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First Faculty of Medicine

Short review of the Ph.D. Thesis

Phenotypical characterization of the healthy human cornea and the alterations caused by posterior polymorphous corneal dystrophy

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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

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

2 LITERAL INTRODUCTION

2.1 Corneal anatomy and physiology

The cornea is a colorless transparent and avascular tissue which forms the outermost surface of the eyeball. Transparency together with surface smoothness, contour and refractive index determines the optical properties of the cornea. The anterior corneal surface is covered by the tear film and the posterior surface is bathed by the aqueous humor. The cornea consists of six different individual layers: the multilayered epithelium and its basal membrane (BME), the Bowman layer, the corneal stroma, the Descemet membrane (DM) and the corneal endothelium (Nishida, 2005).

2.1.1 Corneal epithelium and limbus

The corneal epithelium is approximately 50 μm thick and is composed of nonkeratinized, stratified squamous epithelial cells. The corneal epithelium is renewed throughout life from basal epithelial cells and from the population of limbal epithelial stem cells (LESCs), which proliferate and migrate centripetally to the central epithelium (Davanger and Evenson, 1971; Dua et al., 2009; Thoft and Friend, 1983). These cells, 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, where one cell remains undifferentiated and stays as a stem cell while another fast-dividing progenitor cells, named transit amplifying cells, begin to divide and differentiate into the suprabasal and superficial cells of the corneal epithelium (Hall and Watt, 1989; Schlötzer-Schrehardt and Kruse, 2005). Despite the best efforts of all researchers, it is still not possible to identify and isolate LESCs with total certainty and success.

2.1.2 Corneal endothelium

The endothelium is a monolayer of flat, mostly hexagonal cells which are high metabolically active. 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). The endothelial damage is repaired by the migration and enlargement of the remaining endothelial cells. The most important physiological function of the corneal endothelium is the regulation of 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).

The corneal endothelium and keratocytes originate from the neural crest and the lateral plate mesoderm, which together form the periocular mesenchyme (Cvekl and Tamm, 2004; Gage et al., 2005; Reneker et al., 2000). Accordingly, human corneal endothelial cells show distinctive phenotypical heterogeneity. Neural cell markers (neuron-specific enolase, S-100 protein, neuron cell adhesion molecule and neurofilaments) were detected in the corneal endothelium (Foets et al., 1992a; 1992b; Hayashi et al., 1986; Shamsuddin et.al., 1986). Mesenchymal cell marker – vimentin was observed in the corneal endothelium as well (Foets et al., 1990; Hayashi et al., 1986) but little is known about other mesothelial cell markers and their presence in the adult human cornea. Moreover, the expression of epithelial cell markers – cytokeratins (CKs) 8 and 18 in the corneal endothelium is a matter of some controversy (Cockerham et al., 2002; Kasper et al., 1992; Kramer et al., 1992; Levy et al., 1995; Wollensak and Witschel, 1996).

2.2 Corneal dystrophies

According to the traditional classification based on the anatomic location of the dystrophies, we recognize three groups of corneal dystrophies: anterior corneal dystrophies of the epithelium and Bowman layer, stromal dystrophies and posterior dystrophies of the DM and endothelium (Aldave and Sonmez, 2007; Pieramici and Afshari, 2006). The endothelial corneal dystrophies group includes Fuchs endothelial corneal dystrophy (FECD), posterior polymorphous corneal dystrophy (PPCD), congenital hereditary endothelial dystrophy (CHED) and X-linked endothelial corneal dystrophy (XECD). All are thought to represent defects of neural crest terminal differentiation (Bahn et al., 1984). The group shares many features including altered morphology of endothelial cells, secretion of an abnormal posterior collagenous layer (PCL) in the posterior side of DM and consequently corneal decompensation (Levy et al., 1996).

2.2.1 Posterior polymorphous corneal dystrophy

PPCD is a rare, bilateral autosomal dominant disorder primarily affecting the corneal endothelium and DM (Hogan and Bietti, 1969). The epithelization and proliferation of the pathologic endothelium of PPCD corneas are the most common findings at the cellular level (Boruchoff and Kuwabara, 1971; Krachmer, 1985; Rodrigues et al., 1981). Epithelization was also confirmed by the detection of CKs, typical epithelial proteins (Rodrigues et al., 1980).

2.2.1.1 Clinical findings

PPCD is characterized biomicroscopically (slit-lamp examination) by vesicular lesions, 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).

As the disease is usually 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 (Cibis et al., 1977). The aberrant endothelium grows through a trabecular meshwork, which can lead to iridocorneal adhesion, iris atrophy and increased intraocular pressure, which causes a secondary glaucoma (Cibis et al., 1977; Krachmer, 1985). Although PPCD is widely believed to be an inherited defect in the corneal endothelium or DM, the first recorded recurrence of PPCD after penetrating keratoplasty was published by Boruchoff et al. (1990). A few years later, Sekundo et al. (1994) described the repopulation of the posterior surface of three donor corneas by the host pathological endothelium.

2.2.1.2 Laboratory observations

The corneal epithelium is mostly of normal thickness, but thin fibrocellular tissue (fibrous pannus) between the epithelium and Bowman layer was observed occasionally (Feil et al., 1997; Grayson et al., 1974). A large amount of wide-spaced collagen is deposited posterior to DM forming an abnormal PCL (Johnson and Brown, 1978; Waring, 1982). Epithelial-like abnormal endothelial cells with prominent microvilli, abundant keratofibrils, desmosomes and sparse microorganelles (all characteristics of epithelial cells) have been well documented in PPCD (Boruchoff and Kuwabara, 1971; Feil et al., 1997; Krachmer, 1985; Rodrigues et al., 1981).

2.2.1.3 Genes implicated in PPCD

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 to be disease-causing (Héon et al., 2002) although evidence exists that in the linked families (from the Czech Republic) another undiscovered PPCD gene at 20p11.2 is implicated (Aldave et al., 2009; Gwilliam et al., 2005). PPCD2 (OMIM, #609140) together with FECD 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; Vincent et al., 2009).

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 were used. All samples were obtained from the Ocular Tissue Bank Prague, General Faculty Hospital, Prague, Czech Republic.

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. 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. All pathological corneal explants were obtained from the Department of Ophthalmology, First Medical Faculty of Charles University and General Faculty Hospital in Prague.

3.1.3 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). Corneal discs were dissected and used for preparation of radial and tangential cryosections (7 µm thick), (Chen et al., 2004). 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). Pathological corneal explants 7 – 8 mm large were used for cryosections only.

3.2 General techniques

3.2.1 Histochemistry

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

3.2.2 Fluorescent immunohistochemistry and immunocytochemistry

After fixation, rinsing, permeabilization (only in the case of immunocytochemistry) and blocking, slides and membranes were incubated with primary antibodies for 1 hour at room temperature. After washing, the slices were incubated with appropriate FITC-conjugated and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 hour at room temperature. After rinsing the slices were mounted to counterstain the DNA within the nuclei.

3.2.3 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 was applied to the sections in one step, followed by a mixture of FITC-conjugated and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA).

3.2.4 Enzymatic immunohistochemistry and immunocytochemistry

After fixation, rinsing, permeabilization (only in the case of immunocytochemistry) and blocking, the slides and membranes were incubated with primary antibodies for 1 hour at room temperature. After washing the slices were incubated with appropriate biotinylated secondary antibodies and then the tertiary complex was applied (UltraTech HRP AEC kit, Immunotech, France or SABcomplex/AP, DakoCytomation, Glostrup, Denmark). The staining was visualized by 3-amino-9-ethylcarbazole (AEC), (Immunotech) or 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).

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

After total RNA isolation (Rneasy Plus Microkit, Qiagen, Hilden, Germany or TRI Reagent, Molecular Research Center, Cincinnati, OH) RNA was reverse transcribed into cDNA (SuperScript II Reverse Transcriptase or SuperScript III/RNase OUT Enzyme Mix).

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.

3.2.6 Western blot

After lysis the protein concentration was determined using a commercial BCA Protein Assay Kit (Pierce, Rockford, USA). 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 a SuperSignal® West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology) and a Syngene membrane documentation system Chemigeniius-Q and GeneSnap program (Synoptics Ltd., Cambridge, UK).

3.2.7 Fluorescence in situ hybridization (FISH) 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).

3.2.8 Microscopic techniques

The specimens were examined by light and fluorescent microscopy using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) and a 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.

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. None of the detected CKs were expressed by keratocytes in the stroma.

4.2 Detection of CK8 in the limbal basal cells

Sixty percent of the cadaveric corneo-scleral samples 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 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 (Fig. 1).

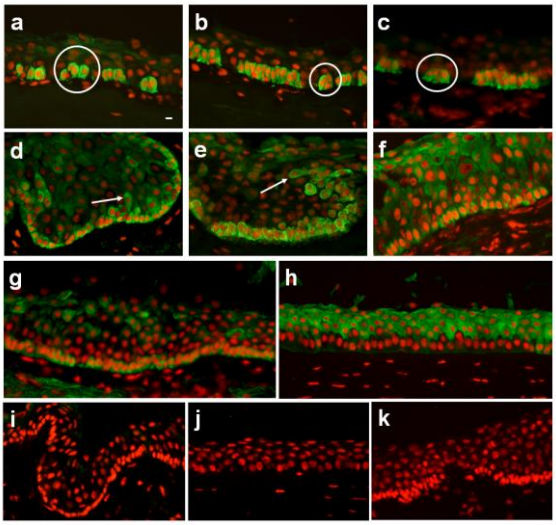

Figure 1: Immunolocalization of CK8 in radial sections of the limbus and cornea. CK8 expression in the limbus: positive limbal basal cells forming clusters (circles; **a-c**) or lines with clearly visible elongated CK8-positive cells projecting from the basal layer (arrows; **d, e**); limbal specimen 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 represents 10 μm.

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 (Fig. 2). CK8 expression in the cornea, limbus and conjunctiva was confirmed using RT-PCR.

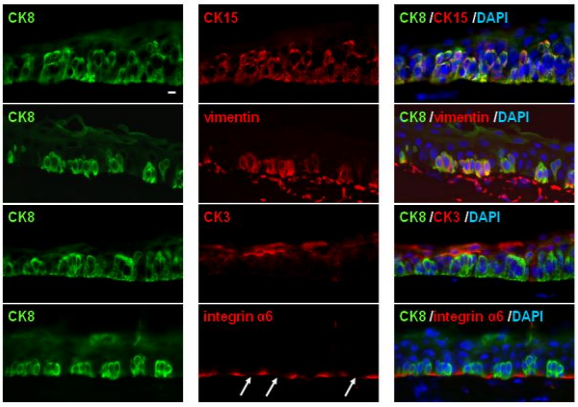

Figure 2: Immunolocalization of CK8 with CK15, vimentin, CK3 or integrin α6 in the basal layer of the limbus on radial sections. CK8 (green, FITC) colocalized with CK15 and with vimentin (red, TRITC). CK3 (red, TRITC) was completely absent from the basal cells of the limbus, whereas a few suprabasal 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 represents 10 μm.

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 (Fig. 3). No signal was detected when 4% formalin or 10% paraformaldehyde was used as a fixative, irrespective of the antibody used.

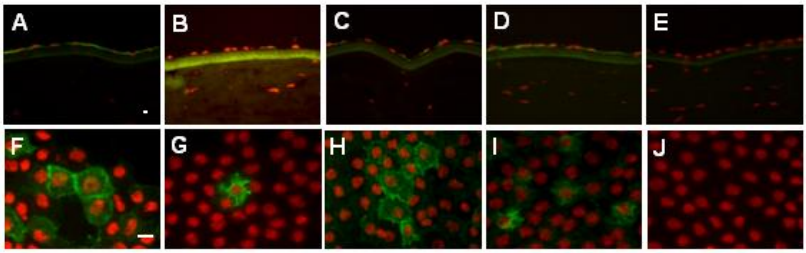

Figure 3: Indirect immunofluorescent staining of the adult human corneal endothelium. Expression of cytokeratins 8 and 18 on cryosections (A-E) and on endothelial imprints (F-J). Immunostaining for CK 8 (A, F), CK 18 – Dako (B, G), CK 18 – Sigma (C, H), the CK pair 8/18 (D, I), and the negative control (primary antibody omitted) (E, J). Scale bar represents 10 µm.

CK8 and CK18 proteins and mRNA were detected in the endothelium of all tested corneas by Western blot or semi-quantitative RT-PCR, respectively (Fig. 4).

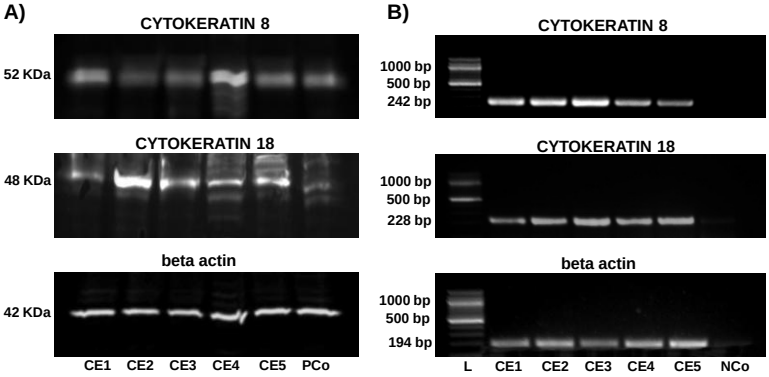

Figure 4: Expression of the cytokeratin 8 and 18 proteins and genes in the corneal endothelium (CE) determined by Western blot (A) and RT-PCR (B) . Beta actin was used as an internal control. PCo – positive control (corneal epithelium), NCo – negative control, (reaction without sample cDNA) a marker for internal contamination, L – ladder.

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 (Fig. 5). 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 (Fig. 6). 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 (Fig. 7), (all results from qRT-PCR were obtained from Mgr. Ales Neuwirth, Institute of Molecular Genetics AS CR, Prague),

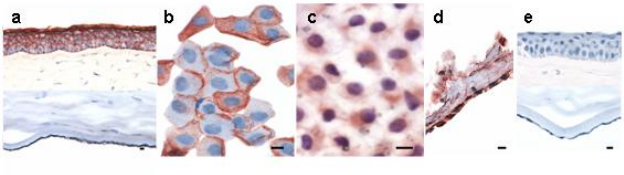

Figure 5: Enzymatic immunohistochemical detection of mesothelin in the human cornea. Positivity in the epithelium and endothelium on cryosections (a). Detail of the membrane-bound signal in the epithelial imprints (b) and the cytoplasmic signal in the endothelial cells of lamella (c). Surface cells of the peritoneum as a positive control (d) and corneal negative control (e). Scale bar represents 10 µm.

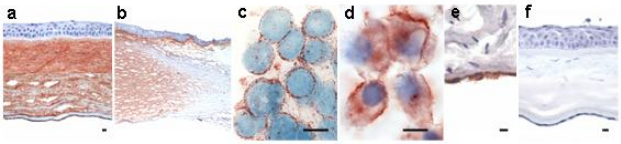

Figure 6: Enzymatic immunohistochemical detection of HBME-1 protein. A strong signal in the stroma and endothelium of corneal cryosections (a). A sharp border between the corneal (positive) and scleral (negative) stroma (b). A clear brush border pattern in the endothelial imprints (c) and strong cytoplasmic 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.

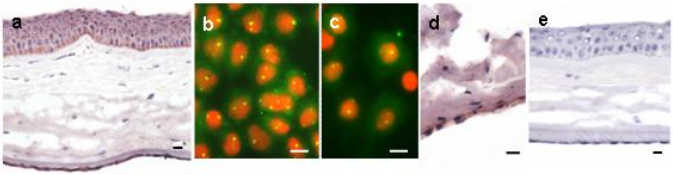

Figure 7: Immunohistochemical detection of calbindin 2. Positivity in the epithelium and endothelium of corneal cryosections (a). Calbindin 2-positive intranuclear dots in the epithelial (b) and endothelial (c) imprints. Surface cells of the peritoneum as a positive control (d) and corneal negative control (e). Scale bar represents 10 µm.

4.5 Cytokeratin expression in the 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 (Fig. 8).

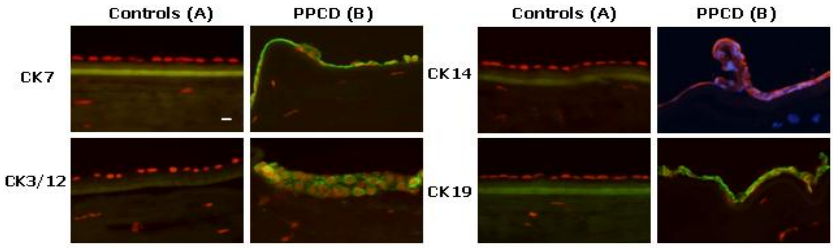

Figure 8: Immunolocalization of various CKs in control (A) and PPCD (B) corneal sections. Cell nuclei were stained with propidium iodide or DAPI. Scale bar represents 10 µm.

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), (Fig. 9). 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.

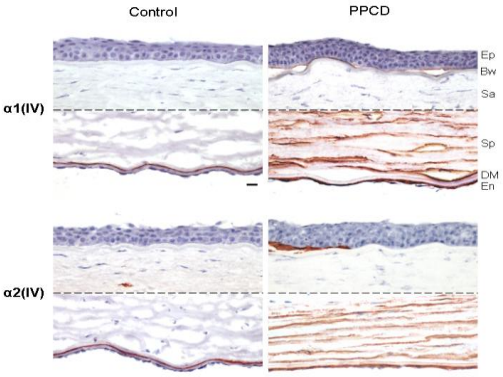

Figure 9: The immunohistochemical localization of the α1 and α2 collagen IV chains in the control corneas and corneas obtained from PPCD patients. Scale bar represents 10 µm. Ep – epithelium, Bw – Bowman layer, Sa – anterior part of the stroma, Sp – posterior part of the stroma, DM – Descemet membrane, En – endothelium. ----- line represents a part of the corneas which are not included in the figure.

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 (Fig. 10). In most

CK19-positive cells, both X and Y chromosomes were simultaneously detected using FISH (Fig. 11). 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).

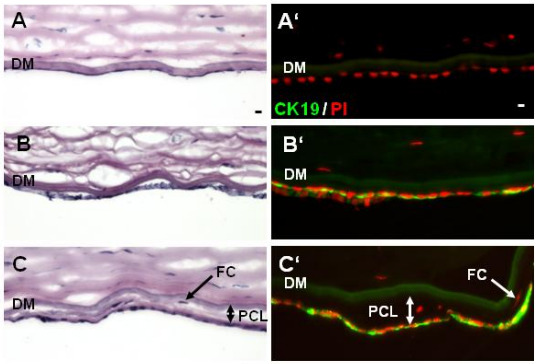

Figure 10: Morphology of the posterior corneal layers of the control (A, A'), original (B, B') and failed cornea (C, C') obtained from a patient with posterior polymorphous corneal dystrophy. An abnormal posterior collagenous layer (PCL) with scattered cells of fibroblast-like shape (FC) was detected 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.

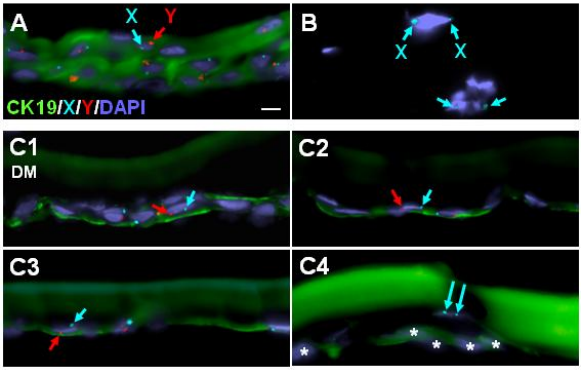

Figure 11: Combination of fluorescent immunohistochemistry and fluorescence in situ hybridization of the X and Y chromosomes in a failed sex-mismatched corneal graft showing the recurrence of PPCD at the cellular-ultrastructural level. Corneal epithelial cells were positive for cytokeratin 19 (CK19 - 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.

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 (*Merjava et al., 2010, Histol. Histopathol.*).

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 transit amplifying cells, 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 (Schlötzer-Schrehardt and Kruse, 2005). 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 (*Merjava et al., 2011, Invest. Ophthalmol. Vis. Sci*).

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., 1995). 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 (*Merjava et al., 2009b, Exp. Eye Res.*). 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).

The fact that the mammalian endothelium originates from both neural and mesodermal cells (Gage et al., 2005), 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 (*Jirsova et al., 2010, Exp. Eye Res.*).

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), (*Jirsova et al., 2007, Exp. Eye Res.*).

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), it may happen that the proliferation of endothelial cells of PPCD patients is induced by the collagen VIII deposited in the PCL as well (*Merjava et al., 2009a; Exp. Eye Res.*).

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., 2007). 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 (*Merjava et al., 2011, under revision*). 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.

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8 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.

8.1 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. **Klintworth GK,** 2009. **Omary MB,** 2009. **Clausen I,** 2009. **Vincent AL,** 2009. **Kannabiran C,** 2009. **Pineda R,** 2010. **Jakobiec FA,** 2010. **Shen AL,** 2010. **Hillenaar T,** 2010.

[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.* 2009a;88:945-952. (**IF = 2.538)**

cited by: **Jakobiec FA,** 2010. **Saito K,** 2011. **Herwig MC,** 2011.

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

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[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 J.T., Jirsova K. Cytokeratin 8 is expressed in human corneo-conjunctival epithelium, particularly in limbal epithelial cells. *Invest Ophthalmol Vis Sci*. 2011;52:787-794. (**IF = 3.431)**

[11] **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. *Histol Cell Biol.* 2011. Submitted.

8.2 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

8.3 Other selected presentations

[20] Jirsová K, **Merjavá S,** Martincová R, et al. 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, et al. 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, et al. 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, et al. 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.

8.4 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.