Charles University in Prague

1st Faculty of Medicine

Autoreport of the Dissertation Theses

Cell and Molecular Characterization of Failed Human Corneal Grafts.

The Role of Matrix Metalloproteinases in Recurrent Corneal Melting.

Buněčná a molekulární charakterizace selhaných transplantátů lidské rohovky.

Role matrix metaloproteináz při opakované keratolýze lidské rohovky.

Kristýna Brejchová

2011

Doctoral Educational Programmes in Biomedicine

Charles University in Prague
and The Academy of Sciences of the Czech Republic

Educational program:	Biology and pathology of the cell
Chairman of the educational program:	Prof. MUDr. Milan Elleder, DrSc.
Place of work:	Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and First Faculty of Medicine, Charles University
Supervisor:	Mgr. Kateřina Jirsová, Ph.D.
Second supervisor:	Prof. MUDr. Martin Filipec, CSc.

Table of Contents

Abstract

The aim of this work was to investigate the contribution of matrix metalloproteinases (MMPs) to recurrent corneal melting. Twenty three melted corneas from seven patients were separated into three groups: a) patients with primary Sjögren's syndrome, b) rheumatoid arthritis and c) other corneal melting underlying pathologies. Eleven cadaverous corneas served as controls. The presence of MMP-1, -2, -3, -7, -8, -9, and -13 was detected using indirect enzyme immunohistochemistry. The active forms of MMP-2 and -9 and MMP-3 and -7 were examined by gelatin and casein zymography, respectively. The concentrations of active MMP-1 and -3 were measured using activity assays. Increased immunostaining intensity for MMP-1, -2, -3, -7, -8 and -9 was shown in the corneal epithelium and the stroma of almost all melted corneas from all three groups compared to the negative or slightly positive staining of the controls. In the endothelium immunostaining for MMP-2 and MMP-9 was increased in most specimens of group II and III and group I, respectively. A markedly higher level of active MMP-2 was detected in six and active MMP-9 in all pathologic specimens compared to controls. In contrast to completely negative controls, the proenzymes of MMP-3 and -7 were detected in almost all melted corneas from all three groups. Active MMP-3 and -7 was found each in one specimen from group I. Significantly increased concentrations of active MMP-1 and -3 were also found in the melted corneas. The increased expression and activity of a wide range of MMPs in melted cornea samples suggest that although different stimuli may trigger the pathways leading to the destruction of the cornea, these enzymes could partake mainly in the operational stage of this process, in which the massive degradation of the extracellular matrix has taken place.

Abstrakt

Cílem této práce bylo studium vlivu matrix metaloproteináz (MMP) na opakovanou keratolýzu lidské rohovky. Dvacet tři rohovek s keratolýzou získaných od sedmi pacientů bylo rozděleno do tří skupin: a) pacienti s primárním Sjögrenovým syndromem, b) pacienti s revmatoidní artritidou, c) pacienti s dalšími keratolýzu doprovázejícími chorobami. Kontrolní tkáň tvořilo jedenáct kadaverózních rohovek. Přítomnost MMP-1, -2, -3, -7, -8, -9, a -13 byla detekována metodou nepřímé imunohistochemie. Aktivita MMP-2 a -9 byla zjišťována želatinovou a aktivita MMP-3 a -7 kaseinovou zymografií. Koncentrace aktivní MMP-1 a -3 byla stanovena aktivačními analýzami. Při imunodetekci MMPs byla v porovnání s negativními či slabě pozitivními kontrolami pozorována zvýšená intenzita signálu MMP-1 -2, -3, -7, -8 a -9 v epitelu a stromatu téměř u všech patologických vzorků. V endotelu byla u většiny vzorků zaznamenána zvýšená hladina MMP-2 ve skupině II a III a MMP-9 ve skupině I. Želatinová zymografie prokázala zvýšenou aktivitu MMP-2 u šesti a MMP-9 u všech rohovek s keratolýzou. Neaktivní formy MMP-3 a -7 byly přítomny u většiny patologických vzorků všech tří skupin, aktivní formy těchto enzymů byly zaznamenány každá pouze u jednoho vzorku ze skupiny I, zatímco kontrolní vzorky byly negativní. Signifikantně zvýšená koncentrace aktivní MMP-1 a -3 byla u patologických vzorků prokázána i pomocí aktivačních analýz. Zvýšená exprese a aktivita řady MMP u rohovek s keratolýzou podporuje hypotézu, že ačkoliv stimuly spouštějící dráhy vedoucí k destrukci rohovky mohou být různé, tyto enzymy se uplatňují zejména ve fázi výkonné, při které dochází k masivní degradaci extracelulární matrix.

1 Introduction

1.1 Transplantation of the Human Cornea

The cornea is the transparent avascular front part of the eye composed of six layers (the epithelium, the basement membrane, Bowman's layer, the stroma, Descemet's membrane and the endothelium). It forms a mechanical and chemically impermeable barrier between the external and internal environment together with the conjunctiva, sclera and tear film. The cornea serves as the gateway into the eye for external images. Its optical properties are determined by its transparency, surface smoothness, contour, and refractive power [1].

Corneal transplantation is the most frequently and successfully performed grafting procedure in which either a full thickness host cornea is replaced by a donor corneal button (penetrating keratoplasty) [2] or the affected layers of the host corneal tissue is substituted with lamella prepared from the donor cornea (lamellar keratoplasty) [3]. Although the reported survival rate of penetrating keratoplasty is about 90% for uncomplicated first grafts one year after transplantation is performed in normal, nonvascularized, low-risk beds [4, 5], the graft survival and success depend on a number of factors related to both the donor tissue and recipient [2, 6]. Graft failure occurred in 70 - 90% of high-risk patients [7, 8]. The success of these grafts depends particularly on immunological risk factors such as vascularization, previous graft rejection as well as nonimmunologic factors (ocular surface diseases, the presence of glaucoma or previous surgery, etc.) [5, 6, 9]. Except for less common reasons of corneal graft failure (such as melting, primary graft failure, endophtalmitis, disease recurrence or late endothelial failure) [10-12], the main cause of this event remains immune-mediated rejection characterized by delayed-type hypersensitivity to donor alloantigens and leukocytic cellular infiltration of the graft site [12, 13].

1.2 Corneal Melting

Corneal melting (keratolysis) is one of the uncommon but very severe reasons for graft failure[10, 14]. It is a difficult-to-treat and occasionally recurrent condition, which can affect native cornea as well as corneal grafts. This pathologic process involves the development of epithelial defects and gradual stromal thinning caused by the destruction of the extracellular matrix (ECM). Finally, descemetocele may be formed, and subsequent perforation of the cornea follows [15-18].

Keratolysis could be associated with infectious [19, 20], non-infectious inflammatory [21-23], traumatic [24, 25], or trophic causes [26], but may also arise for no apparent reason [27].

Non-infectious corneal melting could occur as an isolated ocular problem; however, it is more often linked to systemic diseases (rheumatoid arthritis - RA, Sjögren's syndrome, Wegener's granulomatosis and also rarely to ocular cicatricial pemphigoid - OCP) [15, 21, 23, 28]. RA is a chronic inflammatory disease with progressive articular damage often associated with systemic manifestations in different tissues [29]. Ocular involvement, including dry eye syndrome, secondary Sjögren's syndrome, scleritis and keratolysis, occurs in 25 - 50 % of RA patients, most often in individuals suffering from the severe form of the disease [22, 29, 30]. Sjögren's syndrome, which occurs as a solitary process, is classified as primary Sjögren's syndrome (pSS) and its estimated prevalence is about 0.5% [31]. However, both of them are characterized by the destruction of the lacrimal and salivary glands, resulting in dry eye and xerostomia [31-33]. Lymphocytic infiltration occurs in the exocrine glands producing various autoantibodies [32, 34, 35]; however this autoimmune disease may affect several other tissues and organs as well [33, 36-38].

Management of corneal melting is very difficult and especially severe cases require performance of penetrating keratoplasty [16-18]. Unfortunately, some corneal grafts recurrently fail by melting as well [16, 21].

1.3 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of 23 structurally and functionally related endopeptidases, which degrade various components of the ECM [39, 40]. Depending on their substrate preference, and structural organisation, they are grouped into six subfamilies: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (e.g., MMP-3, -10, -11), matrilysins (e.g., MMP-7, -26), membrane type MMPs (MT1 - MT6-MMPs) and others (e.g., macrophage metalloelastase - MMP-12 or enamelysin - MMP-20) [41].

The structure of MMPs is organized into three basic domains: an amino-terminal propeptide; a catalytic domain; and a hemopexin-like domain at the carboxy-terminal [39, 42, 43]. The propeptide contains a cysteine residue interacting with the catalytic zinc atom, which is essential in the catalytic processes of the MMPs. Latent MMPs are therefore activated by removal of this propeptide domain. The hemopexin-like domain of MMPs has been shown to play a functional role in substrate binding and/or in interactions with the tissue inhibitors of metalloproteinases (TIMPs) [40, 44, 45]. In addition to these basic domains, the family of MMPs evolves into different subgroups by incorporating and/or deleting structural and functional

domains (e.g. the fibronectin type-II like domain in gelatinases, the transmembrane domain and recognition motif for furin-like convertases in MT-MMPs, MMP-11 etc.) [42, 46-48].

MMPs are regulated at four levels: gene expression [49], compartmentalization (anchoring MMPs to the cell membrane, or some other molecules in the pericellular space) [50], delocalization of the prodomain from the catalytic site of MMPs [42, 43, 50, 51] and enzyme inactivation by inhibitors (TIMPs and others e.g. α2-macroglobulin) [49].

Although MMPs are able to cleave almost all components of ECM and a large number of non-ECM proteins (grow-factors, cytokines, cell receptors, other MMPs etc.) [42]; members of each subfamily are more or less substrate specific. In the cornea the most abundant proteins are fibrillar collagens types I and III present in the stroma, which are cleaved by all members of the subfamily collagenases [52, 53]. Gelatinases are able to degrade especially basement membrane components of the corneal epithelium (collagen types IV and VII, fibronectin and laminin) and stromal collagen types V and VI, core protein decorin and denatured collagens [52-59]. Very low levels of collagenases MMP-1, -8 and -13 in the epithelium as well as low level of MMP-2 in both the epithelium and anterior stroma is normally present [60-63]. Stromelysins and matrilysin have similar substrate preferences and cleave ECM components constitutively present in the stroma and corneal basement membranes (type IV collagen, procollagens, collagen cross-links, fibronectin, laminin) [39, 52, 57, 64]. The presence of these enzymes in the normal human cornea is still uncertain.

1.4 Matrix Metalloproteinases in Non-Infectious Corneal Melting

As corneal melting is a result of the destruction and loss of the ECM, it is often linked to the over-expression and increased activity of MMPs [62, 63, 65, 66].

Elevated levels of MMP-1 were found in the corneal epithelium and stroma and both gelatinases (MMP-2 and -9) in tears of patients with noninfectious ulcerative keratolysis associated with RA [63, 67]. Increased expression of MMP-2, -3, -8 -9 was also described in melted corneas of patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs) after photorefractive keratectomy or cataract surgery [62, 65, 68].

1.5 Induction Mechanisms of Corneal Melting

Noninfectious corneal melting is very often associated with an autoimmune disease (e.g. RA and Sjögren's syndrome) [16, 63, 69, 70] and immune mediated inflammation is suspected of playing an important part in this process [16, 69]. A disturbed epithelial barrier caused by dry eye, which is very often present in RA or Sjögren's syndrome, allows some

immunomodulatory mediators from tears or from the conjunctival vasculature, in cases of marginal melting, to enter into the cornea [69]. It is supposed that lymphocytic (polymorphonuclear neutrophils and macrophages) and leukocytic (T cells) infiltrates [14, 62, 69] secrete pro-inflammatory cytokines in melted corneas as well, as is described in the pathophysiology of pSS in salivary and lacrimal glands [31] or in synovial fluid of RA patients[49]. Pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β, of which levels are markedly increased in the keratocytes of corneas with rheumatoid corneal ulceration [71] and in the tears of pSS patients [70], respectively, initiate MMP expression in various tissues via the mitogen-activated protein kinases pathway [39, 72]. However, MMPs are directly produced by activated neutrophils and macrophages as well [73].

Except for epithelial erosion, dry eye conditions also cause desiccation and hyperosmolar stress leading to the stimulation of pro-inflammatory cytokines and in consequence to the induction of MMP over-expression [72, 74].

2 Hypotheses and Aims of the Work

We hypothesized that non-infectious inflammatory corneal melting causing graft failure in patients with different underlying diseases is mainly inflicted by MMPs.
The aims of this work are:

1. to increase our understanding of the major MMPs' role in the process of corneal melting and identify potential targets for the prevention and treatment of devastating corneal keratolysis

2. immunohistochemical localization of some members of all the MMPs' main groups - collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9) stromelysins (MMP-3) and matrilysins (MMP-7) in cadaverous corneas, which served as controls, melted native corneas and in grafts recurrently failed due to corneal melting obtained from patients from three groups: a) patients with primary Sjögren's syndrome, b) patients suffering from rheumatoid arthritis, and c) patients with other underlying pathologies

3. to compare the presence, localization and staining intensity of all above mentioned MMPs in control and melted corneas

4. to determine the activity of selected members of all the main MMP groups – collagenases (MMP-1), gelatinases (MMP-2 and -9) stromelysins (MMP-3) and matrilysins (MMP-7) in controls, melted corneas and grafts

5. to compare activities of MMP-1, -2, -3, -7 and -9 in control and melted specimens

6. to compare MMP expression and activity in specimens among all three patient groups and to ascertain whether the underlying diseases have some influence on individual MMP production and activation.

3 Material and Methods

3.1 Patients and Specimens

The study adhered to the tenets set out in the Declaration of Helsinki. Local Ethics Committee approval was granted. Twenty three melted corneas (including regrafts) from seven patients were obtained during penetrating keratoplasty for corneal melting. All of these explants were collected between July 2002 and December 2007 from the Department of Ophthalmology, General Teaching Hospital and 1st Faculty of Medicine, Charles University in Prague. The patients were divided into three groups according to their diagnosis (Group I – III).

Group I included 11 specimens of two patients with pSS. Systemic immunosuppression was administered to both patients. The presence of severe dry eye was diagnosed in both cases bilaterally. Case 1 suffered from numerous episodes of corneal melting in both the right eye (six grafts were used as specimens P0-2, P0-3, P0-4, P0-6, P0-7, and P0-8) and left eye (her native cornea – P0-1 and one graft as specimen P0-5) requiring a number of surgical procedures including penetrating keratoplasties. Peripheral ulcerative keratitis was observed in both eyes of case 2, who developed corneal thinning in her right eye (specimens P0-9, P0-10 and P0-11) followed by penetrating keratoplasties.

Group II contained seven melted explants of three patients with RA, all of whom were evaluated by rheumatologists, and the severity of their RA was classified into four stages (http://www.wheelessonline.com). All the patients were administered systemic immunosuppressives. Full thickness grafts were transplanted due to keratolysis, which occurred in all native corneas (specimen P2-1) and grafts (specimens P1-1, P1-2, P1-3, P2-2, P2-3, P3) either centrally or paracentrally. The presence of accompanying keratoconjunctivitis sicca (KCS) was classified as moderate in P1, and severe in P2 and P3 [75].

Group III included five melted corneas of two patients: P4 suffered from OCP and idiopathic autoimmune hemolytic anemia and P5 who underwent full thickness and patch keratoplasty, but no systemic disorder explaining the observed keratolysis was detected. She suffers from chronic entropion. Corneal melting occurred in both the native corneas (P4-2, P5-2) and grafts (P4-1, P4-3, P5-1), either centrally or paracentrally. KCS was classified as severe in P4 [75].

3.2 Specimen Preparation

Eleven unaffected donor corneal buttons (mean age 59.8 ± 16.9 years) that were unsuitable for transplantation due to their low endothelial cell density, served as controls (obtained from

the Ocular Tissue Bank, General Teaching Hospital, Prague). All specimens were dissected into two halves, snap-frozen in liquid nitrogen, and stored at -70 ºC. Prior to freezing, one-half was embedded in Optimal Cutting Temperature Compound (OCT, Christine Gröpl, Tulln, Austria).

Before the activity assessment the specimens were processed as previously described [76]. In brief, they were thawed and homogenized in cacodylate buffer (0.1 M cacodylic acid, 0.15 M NaCl, 0.01 M $CaCl_2$, 1.5 mM NaN_3, 0.005% Triton X-100, and 0.1 nM $ZnCl_2$). Subsequently all samples underwent protein extraction for two days at 4 °C, followed by centrifugation for 30 minutes at 10,000× g. The supernatants were removed and frozen at -20 °C. Specimen P5-1 (Group III) was used only for immunohistochemistry and the MMP-1 activity assay couldn't be done in specimen P0-3 (Group I) due to a lack of material. The Thermo Scientific Pierce BCA Protein Assay (Pierce Biotechnology Inc., Rockford, IL, USA) was used for the colorimetric detection and quantitation of total protein in all of our specimens.

3.3 Indirect Enzyme Immunohistochemistry

A seven-μm thick cryosections from each of the OCT-embedded control and melted specimens were placed on gelatin-coated glass slides and stored at -20°C. The slides were defrosted, fixed with cold acetone and re-hydrated in phosphate-buffered saline (PBS), both for 10 minutes. Endogenous peroxidase was blocked by a 30-minute incubation in 3% hydrogen peroxide in PBS. After washing in PBS the specimens were blocked for 30 minutes with 2.5% bovine serum albumin in PBS. The sections were subsequently immunolabeled (1 hour at room temperature) with primary antibodies (polyclonal rabbit anti-human MMP-1 and MMP-8, monoclonal mouse anti-human MMP-2, MMP-7, MMP-9 and MMP-13 all from Chemicon Intl. Inc, Temecula, CA, USA and polyclonal rabbit anti-human MMP-3 from AnaSpec Inc., San Jose, CA, USA). Subsequently, the slides were washed in PBS and the secondary antibodies (polyclonal rabbit anti-mouse IgG and swine anti-rabbit IgG conjugated with biotin; DakoCytomation, Glostrup, Denmark) were applied for 1 hour. After rinsing in PBS, streptavidin/horseradish peroxidase (DakoCytomation, Glostrup, Denmark) was added for 30 minutes. Finally, the slides were developed with 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in PBS, counterstained with Harris hematoxylin, and mounted with Eukit (Fluka, Buchs, Switzerland). One section on each slide, where the primary antibody was omitted, served as a negative control. The intensity of the signal was assessed separately in the epithelium, anterior stroma, posterior stroma, and endothelium using five grades of positivity: 0 (negative), 1 (weak), 2 (moderate), 3 (intense),

4 (very intense). The mean average positivity was calculated from at least three sections of two independent experiments.

3.4 Gelatin and Casein Substrate Zymography

Gelatin and casein zymography were used for the detection of MMP-2 and -9, and MMP-3 and -7, respectively. All specimens (native cellular protein quantity, 8.5 μg) were treated with a sample buffer (1.5% sodium dodecyl sulfate - SDS, 15% glycerol, and 0.005% bromphenol blue). Both zymographies were carried out as described previously [77] with slight modifications. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% SDS-polyacrylamide gel containing 0.1% gelatin (AppliChem GmbH, Darmstadt, Germany) or a 12% gel containing 0.09% casein (Sigma-Aldrich, St. Louis, MO, USA). In brief, SDS-PAGE was performed for 60 minutes at 200 V at 4 °C for gelatin zymography, and for 90 minutes at 20 mA at 4 °C for casein zymography. After rinsing twice in 2.5% Triton X-100 for 30 minutes at room temperature, the gels were incubated in a reaction buffer (50 mM Tris-HCl, pH 7.5; 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% 23 lauryl ether - Brij-35) at 37 °C overnight. The gels were stained for 1 hour at room temperature in 0.5% Coomassie brilliant blue R-250 (Serva Electrophoresis, Heidelberg, Germany) in 40% methanol and 10% acetic acid, and then destained with a mixture of 40% methanol and 10% acetic acid. Proteolytic activities appeared as clear bands of lysis against a dark background of stained gelatin or casein. All experiments were performed in duplicate. To confirm that the bands were MMPs, separate gels were treated overnight with a buffer lacking calcium and containing 20 mM ethylenediaminetetraacetic acid (EDTA), and afterwards processed as described above.

3.5 Matrix Metalloproteinase-1 and -3 Activity Assay

The concentrations of the active forms of MMP-1 and -3 were determined using a commercial kit (Amersham matrix metalloproteinase-1 and Amersham matrix metalloproteinase-3 Biotrak Activity assay system, Amersham Biosciences, Amersham, UK) according to the manufacturer's protocol and as described previously [78]. The values of the color reaction of the assays were read at 405 nm on a SUNRISE ELISA Reader (Tecan Trading AG, Männedorf, Switzerland). MMP-1 and -3 were determined in samples by interpolation from the standard curve. The MMP-1 activity assay could not be performed in P5-1 (Group III) and P0-3 (Group I), and concentrations of the active MMP-3 was measured only in samples of Groups II and III due to a lack of sufficient quantities of the specimens. Owing to the limited amount of all sample material available, we performed this experiment only once.

3.6 Statistical Analysis

The Mann-Whitney U test was used to analyze the differences between the control and the experimental groups. A p value <0.05 was considered to indicate a statistical significance.

4 Results

4.1 Localization of Matrix Metalloproteinases in Melted and Control Corneas

All control corneas exhibited regular morphology, with a five- to six-layer epithelium, stroma of normal thickness, an unaffected Descemet's membrane and a monolayer endothelium. Severe damage was observed in most of the pathological specimens including the complete absence of the epithelial layers and, in some specimens, a partly dissolved Bowman's layer and a partly destroyed edge of the anterior stroma in the area of the lesions or throughout the whole specimen. Also the endothelium was destroyed in most melted corneas from all three groups.

In controls, moderate staining for MMP-1 and -2, and weak staining for MMP-8, were detected in the epithelium. A weak signal for MMP-2 was observed in the anterior stroma. The endothelium revealed weak staining for MMP-1 and -8 (Fig. 1). Staining intensities of particular MMPs were averaged as no prominent differences in MMP staining were found among the individual control specimens for any of the MMPs tested (Tab. 1).

Staining intensity for MMP-1 and -9 was increased in the corneal epithelium and the stroma of all patient groups. Immunostaining for MMP-2, -3, -7 and -8 was elevated in both these corneal layers in almost all melted specimens (74 - 96%) from all three groups, in contrast to the controls. In the endothelium, immunostaining for MMP-2 was increased in almost all specimens of groups I, II and III (55, 71 and 60%), respectively. MMP-9 staining was elevated in the endothelium of specimens from group I (60%). In all pathologic samples pronounced differences in the staining pattern of individual MMPs were observed. The staining intensity of individual MMPs in melted specimens from all three patient groups is summarized in table 1, and representative pictures of some melted corneas are shown in figure 1. The influence of the underlying diseases on the localization and staining intensity of individual MMPs seems to be rather negligible, as no trend indicating the increased presence of some unique combination of MMPs in samples obtained from individual groups was shown.

Table 1. The immunohistochemical staining intensities of individual MMPs in the corneal specimens from all three groups and their average values in all controls (C).

MMP	CL	Group I											Group II							Group III					C
		P0-1	P0-2	P0-3	P0-4	P0-5	P0-6	P0-7	P0-8	P0-9	P0-10	P0-11	P1-1	P1-2	P1-3	P2-1	P2-2	P2-3	P3	P4-1	P4-2	P4-3	P5-1	P5-2	
MMP 1	**Ep**	4	4	D	D	D	2	D	4	4	D	D	4	3	D	4	3	4	4	3	4	4	3	3	2
	AS	3	4	3	2	3	1	4	4	4	2	3	4	2	3	4	3	3	4	2	4	3	2	4	0
	PS	2	3	2	2	4	2	3	4	4	1	3	3	2	0	4	0	2	4	1	4	2	0	4	0
	En	0	3	D	D	0	0	D	D	2	D	D	3	D	0	3	0	1	D	0	4	3	0	D	1
MMP 2	**Ep**	3	3	D	D	D	4	3	2	4	D	D	1	D	D	4	3	3	3	1	4	3	4	D	2
	AS	4	2	2	0	4	2	2	3	3	2	3	3	3	2	4	3	3	4	0	2	2	4	2	1
	PS	3	2	1	0	1	0	0	3	0	2	1	1	2	0	2	0	1	3	0	2	0	1	1	0
	En	2	1	0	0	3	2	0	D	1	0	0	3	D	0	2	2	2	2	0	2	2	1	0	0
MMP 3	**Ep**	2	2	D	D	D	2	0	2	3	2	3	1	D	D	3	3	1	4	3	3	2	3	3	0
	AS	2	2	1	0	2	1	4	2	4	0	2	2	2	4	3	2	0	3	0	2	2	0	3	0
	PS	1	0	2	0	2	1	4	2	4	0	2	1	1	0	2	0	0	3	0	2	1	0	2	0
	En	D	0	0	0	1	0	D	D	2	D	D	D	0	0	1	0	0	0	0	D	1	D	3	0
MMP 7	**Ep**	0	4	D	D	D	4	3	4	0	D	4	3	D	D	3	4	1	4	3	2	0	4	3	0
	AS	0	1	2	0	3	2	1	3	0	0	2	2	2	3	2	1	0	1	0	2	1	1	2	0
	PS	0	1	2	0	3	1	1	3	0	0	2	1	0	0	2	0	0	1	0	2	1	0	2	0
	En	D	D	D	0	2	1	D	D	0	D	D	D	0	0	1	0	0	0	0	2	0	D	0	0
MMP 8	**Ep**	2	4	D	D	D	1	0	2	2	D	D	3	D	D	4	1	3	1	1	4	3	3	1	1
	AS	2	1	1	2	3	2	0	2	1	2	4	3	0	1	3	1	0	1	0	3	3	2	1	0
	PS	0	1	0	2	3	1	0	2	1	1	4	1	0	0	2	0	0	1	0	1	1	1	0	0
	En	D	D	0	D	2	0	0	D	2	0	D	D	1	0	2	0	0	0	0	D	0	D	0	1
MMP 9	**Ep**	2	4	D	D	D	1	2	2	4	D	3	4	2	D	4	D	2	1	2	4	4	3	2	0
	AS	3	3	1	3	4	4	2	3	1	2	3	4	4	3	3	D	3	3	1	3	3	3	2	0
	PS	1	3	0	1	2	3	2	3	1	1	4	1	1	1	2	4	0	2	0	1	3	0	1	0
	En	4	2	D	1	2	1	D	D	1	D	0	D	D	0	3	0	0	0	0	1	0	D	0	0
MMP 13	**Ep**	0	0	D	D	D	1	0	1	0	D	D	0	D	D	1	0	0	0	0	0	0	0	D	0
	AS	0	0	1	0	2	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
	PS	0	0	1	0	2	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	En	D	0	0	0	1	0	D	D	0	0	D	0	0	0	1	0	0	0	0	0	0	D	0	0

The scale used for the intensity of the signal: 0 - negative, 1 - weak, 2 - moderate, 3 - intense, 4 - very intense positivity. CL = corneal layer, D = destroyed tissue, Ep = epithelium, AS = anterior stroma, PS = posterior stroma and En = endothelium.

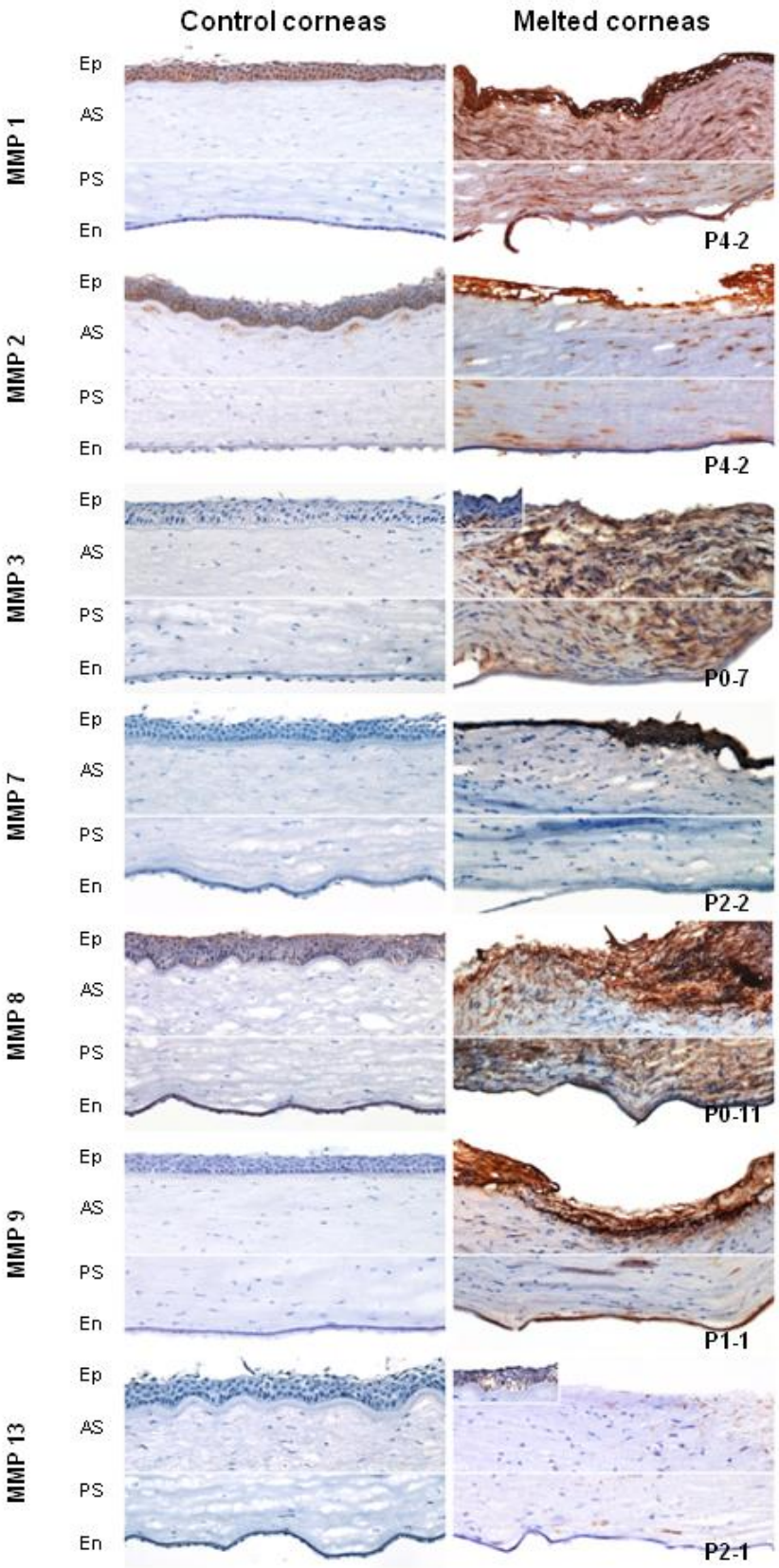

Figure 1. Immunostaining of MMP-1, -2, -3, -7, -8, -9 and 13 in representative samples of melted corneas obtained from patients from groups I, II and III and controls.

Original magnification, 100×.

Ep = epithelium,

AS = anterior stroma,

PS = posterior stroma,

En = endothelium.

4.2 Detection of Matrix Metalloproteinase-2 and -9 Activities

Control samples revealed a moderate level of the partly cleaved MMP-2 proenzyme (67 kDa band) in all specimens, whereas the active form (62 kDa band) was either not present or very faint, except in samples C1, C3, and C11. In group I, melted specimens P0-1 and P0-2 showed extremely high levels of both partly cleaved proenzyme and the active form of MMP-2. Specimens P0-7, P0-9, and P0-11 revealed distinct levels of both forms of this enzyme and other samples displayed either no, or low levels of, both forms of this enzyme. In group II, prominent bands and faint bands of both the partly cleaved proenzyme and the active MMP-2 were detected in four (P1-1, P2-1, P2-2, P3) and one (P1-3) melted corneas, respectively. Two samples showed the presence of the active form only (P1-2 and P2-3). The sample P4-3 and specimens P4-1, P5-2 from group III exhibited prominent bands and faint bands of both MMP-2 forms (the partly cleaved proenzyme and the active enzyme), respectively. The graft P4-2 exhibited a prominent band of the inactive and a faint band of active MMP-2.

The presence of MMP-9 in control corneas was not shown, except for faint bands of MMP-9 proenzyme (95 kDa band) in samples C3 and C7. Levels of MMP-9 proenzyme and the active form (82 kDa band) were extremely high in seven (P0-4, P0-6 - P0-11), and prominent in one (P0-5), samples of group I. Three melted specimens (P0-1 - P0-3) showed weak bands for both MMP-9 forms. In group II, markedly elevated levels either of both forms of MMP-9, or of only the active form, were observed in five melted specimens (P1-1, P1-2, P1-3, P2-2, P2-3) and two melted corneas (P2-1 and P3), respectively. Increased levels and low levels of both MMP-9 forms were detected in three (P4-1, P4-2, P4-3) and one (P5-2) melted specimens of group III, respectively.

4.3 Detection of Matrix Metalloproteinase-3 and -7 Activities

Casein zymography revealed neither the proenzyme (60 kDa) nor the active enzyme of MMP-3 (47 kDa) in any of the control specimens. Negligible levels of the pro-MMP-3 were found in five melted specimens (P0-1, P0-2, P0-6, P0-7, and P0-9), and levels of both the proenzyme and active MMP-3 were found in specimen P0-5 of patients from group I. Three melted corneal samples (P1-1, P1-2, and P2-3) of group II and two melted corneal samples (P4-2, P4-3) of group III exhibited low levels of the MMP-3 proenzyme.

No form of MMP-7 was observed in any of the control specimens. Dense bands and faint bands migrating at 30 kDa and 28 kDa, corresponding to the proenzyme of MMP-7 and its intermediate cleavage product in nine (P0-2 - P0-10) and two melted corneas (P0-1 and P0-11) of group I, were observed, respectively. A very slight band of the active MMP-7 (20 kDa)

was found in specimen P0-7 only. High levels of inactive MMP-7 forms (intermediate cleavage products - 28 and 25 kDa) were found in five (P1-1, P1-2, P1-3, P2-2, P2-3) melted corneas of group II, whereas the sample P2-1 of group II, and all four tested pathologic samples of group III (P4-1, P4-2, P4-3, and P5-2), exhibited faint bands of these two MMP-7 forms.

4.4 Evaluation of Active Matrix Metalloproteinase-1 and -3 Concentration

The active form of MMP-1 was not found in any control corneas. In group I, the active MMP-1 was found in eight of ten melted corneas ($p=0.0011$). Significantly higher MMP-1 activity was also shown in all melted corneas from group II and three of four tested pathologic samples from group III compared to controls (p-value < 0.001). Specimens P0-3 and P5-1 couldn't be evaluated due to limited amounts of available tissue.

The concentration of active MMP-3 in the melted specimens of groups II and III was significantly higher (range, 12.6-106.22, p-value < 0.001) than in controls, ten of which displayed low levels of the active MMP-3 (range, 0-32.4 ng/ml). Group I was not included in the MMP-3 activity assay evaluation due to insufficient amounts of the sample.

5 Discussion

In this work, which summarizes results about different MMP expression and activity in healthy and melted corneas or grafts, we have attempted to illustrate the exact contribution of individual MMPs (MMP-1, -2, -3, -7, -8, -9, and 13) in the corneal melting process in individual corneal layers.

5.1 Matrix Metalloproteinases in Process of Corneal Melting

Although the expression of some MMPs during corneal melting associated with various pathologies (RA, cataract surgery treated with NSAIDs) has already been reported [62, 63, 65, 68], the exact mechanism of corneal melting and MMP participation in this process are yet to be satisfactorily described.

The process of keratolysis usually precedes epithelial defect formation (see above), which is subsequently followed by epithelial basement membrane and stromal degradation [79]. The failure to re-epithelialize, the delay in basement membrane repair and the degradation of stromal components are thought to be caused by the proteolytic activity of MMPs [62, 63, 65, 80]. Gelatinases, the presence of which in melted corneas has been shown in this study as well as in previously reported works [62, 65], may play an important role in the first step of this process. Strong activity as well as immunostaing of MMP-9 in almost all samples of all three patient groups clearly corresponds to a recently reported hypothesis that MMP-9 participates in the degradation of the corneal basement membrane during the process of corneal melting [79]. MMP-2 is involved in the attempts of the melted tissue to regenerate as its synthesis is delayed and limited to a short time period [79]. This could explain the variable MMP-2 activity detected in our melted corneas, most probably obtained in different stages of melting. Discrepancies among several studies reporting on MMP-2 expression in ulcerated corneas also support this theory [62, 65, 66, 81]. Other possible candidates capable of cleaving components of the epithelial basement membrane during corneal melting are MMP-3 and -7 [39, 52]. Our study revealed the presence of MMP-3 in the epithelium of melted corneas and, moreover, a significant increase in MMP-3 activity in groups II and III compared to control corneas. Unfortunately, specimens in group I could not be tested by MMP-3 activity assay due to insufficient amounts of the material. The casein zymography did not confirm results of MMP-3 activity assay in specimens from groups II and III, most probably due to the lower sensitivity of casein zymography for this enzyme [82]. Discrepancies in the MMP-3 activity results, as well as between MMP-3 activity and the immunostaining, may also be caused by

the possible cross-reactivity of MMP-3 with MMP-10. As these two MMPs have almost identical substrate specificities [39], an activity assay may recognize both the active MMP-3 and -10. To the best of our knowledge, expression of MMP-7 in melted corneas has not been previously shown by other researchers. Although we detected the active form of MMP-7 in only one specimen from group I, we found a large quantity of MMP-7 proenzyme and its partially activated form [83] in almost all specimens of all three groups.

As collagens, especially collagen types I and III, are the main ECM elements composing the Bowman's layer and corneal stroma [53], collagenases are the likeliest candidates for degradation of these layers during corneal melting. Our study confirmed the previously found[63] high level of MMP-1 in the melted corneas, and, moreover, demonstrated a statistically significantly higher MMP-1 activity. However, the concentration of active MMP-1 in the melted specimens was lower than expected based on immunostaining results. This could be explained by the presence of some TIMPs or other protein inhibitors (a2-macroglobulin, etc.), which are physiologically expressed in corneal tissue [84, 85].

Perceptible differences in immunostaining for MMP-8 in the stroma of all melted specimens could be explained by the fact that this enzyme is mainly produced by neutrophils, the distribution of which in corneal stroma may vary among melted corneas [14, 39, 62, 86]. The presence of MMP-8 in the corneal stroma in areas of leukocyte infiltration was found in some of our specimens from group I (data not shown), as well as having been previously described in ulcerative keratolysis [62]. As far as we understand, the presence of MMP-13 is still unknown. We found a negligible level of this collagenase in the stroma of some samples from all three groups. This could be explained by the fact that MMP-13 preferably cleaves collagen type II, which is not present in any corneal layer [53]. Gelatinases represent another group of collagenolytic enzymes capable of cleaving collagen type V, VI and denatured collagens present in corneal stroma [52, 58, 59]. Except for collagens, it digests the stromal core protein decorin [56]. Our study demonstrated strong expression of both gelatinases in the stroma, as well as a high level of active MMP-9 in almost all tested specimens from all three groups. In addition, the MMP-3 and -7, present in the stroma of almost all specimens from all three groups, may also assist in the degradation of corneal stroma due to their effectiveness at degrading some stromal proteoglycans [52, 87, 88], laminin and fibronectin [52, 57]. Moreover MMP-3 cleaves procollagen peptides and collagen type V [39, 41, 52].

Finally, once the stroma is completely lost, a descemetocele is formed. As the Descemet's membrane is composed mainly of collagen IV and VIII, which are cleaved by MMP-2, -3, -7,

-9 and MMP-1, respectively, and because it's a very fragile layer, the rupture of this destructed cornea is very imminent [39, 89].

5.2 Variability of Matrix Metalloproteinases Expression in Individual Specimens

Some variability in the expression of individual MMPs was found among our specimens in all three groups. No trend towards an increase or decrease of individual MMP expression or activity was found over time, or of disease progression, in any of the consecutive patient samples. Instead, the level and activity of individual MMPs detected seemed rather to vary in each specimen. This phenomenon could have a number of causes such as the different stages of melting at which the explants were obtained or some heterogeneity in the expression of MMPs showing local variations (which depends on the distance from the focus of the melting) within individual specimens. As our specimens were cut into two halves for subsequent use in immunohistochemical staining and the activity assay, thus any variations found in the results could reflect the diversity of MMP distribution or the distance from the focus of the melting. On the other hand, the immunostaining of individual MMPs was similar for consecutive sections obtained from each cornea.

From the clinical course of corneal melting we know that the speed of corneal melting differs tremendously. In some patients with the same diagnosis, the cornea can melt and perforate within a few days whereas in others it can take weeks or even months. Different levels of MMP expression can therefore explain the very different speed of the process of keratolysis in individual cases which is clinically striking.

With respect to our results, it seems that the influence of the diverse underlying diseases on the expression of individual MMPs is negligible as we were not able to show any trend towards the increased expression of some unique combination of MMPs in samples from appropriate groups.

Discrepancies in results from immunostaining and the activity of some MMPs could be explained by another reason, namely the different levels of the active and inactive forms of individual MMPs. The antibodies used for immunohistochemistry do not distinguish between the zymogen and active MMPs, in contrast to the activity assays. In regards to the methods used, we cannot say that the level of the active enzyme is directly proportional to the level of the inactive form of the same enzyme in each specimen. The ratio between the two forms could be affected by the presence of MMP inhibitors and the quantitative differences between their samples. Therefore, the staining results do not have to necessarily correlate with the results from the activity assay or zymography.

5.3 Future Possible Treatment of Corneal Melting

Current management of keratolysis includes several surgical techniques (the application of tissue adhesives, amniotic membrane transplantation, penetrating keratoplasty) used according to the stage of this destructive process [17, 22]. However, in severe cases of keratolysis the treatment is rather unsuccessful [16, 21, 28, 63].

None of the disease-modifying therapies used in our patients were effective in regulating MMP production and corneal melting. According to our results, novel treatment strategies intent on the inhibition of MMP activity or expression should be considered in similar cases. Agents inhibiting MMP activity, such as recombinant TIMPs or another synthetic matrix metalloproteinase inhibitor (Galardin), were previously successfully used to treat ulcerated rabbit corneas [19, 90]. The direct inhibition of MMP activity can also be achieved by ion-chelating agents such as cysteine or EDTA, and by tetracyclines, which also have chelating properties and furthermore inhibit phagocytosis [91-93]. From agents known to inhibit MMP expression, TNF-α antagonist infliximab has been shown to have a beneficial effect in the treatment of progressive RA-associated corneal ulceration [94]. Infliximab inhibits TNF-α which is known to activate MMP production [39, 72, 94]. Finally, an alternative method of keratolysis management could be focused on the recovery and strengthening of the stromal collagen structure by collagen cross-linking. Corneal cross linking is used to treat initial stages of keratoconus; however, some success in the treatment of several cases of refractive corneal melting has already been reported [95, 96].

6 Conclusions and Future Work

In this study, we present three groups of patients suffering from pSS (group I), RA (group II) and patients with other underlying pathologies (group III), who underwent rapidly progressive recurrent corneal melting despite receiving all available treatment. We obtained 23 tissue specimens in total from all of the seven patients investigated (five melted native corneas and 18 corneal grafts failed due to corneal melting).

Our findings clearly show which members of the large MMP family participate in corneal melting and in which corneal layer they mainly take effect. We confirmed partial results of other studies supposing the role of gelatinases (MMP-2 and-9), collagenases (MMP-1 and -8) and stromelysin MMP-3 [62, 63, 65, 68] in corneal melting; but moreover, this is the first comprehensive study describing the presence of all the main MMPs. In addition it shows the presence of matrilysin MMP-7 and a negligible level of collagenase MMP-13 in melted corneas [97, 98]. To the best of our knowledge, we are the first to determine the activity of MMP-1, -3 and -7 in melted corneal specimens [97, 98].

Although we used specimens from patients with different underlying diseases, in whom the exact trigger mechanisms of corneal melting may vary, the final progress of corneal degradation concerning MMP expression and activation was shown to be more or less similar in all the patients' specimens. These findings are in compliance with the complexity of keratolysis and implicate the participation of all the main MMPs in this process.

Even though we showed the expression of a wide spectrum of MMPs in melted and normal corneas, there are still other MMPs which may have an important influence on corneal melting but which have still not been studied. MMP-10 (stromelysin-2) is a good candidate for our future work focusing on this topic because of its similarity to MMP-3 and its similar substrate specifity, which probably cause discrepancies between the MMP-3 activity results as well as differences between MMP-3 activity and the immunostaining.

Despite the research which we performed, the disease-initiating mechanisms are still unclear and need to be clarified. Our future work will mainly be focused on the elucidating of immune-mediated initiation of this destructive process. Since the TIMPs and other MMP inhibitors and the regulation of their expression may have an important influence on corneal melting, their investigation is also one point included in our future plans.

7 References

1. Nishida T. Cornea. second edition ed. Philadephia, Edinburgh, London, New york, Oxford, St Luis. Sydney, Toronto: Elsevier Inc.; 2005:1409.
2. Verdier DD. Penetrating Keratoplasty. second ed. Philadephia: Elsevier Mosby; 2005:2087.
3. Aggarwal RK. Deep lamellar keratoplasty--an alternative to penetrating keratoplasty. Br J Ophthalmol 1997;81:178-179.
4. Report of the organ transplant panel. Corneal transplantation. Council on Scientific Affairs. Jama 1988;259:719-722.
5. Arentsen JJ. Corneal transplant allograft reaction: possible predisposing factors. Trans Am Ophthalmol Soc 1983;81:361-402.
6. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group. Ophthalmology 1994;101:1536-1547.
7. Beauregard C, Huq SO, Barabino S, Zhang Q, Kazlauskas A, Dana MR. Keratocyte apoptosis and failure of corneal allografts. Transplantation 2006;81:1577-1582.
8. Joseph A, Raj D, Shanmuganathan V, Powell RJ, Dua HS. Tacrolimus immunosuppression in high-risk corneal grafts. Br J Ophthalmol 2007;91:51-55.
9. Boisjoly HM, Tourigny R, Bazin R, et al. Risk factors of corneal graft failure. Ophthalmology 1993;100:1728-1735.
10. MacEwen CJ, Khan ZU, Anderson E, MacEwen CG. Corneal re-graft: indications and outcome. Ophthalmic Surg 1988;19:706-712.
11. Patel NP, Kim T, Rapuano CJ, Cohen EJ, Laibson PR. Indications for and outcomes of repeat penetrating keratoplasty, 1989-1995. Ophthalmology 2000;107:719-724.
12. Williams KA, Roder D, Esterman A, Muehlberg SM, Coster DJ. Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry. Ophthalmology 1992;99:403-414.
13. Inoue K, Amano S, Oshika T, Sawa M, Tsuru T. A 10-year review of penetrating keratoplasty. Jpn J Ophthalmol 2000;44:139-145.
14. Kuffova L, Holan V, Lumsden L, Forrester JV, Filipec M. Cell subpopulations in failed human corneal grafts. Br J Ophthalmol 1999;83:1364-1369.
15. Malik R, Culinane AB, Tole DM, Cook SD. Rheumatoid keratolysis: a series of 40 eyes. Eur J Ophthalmol 2006;16:791-797.
16. Palay DA, Stulting RD, Waring GO, 3rd, Wilson LA. Penetrating keratoplasty in patients with rheumatoid arthritis. Ophthalmology 1992;99:622-627.
17. Perez VL, Azar DT, Foster CS. Sterile corneal melting and necrotizing scleritis after cataract surgery in patients with rheumatoid arthritis and collagen vascular disease. Semin Ophthalmol 2002;17:124-130.
18. Pleyer U, Bertelmann E, Rieck P, Hartmann C. Outcome of penetrating keratoplasty in rheumatoid arthritis. Ophthalmologica 2002;216:249-255.
19. Barletta JP, Angella G, Balch KC, et al. Inhibition of pseudomonal ulceration in rabbit corneas by a synthetic matrix metalloproteinase inhibitor. Invest Ophthalmol Vis Sci 1996;37:20-28.
20. McElvanney AM. Doxycycline in the management of pseudomonas corneal melting: two case reports and a review of the literature. Eye Contact Lens 2003;29:258-261.
21. Bernauer W, Ficker LA, Watson PG, Dart JK. The management of corneal perforations associated with rheumatoid arthritis. An analysis of 32 eyes. Ophthalmology 1995;102:1325-1337.
22. Donzis PB, Mondino BJ. Management of noninfectious corneal ulcers. Surv Ophthalmol 1987;32:94-110.

23. Vivino FB, Minerva P, Huang CH, Orlin SE. Corneal melt as the initial presentation of primary Sjogren's syndrome. J Rheumatol 2001;28:379-382.

24. Joseph A, Dua HS, King AJ. Failure of amniotic membrane transplantation in the treatment of acute ocular burns. Br J Ophthalmol 2001;85:1065-1069.

25. Liu H, Zhang W, Pan Z, Wu Y. [Experimental study on the treatment of corneal melting after alkali burn with GM 6001]. Zhonghua Yan Ke Za Zhi 2002;38:539-542.

26. Vajpayee RB, Vanathi M, Tandon R, Sharma N, Titiyal JS. Keratoplasty for keratomalacia in preschool children. Br J Ophthalmol 2003;87:538-542.

27. Hagen KB, Waring GO, 3rd, Johnson-Wint B. Progressive nonulcerative paracentral keratolysis associated with elevated corneal metalloproteinases. Cornea 1997;16:486-492.

28. Solomon A, Meller D, Prabhasawat P, et al. Amniotic membrane grafts for nontraumatic corneal perforations, descemetoceles, and deep ulcers. Ophthalmology 2002;109:694-703.

29. Turesson C, O'Fallon WM, Crowson CS, Gabriel SE, Matteson EL. Extra-articular disease manifestations in rheumatoid arthritis: incidence trends and risk factors over 46 years. Ann Rheum Dis 2003;62:722-727.

30. Hamideh F, Prete PE. Ophthalmologic manifestations of rheumatic diseases. Semin Arthritis Rheum 2001;30:217-241.

31. Garcia-Carrasco M, Fuentes-Alexandro S, Escarcega RO, Salgado G, Riebeling C, Cervera R. Pathophysiology of Sjogren's syndrome. Arch Med Res 2006;37:921-932.

32. Fox PC. Autoimmune diseases and Sjogren's syndrome: an autoimmune exocrinopathy. Ann N Y Acad Sci 2007;1098:15-21.

33. Rehman HU. Sjogren's syndrome. Yonsei Med J 2003;44:947-954.

34. Fox RI, Michelson P, Casiano CA, Hayashi J, Stern M. Sjogren's syndrome. Clin Dermatol 2000;18:589-600.

35. Hansen A, Lipsky PE, Dorner T. Immunopathogenesis of primary Sjogren's syndrome: implications for disease management and therapy. Curr Opin Rheumatol 2005;17:558-565.

36. Haga HJ, Peen E. A study of the arthritis pattern in primary Sjogren's syndrome. Clin Exp Rheumatol 2007;25:88-91.

37. Kaplan MJ, Ike RW. The liver is a common non-exocrine target in primary Sjogren's syndrome: a retrospective review. BMC Gastroenterol 2002;2:21.

38. Muller K, Oxholm P, Mier-Madsen M, Wiik A. Circulating IgA- and IgM-rheumatoid factors in patients with primary Sjogren syndrome. Correlation to extraglandular manifestations. Scand J Rheumatol 1989;18:29-31.

39. Birkedal-Hansen H, Moore WG, Bodden MK, et al. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993;4:197-250.

40. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. Amino Acids.

41. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827-839.

42. Hadler-Olsen E, Fadnes B, Sylte I, Uhlin-Hansen L, Winberg JO. Regulation of matrix metalloproteinase activity in health and disease. Febs J 278:28-45.

43. Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. Faseb J 1998;12:1075-1095.

44. Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr 1997;7:159-178.

45. Gomis-Ruth FX, Maskos K, Betz M, et al. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature 1997;389:77-81.

46. Murphy G, Nguyen Q, Cockett MI, et al. Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. J Biol Chem 1994;269:6632-6636.

47. Puente XS, Pendas AM, Llano E, Velasco G, Lopez-Otin C. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. Cancer Res 1996;56:944-949.

48. Sato H, Kinoshita T, Takino T, Nakayama K, Seiki M. Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. FEBS Lett 1996;393:101-104.

49. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529-543.

50. Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol 2007;26:587-596.

51. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999;274:21491-21494.

52. Cawston TE. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Pharmacol Ther 1996;70:163-182.

53. Marshall GE, Konstas AG, Lee WR. Immunogold fine structural localization of extracellular matrix components in aged human cornea. I. Types I-IV collagen and laminin. Graefes Arch Clin Exp Ophthalmol 1991;229:157-163.

54. Funderburgh JL, Hevelone ND, Roth MR, et al. Decorin and biglycan of normal and pathologic human corneas. Invest Ophthalmol Vis Sci 1998;39:1957-1964.

55. Giannelli G, Pozzi A, Stetler-Stevenson WG, Gardner HA, Quaranta V. Expression of matrix metalloprotease-2-cleaved laminin-5 in breast remodeling stimulated by sex steroids. Am J Pathol 1999;154:1193-1201.

56. Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y. Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochem J 1997;322 (Pt 3):809-814.

57. Ljubimov AV, Burgeson RE, Butkowski RJ, Michael AF, Sun TT, Kenney MC. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab Invest 1995;72:461-473.

58. Marshall GE, Konstas AG, Lee WR. Immunogold fine structural localization of extracellular matrix components in aged human cornea. II. Collagen types V and VI. Graefes Arch Clin Exp Ophthalmol 1991;229:164-171.

59. Myint E, Brown DJ, Ljubimov AV, Kyaw M, Kenney MC. Cleavage of human corneal type VI collagen alpha 3 chain by matrix metalloproteinase-2. Cornea 1996;15:490-496.

60. Fini ME, Yue BY, Sugar J. Collagenolytic/gelatinolytic metalloproteinases in normal and keratoconus corneas. Curr Eye Res 1992;11:849-862.

61. Mackiewicz Z, Maatta M, Stenman M, Konttinen L, Tervo T, Konttinen YT. Collagenolytic proteinases in keratoconus. Cornea 2006;25:603-610.

62. O'Brien TP, Li QJ, Sauerburger F, Reviglio VE, Rana T, Ashraf MF. The role of matrix metalloproteinases in ulcerative keratolysis associated with perioperative diclofenac use. Ophthalmology 2001;108:656-659.

63. Riley GP, Harrall RL, Watson PG, Cawston TE, Hazleman BL. Collagenase (MMP-1) and TIMP-1 in destructive corneal disease associated with rheumatoid arthritis. Eye (Lond) 1995;9 (Pt 6):703-718.

64. Imai K, Yokohama Y, Nakanishi I, et al. Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J Biol Chem 1995;270:6691-6697.

65. Gabison EE, Chastang P, Menashi S, et al. Late corneal perforation after photorefractive keratectomy associated with topical diclofenac: involvement of matrix metalloproteinases. Ophthalmology 2003;110:1626-1631.

66. Ollivier FJ, Gilger BC, Barrie KP, et al. Proteinases of the cornea and preocular tear film. Vet Ophthalmol 2007;10:199-206.

67. Geerling G, Joussen AM, Daniels JT, Mulholland B, Khaw PT, Dart JK. Matrix metalloproteinases in sterile corneal melts. Ann N Y Acad Sci 1999;878:571-574.

68. Hargrave SL, Jung JC, Fini ME, et al. Possible role of the vitamin E solubilizer in topical diclofenac on matrix metalloproteinase expression in corneal melting: an analysis of postoperative keratolysis. Ophthalmology 2002;109:343-350.

69. Kervick GN, Pflugfelder SC, Haimovici R, Brown H, Tozman E, Yee R. Paracentral rheumatoid corneal ulceration. Clinical features and cyclosporine therapy. Ophthalmology 1992;99:80-88.

70. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. Invest Ophthalmol Vis Sci 2001;42:2283-2292.

71. Prada J, Noelle B, Baatz H, Hartmann C, Pleyer U. Tumour necrosis factor alpha and interleukin 6 gene expression in keratocytes from patients with rheumatoid corneal ulcerations. Br J Ophthalmol 2003;87:548-550.

72. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. Invest Ophthalmol Vis Sci 2004;45:4293-4301.

73. Webster NL, Crowe SM. Matrix metalloproteinases, their production by monocytes and macrophages and their potential role in HIV-related diseases. J Leukoc Biol 2006;80:1052-1066.

74. Corrales RM, Stern ME, De Paiva CS, Welch J, Li DQ, Pflugfelder SC. Desiccating stress stimulates expression of matrix metalloproteinases by the corneal epithelium. Invest Ophthalmol Vis Sci 2006;47:3293-3302.

75. Lemp MA. Advances in understanding and managing dry eye disease. Am J Ophthalmol 2008;146:350-356.

76. Vajtr D, Kukacka J, Adamek T. [Findings in myocardial extracellular matrix in fatal poisoning with addictive substances]. Soud Lek 2002;47:54-58.

77. Mesa C, Jr., Mirza M, Mitsutake N, et al. Conditional activation of RET/PTC3 and BRAFV600E in thyroid cells is associated with gene expression profiles that predict a preferential role of BRAF in extracellular matrix remodeling. Cancer Res 2006;66:6521-6529.

78. Li DQ, Shang TY, Kim HS, Solomon A, Lokeshwar BL, Pflugfelder SC. Regulated expression of collagenases MMP-1, -8, and -13 and stromelysins MMP-3, -10, and -11 by human corneal epithelial cells. Invest Ophthalmol Vis Sci 2003;44:2928-2936.

79. Matsubara M, Zieske JD, Fini ME. Mechanism of basement membrane dissolution preceding corneal ulceration. Invest Ophthalmol Vis Sci 1991;32:3221-3237.

80. Fini ME, Cook JR, Mohan R. Proteolytic mechanisms in corneal ulceration and repair. Arch Dermatol Res 1998;290 Suppl:S12-23.

81. Fini ME, Parks WC, Rinehart WB, et al. Role of matrix metalloproteinases in failure to re-epithelialize after corneal injury. Am J Pathol 1996;149:1287-1302.

82. Quesada AR, Barbacid MM, Mira E, Fernandez-Resa P, Marquez G, Aracil M. Evaluation of fluorometric and zymographic methods as activity assays for stromelysins and gelatinases. Clin Exp Metastasis 1997;15:26-32.

83. Yu WH, Woessner JF, Jr. Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7). J Biol Chem 2000;275:4183-4191.

84. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002;115:3719-3727.

85. Twining SS, Fukuchi T, Yue BY, Wilson PM, Zhou X, Loushin G. Alpha 2-macroglobulin is present in and synthesized by the cornea. Invest Ophthalmol Vis Sci 1994;35:3226-3233.

86. Hsu JK, Johnston WT, Read RW, et al. Histopathology of corneal melting associated with diclofenac use after refractive surgery. J Cataract Refract Surg 2003;29:250-256.

87. Akhtar S, Kerr BC, Hayes AJ, Hughes CE, Meek KM, Caterson B. Immunochemical localization of keratan sulfate proteoglycans in cornea, sclera, and limbus using a keratanase-generated neoepitope monoclonal antibody. Invest Ophthalmol Vis Sci 2008;49:2424-2431.

88. Murphy G, Cockett MI, Ward RV, Docherty AJ. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). Biochem J 1991;277 (Pt 1):277-279.

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

90. Paterson CA, Wells JG, Koklitis PA, Higgs GA, Docherty AJ. Recombinant tissue inhibitor of metalloproteinases type 1 suppresses alkali-burn-induced corneal ulceration in rabbits. Invest Ophthalmol Vis Sci 1994;35:677-684.

91. Brown SI, Weller CA. Collagenase inhibitors in prevention of ulcers of alkali-burned cornea. Arch Ophthalmol 1970;83:352-353.

92. Couture S, Doucet M, Moreau M, Carrier M. Topical effect of various agents on gelatinase activity in the tear film of normal dogs. Vet Ophthalmol 2006;9:157-164.

93. Seedor JA, Perry HD, McNamara TF, Golub LM, Buxton DF, Guthrie DS. Systemic tetracycline treatment of alkali-induced corneal ulceration in rabbits. Arch Ophthalmol 1987;105:268-271.

94. Thomas JW, Pflugfelder SC. Therapy of progressive rheumatoid arthritis-associated corneal ulceration with infliximab. Cornea 2005;24:742-744.

95. Koller T, Seiler T. [Therapeutic cross-linking of the cornea using riboflavin/UVA]. Klin Monbl Augenheilkd 2007;224:700-706.

96. Tomkins O, Garzozi HJ. Collagen cross-linking: Strengthening the unstable cornea. Clin Ophthalmol 2008;2:863-867.

97. Brejchova K, Liskova P, Cejkova J, Jirsova K. Role of matrix metalloproteinases in recurrent corneal melting. Exp Eye Res 90:583-590.

98. Brejchova K, Liskova P, Hrdlickova E, Filipec M, Jirsova K. Matrix metalloproteinases in recurrent corneal melting associated with primary Sjorgen's syndrome. Mol Vis 2009;15:2364-2372.

Web publications:

Wheeless, C.R., Nunley, J.A., Urbaniak, J.R., 1996e2009. Wheeless' Textbook of Orthopaedics. Duke Orthopaedics, Duke University Medical Center's Division of Orthopaedic Surgery, in Conjunction with Data Trace Internet Publishing, LLC, Durham, LC, USA. http://www.wheelessonline.com.

8 List of Author's Publications

8.1 Publications Supporting Dissertation Thesis

Brejchova K, Liskova P, Hrdlickova E, Filipec M, Jirsova K. Matrix metalloproteinases in recurrent corneal melting associated with primary Sjörgen's syndrome. Mol Vis. 2009 Nov;15:2364-2372. PMID: 19936308 (impact factor 2.464)

Brejchova K, Liskova P, Cejkova J, Jirsova K. Role of matrix metalloproteinases in recurrent corneal melting. Exp Eye Res. 2010 May;90(5):583-590. PMID: 20153319 (impact factor 2.651)

8.2 Other Publications

8.2.0 Publications with Impact Factor

Jirsova K, Juklova K, Vesela V, Filipec M. Morphological and immunocytochemical characterization of snake-like chromatin cells. *Histol Histopathol*. 2006 Apr;21(4):355-60. PMID: 16437380 (impact factor 2.194)

Jirsova K, Juklova K, Aref Alfakih, Filipec M. Presence of Snake-like Chromatin in Epithelial Cells of Keratoconjunctivitis Sicca Followed by a Large Number of Micronuclei. *Acta Cytol.* 2007 Jul-Aug;51(4):541-6. PMID: 17718118 (impact factor 0.835)

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 Jun;88(4):413-9. PMID: 19604163 (impact factor 2.138)

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 Feb 9;52(2):787-94. PMID: 20926822 (impact factor 3.528)

8.2.1 Publications without Impact Factor

Jirsová K, Hrdlicková E, Alfakih A, Juklová K, Filipec M, Faltus V, Veselá V. [The application of the autologous serum eye drops results in significant improvement of the conjunctival status in patients with the dry eye syndrome]. Cesk Slov Oftalmol. 2008 Mar;64(2):52-6. Czech. PMID: 18419102 (IF 0)

Katerina Jirsova, Gabriela Jechova and Kristyna Juklova. Snake-like chromatin is not a form of apoptosis. Kapitola v knize: Cell Apoptosis Research. Scott R. Erlich (editor), New York, Nova Science Publishers, 2007, 203-212.