. Boubelik, M.D. for kindly supplying tissue for positive controls and to Prof. V. Mandys for a critical review of the manuscript.

References

Biswas, S., Munier, F.L., Yardley, J., Hart-Holden, N., Perveen, R., Cousin, P., Sutphin, J.E., Noble, B., Batterbury, M., Kielty, C., Hackett, A., Bonshek, R., Ridgway, A., McLeod, D., Sheffield, V.C., Stone, E.M., Schorderet, D.F., Black, G.C., 2001. Missense mutations in COL8A2, the gene encoding the alpha2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. Hum. Mol. Genet. 10, 2415–2423.

Boruchoff, S.A., Kuwabara, T., 1971. Electron microscopy of posterior polymorphous degeneration. Am. J. Ophthalmol. 72, 879–887.

Cibis, G.W., Krachmer, J.A., Phelps, C.D., Weingeist, T.A., 1977. The clinical spectrum of posterior polymorphous dystrophy. Arch. Ophthalmol. 95, 1529–1537.

Cockerham, G.C., Laver, N.V., Hidayat, A.A., McCoy, D.L., 2002. An immunohistochemical analysis and comparison of posterior polymorphous dystrophy with congenital hereditary endothelial dystrophy. Cornea 21, 787–791.

Diebold, Y., Calonge, M., Fernandez, N., Lazaro, M.C., Callejo, S., Herreras, J.M., Pastor, J.C., 1997. Characterization of epithelial primary cultures from human conjunctiva. Graefe's Arch. Clin. Exp. Ophthalmol. 235, 268–276.

Gwilliam, R., Liskova, P., Filipec, M., Kmoch, S., Jirsova, K., Huckle, E.J., Stables, C.L., Bhattacharya, S.S., Hardcastle, A.J., Deloukas, P., Ebenezer, N.D., 2005. Posterior polymorphous corneal dystrophy in Czech families maps to chromosome 20 and excludes the VSX1 gene. Investig. Ophthalmol. Vis. Sci. 46, 4480–4484.

Heon, E., Greenberg, A., Kopp, K.K., Rootman, D., Vincent, A.L., Billingsley, G., Priston, M., Dorval, K.M., Chow, R.L., McInnes, R.R., Heathcote, G., Westall, C., Sutphin, J.E., Semina, E., Bremner, R., Stone, E.M., 2002. VSX1: a gene for posterior polymorphous dystrophy and keratoconus. Hum. Mol. Genet. 11, 1029–1036.

Hirst, L.W., 1983. Congenital anterior segment epithelialisation (case). Aust. J. Ophthalmol. 11, 209–213.

Hirst, L.W., Waring III, G.O., 1983. Clinical specular microscopy of posterior polymorphous endothelial dystrophy. Am. J. Ophthalmol. 95, 143–155.

Hirst, L.W., Bancroft, J., Yamauchi, K., Green, W.R., 1995. Immunohistochemical pathology of the corneal endothelium in iridocorneal endothelial syndrome. Investig. Ophthalmol. Vis. Sci. 36, 820–827.

Hogan, M.J., Bietti, G., 1969. Hereditary deep dystrophy of the cornea (polymorphous). Am. J. Ophthalmol. 68, 777–788.

Joyce, N.C., Meklir, B., Joyce, S.J., Zieske, J.D., 1996. Cell cycle protein expression and proliferative status in human corneal cells. Investig. Ophthalmol. Vis. Sci. 37, 645–655.

Kasper, M., Stosiek, P., Lane, B., 1992. Cytokeratin and vimentin heterogeneity in human cornea. Acta Histochem. 93, 371–381.

Kramer, T.R., Grossniklaus, H.E., Vigneswaran, N., Waring, G.O., Kozarsky, A., 1992. Cytokeratin expression in corneal endothelium in the iridocorneal endothelial syndrome. Investig. Ophthalmol. Vis. Sci. 33, 3581–3585.

Krachmer, J.H., 1985. Posterior polymorphous corneal dystrophy: a disease characterized by epithelial-like endothelial cells which influence management and prognosis. Trans. Am. Ophthalmol. Soc. 83, 413–475.

Krafchak, C.M., Pawar, H., Moroi, S.E., Sugar, A., Lichter, P.R., Mackey, D.A., Mian, S., Nairus, T., Elner, V., Schteingart, M.T., Downs, C.A., Kijek, T.G., Johnson, J.M., Trager, E.H., Rozsa, F.W., Mandal, M.N., Epstein, M.P., Vollrath, D., Ayyagari, R., Boehnke, M., Richards, J.E., 2005. Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells. Am. J. Hum. Genet. 77, 694–708.

Kurpakus, M.A., Stock, E.L., Jones, J.C., 1992. The role of the basement membrane in differential expression of keratin proteins in epithelial cells. Dev. Biol. 150, 243–255.

Lauweryns, B., van den Oord, J.J., De Vos, R., Missotten, L., 1993. A new epithelial cell type in the human cornea. Investig. Ophthalmol. Vis. Sci. 34, 1983–1990.

Levy, S.G., McCartney, A.C., Baghai, M.H., Barrett, M.C., Moss, J., 1995. Pathology of the iridocorneal-endothelial syndrome. The ICE-cell. Investig. Ophthalmol. Vis. Sci. 36, 2592–2601.

Levy, S.G., Moss, J., Noble, B.A., McCartney, A.C., 1996. Early-onset posterior polymorphous dystrophy. Arch. Ophthalmol. 114, 1265–1268.

Mandys, V., Lukas, K., Revoltella, R., 2003. Different patterns of cytokeratin expression in Barrett's esophagus – what is beyond? Pathol. Res. Pract. 199, 581–587.

Matsumoto, K., Weber, P.A., Makley Jr., T.A., 1988. Posterior polymorphous dystrophy – a histopathologic presentation. Ann. Ophthalmol. 20, 388–390 (393).

Molia, L.M., Lanier, J.D., Font, R.L., 1999. Posterior polymorphous dystrophy associated with posterior amyloid degeneration of the cornea. Am. J. Ophthalmol. 127, 86–88.

Moll, R., Franke, W.W., Schiller, D.L., Geiger, B., Krepler, R., 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11–24.

Moroi, S.E., Gokhale, P.A., Schteingart, M.T., Sugar, A., Downs, C.A., Shimizu, S., Krafchak, C., Fuse, N., Elner, S.G., Elner, V.M., Flint, A., Epstein, M.P., Boehnke, M., Richards, J.E., 2003. Clinicopathologic correlation and genetic analysis in a case of posterior polymorphous corneal dystrophy. Am. J. Ophthalmol. 135, 461–470.

Ramaekers, F., Huysmans, A., Schaart, G., Moesker, O., Vooijs, P., 1987. Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp. Cell Res. 170, 235–249.

Rodrigues, M.M., Sun, T.T., Krachmer, J., Newsome, D., 1980. Epithelialization of the corneal endothelium in posterior polymorphous dystrophy. Investig. Ophthalmol. Vis. Sci. 19, 832–835.

Rodrigues, M.M., Newsome, D.A., Krachmer, J.H., Sun, T.T., 1981. Posterior polymorphous dystrophy of the cornea: cell culture studies. Exp. Eye Res. 33, 535–544.

Ross, J.R., Foulks, G.N., Sanfilippo, F.P., Howell, D.N., 1995. Immunohistochemical analysis of the pathogenesis of posterior polymorphous dystrophy. Arch. Ophthalmol. 113, 340–345.

Tetsumoto, K., Kubota, T., Rummelt, V., Holbach, L.M., Naumann, G.O., 1993. Epithelial transformation of the corneal endothelium in forceps birth-injury-associated keratopathy. Cornea 12, 65–71.

van der Velden, L.A., Manni, J.J., Ramaekers, F.C., Kuijpers, W., 1999. Expression of intermediate filament proteins in benign lesions of the oral mucosa. Eur. Arch. Otorhinolaryngol. 256, 514–519.

Wollensak, G., Witschel, H., 1996. Vimentin and cytokeratin pattern in granular corneal dystrophy. Graefe's Arch. Clin. Exp. Ophthalmol. 234 (Suppl. 1), S110–S114.

Experimental Eye Research 88 (2009) 945–952

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Changes in the localization of collagens IV and VIII in corneas obtained from patients with posterior polymorphous corneal dystrophy

Stanislava Merjava [a], Petra Liskova [a], Yoshikazu Sado [c,d], Paul F. Davis [e], Nicholas S. Greenhill [e], Katerina Jirsova [a,b,*]

[a] Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Czech Republic
[b] Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Czech Republic
[c] Division of Ultrastructural Biology, Shigei Medical Research Institute, Yamada, Okayama 701-0202, Japan
[d] Division of Immunology, Shigei Medical Research Institute, Yamada, Okayama 701-0202, Japan
[e] Bioactivity Research Unit, Department of Medicine, Wellington School of Medicine, P.O. Box 7343, Wellington South, New Zealand

ARTICLE INFO

Article history:
Received 8 September 2008
Accepted in revised form 9 December 2008
Available online 9 January 2009

Keywords:
posterior polymorphous corneal dystrophy
collagen IV
collagen VIII
cornea
immunohistochemistry

ABSTRACT

Posterior polymorphous corneal dystrophy (PPCD) is a hereditary bilateral disorder affecting primarily the endothelium and Descemet's membrane (DM). The aim of this study was to determine the changes in the presence and localization of the α1–α6 collagen IV chains and α1, α2 collagen VIII chains in Czech patients with PPCD. Twelve corneal buttons from ten PPCD patients who underwent corneal grafting, as well as eight unaffected corneas, were used. Enzymatic indirect immunohistochemistry was performed on cryosections using antibodies against the α1–α6 collagen IV chains and α1, α2 collagen VIII chains. The intensity of the signal was examined separately in the basal membrane of the epithelium (BME), stroma and DM. More than 50% of PPCD specimens exhibited positivity for α1 and α2 collagen IV chains in the BME and in the posterior stroma, while no staining was detected in these areas in control specimens. The signal for the α1 and α2 collagen IV chains was more intense in DM of PPCD corneas compared to controls and it was shifted from the stromal side (in control tissue) to the endothelial side of DM (in the patients). A less intensive signal in PPCD corneas for the α3 and α5 chains in DM and an accumulation of α3–α5 in the posterior stroma in diseased corneas were the only differences in staining for the α3–α6 collagen IV chains. The α1 collagen VIII chain was detected on both the endothelial and the stromal sides of DM in 90% of patients with PPCD, compared with the prevailing localization on the stromal side of DM in control corneas. A change in the localization of the α2 collagen VIII chain in DM from vertically striated features in control specimens to double line positivity in the DM of PPCD corneas and positive staining in the posterior collagenous layer of four patients were also detected. In three PPCD patients a fibrous pannus located under the BME, positive for α1–α3, α5 collagen IV chains and α1 collagen VIII chain, was observed. The increased expression of the α1, α2 collagen IV and α1 collagen VIII chains and the change in their localization in DM may contribute to the increased endothelial proliferative capacity observed in PPCD patients.

1. Introduction

Posterior polymorphous corneal dystrophy (PPCD) is a bilateral, autosomal dominant disorder affecting primarily the corneal endothelium and Descemet's membrane (DM) (Cibis et al., 1976; Hogan and Bietti, 1969). The epithelization and proliferation of the pathologic endothelium of PPCD corneas are the most common findings at the cellular level, characterized in detail using light and electron microscopy (Boruchoff and Kuwabara, 1971; Krachmer, 1985). Epithelization was also confirmed by the detection of a wide cytokeratin spectrum, typical epithelial proteins (Jirsova et al., 2007; Rodrigues et al., 1980).

At least four different genes are implicated in PPCD, of which three are known. PPCD1 (OMIM #122000) is linked to chromosome 20 (Héon et al., 1995), and the visual system homeobox gene 1 (*VSX1*; OMIM *605020) was reported as disease-causing (Héon

* Corresponding author. Laboratory of the Biology and Pathology of the Eye and Ocular Tissue Bank, VFN, U nemocnice 2, 128 08 Prague, Czech Republic. Tel.: +420 224 963 008; fax: +420 224 962 338.
E-mail address: katerina.jirsova@vfn.cz (K. Jirsova).

0014-4835/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2008.12.017

et al., 2002) although evidence exists that in the linked families another undiscovered PPCD gene at 20p11.2 is implicated (Gwilliam et al., 2005). PPCD2 (OMIM #609140) is caused by the gene encoding the α2 collagen VIII chain (*COL8A2*; OMIM *120252) on chromosome 1 (Biswas et al., 2001). Finally, the human zinc finger E-box binding homeobox 1 gene (*ZEB1* also known as *TCF8*; OMIM *189909) on chromosome 10 is implicated in PPCD3 (OMIM #609141) (Krafchak et al., 2005; Liskova et al., 2007).

Collagens fibers are a basic component of the extracellular stromal matrix. Their architecture together with a functional intact endothelium are the main factors responsible for the regulation of corneal hydration and transparency (Maurice, 1957; Scott, 1998). Type IV collagen, aside from minor expression in the corneal stroma, is the major structural component of basement membranes (BMs), including both corneal BMs: the BM of the epithelium (BME) and the DM (Hudson et al., 1993; Kefalides, 1973). To date, six α chains of collagen IV (α1–α6) have been identified (Leinonen et al., 1994). Trimers of α1 (IV) and α2 (IV) chains ([α1]2α2 and [α1]3) are ubiquitous and form the major component of BMs (Timpl, 1989), whereas chains α3 (IV), α4 (IV), α5 (IV) and α6 (IV) represent only a minor component of BMs with restricted tissue distribution (Sanes et al., 1990). Each of these chains was observed in normal human cornea (Kabosova et al., 2007; Ljubimov et al., 1995; Tuori et al., 1997). Type IV collagen provides not only an architectural network, but the different NC1 domains of its α chains also regulate cellular behavior including antiangiogenic or antitumor properties (Floquet et al., 2004). It has been shown that the α1 and α2 chains of collagen IV favor cell migration, whereas the α3 chain limits the invasive phenotype (Ortega and Werb, 2002).

Type VIII collagen is a non-fibrillar, short chain collagen. Its exact function, except for its structural properties, is still unclear; however, studies suggest its importance in cell differentiation and a role in determining cell phenotype (Shuttleworth, 1997). It consists of two collagenous polypeptides, α1 (VIII) and α2 (VIII), which form two distinct homotrimeric or heterotrimeric proteins (Greenhill et al., 2000; Illidge et al., 2001). Collagen VIII is considered to be the major protein of mammalian DM (Kapoor et al., 1988; Tamura et al., 1991).

In healthy human corneas DM consists of two morphologically separated regions: the anterior banded zone (ABZ – average thickness is 3 μm) formed during intrauterine life and the posterior non-banded zone (PNBZ) which is synthesized after birth (thickness increases with age from 2 to 10 μm) (Johnson et al., 1982). The ABZ is mostly composed of wide-spaced collagen, which is characterized by ultrastructural labeling as collagen type VIII, whereas the PNBZ is a broad layer of amorphous extracellular matrix (Levy et al., 1996; Sawada et al., 1990). In horizontal sections of the ABZ, collagen VIII forms a hexagonal lattice (Sawada, 1982). As well as collagens VIII and IV, DM contains laminin and fibronectin (Ben-Zvi et al., 1986).

In various corneal endotheliopathies including Fuchs' endothelial corneal dystrophy, congenital hereditary endothelial dystrophy and PPCD, a large amount of wide-spaced collagen is deposited posterior to DM forming a posterior collagenous layer (PCL). This abnormal layer varies in composition and structure depending on the type of pathology with strong individual differences (Waring, 1982). Collagens I, III–VI, VIII, laminin, tenascin and fibronectin were detected using immunoelectron microscopy in PCL (Gottsch et al., 2005; Levy et al., 1995). In patients with PPCD (similarly as in the other endotheliopathies) three types of PCL (banded, fibrillar and fibrocellular) have been described (Waring, 1982).

The aim of this study was to map the occurrence of all six collagen IV chains, as well as the α1 and α2 collagen VIII chains, in normal adult corneas and in corneas obtained from PPCD patients where the structural changes in DM may be reflected by alterations in collagen composition.

2. Materials and methods

2.1. Patients and specimens

The study followed the ethical standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki. The diagnosis of PPCD was based on the presence of characteristic bilateral vesicular lesions, bands and geographic opacities observed on slit-lamp microscopy together with positive family history. All PPCD corneal explants were obtained from the Department of Ophthalmology, 1st Medical Faculty of Charles University and General Teaching Hospital in Prague.

Twelve corneal buttons from ten PPCD patients (4 males and 6 females) of Czech origin, ranging in age from 14 to 71 years with a mean age of 40 ± 16.4 years, were investigated. All but one eye (Table 1; P4) requiring corneal transplantation developed stromal and epithelial oedema. Three of the PPCD patients exhibited a keratoconus pattern on videokeratography (without the typical thinning of the cornea); three had bullous keratopathy (Table 1). Two corneas were obtained from members of the family previously mapped to 20p11.2 (P1, P10) (Gwilliam et al., 2005), while nine were from seven members of five families originating in the same small geographic area within the Czech Republic. Preliminary unpublished genotyping data in these families also suggest linkage to the PPCD1 locus. One cornea was from a patient with an identified disease-causing mutation in the *ZEB1* gene (P4) (Liskova et al., 2007).

Eight corneal discs (7 males and 1 female, age from 38 to 74, mean age of 60 ± 13.3 years), not acceptable for transplantation because of a positive serology of the donor or the endothelial quality, obtained from the Ocular Tissue Bank Prague were used as controls.

Corneal buttons were dissected, snap frozen in liquid nitrogen and embedded in Optimal Cutting Temperature Compound. Tissue was cryosectioned at a thickness of 7 μm and four slices were mounted per slide.

2.2. Enzymatic indirect immunohistochemistry

The specimens were stained with each antibody, and each staining was performed in duplicate. A negative control (primary antibody omitted) was included on every slide.

2.2.1. Collagen IV

The tissue was fixed with cold acetone for 10 min and rinsed with phosphate buffered saline (PBS). The slices for α5 collagen IV chain staining were denatured in a cold glycine/urea solution (Wielisa Alports KIT, Weislab, Sweden) for 5 min; slices for α4 and α6 chains were incubated in 0.1 M KCl–HCl buffer (pH 1.5) for 10 min. After rinsing (3 × 5 min PBS) the endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature and rinsed. The slides were pretreated with protein blocking antigen (UltraTech HRB AEC kit, Immunotech, France) for 5 min and then incubated with primary antibodies diluted in PBS containing 1% bovine serum albumin for 1 h. The following mouse or rat monoclonal antibodies and dilutions were used: α1 collagen

Table 1
Clinical findings accompanying PPCD in our cohort of patients studied.

Patients (P)	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Keratoconus pattern	–	–	–	+	–	+	+	–	–	–
Bullous keratopathy	–	–	–	–	+	+	+	–	–	–

IV chain (1:25), α3 collagen IV chain (1:50) and α5 collagen IV chain (1:50) (all from Wielisa Alports KIT); α2 collagen IV chain (1:300) (Chemicon International Inc., Temecula, USA); α4 collagen IV chain (1:25) and α6 collagen IV chain (1:50) (Y. Sado, Japan). After rinsing, biotinylated secondary antibody and then the tertiary complex (streptavidin/horse radish peroxidase) were applied, both for 30 min. Immunostaining was detected by 3-amino-9-ethylcarbazol for 1 min (Immunotech). The slides were rinsed in distilled water, counterstained with Harris hematoxylin and mounted in Aquatex medium (Merck KGaA, Germany).

2.2.2. α1 collagen VIII chain

The tissue was fixed with cold acetone for 10 min and rinsed in Tris buffered saline (TBS). The slices were incubated with primary mouse antibody against the α1 collagen VIII chain (1:15) (N.S. Greenhill, New Zealand) diluted in TBS for 1 h at room temperature. After rinsing the slices were incubated with biotinylated rabbit anti-mouse antibody (1:200) (DakoCytomation, Glostrup, Denmark) for 30 min. After washing in TBS, the SAB-complex/AP (DakoCytomation) was applied for 30 min. The staining was detected by a mixture of naphtol, levamizol, Fast Red and veronal acetate buffer (all from Sigma, St. Louis, USA) for 10 min. Slides were counterstained and mounted as described above.

2.2.3. α2 collagen VIII chain

The tissue was fixed with cold acetone for 10 min; the endogenous peroxidase was blocked with 0.3% hydrogen peroxide in TBS for 20 min at room temperature and rinsed. The slices were incubated overnight at 4 °C with primary rabbit antibody against α2 collagen VIII chain (1:1000) (N.S. Greenhill, New Zealand) diluted in TBS containing 1% bovine serum albumin. After rinsing in TBS the slices were incubated with biotinylated swine anti-rabbit antibody (1:50) (DakoCytomation) diluted in 0.5% casein in TBS for 30 min. After washing, the tertiary complex streptavidin/horse radish peroxidase (1:50) (DakoCytomation) diluted in 0.5% casein in TBS was applied for 30 min and collagen staining was detected by 3-amino-9-ethylcarbazol for 1 min. Slides were counterstained and mounted as described above.

2.3. Specimen assessment

Slices were examined by light microscopy using an Olympus BX51 (Olympus Co., Tokyo, Japan) at a magnification of 200–400×. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany). The thickness of DM was measured three times in each specimen (the average was calculated) using a NIS Elements image analysis system (Laboratory Imaging, Czech Republic). The staining intensity was assessed separately in the BME, stroma and DM, and graded using a scale: −, negative; ±, occasional positivity; +, mild; ++, moderate; and +++, intense staining. The mean range was calculated from three sections and two experiments.

3. Results

3.1. Morphology

Fibrous pannus, a fibrocellular layer populated by fibroblast-like cells, was observed between the BME and Bowman's layer in three PPCD patients. The mean thickness of DM in the control specimens was 10.0 ± 1.3 μm (from 7.3 to 15.0 μm); the thickness of DM measured in PPCD specimens was irregular: 4.8–20.0 μm thick with large intra- and interindividual differences (average of 10.3 ± 3.0 μm). An abnormal PCL was observed in six PPCD patients. The endothelium of all control specimens formed one layer of flat cells tightly attached to DM. PPCD endothelium consisted of one or two cell layers, and focal prominences up to six cell layers thick were observed.

3.2. Enzymatic indirect immunohistochemistry of collagen IV

The localization of the α1–α6 collagen IV chains in normal and PPCD corneas is shown in Table 2.

The α1 and α2 collagen IV chains were not detected in the BME or the stroma of any control cornea, but intense staining was found in the BM of the limbus and conjunctiva (data not shown). The α1 (IV) chain was present in one line on the stromal side of the peripheral zone of DM in one case (Table 2; Co7) or on the stromal

Table 2
The immunohistochemical localization of α1–α6 collagen IV chains in the control corneas and corneas obtained from PPCD patients.

Controls/patients	α1 (IV)			α2 (IV)			α3 (IV)			α4 (IV)			α5 (IV)			α6 (IV)		
	BME	S	DM	BME	S	DM	BME	S	DM	BME	S	DM	BME	S	DM	BME	S	DM
Co1	−	−	−	−	−	++	+	++	++	+	+	+++	+++	+	++	++	−	+
Co2	−	−	−	−	−	+	++	++	+++	+	++	+++	+++	+	++	+++	−	+
Co3	−	−	−	−	−	+	+	+	+++	+	+	+++	+++	−	++	++	−	+
Co4	−	−	+	−	−	++	++	+	++	+	+	+++	+++	+	++	++	−	+
Co5	−	−	−	−	−	+	−	+	+++	−	−	+++	+++	−	+++	++	−	+
Co6	−	−	−	−	−	+	−	+	+++	−	−	+++	+++	+	++	+	−	+
Co7	−	−	++	−	−	++	+	++	+++	+	++	+++	+++	+	+++	++	−	+
Co8	−	−	++	−	−	++	−	+	+++	−	+	+++	+++	−	+++	+	−	+
P1	−	−	++	−	−	+	+++	+	++	+	−	++	+++	+	+	+	−	+
P2	−	+	+	−	++	++	+	+	++	−	−	+++	+++	+	++	+	−	+
P3	+	−	++	+	−	++	+	+	++	−	−	++	+++	−	++	+	−	+
P4	++	+++[a]	++	+	+++[a]	+	++	+++[a]	++	+	++	++	++	+[a]	++	+	−	+
P5 RE	++	+	++	++	+	++	+++	++	+	+	++	++	+++	+	++	+	−	+
P5 LE	−	−	+	−	−	+	+	+	++	+	+	++	++	−	++	−	−	+
P6 RE	+	−	++	+	−	++	++	−	++	−	−	++	+++	−	++	−	−	+
P6 LE	−	−	+	−	−	+	+	−	+	−	+	+	++	−	+	+	−	+
P7	++	+++[a]	++	++	+++[a]	++	+++	+++[a]	+++	+	++	+++	++	+++[a]	+++	++	−	+
P8	−	−	+	−	++	++	−	+	++	−	+	++	+++	+	++	+	−	+
P9	+	−	+	+	+	+	+++	+	−	++	++	−	+++	+	+	+	−	−
P10	+	++	++	−	++	+++	+	++	+++	+	+	+++	+++	+	+++	+	−	+

The positivity in the basal membrane of the epithelium (BME), stroma (S) and Descemet's membrane (DM) in control corneas (Co) and corneas obtained from PPCD patients (P). A scale for the intensity of the signal: −, negative; +, mild; ++, moderate; and +++, intense positivity. RE, right eye; LE, left eye.

[a] Staining in fibrous pannus.

and endothelial sides in two (Co4, Co8) of eight control corneas. The α2 (IV) chain was present only on the stromal side of DM in all control corneas (Fig. 1). The α1 (IV) and α2 (IV) chains were found in the BME of 58% and 50% of PPCD specimens, respectively. Both chains were localized in the posterior 1/10–1/3 of the stroma. DM of all patients revealed mild to intense positivity for the α1 and α2 (IV) chains on the endothelial (eight patients for α1, six for α2) (Fig. 1) or the endothelial and stromal sides of DM (two patients for α1, four for α2). The immunostaining was observed as one or two lines in DM or else the entire PCL was stained (Table 2; P4, P7).

A mild to moderate signal for the α3 collagen IV chain was observed in the BME of 63% of the control corneas. BM positivity decreased in the limbus, and the BM of the conjunctiva was negative. The stroma was diffusely positive, and DM was intensively stained on the endothelial side of all control corneas. Mild to intense positivity for the α3 chain was observed in the BME of 90% of PPCD patients. The stroma of seven PPCD specimens was positive in its posterior part (1/10–1/3) (P3–P5, P7, P9, P10), three PPCD specimens were diffusely positive in the stroma without any gradient (P1, P2, P8) and two specimens were negative (P6RE, P6LE). 90% of PPCD patients had mild to intense staining in one line on the endothelial side of DM, while in two of these patients the positive line was doubled (P4, P7). An intense signal was observed in the DM of most of the control specimens; in the PPCD specimens moderate positivity prevailed.

From no signal through to a moderate signal for the α4 collagen IV chain was present in control as well as in PPCD specimens in both the BME and the stroma, with the only difference being that a diffuse pattern was observed in control stroma and the posterior 1/10–1/3 was positive in PPCD specimens. The positivity in DM was intense in control specimens and was negative to intense in PPCD specimens.

Mild to intense staining for the α5 collagen IV chain was observed in the BME and DM of control as well as PPCD specimens. Mild diffuse staining was found in the stroma of control specimens. The stroma of four PPCD patients was positive in its posterior part (1/10–1/3) (P4, P5, P7, P9). The other four PPCD patients were diffusely positive in the stroma without any gradient (P1, P2, P8, P10).

Mild to intense positivity was detected for the α6 collagen IV chain in the BME in each control cornea and weaker positivity in the BME was observed in the PPCD specimens. The stroma and DM showed a very similar staining pattern for the α6 chain in control and PPCD corneas; the stroma was negative and DM showed only mild positivity.

None of the tested collagen IV chains was detected in epithelial, stromal or endothelial cells.

3.3. Enzymatic indirect immunohistochemistry of collagen VIII

The α1 collagen VIII chain was not detected in the BME or the stroma of any specimen, except for three pathological specimens (Table 3; P4, P5RE, P7) in which staining was found in the fibrous pannus (P5RE) and in the posterior part of the stroma (P4, P7). The α1 chain was present in the stroma of the limbus and the conjunctiva of all control specimens (data not shown). In all control specimens the α1 (VIII) chain was localized on the stromal side of DM (Fig. 2) and in four cases (Co3, Co5, Co7, Co8) on the endothelial side as well. DM of all patients showed mild to intense positivity on

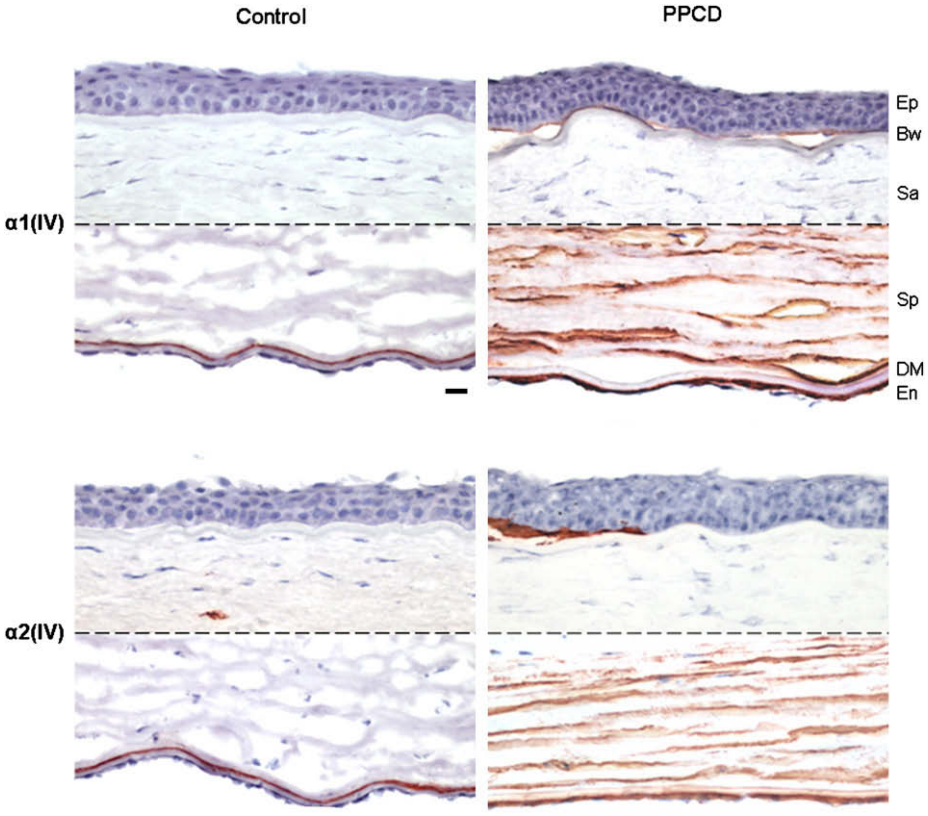

Fig. 1. The immunohistochemical localization of the α1 and α2 collagen IV chains in the control corneas and corneas obtained from PPCD patients. Scale bar represents 10 μm. Ep – epithelium, Bw – Bowman's layer, Sa – anterior part of the stroma, Sp – posterior part of the stroma, DM – Descemet's membrane, En – endothelium. ——— line represents a part of the corneas which are not included in the figure.

Table 3
The immunohistochemical localization of the α1 collagen VIII chain in the control corneas and corneas obtained from PPCD patients.

Controls/patients		Co1	Co2	Co3	Co4	Co5	Co6	Co7	Co8	P1	P2	P3	P4	P5 RE	P5 LE	P6 RE	P6 LE	P7	P8	P9	P10
α1 (VIII)	BME	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	–	–	–	–	–	–	–	–	–	–	–	+++[a]	–[a]	–	–	–	+[a]	–	–	–
	DM/s	+++	+++	++	+++	++	++	++	++	+	+++	+++	+++	+++	+++	++	++	+++	++	++	+
	DM/e	–	–	++	–	++	–	++	++	+	+++	+++	–	+++	+++	++	++	+++	++	++	+

The positivity in the basal membrane of the epithelium (BME), stroma (S), stromal side of Descemet's membrane (DM/s) and endothelial side of Descemet's membrane (DM/e) in the control corneas (Co) and corneas obtained from PPCD patients (P). A scale for the intensity of the signal: –, negative; +, mild; ++, moderate; and +++, intense positivity. RE, right eye; LE, left eye.
[a] Staining in fibrous pannus.

the stromal (P4) or on both the endothelial and the stromal sides of DM (P1–P3, P5–P10) (Fig. 2).

The α2 collagen VIII chain was detected in the stroma and DM of all control specimens. The staining in DM was localized mainly on the stromal side, but a vertically striated pattern was present in 63% of control corneas (Table 4; Co1, Co3, Co5, Co6, Co8) (Fig. 3). Very strong positivity was observed in the endothelial cells of most control specimens. In PPCD corneas, the α2 (VIII) chain was present in 83% on the stromal and 67% on the endothelial side of DM, Table 4. The vertical staining pattern of DM was observed occasionally in 33% of PPCD corneas (P4, P5, P6RE). A double positive line (P2, P8) and an intensively positive PCL (P2, P5LE, P8, P10) in the DM of PPCD corneas were detected (Fig. 3). The staining of endothelial cells in the PPCD corneas was weaker compared to control endothelium. Epithelial-like cells were less positive than other cells with an endothelial phenotype in PPCD patients.

Subepithelial fibrous pannus, found in three pathological corneas (Table 3; P4, P5 RE, P7), revealed positivity for the α1–α3 and α5 collagen IV chains (Table 2; P4, P7) (Fig. 4a, b) and the α1 collagen VIII chain (Table 3; P4, P5 RE, P7) (Fig. 4c). No differences between control and PPCD corneas were detected in Bowman's layer.

4. Discussion

Morphological as well as functional changes in the posterior part of corneas is the main feature of PPCD corneas (Boruchoff and Kuwabara, 1971; Hogan and Bietti, 1969; Jirsova et al., 2007; Rodrigues et al., 1980). Herein we describe not only alterations of the endothelium and DM, but also changes in the composition of the BME and the anterior stroma of PPCD corneas. The most striking difference identified was the presence of the α1 and α2 collagen IV chains in the BME and the posterior part of the stroma in PPCD corneas, whereas no positive staining was seen in normal tissue. Interestingly, the positivity for the α1 collagen IV chain was not detected in the BME in our preliminary study, in which the presence of the α1 and α2 collagen IV chains was assessed by means of standard fluorescent immunohistochemistry in seven PPCD corneas (Merjava et al., 2008). Enzyme immunohistochemistry, as used in this study, has therefore been shown to be a more appropriate method. Additionally, this approach allows a more detailed morphological assessment compared to fluorescent immunohistochemistry.

More intensive staining for α1 and α2 collagen IV chains and their localization on the endothelial side of DM were further observed in diseased corneas compared to their presence mostly on the stromal part of DM in control specimens. The changes in the composition of this DM part (PNBZ), formed after birth, may reflect the abnormal secretion of pathological PPCD endothelium. Because the localization of the α1 (IV) and α2 (IV) chains in cornea is normally restricted to the BM of the limbus and conjunctiva (Kabosova et al., 2007), i.e. areas with cells showing marked proliferative activity, their occurrence on the endothelial side of DM in PPCD patients may play a role in stimulating the proliferative activity of the aberrant endothelium. Changes in BM composition may further lead to alterations in cytokeratin expression in adjacent cells (Kurpakus et al., 1992). We could not detect any correlation between the increased expression of collagen IV or VIII chains and the expression of different cytokeratins in the pathological endothelium as reported in our previous study (Jirsova et al., 2007). Similarly, the thickness of the PCL did not depend on either abnormal cytokeratin expression or on the patient's age. The thickest PCL was observed in patients P8, aged 21 years, and P2, who was 71 years old. An increased thickness of DM with age was

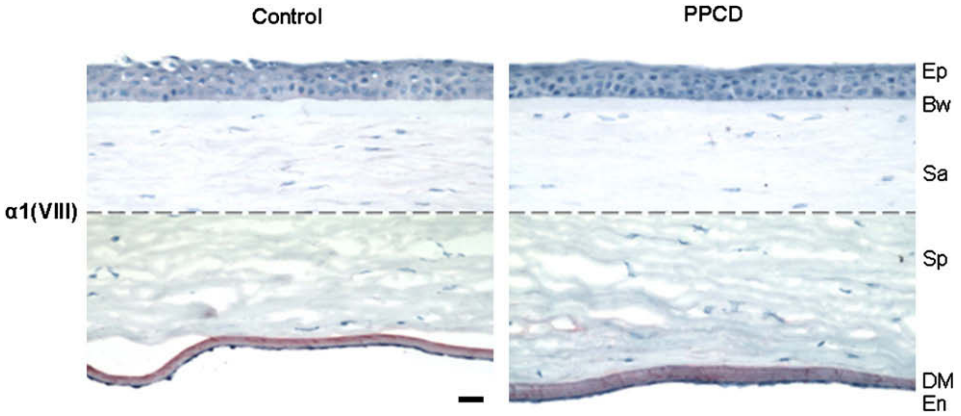

Fig. 2. The immunohistochemical localization of the α1 collagen VIII chain in control corneas and corneas obtained from PPCD patients. Scale bar represents 10 μm. Ep – epithelium, Bw – Bowman's layer, Sa – anterior part of the stroma, Sp – posterior part of the stroma, DM – Descemet's membrane, En – endothelium. ——— line represents a part of the corneas which are not included in the figure.

Table 4
The immunohistochemical localization of the α2 collagen VIII chain in the control corneas and corneas obtained from PPCD patients.

Controls/patients		Co1	Co2	Co3	Co4	Co5	Co6	Co7	Co8	P1	P2	P3	P4	P5 RE	P5 LE	P6 RE	P6 LE	P7	P8	P9	P10
α2 (VIII)	S	++	++	++	+	±	+	+	+	+	++	+	+	++	−	++	+	±	−	+	−
	DM/s	++	++	++	+++	++	++	±	±	+	++	++	+	+	−	+	+	+	+	+	−
	DM/e	±	±	±	−	−	−	−	±	+	++[a]	++	−	−	++[a]	−	+	++	−[a]	+	+++[a]
	END	+++	+++	+++	++	±	+++	+++	±	++	++	+++	++	++	±	++	±	++	+	−	−

The positivity in the stroma (S), stromal side of Descemet's membrane (DM/s), endothelial side of Descemet's membrane (DM/e) and endothelium (END) in the control corneas (Co) and corneas obtained from PPCD patients (P). A scale for the intensity of the signal: −, negative; ±, occasional positivity; +, mild; ++, moderate; and +++, intense positivity. RE, right eye; LE, left eye.
[a] Occurrence of positive PCL.

observed in both control and PPCD corneas. Although the first attack leading to the alteration of the endothelial phenotype in PPCD has not been characterized yet, the modification in DM composition may enhance these changes.

An accumulation of α1 and α2 and a loss of α3 and α5 collagen IV chains in the BME of patients with keratoconus and an accumulation of the α1 and α2 chains in DM in patients with bullous keratopathy have been previously described (Deng et al., 2001; Kenney et al., 1997; Ljubimov et al., 1996; Tuori et al., 1997). In our study the level of the α1–α3 and α5 collagen IV chains in the BME and in DM in the three PPCD patients with a keratoconus pattern on videokeratography (Table 1, P4, P6, P7) and in three patients with bullous keratopathy (P5–P7) did not markedly differ from the level in other PPCD patients, suggesting that a different mechanism underlies the origin of the changes in collagen IV expression in these patients.

No ectopic expression of the α3 collagen IV chain in pathological endothelium was found in our patient with a known disease-causing mutation in the *ZEB1* gene (Liskova et al., 2007), which is contrary to the results obtained previously in one patient as well as in the abnormal endothelium of *ZEB1* null mice (Krafchak et al., 2005; Liu et al., 2008). An explanation could lie in the different expression patterns of these cells at various stages of the disease.

In this study an accumulation of the α1 (IV) and α2 (IV) chains as well as the α1 (VIII) and α2 (VIII) chains was observed in PCL, which is a common feature of PPCD corneas (Waring, 1982). However, besides a single mutation in *COL8A2* in two PPCD patients (Biswas et al., 2001), other studies failed to identify disease-causing changes in PPCD patients in the *COL8A1* or *COL8A2* genes (Liskova et al., 2007; Urquhart et al., 2006; Yellore et al., 2005). Our cases are not expected to show pathogenic mutations in the *COL8A2* gene responsible for PPCD2 either. All but P4 show a linkage to the PPCD1 locus on chromosome 20p11.2 (unpublished data). P4 was screened for changes in the coding sequences of all three genes currently known to be implicated in PPCD, and a disease-causing mutation was found in *ZEB1* (Liskova et al., 2007).

In *COL8A1*$^{-/-}$/*COL8A2*$^{-/-}$ null mice a markedly thinner DM lacking the ABZ was observed together with low density, polymegathism and polymorphism of the endothelium (Hopfer et al., 2005). Changes in the localization and distribution of collagen VIII were previously detected in patients with Fuchs' endothelial corneal dystrophy, both with and without disease-causing mutations in the *COL8A2* gene (Gottsch et al., 2005). We have observed a vertically striated pattern of DM in most of the control corneas and occasionally in pathological ones, contrary to the results of Gottsch et al. (2005), who found a striated pattern of DM in pathological but not control specimens. As collagen VIII is expressed in rapidly proliferating cells such as different tumor cells and endothelial cells during angiogenesis (Paulus et al., 1991; Sage and Iruela-Arispe, 1990), it may be that the proliferation of endothelial cells of PPCD patients is induced by the collagen VIII deposited in the PCL. Finally, we detected the presence of the α1 collagen VIII chain in the posterior stroma of two patients with PPCD (P4, P7). Interestingly, the localization of this positivity colocalized with the α1–α5 collagen IV chains.

We have detected fibrous pannus positive for the α1–α3 and α5 collagen IV chains and the α1 collagen VIII chain. Besides collagens IV and VIII, laminin and tenascin were detected in subepithelial fibrosis areas of patients with bullous keratopathy (Ljubimov et al., 1996). We hypothesize that this structure, first described in PPCD by Grayson (Grayson, 1974), is formed secondarily as a consequence of bullous keratopathy, which was, in our study, clinically confirmed in two cases out of three with this abnormal layer. Opacity under the epithelium observable on slit-lamp examination was noted in the patient providing the third specimen (P4). In our opinion, fibrous pannus formation reflects secondary changes as a reaction to the compromised function of the endothelium.

In conclusion we suggest that changes in collagens IV and VIII chains in PPCD corneas may contribute to the morphological changes in the abnormal endothelium as well as to the stimulation of its proliferative capacity.

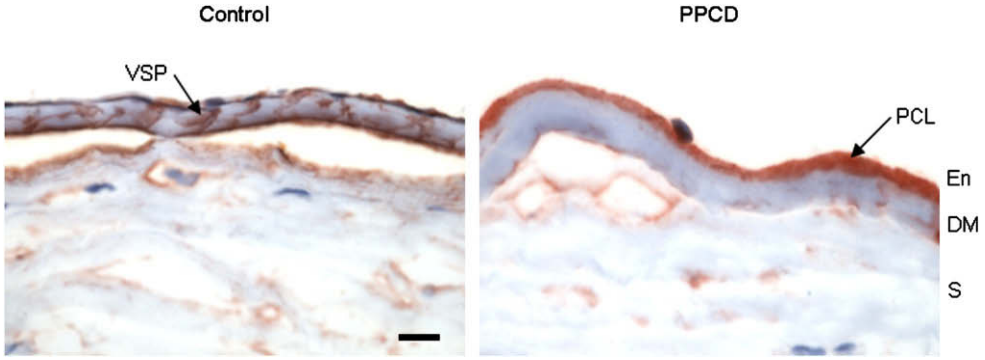

Fig. 3. The immunohistochemical localization of the α2 collagen VIII chain in control cornea and cornea obtained from PPCD patient. Scale bar represents 10 μm. S – stroma, DM – Descemet's membrane, PCL – posterior collagenous layer, En – endothelium, VSP – vertically striated pattern.

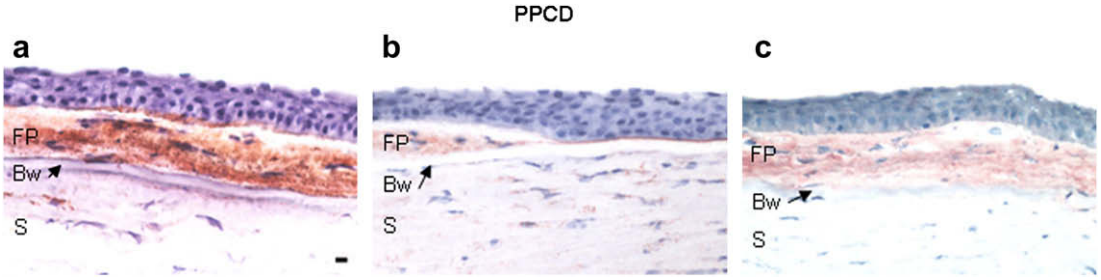

Fig. 4. The immunohistochemical localization of the α1 (IV) (a), α3 (IV) (b) and α1 (VIII) (c) collagen chains in the fibrous pannus (FP), detected in corneas obtained from PPCD patients. Scale bar represents 10 μm. S – stroma, Bw – Bowman's layer.

Acknowledgements

This work was supported by the Czech Ministry of Education, Youth and Sports research project 0021620806/20610011. We would like to thank Dr. V. Vesela for excellent technical assistance with the preparation of the specimens.

References

Ben-Zvi, A., Rodrigues, M.M., Krachmer, J.H., Fujikawa, L.S., 1986. Immunohistochemical characterization of extracellular matrix in the developing human cornea. Curr. Eye Res. 5, 105–117.

Biswas, S., Munier, F.L., Yardley, J., Hart-Holden, N., Perveen, R., Cousin, P., Sutphin, J.E., Noble, B., Batterbury, M., Kielty, C., Hackett, A., Bonshek, R., Ridgway, A., McLeod, D., Sheffield, V.C., Stone, E.M., Schorderet, D.F., Black, G.C., 2001. Missense mutations in *COL8A2*, the gene encoding the α2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. Hum. Mol. Genet. 10, 2415–2423.

Boruchoff, S.A., Kuwabara, T., 1971. Electron microscopy of posterior polymorphous degeneration. Am. J. Ophthalmol. 72, 879–887.

Cibis, G.W., Krachmer, J.H., Phelps, C.D., Weingeist, T.A., 1976. Iridocorneal adhesions in posterior polymorphous dystrophy. Trans. Sect. Ophthalmol. Am. Acad. Ophthalmol. Otolaryngol. 81, 770–777.

Deng, Y., Liu, S., Cai, R., 2001. Immunohistochemistry research on keratoconus. Yan Ke Xue Bao 17, 65–67. 75.

Floquet, N., Pasco, S., Ramont, L., Derreumaux, P., Laronze, J.Y., Nuzillard, J.M., Maquart, F.X., Alix, A.J., Monboisse, J.C., 2004. The antitumor properties of the α3(IV)-(185–203) peptide from NC1 domain of type IV collagen (tumstatin) are conformation-dependent. J. Biol. Chem. 279, 2091–2100.

Gottsch, J.D., Zhang, Ch., Sundin, O.H., Bell, W.R., Stark, W.J., Green, W.R., 2005. Fuchs corneal dystrophy: aberrant collagen distribution in an L450W mutant of the *COL8A2* gene. Invest. Ophthalmol. Vis. Sci. 46, 4504–4511.

Grayson, M., 1974. The nature of hereditary deep polymorphous dystrophy of the cornea: its association with iris and anterior chamber dysgenesis. Trans. Am. Ophthalmol. Soc. 72, 516–559.

Greenhill, N.S., Rüger, B.M., Hasan, Q., Davis, P.F., 2000. The alpha1(VIII) and alpha2(VIII) collagen chains form two distinct homotrimeric proteins in vivo. Matrix Biol. 19, 19–28.

Gwilliam, R., Liskova, P., Filipec, M., Kmoch, S., Jirsova, K., Huckle, E.J., Stables, C.L., Bhattacharya, S.S., Hardcastle, A.J., Deloukas, P., Ebenezer, N.D., 2005. Posterior polymorphous corneal dystrophy in Czech families maps to chromosome 20 and excludes the *VSX1* gene. Invest. Ophthalmol. Vis. Sci. 46, 4480–4484.

Héon, E., Mathers, W.D., Alward, W.L.M., Weisenthal, R.W., Sunden, S.L.F., Fishbaugh, J.A., Taylor, Ch.M., Krachmer, J.H., Scheffield, V.C., Stone, E.M., 1995. Linkage of posterior polymorphous corneal dystrophy to 20q11. Hum. Mol. Genet. 4, 485–488.

Héon, E., Greenberg, A., Kopp, K.K., Rootman, D., Vincent, A.L., Billingsley, G., Priston, M., Dorval, K.M., Chow, R.L., McInnes, R.R., Heathcote, G., Westall, C., Sutphin, J.E., Semina, E., Bremner, R., Stone, E.M., 2002. *VSX1:* a gene for posterior polymorphous dystrophy and keratoconus. Hum. Mol. Genet. 11, 1029–1036.

Hogan, M.J., Bietti, G., 1969. Hereditary deep dystrophy of the cornea (polymorphous). Am. J. Ophthalmol. 68, 777–788.

Hopfer, U., Fukai, N., Hopfer, H., Wolf, G., Joyce, N., Li, E., Olsen, B., 2005. Targeted disruption of *Col8a1* and *Col8a2* genes in mice leads to anterior segment abnormalities in the eye. FASEB J. 19, 1232–1244.

Hudson, B.G., Reeders, S.T., Tryggvason, K., 1993. Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J. Biol. Chem. 268, 26033–26036.

Illidge, Ch., Kielty, C., Shuttleworth, A., 2001. Type VIII collagen: heterotrimeric chain association. Int. J. Biochem. Cell Biol. 33, 521–529.

Jirsova, K., Merjava, S., Martincova, R., Gwilliam, R., Ebenezer, N.D., Liskova, P., Filipec, M., 2007. Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. Exp. Eye Res. 84, 680–686.

Johnson, D.H., Bourne, W.M., Campbell, R.J., 1982. The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas. Arch. Ophthalmol. 100, 1942–1947.

Kabosova, A., Azar, D.T., Bannikov, G.A., Campbell, K.P., Durbeej, M., Ghohestani, R.F., Jones, J.C., Kenney, M.C., Koch, M., Ninomiya, Y., Patton, B.L., Paulsson, M., Sado, Y., Sage, E.H., Sasaki, T., Sorokin, L.M., Steiner-Champliaud, M.F., Sun, T.T., Sundarraj, N., Timpl, R., Virtanen, I., Ljubimov, A.V., 2007. Compositional differences between infant and adult human corneal basement membranes. Invest. Ophthalmol. Vis. Sci. 48, 4989–4999.

Kapoor, R., Sakai, L.Y., Funk, S., Roux, E., Bornstein, P., Sage, E.H., 1988. Type VIII collagen has a restricted distribution in specialized extracellular matrices. J. Cell Biol. 107, 721–730.

Kefalides, N.A., 1973. Structure and biosynthesis of basement membranes. Int. Rev. Connect. Tissue Res. 6, 63–104.

Kenney, M.C., Nesburn, A.B., Burgeson, R.E., Butkowski, R.J., Ljubimov, A.V., 1997. Abnormalities of the extracellular matrix in keratoconus corneas. Cornea 16, 345–351.

Krachmer, J.H., 1985. Posterior polymorphous corneal dystrophy: a disease characterized by epithelial-like endothelial cells which influence management and prognosis. Trans. Am. Ophthalmol. Soc. 83, 413–475.

Krafchak, C.M., Pawar, H., Moroi, S.E., Sugar, A., Lichter, P.R., Mackey, D.A., Mian, S., Nairus, T., Elner, V., Schteingart, M.T., Downs, C.A., Kijek, T.G., Johnson, J.M., Trager, E.H., Rozsa, F.W., Ali Mandal, M.N., Epstein, M.P., Vollrath, D., Ayyagari, R., Boehnke, M., Richards, J.E., 2005. Mutations in *TCF8* cause posterior polymorphous corneal dystrophy and ectopic expression of *COL4A3* by corneal endothelial cells. Am. J. Hum. Genet. 77, 694–708.

Kurpakus, M.A., Stock, E.L., Jones, J.C., 1992. The role of the basement membrane in differential expression of keratin proteins in epithelial cells. Dev. Biol. 150, 243–255.

Leinonen, A., Mariyama, M., Mochizuki, T., Tryggvason, K., Reeders, S.T., 1994. Complete primary structure of the human type IV collagen alpha4 (IV) chain. Comparison with structure and expression of the other alpha (IV) chains. J. Biol. Chem. 269, 26172–26177.

Levy, S.G., McCartney, A.C., Sawada, H., Dopping-Hepenstal, P.J., Alexander, R.A., Moss, J., 1995. Descemet's membrane in the iridocorneal-endothelial syndrome: morphology and composition. Exp. Eye Res. 61, 323–333.

Levy, S.G., Moss, J., Sawada, H., Dopping-Hepenstal, P.J., McCartney, A.C., 1996. The composition of wide-spaced collagen in normal and diseased Descemet's membrane. Curr. Eye Res. 15, 45–52.

Liskova, P., Tuft, S.J., Gwilliam, R., Ebenezer, N.D., Jirsova, K., Prescott, Q., Martincova, R., Pretorius, H., Sinclair, N., Boase, D.L., Jeffrey, M.J., Deloukas, P., Hardcastle, A.J., Filipec, M., Bhattacharya, S.S., 2007. Novel mutations in the *ZEB1* gene identified in Czech and British patients with posterior polymorphous corneal dystrophy. Hum. Mutat. 28, 638.

Liu, Y., Peng, X., Tan, J., Darling, D.S., Kaplan, H.J., Dean, D.C., 2008. *Zeb1* mutant mice as a model of posterior corneal dystrophy. Invest. Ophthalmol. Vis. Sci. 49, 1843–1849.

Ljubimov, A.V., Burgeson, R.E., Butkowski, R.J., Michael, A.F., Sun, T.T., Kenney, M.C., 1995. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab. Invest. 72, 461–473.

Ljubimov, A.V., Burgeson, R.E., Butkowski, R.J., Couchman, J.R., Wu, R.R., Ninomiya, Y., Sado, Y., Maguen, E., Nesburn, A.B., Kenney, M.C., 1996. Extracellular matrix alteration in human corneas with bullous keratopathy. Invest. Ophthalmol. Vis. Sci. 37, 997–1007.

Maurice, D.M., 1957. The structure and transparency of the cornea. J. Physiol. 136, 263–286.

Merjava, S., Liskova, P., Jirsova, K., 2008. Immunohistochemical characterization of collagen IV in control corneas and in corneas obtained from patients suffering from posterior polymorphous corneal dystrophy. Cesk. Slov. Oftalmol. 64, 115–119.

Ortega, N., Werb, Z., 2002. New functional roles for non-collagenous domains of basement membrane collagens. J. Cell Sci. 115, 4201–4214.

Paulus, W., Sage, E.H., Liszka, U., Iruela-Arispe, M.L., Jellinger, K., 1991. Increased levels of type VIII collagen in human brain tumours compared to normal brain tissue and non-neoplastic cerebral disorders. Br. J. Cancer 63, 367–371.

Rodrigues, M.M., Sun, T.T., Krachmer, J.H., Newsome, D.A., 1980. Epithelization of the corneal endothelium in posterior polymorphous dystrophy. Invest. Ophthalmol. Vis. Sci. 19, 832–835.

Sage, H., Iruela-Arispe, M.L., 1990. Type VIII collagen in murine development. Association with capillary formation in vitro. Ann. N. Y. Acad. Sci. 580, 17–31.
Sanes, J.R., Engvall, E., Butkowski, R., Hunter, D.D., 1990. Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J. Cell Biol. 111, 1685–1699.
Sawada, H., 1982. The fine structure of bovine Descemet's membrane with special reference to biochemical nature. Cell Tissue Res. 226, 241–255.
Sawada, H., Konomi, H., Hirosawa, K., 1990. Characterization of the collagen in the hexagonal lattice of Descemet's membrane: its relation to type VIII collagen. J. Cell Biol. 110, 219–227.
Scott, J.E., 1998. How rational histochemistry produced order out of chaos in the "amorphous ground substance" (with a little help from biochemistry, biophysics etc.). Eur. J. Histohem. 42, 29–34.
Shuttleworth, C.A., 1997. Type VIII collagen. Int. J. Biochem. Cell Biol. 29, 1145–1148.
Tamura, Y., Konomi, H., Sawada, H., Takashima, S., Nakajima, A., 1991. Tissue distribution of type VIII collagen in human adult and fetal eyes. Invest. Ophthalmol. Vis. Sci. 32, 2636–2644.
Timpl, R., 1989. Structure and biochemical activity of basement membrane proteins. Eur. J. Biochem. 180, 487–502.
Tuori, A.J., Virtanen, I., Aine, E., Kalluri, R., Miner, J.H., Uusitalo, H.M., 1997. The immunohistochemical composition of corneal basement membrane in keratoconus. Curr. Eye Res. 16, 792–801.
Urquhart, J.E., Biswas, S., Black, G.C., Munier, F.L., Sutphin, J., 2006. Exclusion of *COL8A1*, the gene encoding the alpha2(VIII) chain of type VIII collagen, as a candidate for Fuchs endothelial dystrophy and posterior polymorphous corneal dystrophy. Br. J. Ophthalmol. 90, 1430–1431.
Waring, G.O., 1982. Posterior collagenous layer of the cornea. Ultrastructural classification of abnormal collagenous tissue posterior to Descemet's membrane in 30 cases. Arch. Ophthalmol. 100, 122–134.
Yellore, V.S., Rayner, S.A., Emmert-Buck, L., Tabin, G.C., Raber, I., Hannush, S.B., Stulting, R.D., Sampat, K., Momi, R., Principe, A.H., Aldave, A.J., 2005. No pathogenic mutations identified in the *COL8A2* gene or four positional candidate genes in patients with posterior polymorphous corneal dystrophy. Invest. Ophthalmol. Vis. Sci. 46, 1599–1603.

Combined indirect fluorescent immunohistochemistry with fluorescence in situ hybridization in a patient with posterior polymorphous corneal dystrophy

Stanislava Merjava [a], Eva Malinova [b], Petra Liskova [a,c], Martin Filipec [d], Zuzana Zemanova [b], Kyra Michalova [b], Katerina Jirsova [a,e]

[a]Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Ke Karlovu 2, 128 00, Prague, Czech Republic.
[b]Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, General University Hospital and First Faculty of Medicine, Charles University in Prague, U nemocnice 2, 128 00, Prague, Czech Republic.
[c]Department of Ophthalmology, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, U nemocnice 2, 128 00, Prague, Czech Republic
[d]Lexum European Eye Clinic, U spolecenske zahrady 3, 140 00, Prague, Czech Republic
[e]Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, U nemocnice 2, 128 00, Prague, Czech Republic.

Corresponding author:
Katerina Jirsova, PhD
Laboratory of the Biology and Pathology of the Eye and Ocular Tissue Bank, VFN,
Ke Karlovu 2, 128 08, Prague, Czech Republic
E-mail: katerina.jirsova@vfn.cz
Tel: +420 224 963 008, Fax: +420 224 962 338

Abstract

Posterior polymorphous corneal dystrophy (PPCD) is a rare, bilateral autosomal dominant disorder affecting primarily the corneal endothelium and Descemet membrane (DM). The aim of this study was to establish the origin of the abnormal endothelium in a patient with PPCD exhibiting cornea graft failure after keratoplasty surgery.

A sex-mismatched graft obtained from a patient with PPCD who underwent repeat penetrating keratoplasty and the patient's original cornea were investigated. Combined fluorescent immunohistochemistry for cytokeratin (CK) 19 (a marker of aberrant PPCD endothelium) with fluorescence in situ hybridization (FISH) of the sex chromosomes were used in order to characterize the cells on the posterior graft surface.

The pathological endothelium of the failed PPCD cornea revealed strong positivity for CK19 using fluorescent immunohistochemistry. In all the CK19-positive cells, both X and Y chromosomes were simultaneously detected using FISH. The results clearly showed that the original cells of the patient (XY) have, within 3.5 years, almost totally overgrown the posterior corneal surface of the graft (XX). Moreover, an abnormal posterior collagenous layer populated by fibroblast-like cells was observed between DM and the endothelium in the failed graft, but its exact origin could not been established due to the low number of cells.

Simultaneous detection of CK19 using fluorescent immunohistochemistry together with the detection of gonosomes using FISH was performed for the first time in the cornea and allowed us to prove that the recurrence of PPCD was caused by the pathological abnormal proliferation and migration of the recipient cells into the donor graft.

Keywords: immunohistochemistry; fluorescence in situ hybridization; posterior polymorphous corneal dystrophy; recurrence

Introduction

Posterior polymorphous corneal dystrophy (PPCD) is a very rare, bilateral autosomal dominant disorder affecting primarily the innermost corneal layers, the Descemet membrane (DM) and the endothelium, biomicroscopically presenting as geographical lesions, bands and vesicles (Hogan and Bietti 1969; Jirsova et al. 2009; Krachmer 1985). Moreover, changes in the posterior stroma and basal membrane of the epithelium were also described in some patients (Merjava et al. 2009). Proliferating epithelial-like endothelial cells with prominent microvilli, abundant keratofibrils, desmosomes and sparse microorganelles (all characteristics of epithelial cells) are the most common findings in PPCD corneas (Boruchoff and Kuwabara 1971; Feil et al. 1997; Krachmer 1985; Matsumoto et al. 1988; Richardson and Hettinger 1985; Rodrigues et al. 1981). Epithelization was also confirmed by the detection of cytokeratins (CKs), typical epithelial proteins (Jirsova et al. 2007; Rodrigues et al. 1980). PPCD is genetically heterogeneous. So far it has been associated with three known genes (Biswas et al. 2001; Heon et al. 2002; Krafchak et al. 2005; Liskova et al. 2007), and evidence has been presented that there is another, as-yet unidentified gene causing this disorder located at locus 20p11.2 (Gwilliam et al. 2005). Despite the identification of some disease-causing genes, the exact mechanisms leading to the development of PPCD have not been elucidated.

The disease has been reported to be static or slowly progressive in most subjects, but occasionally there may be a rapid progression resulting in severe visual impairment from secondary glaucoma or corneal edema, necessitating corneal graft surgery (Krachmer 1985; Weisenthal and Krachmer 1988). The first clinical description of PPCD recurrence after penetrating keratoplasty (PK) was provided by Krachmer (Krachmer 1985). Boruchoff et al. (1990) documented recurrence in an explanted corneal button using light microscopy and histological examination. Sekundo et al. (1994) showed the presence of an epithelium-like endothelium on the posterior surface of three other grafted PPCD specimens using light and electron microscopy. It was suggested that these abnormal cells were host in origin and that they had overgrown onto the donor posterior corneal surface (Boruchoff et al. 1990; Sekundo et al. 1994). However, no direct proof supporting this theory was provided. In order to determine the exact origin of these pathological cells, we have examined a sex-mismatched corneal button explanted from a PPCD patient by a combination of indirect fluorescent immunohistochemistry for CK19 (a marker of aberrant endothelium) and fluorescence in situ hybridization (FISH) of gonosomes. This combined immunophenotyping together with cytogenetic analysis by FISH was described as the method of choice for the assessment of chimerism in a particular cell type after bone marrow sex-mismatched transplantations (Hessel et al. 1996; Trotman et al. 2004), but was not tested in the human cornea up to now.

Materials and methods

The study followed the ethical standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki. Informed consent was obtained from the patient prior to the start of the study.

Specimens

During the last decade, a Czech patient with PPCD was grafted repeatedly in his left eye. The diagnosis of PPCD in this patient was based on family history, clinical examination and, prior to the first PK, a detailed ophthalmological examination including gonioscopy, intraocular pressure measurement and specular microscopy. The original cornea obtained from this patient after his first PK revealed a stratified corneal endothelium with 1-5 layers, expressing a wide range of cytokeratins and changes in the localization of collagens IV and VIII, confirming the diagnosis (Jirsova et al. 2007; Merjava et al. 2009). The patient's family originated from the same geographical region within the Czech Republic and shared part of the same haplotype (unpublished data) as two families previously mapped to 20p11.2.

Two corneal buttons (both 7.0 mm in diameter) obtained from this patient were used in this study. The original cornea was removed from the left eye at the age of 41 years; 3.5 years later a repeat PK in the same eye was performed for corneal graft failure. The donor cornea used for the first PK was sex-mismatched (gender of the patient was male whereas the donor was a 60-year-old female). The donor cornea was obtained from the Ocular Tissue Bank, General Teaching Hospital, Prague. The time from death to preservation was 10 hours, followed by 18 days preservation in tissue culture medium. Endothelial cell density prior to grafting was 2435 cells/mm^2.

Two cadaverous corneo-scleral discs without any ocular pathology (male and female, aged 49 and 61, respectively), not acceptable for transplantation because of low endothelial density (but not under 1000 cells/mm^2), were obtained from the Ocular Tissue Bank and used as controls.

Both pathological buttons (the original cornea and the failed graft) were immediately dissected into four parts after surgery,; snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at -70°C. The same procedure was used for the control corneas. The time between death and the preparation of cryospecimens did not exceed 24 hours. Tissues were cryosectioned radially at a thickness of 7 µm, and three sections were mounted per slide (Superfrost Plus, Menzel GmbH & Co KG, Germany).

Histological evaluation

All pathological as well as control cryosections were stained with haematoxylin and eosin for morphological assessment by light microscopy. The thickness of DM was measured three times in each specimen (the average was calculated) using a NIS Elements image analysis system (Laboratory Imaging, Prague, Czech Republic).

Indirect fluorescent immunohistochemistry

Slices obtained from the two controls, the original cornea and the failed corneal graft of the PPCD patient were used in each experiment. Two cryosections on each slide were stained with primary antibodies. The third section was used as a negative control (primary antibody omitted). Three independent experiments were performed. The tissue was fixed with cold acetone for 10 minutes and rinsed in phosphate buffered saline (PBS). After washing, the slices were exposed to a blocking solution with 2.5% bovine serum albumin (BSA) in PBS for 20 minutes, and then incubated with the primary antibodies diluted in PBS containing 1% BSA for 1 hour at room temperature. Mouse monoclonal antibodies against CK7 (1:50) and CK19 (1:50) (DakoCytomation, Glostrup, Denmark) were used. The specimens were then washed three times with PBS and incubated with the secondary antibody (fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 hour at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide (PI), (Vector Laboratories, Inc. Burlingame, USA) to counterstain the DNA within the nuclei.

Evaluation of indirect fluorescent immunohistochemistry

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200 – 1000x. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Osnabrück, Germany) and JENOPTIK ProgRes C12plus (Jenoptik, Laser Optik Systeme GmbH, Jena, Germany). At least three hundred epithelial cells per section (600/slide) and one hundred endothelial cells per section (200/slide), directly connected to DM, were examined, and the percentage of CK7 and CK19-positive cells was calculated.

FISH analysis of the sex chromosomes

The detection of gonosomes using FISH was performed immediately after staining with CK19 so that the presence of individual X or Y chromosomes in cells that were stained with the anti-CK19 antibody as well as in unstained cells could be evaluated simultaneously.

After immunohistochemistry, slices for FISH evaluation were mounted with Vectashield-medium for fluorescence (Vector Laboratories, Inc. Burlingame, USA), and the fluorescent signal for CK19 was quickly confirmed microscopically before the next FISH procedure was performed. Immediately after that the slides were rinsed in 2xSSC (standard saline citrate) for 10 minutes until the coverslips and mounting medium released spontaneously. The samples were fixed in a 3:1 methanol/acetic acid solution at room temperature for 10 minutes and then dehydrated through increasing ethanol grades (70, 85 and 96%), 2 minutes for each. For the detection of gonosomes, directly labeled VYSIS DNA probes CEP X (DXZ1) Spectrum Aqua Probe and CEP Y (DYZ3) Spectrum Orange Probe (both from Abbott, Des Plaines, IL, USA) were used. The CEP X (α satellite) DNA probe hybridizes to the centromere of human chromosome X (band region Xp11.1 – q11.1, locus DXZ1) and the CEP Y (α satellite) DNA probe hybridizes to the centromere of human chromosome Y (band region Yp11.1 – q11.1, locus DYZ3). Ten µl of the DNA probe mixture (buffer + distilled water + X/Y DNA probe) was placed on each slide. Subsequently, the slides were covered with coverslips and sealed with rubber cement. The reaction was run on a Thermobrite (Abbott Molecular, Des Plaines, IL, USA). The protocol consisted of denaturation of the target and probe simultaneously for 2 minutes at 73°C. Hybridization was done at 42°C overnight. Then the slides were washed at 73°C in 0.4x SSC/0.3% Nonident-P40 (NP-40), pH 7.0 for 2 minutes followed by washing in 2x SSC/0.1% NP-40, pH 7.0 for 1 minute at room temperature. After dehydration in increasing ethanol grades (70, 85, 96% - each for 2 minutes), the samples were allowed to air-dry and the cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), (Vectashield).

Evaluation of combined immunohistochemical and FISH signals

The specimens were examined using a Zeiss AX10 Imager Z1 microscope (Carl Zeiss GmbH, Jena, Germany) at a magnification of 230 – 600x. Images were taken using a CCD ProgRes MF camera (Jenoptik, Laser Optik Systeme GmbH, Jena, Germany) and Isis MetaSystem photo software (MetaSystem, Altlussheim, Germany). A quadrate bandpass DAPI/FITC/ORANGE/AQUA filter (360/490/560/426 nm) was used to view all four fluorescent signals. The examinations were performed by two different researchers (blinded slides), and the results were compared and summarized. Six cross-sections from the pathological and control corneal buttons were used for cell counting. At least 100 cells of the corneal epithelium and stroma and at least 300 endothelial cells were examined altogether (in actuality, each endothelial cell was evaluated).

First, the green fluorescent signal of CK19 staining was assessed in all single cells, particularly in the endothelium. Then the cell nuclei (with a clearly visible nuclear edge) were identified as being male- or female-derived, and five independent categories for gender evaluation were established as follows: 1 - cell nuclei without a signal (excluded from the results), 2 - one orange signal in the cell nucleus (Y, male), 3 - one aqua signal (X, excluded from the results), 4 - both X and Y signals within one cell nucleus (male), 5 - two aqua signals within one cell (female). This categorization allowed us to assess only cells with a clearly discernable sex, excluding cells, for example, in which only one X chromosome was detected or both chromosomes were not present in the sample due to cryosectioning.

Results

Histology

No differences were observed in the corneal epithelium, Bowman layer or the stroma among the control, original and failed PPCD samples using haematoxilin and eosin staining. The mean thickness of DM in the control specimens was 11.0 ± 2.4 µm (range from 8.0 to 14.4 µm); the thickness of DM measured in the original PPCD specimen was 9.8 ± 1.1 µm and in the failed graft 9.4 ± 1.1 µm. An abnormal posterior collagenous layer (PCL), populated by cells with a fibroblast-like characteristic shape, was observed between DM and the endothelium on the periphery of the failed graft, while no PCL was observed in the original PPCD cornea. The thickness of this abnormal PCL varied from 7.6 to 26.7 µm. The endothelium of all control specimens was formed of one layer of flat cells adjoined to DM. The PPCD endothelium of both the original cornea as well as the failed graft consisted predominantly of one or two cell layers, but areas with up to three cell layers were also observed (Fig. 1, panels on the left side).

Detection of CK7

CK7 was completely negative in both control samples (male and female). Moreover, no signal was present in the epithelial cells of the original corneal explant or in the failed corneal graft of the PPCD patient. Eighty and almost 50% of the abnormal endothelial cells revealed CK7-positivity in the native original cornea and the failed corneal graft, respectively.

Detection of CK19

CK19 was detected only in the epithelial cells of the healthy control male and female corneas, where heterogeneous positivity was detected in approximately 50% of the cells in all epithelial layers. No CK19 staining was detected in the stroma or in the endothelial cells of either control cornea (Fig. 1, A'). Heterogeneous positivity for CK19 was observed in almost 30% of the epithelial cells of the original corneal explant as well as in the failed corneal graft; no signal was present in the stroma. Forty-five and almost 100% of the endothelial cells revealed an intense positivity in the native original cornea and the failed corneal graft, respectively (Fig. 1, B', C'). No signal for CK19 was seen in any of the fibroblast-like cells within the abnormal PCL (Fig. 1, C') or in the negative control stainings.

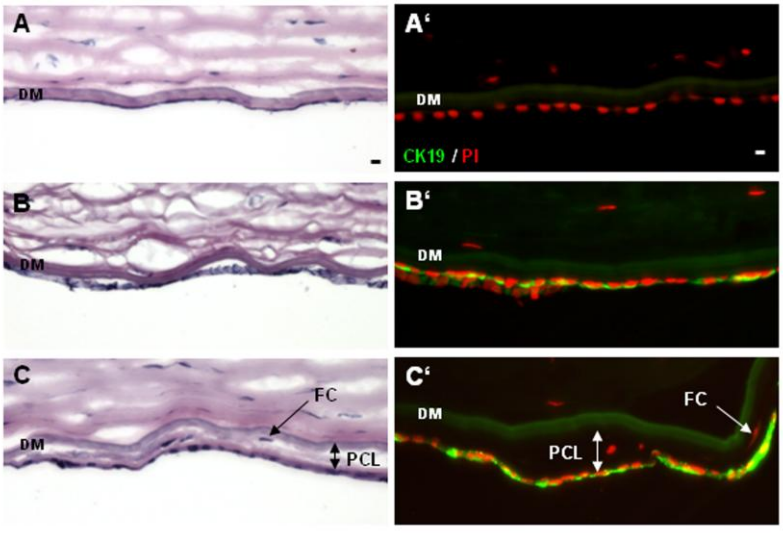

Fig. 1 Morphology of the posterior corneal layers of the control (A, A'), original (B, B') and failed cornea (C, C') obtained from a patient with posterior polymorphous corneal dystrophy. An abnormal posterior collagenous layer (PCL) with scattered cells of fibroblast-like shape (FC) was detected between Descemet membrane (DM) and the endothelium only in the failed graft (C, C'). Panels on the left side are after haematoxilin and eosin staining. Panels on the right side are after fluorescent immunohistochemistry and show a *green* fluorescent signal for CK19 (FITC) if present. Nuclei were counterstained with propidium iodide (PI, *red*). Scale bar represents 10 µm

Detection of X and Y chromosomes

All corneal layers (epithelium, stroma, endothelium) of the control male cornea revealed signals for both X and Y chromosomes. The control female cornea revealed two X signals in all layers (Fig. 2).

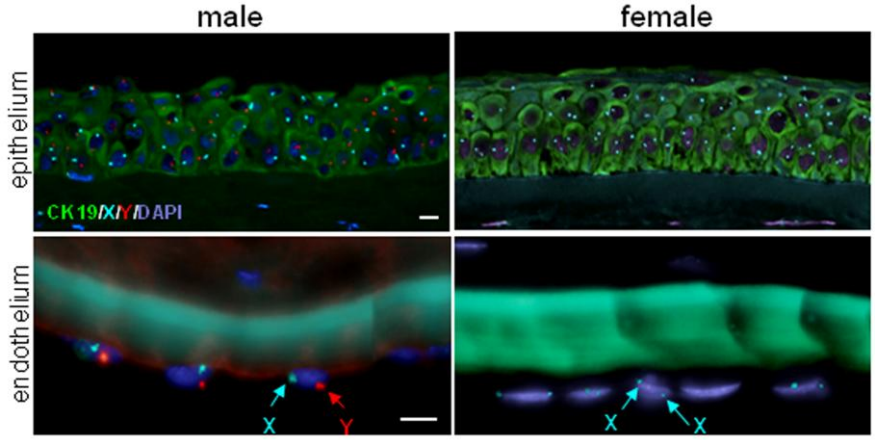

Fig. 2 Combined detection of cytokeratin 19 (*green*, FITC) with X (*blue*, AQUA) and Y (*red*, ORANGE) chromosomes in the control corneas. XY chromosomes were detected in the male corneal epithelium and endothelium. Two X chromosomes were detected in the female corneal epithelium and endothelium. Nuclei were counterstained with DAPI (*violet*). Scale bar represents 10 µm

Both X and Y chromosomes were clearly detectable in the epithelial, stromal and endothelial cells of the original cornea from our patient with PPCD. In the failed corneal graft, an almost total replacement of the female donor cells (XX) by recipient cells (XY) was observed in the epithelium, while 99% of stromal keratocytes were female in origin (XX), (Fig. 3, A, B). Altogether, 558 endothelial cells were evaluated in the failed corneal graft. In 159 of endothelial cells both X and Y chromosomes were detected (Fig. 3, C1-C3). Only in two endothelial cells of the failed cornea, located in the immediate vicinity of DM, were two X chromosomes detected. These cells exhibited endothelial morphology more than an epithelial-like phenotype. They were flat with elongated cell nuclei, and only these two cells revealed no CK19 staining (Fig. 3, C4). No signal for gonosomes was detected in 178 cells, and therefore it was impossible to decide if these cells were male- or female-derived. The same situation arose in a further 110 cells, in which only one X chromosome was detected. These cells were also excluded from the final calculations. In the remaining 109 cells only one Y chromosome was detected within the cell nucleus, suggesting that these cells were of male origin. The assessment of the endothelial cells in the failed corneal graft clearly showed that almost all the endothelial cells of the graft were host in origin (Tab. 1). On the other hand, we were unable to confirm the origin of the cells in the PCL, as in five cells only one X chromosome was observed, in one cell only one Y chromosome was detected and one cell was without gonosomes.

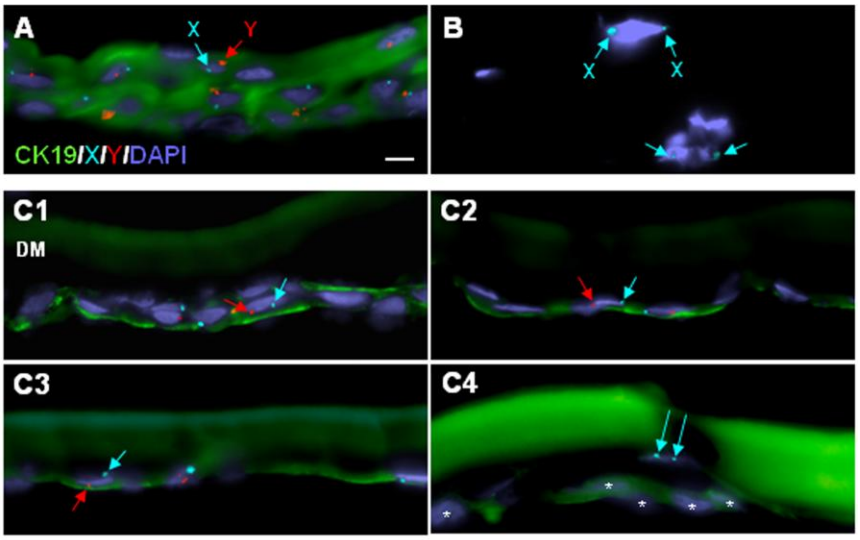

Fig. 3 Combination of fluorescent immunohistochemistry and fluorescence in situ hybridization of the X and Y chromosomes in a failed sex-mismatched corneal graft showing the recurrence of PPCD at the cellular-ultrastructural level. Corneal epithelial cells were positive for cytokeratin 19 (CK19 - *green*, FITC), and both X (*blue*, AQUA) and Y (*red*, ORANGE) chromosomes were detected in these cells (A). The stroma revealed two X signals (B). In pathological CK19-positive endothelial cells, both X and Y chromosomes were detected (C1-C3). In a CK19-negative endothelial cell, two X (*blue*, AQUA) chromosomes within one nucleus were detected (C4). This cell was located in the immediate vicinity of Descemet membrane (DM) and was surrounded by CK19-positive aberrant epithelial-like cells (*). Nuclei were counterstained with DAPI (*violet*). Scale bar represents 10 µm

Table 1 Endothelial cells found on the posterior surface of a sex-mismatched cornea graft obtained from a PPCD patient and their characteristics as detected using FISH and immunohistochemistry. Cells were divided into five categories based on their gender evaluation and positivity or negativity for cytokeratin 19 (CK19). To obtain the final results, only cells in categories 2, 4 and 5 were included.

category	FISH results	number of endothelial cells	CK19 expression
1	**0** - no signal	178	positive
2	**Y** - one orange signal	109	positive
3	**X** - one aqua signal	110	positive
4	**XY** - one orange and one aqua signal	159	positive
5	**XX** - two aqua signals	2	negative

Discussion

Combined immunohistochemistry together with FISH is commonly used for the simultaneous detection of immunophenotypic markers and cytogenetic aberrations present in tumor cells, as well as for the assessment of chimerism in a particular cell type after bone marrow sex-mismatched transplantations (Bzorek et al. 2008; Cook et al. 2006; Hessel et al. 1996; Nolte et al. 1996; Siebert and Weber-Matthiesen 1997; Trotman et al. 2004). A few problems may arise if the combination of these two methods is used. Fixation, protein digestion pretreatment and the working temperatures required for the FISH procedure significantly limit the ability to detect antigens by immunohistochemistry (Trotman et al. 2004). In our study, this particular combination of both methods has been performed in the human cornea for the first time.

Identification of individual donor and host endothelial cells in sex-mismatched corneal grafts using FISH analysis of the gonosomes has only been introduced within the past decade, and relatively very few studies have been performed so far (Lagali et al. 2009; Lagali et al. 2010; Macdonald et al. 2010; Wollensak and Green 1999). The migration of host endothelial cells to the donor's endothelium after grafting has been described; however, as it exhibits a highly variable pattern (Lagali et al. 2009; Lagali et al. 2010; Macdonald et al. 2010; Wollensak and Green 1999), we considered it important to use a more reliable marker than just the identification of the sex chromosomes in order to determine the origin and the proportion of pathological cells in the graft. From a broad spectrum of CKs found in PPCD but not in normal endothelium, we have chosen CK19 because of its strong expression in almost all aberrant PPCD endothelial cells (Jirsova et al. 2007). In order to detect the X and Y chromosomes in already immunohistochemically stained corneal cells from a patient with PPCD and to simultaneously evaluate all signals within one cell, we had to modify the recently published protocols (Donadoni et al. 2004; Hessel et al. 1996; Trotman et al. 2004). This approach allowed us to clearly determine whether the pathological endothelial cells originated from the recipient or whether donor cells had undergone metaplasia.

On the basis of morphology it has been suggested that PPCD recurrence is caused by the migration of the host endothelium (Sekundo et al. 1994), but no proof has so far been provided for this hypothesis, thus the possibility remained that the donor endothelium undergoes metaplasia triggered by unknown factors. As almost all of the assessed immunohistochemicaly stained CK19-positive cells had Y or XY chromosomes, we have concluded that they must be of host origin and that the recurrence of the pathology was the result of the overgrowth of pathological host endothelial cells over the posterior part of the donor graft. Three and a half years after grafting, we have detected only two endothelial cells those were clearly of donor origin; both were CK19-negative. Thus the proportion of surviving donor endothelial cells in the PPCD graft was less than 1%. It was postulated earlier that after keratoplasty of PPCD patients, the healthy donor endothelium could be destroyed by proliferating and migrating host epithelial-like endothelial cells or could be eliminated by immunological mechanisms and then repopulated by the host cells (Boruchoff et al. 1990; Sekundo et al. 1994). We suggest that in our case the major factor contributing to the almost total lack of donor endothelial cells in the graft was the presence of host epithelial-like PPCD cells migrating from the periphery of the recipient's cornea. Although we have not observed clinically any signs of immune-mediated corneal graft rejection, it can not be excluded that immunological mechanisms also contributed to the destruction of the donor endothelium (Bourne 2001; Malik et al. 1968).

Similarly to our results, Sekundo et al. (1994) detected a fibrocellular PCL at the periphery of a failed transplant from a PPCD patient. It was postulated previously that this fibrous layer contains fibroblasts and is formed by aberrant epithelial-like cells (Boruchoff et al. 1990; Sekundo et al. 1994). In our study we were unable to determine the origin of the cells in the PCL. Therefore, we can not exclude the possibility that ingrowing host keratocytes produced this layer together with the aberrant host endothelium.

To eliminate epithelial downgrowth over the host endothelium, which is a very rare event, as a possible explanation for our findings, we investigated the expression of CK7, which is not present in the corneal epithelium (Moroi et al. 2003; Feder and Krachmer 1985), in both of our pathological specimens. The presence of CK7 in about 50% of abnormal endothelial cells in the failed corneal graft indicates that no epithelial downgrowth occurred in our case.

Despite the high mitotic potential of the diseased endothelium in PPCD, there are only a few reports about the recurrence of this disorder. So far 12 cases with recurrence have been clearly documented in the literature, of which only three were examined by light and electron microscopy (Boruchoff et al. 1990; Krachmer 1985; Sekundo et al. 1994). Corneal tissue has been collected so far from 16 Czech PPCD patients, and four of them underwent repeat keratoplasty; however, only one received a sex-mismatched donor cornea. The combination of fluorescent immunohistochemical staining with FISH allowed us to show that in the PPCD case presented here, the proliferation and migration of the original pathological endothelium from the host periphery into the donor graft may have significantly contributed to corneal graft failure. We succeeded in combining and standardizing both methods so that this combined technique can now become a part of routine laboratory work, enlarging the possibilities of its usage in ophthalmology as well.

Acknowledgments

This work was supported by the research project of the Ministry of Education, Youth and Sports of the Czech Republic MSM0021620806, by Project 260501 from Charles University in Prague and by the grant from the Ministry of Health of the Czech Republic MZOVFN2005.

References

Biswas S, Munier FL, Yardley J, Hart-Holden N, Perveen R, Cousin P, Sutphin JE, Noble B, Batterbury M, Kielty C, Hackett A, Bonshek R, Ridgway A, McLeod D, Sheffield VC, Stone EM, Schorderet DF, Black GC (2001) Missense mutations in COL8A2, the gene encoding the alpha2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. Hum Mol Genet 10:2415–2423

Boruchoff SA, Kuwabara T (1971) Electron microscopy of posterior polymorphous degeneration. Am J Ophthalmol 72:879–887

Boruchoff SA, Weiner MJ, Albert DM (1990) Recurrence of posterior polymorphous corneal dystrophy after penetrating keratoplasty. Am J Ophthalmol 109:323–328

Bourne WM (2001) Cellular changes in transplanted human corneas. Cornea 20:560–569

Bzorek M Sr, Petersen BL, Hansen L (2008) Simultaneous phenotyping and genotyping (FICTION-methodology) on paraffin sections and cytologic specimens: a comparison of 2 different protocols. Appl Immunohistochem Mol Morphol 16:279-286

Cook JR, Hartke M, Pettay J, Tubbs RR (2006) Fluorescence in situ hybridization analysis of immunoglobulin heavy chain translocations in plasma cell myeloma using intact paraffin sections and simultaneous CD138 immunofluorescence. J Mol Diagn 8:459-465

Donadoni C, Corti S, Locatelli F, Papadimitriou D, Guglieri M, Strazzer S, Bossolasco P, Salani S, Comi GP (2004) Improvement of combined FISH and immunofluorescence to trace the fate of somatic stem cells after transplantation. J Histochem Cytochem 52:1333–1339

Feder RS, Krachmer JH (1985) The diagnosis of epithelial downgrowth after keratoplasty. Am J Ophthalmol 99:697–703

Feil SH, Barraquer J, Howell DN, Green WR (1997) Extrusion of abnormal endothelium into the posterior corneal stroma in a patient with posterior polymorphous dystrophy. Cornea 16:439–446

Gwilliam R, Liskova P, Filipec M, Kmoch S, Jirsova K, Huckle EJ, Stables CL, Bhattacharya SS, Hardcastle AJ, Deloukas P, Ebenezer ND (2005) Posterior polymorphous corneal dystrophy in Czech families maps to chromosome 20 and excludes the VSX1 gene. Invest Ophthalmol Vis Sci 46:4480–4484

Heon E, Greenberg A, Kopp KK, Rootman D, Vincent AL, Billingsley G, Priston M, Dorval KM, Chow RL, McInnes RR, Heathcote G, Westall C, Sutphin JE, Semina E, Bremner R, Stone EM (2002) VSX1: a gene for posterior polymorphous dystrophy and keratoconus. Hum Mol Genet 11:1029–1036

Hessel H, Mittermüller J, Zitzelsberger H, Weier HU, Bauchinger M (1996) Combined immunophenotyping and FISH with sex chromosome-specific DNA probes for the detection of chimerism in epidermal Langerhans cells after sex-mismatched bone marrow transplantation. Histochem Cell Biol 106:481-485

Hogan MJ, Bietti G (1969) Hereditary deep dystrophy of the cornea (polymorphous). Trans Am Ophthalmol Soc 67:235–264

Jirsova K, Merjava S, Liskova P (2009) Posterior polymorphous corneal dystrophy: A review of current knowledge. In: Korhonen L, Laine E (ed) New Topics in Eye Research. Nova Science Publisher, New York, 49–74

Jirsova K, Merjava S, Martincova R, Gwilliam R, Ebenezer ND, Liskova P, Filipec M (2007) Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. Exp Eye Res 84:680–686

Krachmer JH (1985) Posterior polymorphous corneal dystrophy: a disease characterized by epithelial-like endothelial cells which influence management and prognosis. Trans Am Ophthalmol Soc 83:413–475

Krafchak CM, Pawar H, Moroi SE, Sugar A, Lichter PR, Mackey DA, Mian S, Nairus T, Elner V, Schteingart MT, Downs CA, Kijek TG, Johnson JM, Trager EH, Rozsa FW, Ali Mandal MN, Epstein MP, Vollrath D, Ayyagari R, Boehnke M, Richards JE (2005) Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells. Am J Hum Genet 77:694–708

Lagali N, Stenevi U, Claesson M, Fagerholm P, Hanson C, Weijdegard B (2009). Survival of donor-derived cells in human corneal transplants. Invest Ophthalmol Vis Sci 50:2673–2678

Lagali N, Stenevi U, Claesson M, Fagerholm P, Hanson C, Weijdegård B, Strömbeck AS; Swedish Society of Corneal Surgeons (2010) Donor and recipient endothelial cell population of the transplanted human cornea: a two-dimensional imaging study. Invest Ophthalmol Vis Sci 51:1898–1904

Liskova P, Tuft SJ, Gwilliam R, Ebenezer ND, Jirsova K, Prescott Q, Martincova R, Pretorius H, Sinclair N, Boase DL, Jeffrey MJ, Deloukas P, Hardcastle AJ, Filipec M, Bhattacharya SS (2007) Novel mutations in the ZEB1 gene identified in Czech and British patients with posterior polymorphous corneal dystrophy. Hum Mutat 28:638

Macdonald EC, Gregory ME, Lockington D, Kennedy A, Roberts F, Ramaesh K (2010) Observation of the in vivo movement of host keratocytes into donor tissue following corneal graft; a novel technique. Br J Ophthalmol 94:790–794

Malik SR, Gupta AK, Sota LD (1968) Fate of endothelium in corneal homografts. An experimental study. Br J Ophthalmol 52:893–897

Matsumoto K, Weber PA, Makley TA, Jr. (1988) Posterior polymorphous dystrophy--a histopathologic presentation. Ann Ophthalmol 20:388–390, 393

Merjava S, Liskova P, Sado Y, Davis PF, Greenhill NS, Jirsova K (2009) Changes in the localization of collagens IV and VIII in corneas obtained from patients with posterior polymorphous corneal dystrophy. Exp Eye Res 88:945–952

Moroi SE, Gokhale PA, Schteingart MT, Sugar A, Downs CA, Shimizu S, Krafchak C, Fuse N, Elner SG, Elner VM, Flint A, Epstein MP, Boehnke M, Richards JE (2003) Clinicopathologic correlation and genetic analysis in a case of posterior polymorphous corneal dystrophy. Am J Ophthalmol 135:461–470

Nolte M, Werner M, von Wasielewski R, Nietgen G, Wilkens L, Georgii A (1996) Detection of numerical karyotype changes in the giant cells of Hodgkin's lymphomas by a combination of FISH and immunohistochemistry applied to paraffin sections. Histochem Cell Biol 105:401-404

Richardson WP, Hettinger ME (1985) Endothelial and epithelial-like cell formations in a case of posterior polymorphous dystrophy. Arch Ophthalmol 103:1520–1524

Rodrigues MM, Newsome DA, Krachmer JH, Sun TT (1981) Posterior polymorphous dystrophy of the cornea: cell culture studies. Exp Eye Res 33:535–544

Rodrigues MM, Sun TT, Krachmer J, Newsome D (1980) Epithelialization of the corneal endothelium in posterior polymorphous dystrophy. Invest Ophthalmol Vis Sci 19:832–835

Sekundo W, Lee WR, Aitken DA, Kirkness CM (1994) Multirecurrence of corneal posterior polymorphous dystrophy. An ultrastructural study. Cornea 13:509–515

Siebert R, Weber-Matthiesen K (1997) Fluorescence in situ hybridization as a diagnostic tool in malignant lymphomas. Histochem Cell Biol 108:391-402

Trotman W, Beckett T, Goncz KK, Beatty BG, Weiss DJ (2004) Dual Y chromosome painting and in situ cell-specific immunofluorescence staining in lung tissue: an improved method of identifying donor marrow cells in lung following bone marrow transplantation. Histochem Cell Biol 121:73-79

Weisenthal RW, Krachmer JH (1988) Posterior polymorphous corneal dystrophy: ten years of progress. New York: Raven Press, 457–466.

Wollensak G, Green WR (1999) Analysis of sex-mismatched human corneal transplants by fluorescence in situ hybridization of the sex-chromosomes. Exp Eye Res 68:341–346