Charles University in Prague

First Faculty of Medicine

The Biology and Pathology of the Cell

Mgr. Kristýna Brejchová

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.

Ph.D. thesis

Supervisor: Mgr. Kateřina Jirsová, Ph.D.

Second supervisor: Prof. MUDr. Martin Filipec, CSc

Prague 2011

DECLARATION

I, Kristyna Brejchova, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. I also confirm that the work presented hasn’t been used to achieve another or the same university degree.

I agree with the permanent archiving of the electronic version of my theses in the database system interuniversity project Theses.cz in order to allow permanent inspection of the resemblance of qualification work.

In Prague 27.6.2011

Kristýna Brejchová

Brejchová, Kristýna. Buněčná a molekulární charakterizace selhaných transplantátů lidské rohovky. Role matrix metaloproteináz při opakované keratolýze lidské rohovky. *[Cell and molecular characterization of failed corneal grafts. Role of matrix metalloproteinases in recurrent corneal melting.]*. Prague, 2011. p. 86, two appendices. Dissertation thesis (Ph.D.). Charles University in Prague, 1st Faculty of Medicine, Institute of Inherited Metabolic Disorders. Supervisor Jirsová, Kateřina, second supervisor Filipec, Martin.

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) those with rheumatoid arthritis and c) those with 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 groups 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 the controls. In contrast to the 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 in each 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 takes place.

Key words: corneal melting, corneal ulcer, matrix metalloproteinases, extracellular matrix, primary Sjögren's syndrome, rheumatoid arthritis

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.

Klíčová slova: keratolýza rohovky, rohovkový vřed, matrix metaloproteinázy, mezibuněčná hmota, primární Sjögrenův syndrom, revmatoidní artritida

ACKNOWLEDGEMENTS

I would like to thank all the people who have helped me to get to the point of submitting this thesis.
I have received a great amount of support from both of my supervisors Mgr. Kateřina Jirsová, Ph.D. and Prof. MUDr. Martin Filipec, CSc. for their scientific and critical guidance during my postgraduate studies. I must express my gratitude for the opportunity to work in the ever inspiring environment of the Institute of Inherited Metabolic Disorders and to show my appreciation to all my collaborators from the Laboratory of the Biology and Pathology of the Eye namely MUDr. Petra Lišková, M.D., Ph.D., MUDr. Viera Veselá, Mgr. Stanislava Merjavá, Šárka Kalašová and Mgr. Aleš Neuwirth for their interest in my work, and for creating a pleasant working atmosphere.

I would also like to express many thanks to the Departure of Ophthalmology, General Teaching Hospital for providing clinical figures (figures 3 and 5).

This thesis was elaborated within the postgraduate program for Biology and Pathology of the Cell at the Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University, Prague.

The work was funded by the research project 0021620806 of the Ministry of Education, Youth and Sports of the Czech Republic.

TABLE OF CONTENTS

DECLARATION 2
ABSTRACT 4
ABSTRAKT 5
ACKNOWLEDGEMENTS 6
TABLE OF CONTENTS 7
1 INTRODUCTION 8
1.1 The Human Cornea 8
1.2 Corneal Transplantation 11
1.3 The Immune privilege of corneal grafts and corneal graft failure 12
1.4 Corneal Melting 15
1.5 Matrix metalloproteinases – structure, substrates and presence in normal human cornea 17
1.6 Matrix Metalloproteinases in non-infectious corneal melting 21
1.7 Induction mechanisms of corneal melting 21
2 AIMS 24
3 MATERIAL AND METHODS 25
3.1 Patients and Specimens 25
3.1.0 *Group I* 25
3.1.1 *Group II* 29
3.1.2 *Group III* 29
3.2 Specimen Preparation 31
3.3 Indirect Enzyme Immunohistochemistry 31
3.4 Gelatin and Casein Substrate Zymography 33
3.5 MMP-1 Activity Assay 34
3.6 MMP-3 Activity Assay 34
3.7 Statistical Analysis 35
4 RESULTS 36
4.1 Presence of Matrix Metalloproteinases in Control Corneas 36
4.2 Localization of Matrix Metalloproteinases in Melted Corneas 36
4.3 Detection of Matrix Metalloproteinase-2 and -9 Activities 41
4.4 Detection of Matrix Metalloproteinase-3 and -7 Activities 42
4.5 Evaluation of Active Matrix Metalloproteinase-1 and -3 Concentration 44
5 DISCUSSION 46
5.1 Highlights of the Work Presented 46
5.2 Matrix Metalloproteinases in the Process of Corneal Melting 46
5.2.0 *Loss of the Epithelium and Degradation of the Basement Membrane* 46
5.2.1 *Degradation of Stromal Extracellular matrix and Rupture of the Cornea* 48
5.3 Variability of Matrix Metalloproteinases Expression in Individual Specimens 49
5.4 Future Possible Treatment of Corneal Melting 50
5.5 Conclusions and Future Work 51
6 LIST OF ABBREVIATIONS 53
7 REFERENCES 54
8 LIST OF AUTHOR’S PUBLICATIONS AND PRESENTATIONS 67
8.1 List of Author’s Publications 67
8.2 List of Author’s Presentations 68
9 APPENDED PUBLICATIONS 69

1 INTRODUCTION

1.1 The Human Cornea

The cornea is a transparent avascular tissue located at the anterior part of the human eye (Fig. 1) which is exposed to the external environment. The anterior corneal surface is covered by the tear film, and the posterior surface is washed by the aqueous humor. The transparent cornea merges with the opaque sclera and semitransparent conjunctiva. The transition zone between the cornea and sclera is called the limbus, which contains a reservoir of pluripotential stem cells [1, 2].

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 [3].

The adult cornea measures 11 to 12 mm horizontally and 9 to 11 mm vertically. It is approximately 0.5 mm thick at the centre, and its thickness increases gradually toward the periphery [4]. It is composed of six layers: the epithelium, the basement membrane, Bowman's layer, the stroma, Descemet's membrane and the endothelium [1] (Fig. 2).

The corneal epithelium consists of five to seven layers of nonkeratinized, stratified squamous epithelial cells, which are generally divided into three different layers. The first is a monolayer of columnar basal cells possessing mitotic activity. The second is formed by two to three layers of wing cells, which are in an intermediate state of differentiation between basal and superficial cells. The most superficial layer includes two to three layers of well differentiated non-proliferating superficial cells with low metabolic activity [1, 5]. The epithelium, together with the tear film, contributes to the maintenance of a smooth corneal surface. The corneal epithelium provides a barrier to external biological and chemical insults due to junctional complexes (tight junctions, adherent junctions, desmosomes, and gap junctions) between adjacent corneal epithelial cells [5, 6].

The basement membrane, which is located between the basal epithelium and the underlying Bowman's layer, is a very important layer fixing the polarity of the epithelial cells [3]. The components necessary for its formation (type IV and VII collagen, laminin, fibronectin) [7] are secreted by the basal cells of the corneal epithelium [8]. The two most important functions of the basement membrane are to provide a matrix on which cells can migrate and to help epithelial wound healing [9-12].

Figure 1. Schematic illustration of the structure of the human eye. Adapted from Willoughby et al. [13]

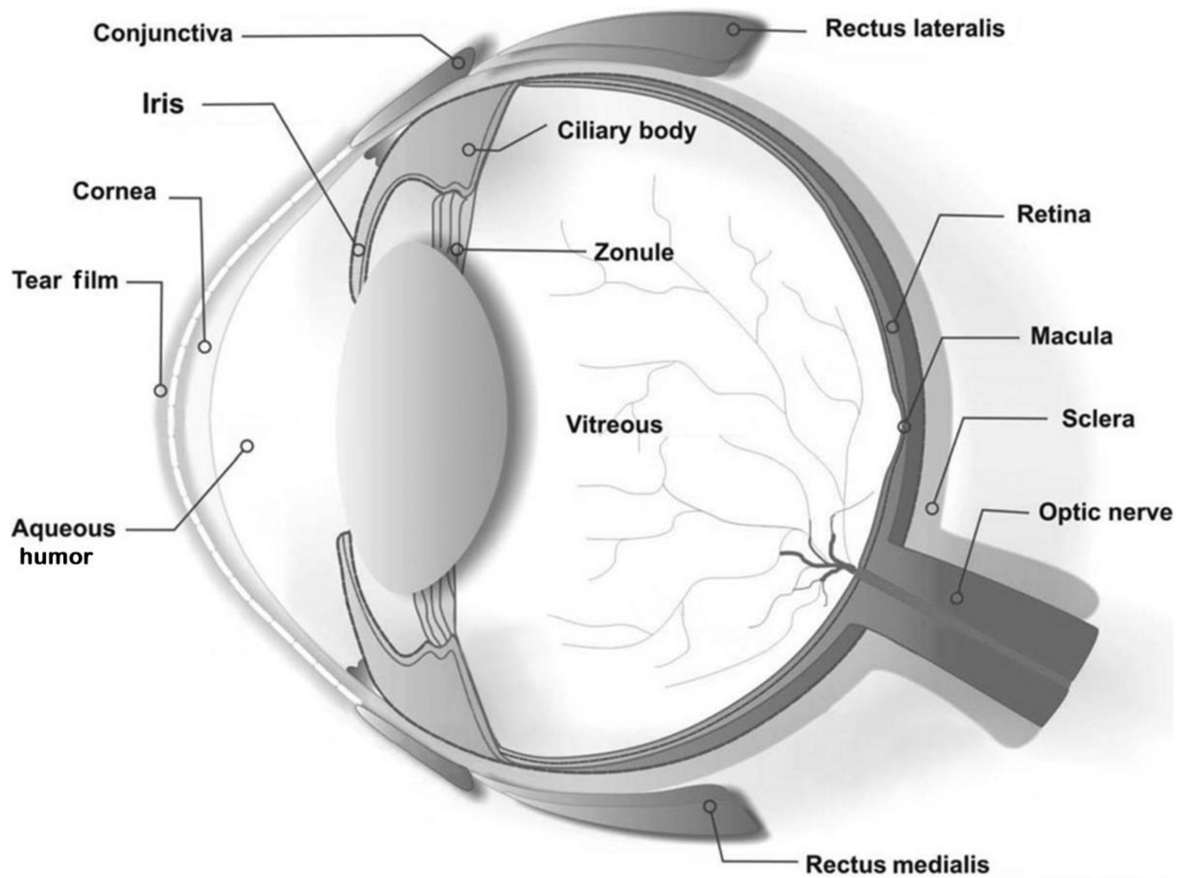
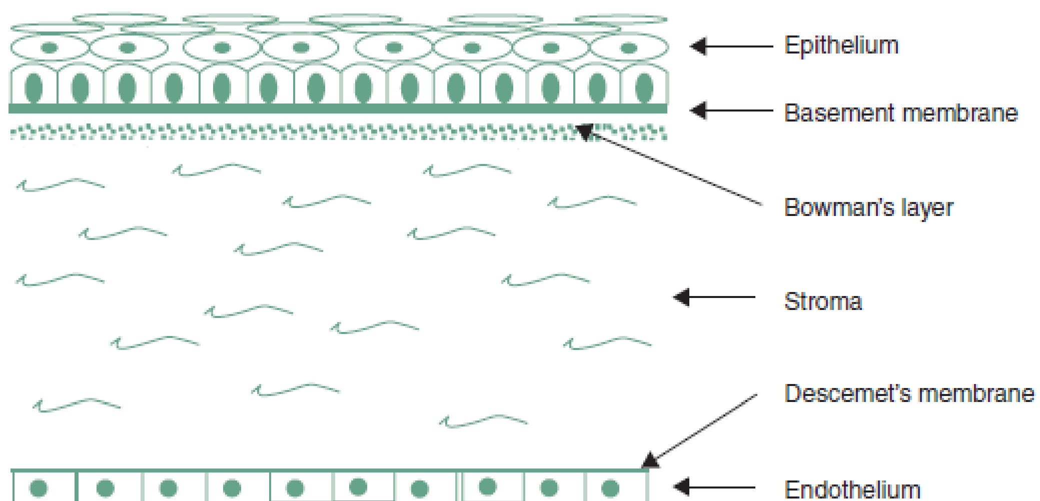


Figure 2. Schematic illustration of the corneal section showing all corneal layers. Adapted from Willoughby et al. [13]



An acellular membrane-like zone between the corneal epithelium and stroma is called Bowman's layer. This 8 - 12 μm thick layer is formed by a random arrangement of proteoglycans and collagen fibers synthesized by stromal keratocytes (corneal fibroblasts)[14-16]. It is composed mainly of collagen types I, III and V. Collagen type VII is also present serving as an anchor between hemidesmosomes and anchoring plaques in the stroma, and thus stabilizing epithelial cells, the basement membrane and Bowman's layer[17-19].

The largest portion of the cornea is formed by the stroma. It consists of an extracellular matrix (ECM), keratocytes and nerves. The cellular components – keratocytes – occupy only 2 - 3% of the total volume of the stroma [20] and their turnover is very slow [21]. The most abundant stromal component is the ECM composed of collagen (collagen type I, III, V and VI) and glycosaminoglycans [12, 17, 18, 22]. The diameter of the collagen fibers and the distance between individual fibers are highly uniform and are less than half of the wavelength of visible light [23, 24]. This regular arrangement is a major determinant of corneal transparency [24]. The turnover of collagen molecules in the cornea is slow, requiring two to three years [25]. Various glycosaminoglycans (keratan sulfate, chondroitin sulfate, and dermatan sulfate) are present between collagen fibers and regulate their spacing thus participating in the maintenance of corneal transparency [14, 26].

Descemet's membrane, the basement membrane of the corneal endothelium, is composed mostly of collagen type IV, VIII and laminin but also contains collagens V, VI and XVIII (reviewed in Jirsova et al.) [18, 27, 28]. This layer is very fragile and it is torn easily on exposure to mechanical stress [3].

The posterior surface of Descemet's membrane covers a single layer of corneal endothelial cells. These cells are polygonal (mostly hexagonal) in shape and on their apical surface there are sparse small microvilli that bulge into the anterior chamber, thereby increasing the surface area exposed to aqueous humor [29, 30]. They contain various junctional complexes (tight and gap junctions). Endothelial cells are metabolically very active but they do not proliferate, or divide very reluctantly, in humans [31-34]. In young adults the endothelial cell density is about 3500 cells/mm^2 and it decreases with age [35]. The most important physiological function of the corneal endothelium is to regulate the water content of the corneal stroma thereby maintaining corneal transparency [36, 37].

The cornea is one of the most heavily innervated and sensitive tissues in the body. Most of the sensory nerves in the cornea are derived from the ciliary nerves of the ophthalmic branch of the trigeminal nerve. Nerve fibers penetrate the cornea in the deep peripheral stroma radially and then course anteriorly, forming a terminal subepithelial plexus. The nerve fibers lose their

myelination within a short distance of their point of entry into the cornea and terminate at the epithelial wing cell level [38, 39]. Loss of the superficial corneal epithelium therefore results in severe ocular pain.

The cornea is one of the few avascular tissues in the body therefore a supply of nutrition (glucose) must be provided by diffusion from the aqueous humor – a liquid filling the anterior chamber (Fig. 1). In contrast, oxygen is supplied to the cornea primarily by diffusion from tear fluid, which absorbs oxygen from the air. A small proportion of the oxygen is met by diffusion from the aqueous humor and limbal circulation [40]. Closure of the eyelids during sleep reduces the amount of oxygen that reaches the cornea. Corneal metabolism thereby changes from aerobic to anaerobic (with consequent accumulation of lactate) during sleep [41, 42].

1.2 Corneal Transplantation

Corneal transplantation is the most frequently performed grafting procedure in which the affected host corneal tissue is replaced with a donor corneal button or lamella.

The techniques of corneal grafting were first reported in 1824 by Reisinger who experimented on rabbits but gained poor results [43]. The first human corneal transplant was performed in 1886, in which a full-thickness rabbit cornea was placed into a human recipient's corneal lamellar bed [44]. It was not until the twentieth century, in 1905, that Edward Konrad Zirm performed the first successful human corneal transplantation called penetrating keratoplasty[45], and since then this method has gradually progressed and been improved, as have techniques employed to store and preserve donor corneas.

The indications for corneal grafting include anatomic, functional (poor visual acuity) and clinical indications (pseudophakic corneal edema, stromal corneal dystrophies, corneal thinning, viral and microbial keratitis, noninfectious ulcerative keratitis or perforation etc.). Anatomic indications of corneal transplantation involve: optical (traumatically distorted or keratoconic corneas), reconstructive (corneal ulceration, melting and perforation), therapeutic (edema, scarring, dystrophy, degeneration and medically unresponsiveness infections) or cosmetic indications (removing unsightly corneal scars or deposits).

Although penetrating keratoplasty is the most successful tissue transplantation procedure (success rates defined by grafts remaining clear after one year approach 90% in low risk patients) [46-50], graft survival and the success of this procedure depend on a number of factors such as the underlying diagnoses and the stage of the disease in the recipient, as well as

immunology, topical and systemic drug administration, surgical technique, and the banking procedures of the corneal graft [50, 51]. The success of high risk penetrating keratoplasties (the success rate of which decreases to about 10 - 30%) [52-54] depends particularly on immunologic risk factors such as vascularization, and previous graft rejection, as well as nonimmunologic factors (ocular surface diseases, glaucoma, decreased sensation etc.) [47, 55-59].

There are two main techniques used in corneal grafting: penetrating and lamellar keratoplasty[60]. Penetrating keratoplasty is a relatively easy surgical procedure and the outcome is generally good [61]. It involves the removal of the whole cornea (all six layers) and its replacement with a full thickness cornea from a donor. The size of the graft depends on several factors including the host corneal size, pathology, and the risk of rejection [51]. The risk of rejection increases with graft sizes outside the range of 7.0 to 8.5 mm diameter, and as the graft-host junction moves closer to the limbus [61-65]. Lamellar keratoplasty involves selective replacements of the diseased corneal layers leaving the rest of the healthy cornea of the patient undisturbed. There are two types: anterior lamellar keratoplasty and posterior lamellar keratoplasty [60, 66]. Anterior lamellar keratoplasty is a procedure in which the epithelium, the basement membrane, Bowman's layer, and the stroma to a variable depth, are replaced by donor tissue. It is used for example in superficial corneal scars or to restore useful vision in various congenital or developmental disorders such as stromal dystrophies [67-69]. Posterior lamellar keratoplasty selectively replaces diseased Descemet's membrane and the endothelium, and it is used for the treatment of disorders affecting these two layers such as endothelial dystrophies (Fuch's endothelial corneal dystrophy, iridocorneal endothelial syndrome, pseudophakic bullous keratopathy etc.) [70-72].

1.3 The Immune privilege of corneal grafts and corneal graft failure

Penetrating keratoplasty is orthotopic corneal transplantation, which is the most common and successful form of solid tissue grafting performed with a survival rate of about 90% in the first year after transplantation for uncomplicated first grafts performed in normal, nonvascularized, low-risk beds [46-50]. This relative success is achieved due to the fact that the corneal allograft, and its graft bed, are endowed with immune privilege. Immune privilege of corneal allografts is based on multiple, overlapping anatomical, physiological and immunomodulatory features of the cornea and the underlying anterior chamber. These include the afferent blockade of the immune response that is provided by a lack of donor mature antigen presenting cells (APC) in the central part of the normal corneal graft. These cells

express major histocompatibility complex class II (MHC II) on their surface, by which they present antigens to T cells. In the cornea, there are three types of APC: MHC class II-positive dendritic cells present in the corneal epithelium and stroma, Langerhan's cells resident in the corneal epithelium, and macrophages mainly populating the posterior stroma [73, 74]. The afferent blockade of the immune response is also assured by the absence of afferent ways of immune response in the graft bed - blood vessels and lymphatics [75]. Other features are mainly associated with deviation of the immune response from a destructive to a tolerant pathway and the blockade of allodestructive immune effector elements including the presence of complement regulatory proteins, immunosuppressive cytokines and neuropeptides in the aqueous humor, and antigen-specific down-regulation of T-helper cell type 1 (Th1) alloimmune response to intraocular antigens. The antigen-specific down-regulation of delayed-type hypersensitivity responses to antigens introduced into the anterior chamber has been termed anterior chamber-associated immune deviation [75-77]. Also important is the Fas ligand (FasL, CD95L) expression on multiple corneal epithelium and particularly on the endothelium. This ligand triggers program cell death in cells bearing a Fas receptor, and therefore effectively kills neutrophils, T-cells and other immune cells [75, 77-80].

Despite the immune privilege in patients with corneal grafts placed in high-risk beds, rates of graft failures are between 70 to 90% [52, 54].

Except for uncommon reasons for corneal graft failure (such as ulceration and corneal melting, primary graft failure, endophtalmitis or disease recurrence) [61, 81, 82], the main cause for this event remains immune-mediated rejection characterized by delayed-type hypersensitivity to donor alloantigens and leukocytic cellular infiltration of the graft site [57, 61].

A number of factors have been found to increase the risk of corneal rejection: rejection associated with vascularisation of the cornea either pre- or post-operatively [47, 55, 59], prior graft failure, particularly due to rejection [56, 58], graft sizes outside the range of 7.0 to 8.5 mm in diameter [54, 61-65], preoperative glaucoma and pre-existing inflammation in the eye or prior anterior segment surgery other than a previous graft [58, 83, 84].

The process of corneal allograft rejection is an inflammatory process which includes an induction phase, called "afferent arm", and an expression phase, called the "efferent arm". In the afferent arm the host becomes sensitized to the donor antigens by means of APC (see above). The allorecognition process involves two different pathways. The direct pathway involves donor APCs that sensitize the host directly after the T cells recognize the donor MHC class II, thus generating direct alloreactive T cells [85]. The indirect pathway involves host APCs invading-migrating into the graft, taking up the donor's antigens, migrating to

draining lymphatic nodes and then presenting their antigens in the context of "self" MHC class II to the receptors of native T cells [86, 87]. The efferent phase is responsible for the actual "attack" on the graft. This phase consists of the proliferation of alloreactive T cells in lymphoid organs, delivery of these cells to the cornea, and the development of a "memory" that can assist the alloimmune response in case of repeated exposure to the same antigen [87].

Clinically, graft rejection is observed as cellular infiltration. Inflammatory cells (macrophages, monocytes and lymphocytes) [88, 89] invade the specific portion of the corneal graft that is being rejected: epithelium, stroma or endothelium. Epithelial and stromal rejection are not common, however, endothelial rejection is the most common of the three types, with reported rates from 8% to 37% of cases who underwent penetrating keratoplasty [47, 48, 90].

There are a variety of strategies which may lead to the prevention of corneal allograft rejection. The most extensive clinical trials have evaluated the role of histocompatibility matching of donor and recipient to prevent graft rejection. Early studies [55, 59] suggested that histocompatibility matching can improve graft survival, particularly in high-risk patients. Retrospective analysis of rejected corneal transplants also demonstrated a decreased frequency of rejection when HLA antigens are shared [91, 92]. Controversial results not confirming the efficacy of HLA antigen matching in immune rejection prevention in high-risk corneas was also suggested by the Collaborative Corneal Transplantation Studies Research Group [93].

The treatment of choice for acute corneal allograft rejection is mainly corticosteroid therapy by topical, periocular, or systemic administration. It is suggested that the use of higher frequency postoperative topical steroids, close follow-up of the patient, and the aggressive treatment of suspected or diagnosed rejection results in graft survival rates [93]. On the other hand, immunosuppression by long-term corticosteroid therapy is associated with unacceptable ocular and systemic side effects. Therefore it is wise to carefully monitor the patient's general health in these cases [94-96]. This is why less toxic combined systemic immunosuppressive therapy with cyclosporine or tacrolimus, mycophenolate mofetyl and corticosteroids is most commonly used in the prevention of immune graft rejection in high-risk corneas. There are other methods of still rather investigational therapies such as monoclonal antibody therapy (using monoclonal antibodies directed at other relevant portions of the molecules involved in the immune response), which also offer promise in the attempt to mitigate graft rejection [97, 98].

1.4 Corneal Melting

Corneal melting (keratolysis) can be a reason for graft failure [81, 88]; however it may affect a native cornea as well [99, 100]. It is a rare, occasionally recurrent and difficult-to-treat condition leading to corneal destruction. It is characterized by the development of epithelial defects and gradual stromal thinning due to the destruction and loss of the ECM, which may lead to descemetocele formation and subsequent perforation of the cornea [100-103] (Fig. 3).

Keratolysis could be associated with infectious [104, 105], non-infectious inflammatory [106-108], traumatic [109, 110], or trophic causes [111], but may also arise for no apparent reason [112].

Infectious corneal melting is caused by bacterial (*Pseudomonas aureginosa*, *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Enterococci* etc.) [105, 113-115], fungal (*Aspergillus flavus, Fusarium solani, Rhodotorula rubra* etc.) [113, 116, 117] or viral (herpes, cytomegalovirus) [118] invasion and colonization of the corneal surface and deeper layers of the cornea, which is followed by immune response, infection and rapid ulceration [104, 105, 116]. The corneal destruction associated with bacterial infection is thought to result from the production of a mixture of proteases (cleaving ECM components) by bacteria, as well as the host's corneal and inflammatory cells [104, 105].

Traumatic corneal melting may occur as a consequence of severe chemical or thermal eye burns. In these pathologic situations toxic substances such as prostaglandins, superoxide radicals and others are released from the burnt cells of the necrotic tissue. An inflammatory response is initiated when they diffuse into surviving tissues [119]. The burned ulcerated corneas produce proteolytic enzymes, originating from both the cornea and invading leukocytes, and are capable of destroying stromal ECM components [107, 120, 121].

Trophic corneal melting can occur in keratomalacia which is a nutritional disorder of the cornea caused by vitamin A deficiency. Although it is rare in the United States or Europe, it poses a severe problem in developing countries affecting mainly children [107]. Except for focal or diffuse corneal melting the manifestation of this disorder includes xerosis and stromal ulcers [111].

Non-infectious corneal melting has many different etiologies. It could occur as an isolated ocular problem; however, it is more often linked to autoimmune diseases such as rheumatoid arthritis (RA), Sjögren's syndrome, Wegener's granulomatosis and, rarely, to ocular cicatricial pemphigoid (OCP) [101, 106, 108, 122].

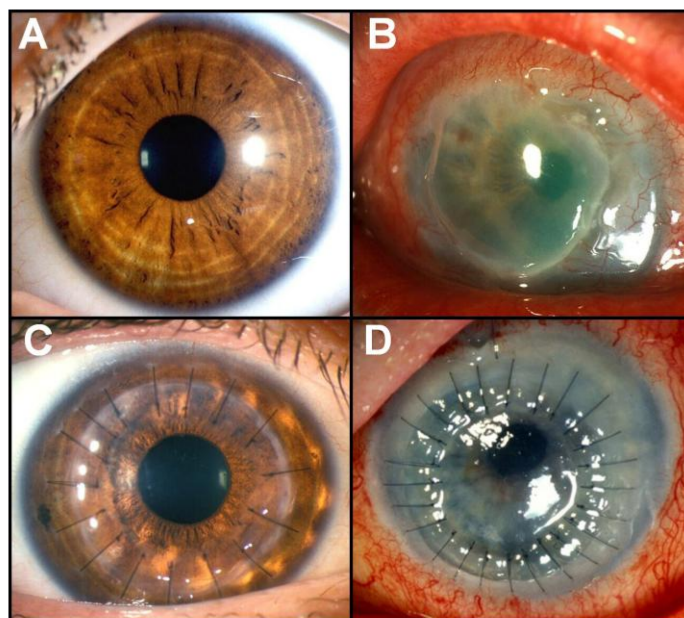
RA afflicts about 1% of the world's population, mainly women [123, 124]. It is a disorder which affects multiple joints throughout the body causing irreversible destruction of the cartilage,

tendon and bone. It is often accompanied by extra-articular manifestations in different tissues [125]. Ocular involvement, including dry eye syndrome, scleritis and keratolysis, occurs in 25 - 50 % of RA patients, most often in individuals suffering from the severe form of the disease [107, 126].

Primary Sjögren's syndrome (pSS), with an estimated prevalence of only about 0.5%, is characterized by the destruction of the lacrimal and salivary glands, resulting in dry eye and xerostomia [127-129]. There is lymphocytic infiltration in the exocrine glands and the production of various autoantibodies [127, 130, 131]; however, this autoimmune disease may affect several other tissues and organs as well [129, 132-134]. Most patients do not exhibit severe ocular complications except for those who suffer, in addition to dry eye, from bacterial keratitis, pannus formation, and sterile corneal melting [108, 135, 136].

Management of corneal melting is difficult and has to be adjusted individually. In less severe cases, operating techniques stabilizing the anatomic integrity of the eye, such as the application of tissue adhesives or amniotic membrane, could be used [102, 107]. In advanced stages of keratolysis, these approaches are frequently unsuccessful [37, 106, 122] and, finally, penetrating keratoplasty has to be performed in cases with imminent corneal perforation [100, 102, 103]. Unfortunately, some transplanted grafts also recurrently fail due to corneal melting [100, 106] (Fig. 3).

Figure 3. Process of recurrent corneal melting. Normal cornea (A), keratolysis of the cornea (B), clear transplanted corneal graft (C), melted corneal graft (D). Photographs were provided by of Ophthalmology, General Teaching Hospital.

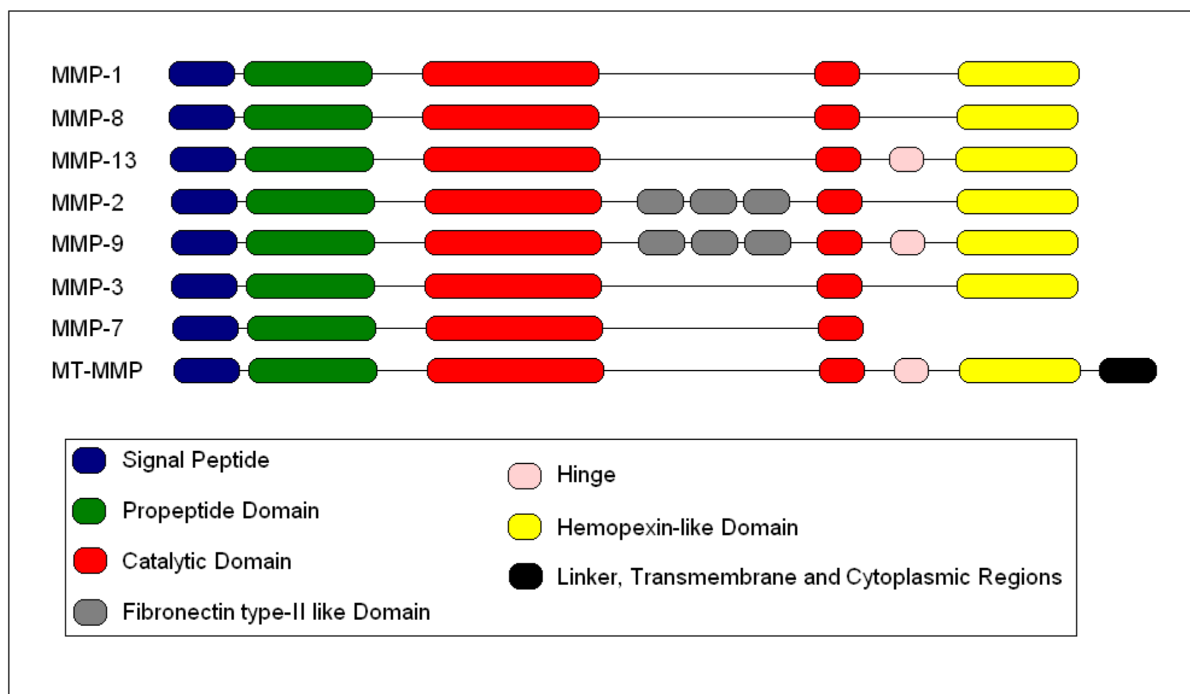


1.5 Matrix metalloproteinases – structure, substrates and presence in normal human cornea

Matrix metalloproteinases (MMPs) are a family of 23 endopeptidases capable of degrading various components of the ECM [137, 138]. These enzymes are grouped on the basis of substrate preference, and domain organization, into six subfamilies: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10), matrilysins (MMP-7, -26), membrane type MMPs (MT-MMPs; MT1 - MT6-MMPs) and others (e.g., macrophage metalloelastase - MMP-12 or enamelysin - MMP-20) [139].

Most of MMPs are synthesized as inactive pre-proenzymes and the N-terminal signal peptide is removed during translation to generate latent proenzyme (zymogen, proMMP). The structure of these proenzymes is organized into three basic, distinctive, and well-conserved domains: an aminoterminalpropeptide; a catalytic domain; and a hemopexin-like domain at the carboxy-terminal (Fig. 4) [137, 140, 141]. The propeptide consists of approximately 80–90 amino acids containing a cysteine residue, which interacts with the catalytic zinc atom.

Figure 4. Schematics of the domain structures of the eight representative MMPs. The catalytic domain (represented by red) has an insertion of a gelatin binding domain (represented in gray) in MMP-2 and MMP-9. In all other MMPs, the catalytic domain is a continuous entity [137, 139, 141, 149, 150].



The catalytic domain contains two zinc ions and at least one calcium ion coordinated to various residues. One of the two zinc ions is present in the active site and is essential to the catalytic processes of the MMPs. The second zinc ion is called "structural" and its role, along with the role of the calcium ion within the catalytic domain, is still uncertain [142, 143]. The hemopexin-like domain of MMPs is highly conserved and shows sequence similarity to the plasma protein, hemopexin. The hemopexin-like domain has been shown to play a functional role in substrate binding and/or in interactions with the tissue inhibitors of metalloproteinases (TIMPs) [138, 144, 145]. In addition to these basic domains, the family of MMPs evolved into different subgroups by incorporating and/or deleting structural and functional domains. For example, fibronectin type-II-like domain (involved in binding to denatured collagen or gelatin) is typical of gelatinases [140, 146]. The transmembrane domain at the carboxy terminus and recognition motif for furin-like convertases at the end of the propeptide domain is a characteristic of the MT-MMPs and three secreted MMPs (MMP-11, -21, -28) [140, 147, 148].

The regulation of MMPs occurs at four levels: gene expression, compartmentalization, physical delocalization of the prodomain from the catalytic site of MMP and enzyme inactivation by inhibitors [140, 151]. As many of MMPs' genes are relatively "inducible", their gene expression is affected by a wide spectrum of effectors like cytokines (e.g. tumor necrosis factor alpha - TNF-α, and interleukin 1 - IL-1), chemical agents (e.g. phorbol esters), physical stress etc. MMP gene expression may be, on the other hand, down-regulated by several suppressive factors [e.g. retinoic acids, glucocorticoids, transforming growth factor beta (TGF-β)] [152]. Recent studies emphasize not only soluble factors but also cell-matrix and cell-cell interactions as keys in gene expression of these enzymes [152, 153]. The signal from all mentioned effectors leads via different signal transduction pathways (e.g. MAPKs), which activate transcription factors (activator protein 1) directly regulating MMP gene expression[123]. Compartmentalization is the second way of MMPs regulation. It seems that cells do not indiscriminately release proteases, which are rather anchored to the cell membrane, or some other molecules in interstitium. This is important to maintain a locally high enzyme concentration and to target their catalytic activity to specific substrates within pericellular space [151]. Except for MT-MMPs, which are bound to the membrane of their original cells, there are other examples like MMP-1 and -2 bound to integrins, MMP-7 bound to surface proteoglycans or MMP-1, -2, -7, -8, -9 and -13 to heparin and heparan sulfate [140, 151, 154-156]. Binding of MMPs to cell membranes may also activate intracellular signaling cascades, an effect independent of their proteolytic activities [140].

Apart from a few members activated by furin (see below), most MMPs are secreted extracellularly as inactive enzymes and activated by the delocalization of prodomain from the catalytic site of MMPs [141, 152]. There are two ways: allosteric activation (interactions between a proMMP and other molecules such as glycosaminoglycans can result in an active MMP without proteolytic cleavage of the propeptide) and activation through proteolytic removal of the prodomain [140]. ProMMPs may be activated autocatalytically or by other MMPs (Tab. 1), plasmin, trypsin, neutrophil elastase, plasma kallikrein and several chemical agents (organomercurials, thiol reagents, oxidants or detergents) [137, 138, 149, 151]. Some MMPs bearing a furin recognition motif (all MT-MMPs, MMP-11 etc.) are processed into the active form intracellularly [157].

In addition, active MMPs may be inactivated by inhibitors. There are two main MMP inhibitors: α_2-macroglobulin and TIMPs. α_2-Macroglobulin, found in blood and tissue fluids, is a general proteinase inhibitor, which has the important role of regulating MMP activities in the fluid phase. It has the "bait" region, by which it attracts proteinases. Cleaving this part of the molecule by a proteinase leads to the structural change of the inhibitors, which trap and thereby neutralize a proteinase [123]. However, the main MMP inhibitors are TIMPs (TIMP-1, -2, -3, -4), which inhibit all MMPs [150, 152]. TIMPs involve non-covalent binding to the target MMP active site with 1:1 stechiometry, in which it chelates the MMP active site zinc ion. However, in addition to blocking MMP enzyme activity, they have been shown to participate in the MMP activation of some MMPs as well (TIMP-2 participate in activation of proMMP-2 by MT1-MMP) [139].

MMPs are able to cleave most ECM components; however, members of each subfamily share different substrate preferences (Tab. 1). In addition, they can process a large number of non-ECM proteins, such as grow-factors, cytokines, chemokines, cell receptors, serine proteinase inhibitors and other MMPs [140].

From substrates present in the normal human cornea, collagenases can cleave fibrillar collagens types I and III, abundant in the stroma [17, 149], and other collagens (collagen type VIII) present in the Descemet's membrane [137, 158]. Gelatinases are especially able to degrade 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 [7, 17, 18, 149, 159-162]. Stromelysins and matrilysin cleave similar ECM components, which are constitutively present in the stroma and corneal basement membranes (type IV collagen, procollagens, collagen cross-links, fibronectin, laminin) [7, 137, 149, 163].

Table 1. Substrates of some representative members of MMP family.
Reference [7, 17, 18, 137, 149, 159-163, 165].

MMP subfamily	MMP number	Protease name	Substrates present in the cornea		proMMP activated
			epithelial basement membrane	stroma	
Collagenases	MMP-1	Collagenase-1 (interstitial collagenase)	-	Collagen types I, III (III>>I)	ProMMP-1, -2
	MMP-8	Collagenase-2 (neutrophil collagenase)	-	Collagen types I, III (I>>III)	ProMMP-8
	MMP-13	Collagenase-3	-	Collagen types I, III (II>>I>>III), gelatin	ProMMP-9, -13
Gelatinases	MMP-2	Gelatinase A	Collagen types IV, VII fibronectin laminin	Collagen types V, VI denatured collagens core protein decorin	ProMMP-1, -2, -13
	MMP-9	Gelatinase B			ProMMP-2, -9, -13
Stromelysins	MMP-3	Stromelysin-1	Collagen type IV, fibronectin laminin	Procollagens collagen cross-links	ProMMP-1, -3, -7, -8, -9, -13
	MMP-10	Stromelysin-2			ProMMP-1, -8, -10
Matrilysins	MMP-7	Matrylisin	Collagen type IV, fibronectin laminin	Procollagens collagen cross-links	ProMMP-1, -2, -7, -9

MMPs play an important physiologic role in tissues where they participate in embryonic growth and tissue morphogenesis, cell migration and matrix microenvironment remodeling, angiogenesis or ovarian and the uterine reproductive cycle [164-166].

In the cornea, where MMPs can be produced by keratocytes, epithelial cells and by immune cells (monocytes and macrophages) [74, 167-171], they probably participate in normal ECM turnover.

In the normal human cornea, very low levels of collagenases MMP-1, -8 and -13 in the epithelium, as well as a low level of MMP-2 in both the epithelium and anterior stroma, are normally present [99, 172-174]. No, or a very low level of, MMP-9 was detected in the epithelium of normal human corneas [175, 176].

The presence of stromelysins MMP-3, -10 and matrilysin MMP-7 in the normal human cornea is a matter of some controversy; in some studies they have been detected in the epithelium [175, 176] while others have not found them in any corneal layer [176-178].

1.6 Matrix Metalloproteinases in non-infectious corneal melting

Although MMPs play an important role in an organism under normal physiological conditions, the loss of their activity control or their over-expression may contribute to the development of various diseases (arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis) [139].

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 [99, 169, 174, 179].

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 [174, 180]. The pathogenesis of rheumatoid corneal melting is unknown, but it is presumed to be an immune-mediated process, associated with an inflammatory response with mononuclear cell infiltration occurring in RA [174].

The increased expression of MMP-2, -3, -8 -9 was also described in the melted corneas of patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs) after photorefractive keratectomy or cataract surgery [99, 179, 181].

1.7 Induction mechanisms of corneal melting

Noninfectious corneal melting is usually associated with an autoimmune disease [100, 174, 182] and immune mediated inflammation is thought to play an important part in this process [100, 182]. The

disturbed epithelial barrier caused by dry eye condition, often associated with autoimmunities such as RA or pSS, or previous surgery promoting epithelial breakdown in such predisposed patients, allows some immunomodulatory mediators to enter from the tears or from the conjunctival vasculature in cases of marginal melting into the cornea [182]. It is supposed that lymphocytic (polymorphonuclear neutrophils - PMN and macrophages) and leukocytic (T cells) infiltrate [88, 99, 182] secrete pro-inflammatory cytokines in melted corneas, and as was described in pathophysiology of pSS in salivary and lacrimal glands [128] or in synovial fluid of RA patients [123]. Pro-inflammatory cytokines TNF-α and IL-1β, the production of which is markedly increased in the corneal keratocytes of patients with rheumatoid corneal ulcerations [183] and in the tears of pSS patients [184], respectively, initiate MMP expression in various tissues via MAPKs [137, 185]. However MMPs are also directly produced by activated neutrophils and macrophages [170].

Apart from epithelial erosion, dry eye conditions contribute to the process of keratolysis due to desiccation and hyperosmolar stress, which lead to the stimulation of pro-inflammatory cytokines and consequently to the induction of MMP over-expression [185, 186].

It has been proposed that NSAIDs treatment could be another trigger mechanism inducing melting [179, 187, 188]. NSAIDs are an important class of anti-inflammatory agents with a wide variety of therapeutic applications throughout medicine (treatment of ocular allergies, prevention of excessive postoperative inflammation, pain and photophobia control following radial keratotomy and excimer laser photorefractive keratectomy) [189]. NSAIDs exert their pharmacologic activity primarily by the inhibition of the cyclooxygenase, which is essential in the biosynthesis of prostaglandins via the arachidonic acid metabolic pathway [187]. There are two different mechanisms which might be involved in NSAIDs-associated corneal melts [190]. The first - an acute toxic one - is associated with the alternative metabolism of arachidonic acid, which is caused by the selective blockade of cyclooxygenase, resulting in the production of leukotrienes, lipoxins and hydroperoxyeicosatetraenoic acids. The resulting accumulation of hydroperoxyeicosatetraenoic acids and leukotrienes provides potent chemoattractants for neutrophils. Furthermore, leukotrienes are also potent stimuli for neutrophil degranulation [179, 187]. The granules released by neutrophils during the inflammatory response contain collagenase as well as other hydrolytic enzymes [170]. When released in the cornea, the accumulation of collagenase may potentiate corneal melting [187]. The second mechanism - a subacute one - is associated with delayed wound healing and localized MMP accumulation at the site of an injury [179, 188, 190]. Diclofenac, one of the NSAIDs, retards corneal epithelial healing [191], decreases the growth of epithelial cells [192] and is associated with a higher

incidence of persistent epithelial defects [193]. On the other hand, corneal melting has not been reproduced in experimental animals with the use of topically applied, commercially available, brand-name NSAIDs. In addition, carefully controlled, prospective, double masked, randomized clinical studies in hundreds of patients indicate that topically applied brand-name NSAIDs appear safe for use in patients [189].

For corneal melting, other agents could also be responsible, such as plasminogen activators or some cathepsins, which may degrade ECM and are able to activate MMPs during this process [194, 195]. The tissue-type plasminogen activator is a thrombolytic agent that degrades fibrin clots through the activation of plasminogen to plasmin [196]. Plasmin has broad substrate specificity. Apart from the lyses of fibrin it contributes to tissue remodeling by degrading ECM proteins, either directly or via the activation of MMPs [194, 197]. Cathepsins B and D are lysosomal enzymes cleaving a wide range of proteins, including corneal proteoglycans, as well as activating a number of zymogens such as procollagenase [195]. They are present in extracellular spaces in various tissues and may be released from PMN and macrophages [198]. Both agents (plasminogen activator and cathepsins) were previously found in ulcerated corneas [194, 195].

2 AIMS

The aims of this study are:

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

2. to immunohistochemically identify 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, and in melted corneas as well as in grafts which recurrently failed due to corneal melting obtained from patients from these three groups: a) patients with primary Sjögren's syndrome, b) patients suffering from rheumatoid arthritis, and c) patients with various other underlying pathologies

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

4. to determine the activity of selected members of all the main MMPs' 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 the activities of MMP-1, -2, -3, -7, -9 detected in control and melted specimens

6. to compare MMP expression and activity in specimens among all three patients' 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 grafts (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 the 1st Faculty of Medicine, Charles University in Prague. The patients were divided into three groups according to their diagnosis (group I – III).

3.1.0 *Group I*

Group I included 11 specimens of two patients with pSS. Case 1, a 77-year-old patient, was diagnosed with pSS elsewhere at the age of 71. She tested positive for anti-SS-A/Ro, anti-SS-B/La, and antinuclear antibodies. No extraglandular manifestations were noted. Systemic immunosuppression administered to the patient included various combinations of cyclophosphamide, prednisolon, azathioprin, methylprednisolon, cyclosporine A, and mycophenolate mofetil. Upon first examination in the Department of Ophthalmology, General Teaching Hospital in Prague in 2002 at age 74, the patient presented bilateral severe dry eye syndrome. In the course of three years, she 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 the left eye (her native cornea was used as specimen P0-1 and one graft as specimen P0-5) requiring a number of surgical procedures including penetrating keratoplasties (Fig. 5, Tab. 2). Despite all the measures undertaken, her condition could not be controlled, and it led to bilateral blindness.

In case 2, the symptoms related to pSS started at the age of 46 when swollen salivary glands, xerostomia, and severe dry eye symptoms were noted by the patient. Histopathology from a labial salivary gland biopsy sample showed focal sialadenitis that was consistent with a diagnosis of pSS. The patient tested positive for rheumatoid factor, antinuclear antibodies, and Scl70 antibodies. Subsequently she also developed arthralgias. Systemic immunosuppression therapy was started at the age of 50. Initially the patient was treated with oral prednisolon; later, various combinations of methotrexate, cyclophosphamide, and methylprednisolon were added.

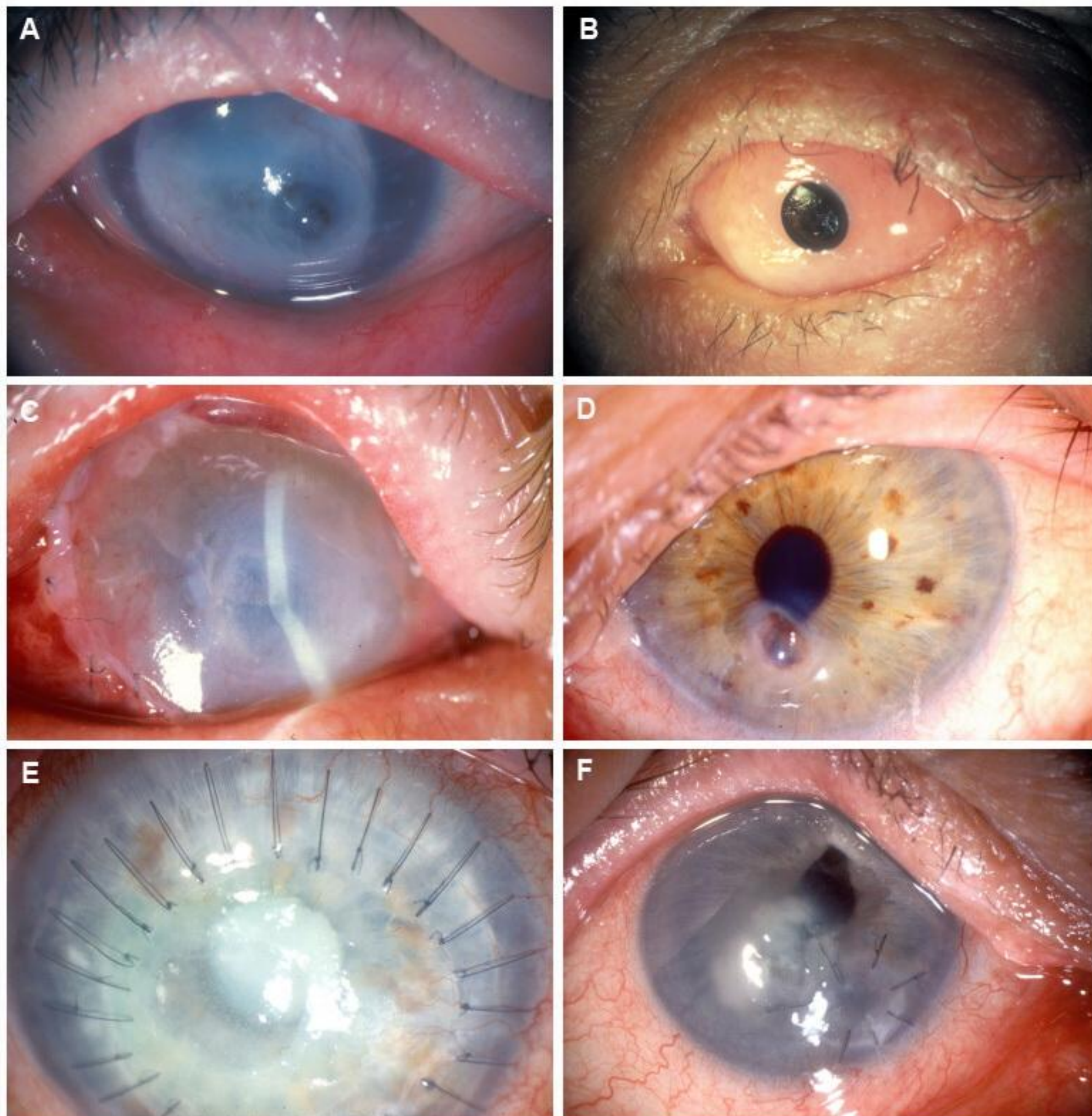
Table 2. Individual surgical interventions performed in case 1 to manage ocular complications related to pSS. Keratolysis preceded all penetrating keratoplasty surgeries. Any occurrence of corneal perforation or descemetocele formation is mentioned specifically in the table. Each date represents one surgical session.

Date	Eye	Procedure	Specimen
1999	Right	Phaco + IOL	
Apr-2002	Left	PK for corneal perforation	
Jun-2002	Left	ECCE + IOL, PK for corneal perforation, AMT	
Sep-2002	Right	Tarsorrhaphy	
	Left	Tarsorrhaphy	
Nov-2002	Right	AMT	
Dec-2002	Right	AMT	
Mar-2003	Right	PK for corneal perforation	
Mar-2003	Left	PK for corneal perforation	P0-1
May-2003	Bilateral	Tarsorrhaphy	
Jun-2003	Left	AMT, tarsorrhaphy	
Aug-2003	Left	AMT, tarsorrhaphy	
Sep-2003	Right	PK, AMT	P0-2
Sep-2003	Left	AMT, tarsorrhaphy	
Oct-2003	Right	AMT, tarsorrhaphy	
Feb2004	Right	Conjunctival flap	
Jul-2004	Right	PK, IOL explantation, AMT	P0-3
	Left	Suture of corneal wound dehiscence, buccal mucosa transplantation	
Aug-2004	Bilateral	AMT, tarsorrhaphy	
Sep-2004	Right	PK, AMT, tarsorrhaphy	P0-4
Sep2004	Left	Necrectomy of the buccal mucosa, PK, AMT	P0-5
Oct-2004	Left	Necrectomy of the buccal mucosa, AMT	
Oct-2004	Right	AMT	
Nov-2004	Right	PK, AMT	P0-6
	Left	Buccal mucosa transplant, tarsorrhaphy	
Nov-2004	Right	AMT	
Nov-2004	Right	AMT	
Dec-2004	Right	AMT	
Dec-2004	Right	PK, AMT, tarsorrhaphy	P0-7
Feb-2005	Right	PK, buccal mucosa transplantation, tarsorrhaphy	P0-8
	Left	Anterior vitrectomy, IOL explantation, iris resection, osteo-odonto-keratoprosthesis implantation, tarsorrhaphy	
Mar-2005	Left	Partial stripping of the buccal mucosa, tarsorrhaphy	
May-2005	Right	Buccal mucosa transplant, tarsorrhaphy	
Jan-2007	Left	Cyclocryotherapy for secondary glaucoma	
Mar-2007	Left	Pars plana vitrectomy for endophthalmitis with bacterial etiology	
Apr+2007	Left	Evisceration and osteo-odonto-keratoprosthesis extraction	
Sep-2007	Right	Osteo-odonto-keratoprosthesis implantation, tarsorrhaphy	
Jan-2008	Right	Osteo-odonto-keratoprosthesis reposition	

IOL = posterior chamber intraocular lens, PK = penetrating keratoplasty, ECCE = extracapsular extraction, Phaco = indicates phacoemulsification, AMT = amniotic membrane transplantation.

Figure 5. Corneal melting of grafts and native cornea of patients from all three groups.

A) Corneal melting of the eye of case 1 from group I, sample P0-1. B) Pintucci's keratoprosthesis in the right eye of case 1, group I. C) Corneal melting of the whole cornea of patient P1, sample P1-1. D) Decemetocele of the left eye, sample P2-1. E) Keratolysis of the graft, sample P4-3. F) Corneal melting at the edge of a patch keratoplasty performed because of melting, sample P5-2. All images were provided by the Department of Ophthalmology, General Teaching Hospital in Prague.



Upon first ocular examination at age 46, she had signs of moderate dry eye syndrome in both eyes. She gradually developed severe dry eye syndrome bilaterally. When the patient reached the age of 58, the first signs of peripheral ulcerative keratitis were observed in the right eye, followed three years later by signs in the left eye. She rapidly developed corneal thinning, and underwent her first keratoplasty in the right eye at the age of 59 (specimen P0-9), followed by numerous other procedures due to complications related to melting of the graft, including two penetrating keratoplasties (specimens P0-10 and P0-11), (Tab. 3). At the last follow up visit, her visual acuity was full light projection in the right eye and hand movements with full projection of light in the left eye.

Table 3. Individual surgical interventions performed in case 2 to manage ocular complications related to pSS. Keratolysis preceded all penetrating keratoplasty surgeries. Any occurrence of corneal perforation or descemetocele formation is mentioned specifically in the table. Each date represents one surgical session.

Date	Eye	Procedure	Specimen
Dec-2003	Right	AMT in the ulcer bed	
Dec-2003	Right	Cyanoacrylate adhesive to the ulcer bed	
Jan-2004	Right	PK, AMT	P0-9
Jan-2004	Right	AMT in the ulcer bed	
Feb-2004	Right	Partial resuture of the corneal graft, AMT in the ulcer bed	
Apr-2004	Right	PK, ECCE, AMT	P0-10
May-2004	Right	AMT	
May-2004	Right	AMT	
May-2004	Right	AMT, tarsorrhaphy	
May-2004	Right	Partial resuture of the corneal graft, AMT in the ulcer bed, AMT	
Jun-2004	Right	Partial resuture of the corneal graft,	
Jun-2004	Right	Conjunctival flap	
Jun-2004	Right	Addition of sutures to the conjunctival flap	
Jul-2004	Right	AMT in the ulcer bed, AMT	
Jul-2004	Right	PK for descemetocele	P0-11
Jul-2004	Right	AMT in the ulcer bed, AMT	
Jul-2004	Right	AMT	
Aug-2004	Right	AMT, tarsorrhaphy	
Aug-2004	Right	AMT	
Sep-2004	Right	Anterior vitrectomy, AMT	
Sep-2004	Right	AMT in the ulcer bed, AMT	
Jan-2005	Right	Cryoablation of the eyelashes	
Mar-2005	Right	Cryoablation of the eyelashes	
Sep-2005	Right	Cryoablation of the eyelashes	

PK = penetrating keratoplasty, ECCE = extracapsular extraction, AMT = amniotic membrane transplantation.

3.1.1 *Group II*

Group II contained seven melted explants of three patients with RA (Tab. 4), all of whom were evaluated by rheumatologists, and the severity of RA was classified into four stages (http://www.wheelessonline.com). All patients were administered systemic immunosuppressives. Full thickness grafts ranging from 7.75 to 8.50 mm in diameter 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 (Fig. 5). The presence of accompanying keratoconjunctivitis sicca (KCS) was classified as mild, moderate or severe [199].

Table 4. Clinical details of patients with recurrent corneal melting.

Age	Sex	Specimen	Eye	PK order in the eye	Autoimmune systemic disorder	Factors influencing the ocular surface	Comments
71	M	P1-1	L	1	RA stage III-IV	Moderate KCS	Descemetocele formation
		P1-2	L	2			
		P1-3	L	3			
77	F	P2-1	L	1	RA stage III	Severe KCS	Corneal perforation
		P2-2	L	3			Descemetocele formation
		P2-3	L	4			Descemetocele formation
80	F	P3	R	2	RA stage IV	Severe KCS	

RA = rheumatoid arthritis, KCS = keratoconjunctivitis sicca, M = male, F = female, L = left, R = right, PK = penetrating keratoplasty.

3.1.2 *Group III*

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 has occurred in both the native corneas (P4-2, P5-2) and grafts (P4-1, P4-3, P5-1) either centrally or paracentrally (Fig. 5, Tab. 5).
KCS was also classified as mild, moderate or severe [199].

Table 5. Clinical details of patients with recurrent corneal melting.

Age	Sex	Specimen	Eye	PK order in the eye	Autoimmune systemic disorder	Factors influencing the ocular surface	Comments
81	F	P4-1	L	2	OCP stage I; idiopathic autoimmune hemolytic anemia	Severe KCS	Descemetocele formation
		P4-2	R	1			Corneal perforation
		P4-3	R	2			
91	F	P5-1	R	2	None detected	Entropion	Lysis of previous patch keratoplasty and perforation of adjacent native cornea
		P5-2	R	3			Descemetocele formation next to the patch keratoplasty; excentric keratoplasty (6.0 mm in diameter) comprising mainly native cornea performed

OCP = ocular cicatricial pemphigoid, KCS = keratoconjunctivitis sicca, M = male, F = female, L = left, R = right, PK = penetrating keratoplasty.

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). The mean time from the donors' deaths to enucleation was 15 hours, and the mean time from death to tissue freezing was 17 hours. Melted grafts were frozen within three hours after surgery. 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 [200]. In brief, they were thawed and homogenized in a 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 the 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. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid (BCA). Different concentrations of bovine serum albumin (25 - 2000 µg/ml) diluted in the cacodylate buffer were used as standards, and the cacodylate buffer as a blank. Twenty-five µl of standards and specimens were each loaded in a separate well of the microplate, covered by 200 µl of a mixture of BCA Reagent A and BCA Reagent B (50:1, Reagent A:B) and gently mixed. The microplate was covered by a lid and incubated at 37°C for 30 minutes. The absorbance at 562 nm was measured on a SUNRISE ELISA Reader (Tecan Trading AG, Männedorf, Switzerland) and the total protein amount was determined by interpolation from the standard curve.

3.3 Indirect Enzyme Immunohistochemistry

Seven-µm thick cryosections from each of the OCT-embedded control and melted specimens were placed on gelatin-coated glass slides (four per slide) and stored at -20°C. The slides were

defrosted, and both fixed with cold acetone and re-hydrated in phosphate-buffered saline (PBS) for 10 minutes. Endogenous peroxidase was blocked by a 30-minute incubation in 3% hydrogen peroxide in PBS. After being washed three times in PBS for 5 minutes, 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 the primary antibodies listed in table 6. According to the manufacturers, each antibody is specific to a single MMP with no cross-reactivity with other MMPs. MMP-1 was detected using two different antibodies (Tab. 6). The specimens from group I were evaluated with antibody AB8105 and from groups II and III using antibody AB806. As the production of antibody AB806 was finished during the time of this study, AB8105 had to be used. Both antibodies were tested on a set of the same specimens to ascertain that they have the same detection manner. Subsequently, the slides were washed three times in PBS for 5 minutes, and the secondary antibodies (polyclonal rabbit anti-mouse IgG and swine anti-rabbit IgG conjugated with biotin, 1:200; DakoCytomation, Glostrup, Denmark) were applied for 1 hour.

Table 6. Matrix metalloproteinase detecting antibodies used for indirect immunohistochemistry.

Antibody	**Catalogue number**	**Concentration**	**Company**
Polyclonal rabbit anti-human MMP-1	AB8105*	1:300	Chemicon Intl. Inc, Temecula, CA, USA
Polyclonal rabbit anti-human MMP-1	AB806**	1:100	Chemicon Intl. Inc, Temecula, CA, USA
Monoclonal mouse anti-human MMP-2	MAB13431	1:350	Chemicon Intl. Inc, Temecula, CA, USA
Polyclonal rabbit anti-human MMP-3	29576	1:50	AnaSpec Inc., San Jose, CA, USA
Monoclonal mouse anti-human MMP-7	MAB13414	1:150	Chemicon Intl. Inc, Temecula, CA, USA
Polyclonal rabbit anti-human MMP-8	AB8115	1:300	Chemicon Intl. Inc, Temecula, CA, USA
Monoclonal mouse anti-human MMP-9	MAB3309	1:150	Chemicon Intl. Inc, Temecula, CA, USA
Monoclonal mouse anti-human MMP-13	MAB13424	1:50	Chemicon Intl. Inc, Temecula, CA, USA

* specimens from the group I, ** specimens from groups II and III

After rinsing in PBS (three times for 5 minutes each), streptavidin/horseradish peroxidase (1:250; 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 antibodies were verified using positive controls that are known to express MMPs: human placenta (MMP-1, -2, -3, -7) and breast carcinoma (MMP-7, -8, -9, -13) [201, 202]. Samples were evaluated using an Olympus BX51 light microscope (Olympus Co., Tokyo, Japan) at a magnification of 100x. Images were taken using a Vosskühler VDS CCD-1300 camera, (VDS Vosskühler GmbH, Osnabrück, Germany). 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 sample buffer (1.5% sodium dodecyl sulfate - SDS, 15% glycerol, and 0.005% bromphenol blue). Both zymographies were carried out as described previously [203] 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, 20 µl of each specimen were loaded onto the gels, and SDS-PAGE was performed 60 minutes at 200 V at 4 °C for gelatin zymography, and 90 minutes at 20 mA at 4 °C for casein zymography. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove the SDS, the proteinases thereby released to fold into their functional conformation. Afterwards they were incubated in the 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 to allow the proteinases to digest their substrates. 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 were 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 MMP-1 Activity Assay

The concentrations of the active forms of MMP-1 were determined using a commercial kit (Amersham matrix metalloproteinase-1 Biotrak Activity assay system, Amersham Biosciences, Amersham, UK) according to the manufacturer's protocol and as described previously [204]. In brief, an anti-MMP-1-coated microplate was allowed to equilibrate to room temperature. One hundred µl of proMMP-1 in concentrations ranging from 0.78 to 12.5 ng/ml served as standards and together with 100 µl of the tissue samples (diluted 1:10) and 100 µl of the assay buffer (served as a blank) were incubated at 4 °C overnight in microplate wells. To measure the total activity of MMP-1 in the standards and in the blank, bound proMMP-1 was activated with 50 µl of 0.025 mM of 1 mM p-aminophenylmercuric acetate (APMA) in the assay buffer. Fifty µl of assay buffer was added to each sample in which the endogenous level of active MMP-1 was measured. A detection reagent was added to each well of the microplate and incubated at 37 °C for 4 hours. Active MMP-1 was evaluated based on the color change resulting from the cleavage of a chromogenic peptide substrate. The values of the color reaction of the assays were read at 405 nm in a SUNRISE ELISA Reader (Tecan Trading AG, Männedorf, Switzerland). MMP-1 in samples was determined by interpolation from the standard curve. The activity assay could not be performed in P5-1 (group III) and P0-3 (group I), due to the lack of a sufficient quantity of specimens. Due to the limited amount of all sample material available, we performed this experiment only once.

3.6 MMP-3 Activity Assay

The commercial kit (Amersham matrix metalloproteinase-3, Biotrak Activity assay system, Biotrak, Amersham Biosciences, UK) was used to evaluate the concentrations of the active MMP-3 in samples of groups II and III. The method was performed according to the manufacturer's protocol as was described previously [204]. Briefly, microplate wells coated with $F(ab')_2$ goat anti-mouse IgG were incubated with an anti-MMP-3 antibody for 3 hours at 37 °C and then rinsed with a wash solution four times. One hundred µl of proMMP-3 in different concentration (0.25-8 ng/ml) were used as standards. All standards, 100 µl of assay buffer as a blank and 100 µl of the tissue samples (diluted 1:10) were incubated at 4 °C overnight in microplate wells. ProMMP-3 was activated with 100 µl of 1 mM APMA in the assay buffer in

all standards and the blank. Each sample in which the endogenous level of active MMP-3 was measured was supplemented with 50 µl of the assay buffer. To activate MMP-3 proenzyme in standards, a 30-minute incubation at 37 °C was used, and all wells were washed four times. The detection reagent was added to each well and incubated at 37 °C for 4.5 hours. The active MMP-3 was also evaluated based on the color change reaction and the resultant color was read at 405 nm in an ELISA Reader (ELISA Reader SUNRISE, Tecan Trading AG, Männedorf, Switzerland) similarly to the MMP-1 activity assay. The activity of MMP-3 in all samples was determined by interpolation from a standard curve. This experiment was carried out only once due to the limited amount of sample material available.

3.7 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 statistical significance.

4 RESULTS

4.1 Presence of Matrix Metalloproteinases in Control Corneas

All the control corneas exhibited regular morphology, with a five- to six-layered epithelium, normal thickness of stroma, unaffected Descemet's membrane and monolayer endothelium.
The staining intensities of particular MMPs in the control samples were averaged as no prominent differences in MMP staining were found among the individual control specimens for any of the MMPs tested (Tab. 7).
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 - in the area adjacent to Bowman's layer. The endothelium of the control specimens also revealed weak staining for MMP-1 and -8. MMP-3, -7, -9, and -13 was not detected in any layer of all control corneas (Fig. 6 and 7).

4.2 Localization of Matrix Metalloproteinases in Melted Corneas

Severe damage was observed in most of the pathological specimens including the complete absence of 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 of the melted corneas from all three groups.
The staining intensity of the different MMPs in melted specimens from all three patient groups is summarized in table 7. Representative pictures of immunohistochemical staining in melted corneas are shown in figures 6 and 7.
Increased MMP-1 staining was found in the epithelial fragments and the anterior stroma of all melted specimens from all groups compared to the controls, except for the epithelium of specimen P0-6 from group I. MMP-1 immunostaining intensity was also elevated in the posterior stroma of all melted corneas from group I, and in almost all from groups II and III (71% and 80% of samples, respectively). The endothelium of 40% of melted specimens was destroyed. Where it was unaffected, almost 50% of specimens from all three groups revealed elevated levels of MMP-1 in this corneal layer.
Compared to the controls, a stronger MMP-2 staining intensity was present in the epithelial fragments of almost all pathologic specimens (about 80-90%) from all three patient groups; only one specimen from each group revealed the same or lower staining pattern (P0-8, P1-1,

P4-1). A similar situation was noted in the anterior stroma, where only two specimens (P0-4 and P4-1 from groups I and III, respectively) were negative. The occurrence of MMP-2 in the posterior stroma was also similar in melted samples among all three groups, where MMP-2 staining was present in approximately 60-70% of all pathologic corneas. In the endothelium, the presence of MMP-2 was detected in about 50% of specimens in groups I and III and in almost all samples (83%) from group II, where the endothelium remained unaffected.

In contrast to the completely negative controls, in the pathologic samples the MMP-3 was detected in the epithelial fragments of all the melted grafts from all three groups except for P0-7 (group I). A similar ratio of samples from groups I and II exhibited MMP-3 staining in the anterior stroma (82% and 86%, respectively), whilst the presence of this enzyme in the posterior stroma was showed in the lower number of pathologic corneas from both these groups (73% and 57%, respectively). About 60% of the samples from group III showed the presence of MMP-3 throughout the whole stroma. The endothelium of almost 35% of the samples used for MMP-3 immunostaining was destroyed. In the remaining specimens, a few samples from all groups revealed MMP-3 expression in this layer (about 20%).

MMP-7 was detected in all layers of almost all melted corneas from group I; only 36% of samples were completely negative. In groups II and III almost all melted corneas were found to express MMP-7 in their epithelium and anterior stroma (group II - 100% in the epithelium and 86% in the stroma; group III - 80% in both corneal layers). About 50% of samples from both of these groups displayed MMP-7 expression in the posterior stroma. Only two samples (each from each group – P2-1 and P4-2) revealed the presence of this enzyme in the endothelium as well.

Increased MMP-8 staining was observed in the epithelium (if it was not damaged) of about 63% of melted specimens from all three groups compared to the control corneas. In the anterior stroma, the increased staining was found in almost all melted grafts in groups I, II and III (91%, 71% and 80% specimens, respectively). MMP-8 was localized in the posterior stroma of 72% of specimens from group I and approximately of 50% of melted corneas from groups II and III. The endothelium of only three samples (P0-5, P0-9 and P2-1 from groups I and II, respectively) revealed increased MMP-8 staining compared to the controls.

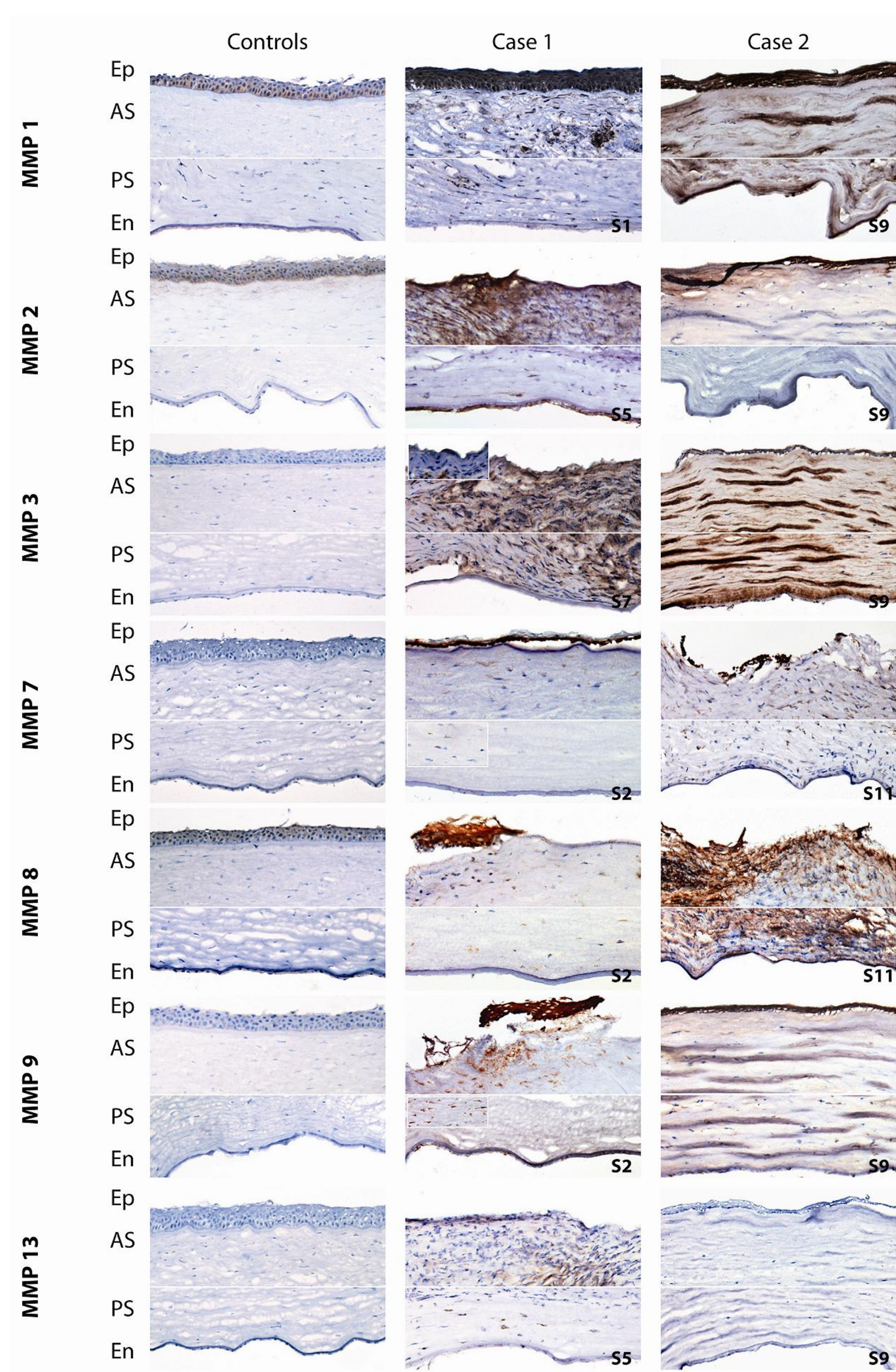
The presence of MMP-9 was detected in the epithelial fragments and the anterior stroma of all pathologic samples and in the posterior stroma of almost all specimens (about 80%) from all three groups.

Table 7. The immunohistochemical localization of individual MMPs in the corneal specimens obtained from patients from groups I, II, and III and the average values of immunohistochemical staining intensities in all the 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 6. Immunohistochemical localization of matrix metalloproteinases in melted and control corneas. Immunohistochemical localization of MMP-1, -2, -3, -7, -8, -9, and -13 in representative images of melted corneal specimens obtained from patients with pSS (group I) and from control corneas. Original magnification: 100×. Ep = epithelium, AS = anterior stroma, PS = posterior stroma, En = endothelium. Assumed from Brejchova et al [205].



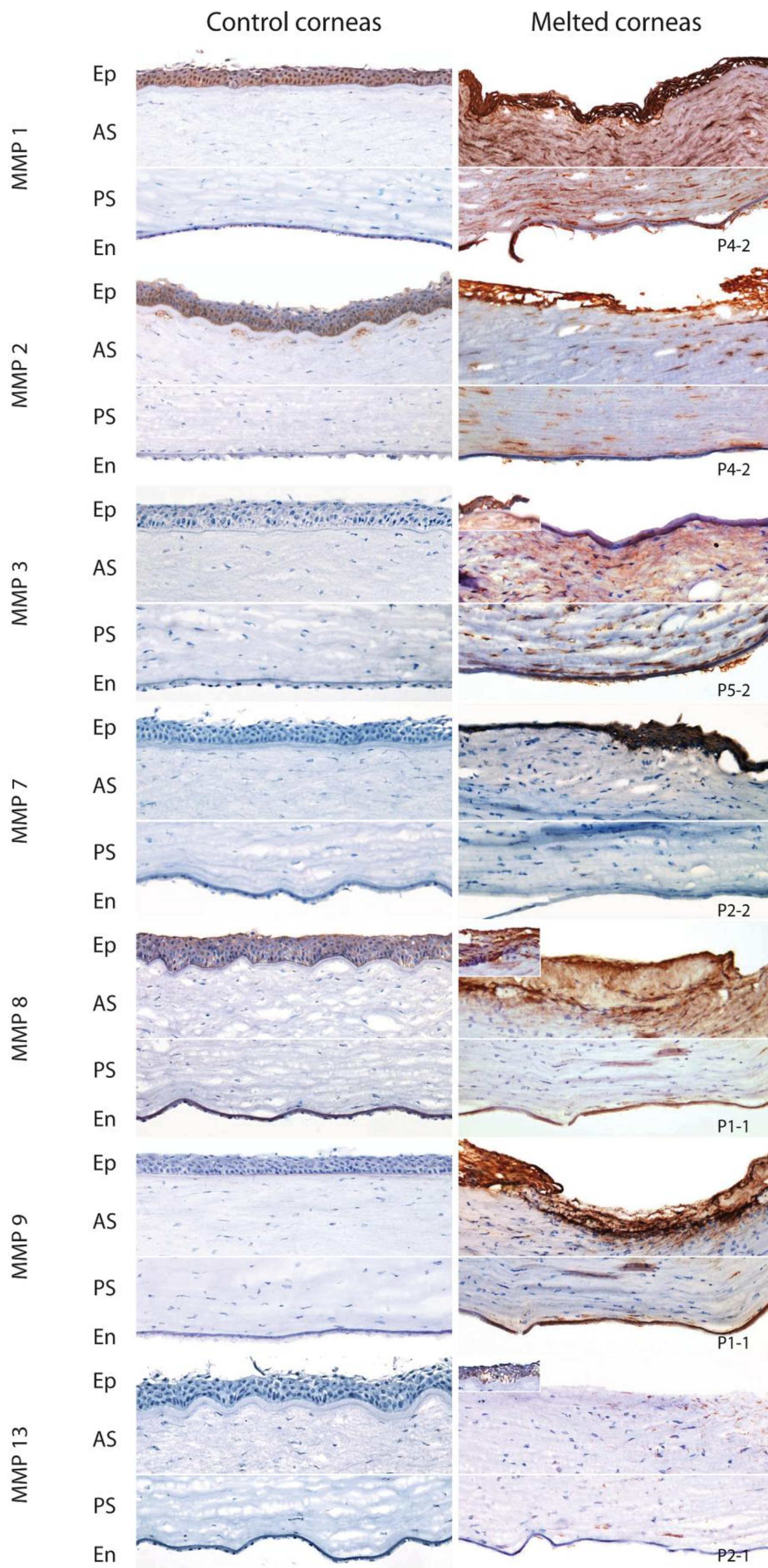


Figure 7. Immunohistochemical localization of matrix metalloproteinases in melted and control corneas. Immunostaining of MMP-1, -2, -3, -7, -8, -9 and 13 in representative samples of melted corneas obtained from controls and patients from groups II and III. The photomicrographs of melted corneas stained for MMP-3, -8 and -13 have insets showing the epithelium of the same specimen. Ep = epithelium, AS = anterior stroma, PS = posterior stroma, En = endothelium. Assumed from Brejchova et al. [206].

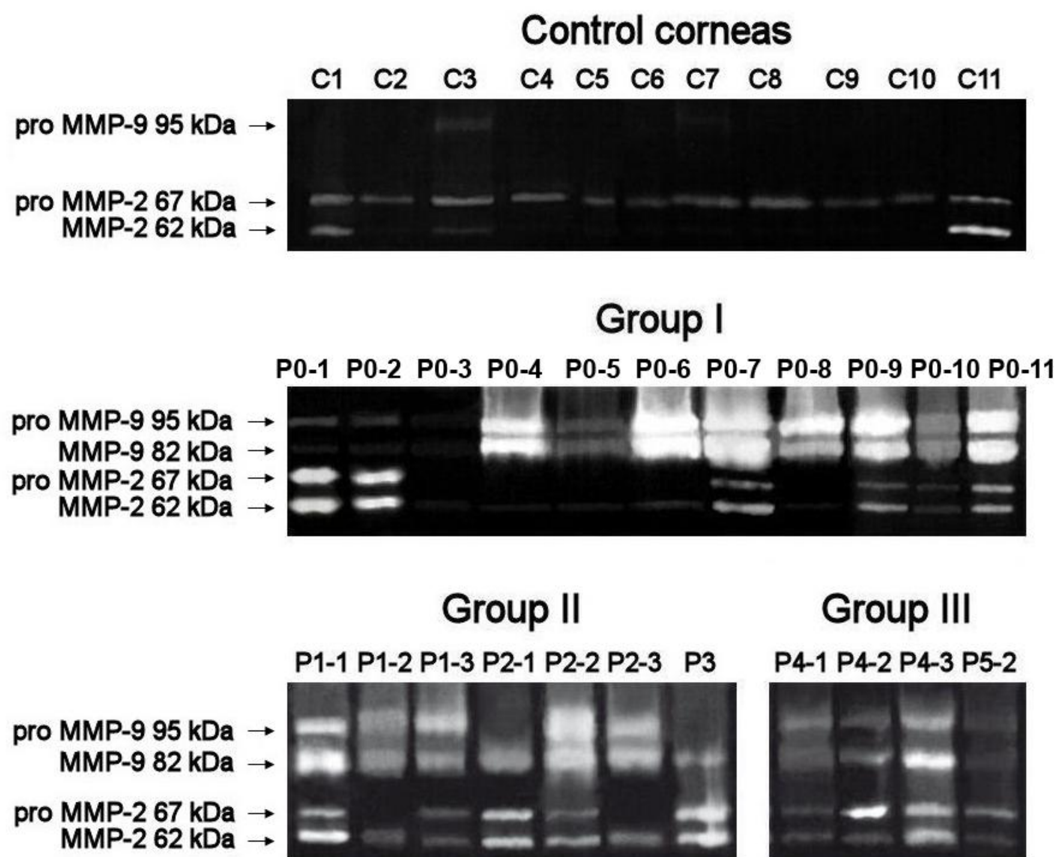
Eighty five percent of the melted corneas from group I with unaffected endothelium exhibited the presence of MMP-9 in this corneal layer, whilst in groups II and III the positive staining of this enzyme was shown only in one specimen from each of these groups (P2-1 and P4-2).
No positivity for MMP-13 was detected in almost all melted corneas except for a low level of this enzyme in 30% of samples from all three groups. Weak to moderate staining was detected in the epithelial fragments and in the stroma of 36% of specimens and in the endothelium of 9% of samples from group I. Weak staining was also found in about 25% of samples from groups II and III (throughout all layers of sample P2-1 and in the anterior stroma of samples P1-1 and P4-3).
In all pathologic samples, pronounced differences in the staining pattern of individual MMPs were observed. No dependence MMP staining pattern and localization in the individual group of patients was found; instead the MMP expression seemed to be rather non-uniform. No immunostaining was present in any of the negative controls.

4.3 Detection of Matrix Metalloproteinase-2 and -9 Activities

A faint band of the partly cleaved form [140], and none, or a very faint band, of the active form of MMP-2 was detected in all the controls, whilst only two controls (C3 and C7) exhibited a faint band of the MMP-9 proenzyme (Fig. 8).
In group I, extremely high and distinct levels of both the partly cleaved and active forms of MMP-2 were found in two (P0-1 and P0-2) and three (P0-7, P0-9 and P0-11) of the 11 melted specimens, respectively. Other samples displayed either no or low levels of both forms of this enzyme, in a similar way to the controls. Levels of MMP-9 proenzyme and the active form were extremely high in seven (P0-4, P0-6 - P0-11) melted grafts and prominent in one (P0-5). Three melted specimens (P0-1 - P0-3) revealed faint bands of both forms of MMP-9 (Fig. 8).
In group II, markedly increased levels of both forms of MMP-2 (the partly cleaved and the active form) were found in four (P1-1, P2-1, P2-2, P3) melted corneas. Two pathologic samples (P1-2, P2-3) showed the presence of the active MMP-2 only, and sample P1-3 displayed a faint band of the partly cleaved proenzyme and a very faint band of the active form of MMP-2. High levels of both forms of MMP-9 and a prominent band of only the active MMP-9 were detected in five (P1-1, P1-2, P1-3, P2-2, P2-3) and two melted corneas (P2-1, P3), respectively, (Fig. 8).

Specimen P4-3 from group III showed markedly increased levels of both the partly cleaved and the active MMP-2, while the sample P4-2 revealed increased expression of the proenzyme only. Two melted corneas (P4-1, P5-2) displayed a faint band of the partly cleaved proenzyme and none of, or a very faint band of, the active form of MMP-2. High levels of both forms of MMP-9 were detected in three (P4-1, P4-2, P4-3) and weak levels in one (P5-2) melted specimen (Fig. 8).

Figure 8. Gelatin zymography in melted specimens of patients from groups I, II and III. Detection of both forms of MMP-2 (the partly cleaved proenzyme - 67 kDa, and the active form of MMP-2 - 62 kDa) as well as both forms of MMP-9 (the proenzyme -95kDa and active MMP-9 - 82 kDa) in the controls and melted specimens.



4.4 Detection of Matrix Metalloproteinase-3 and -7 Activities

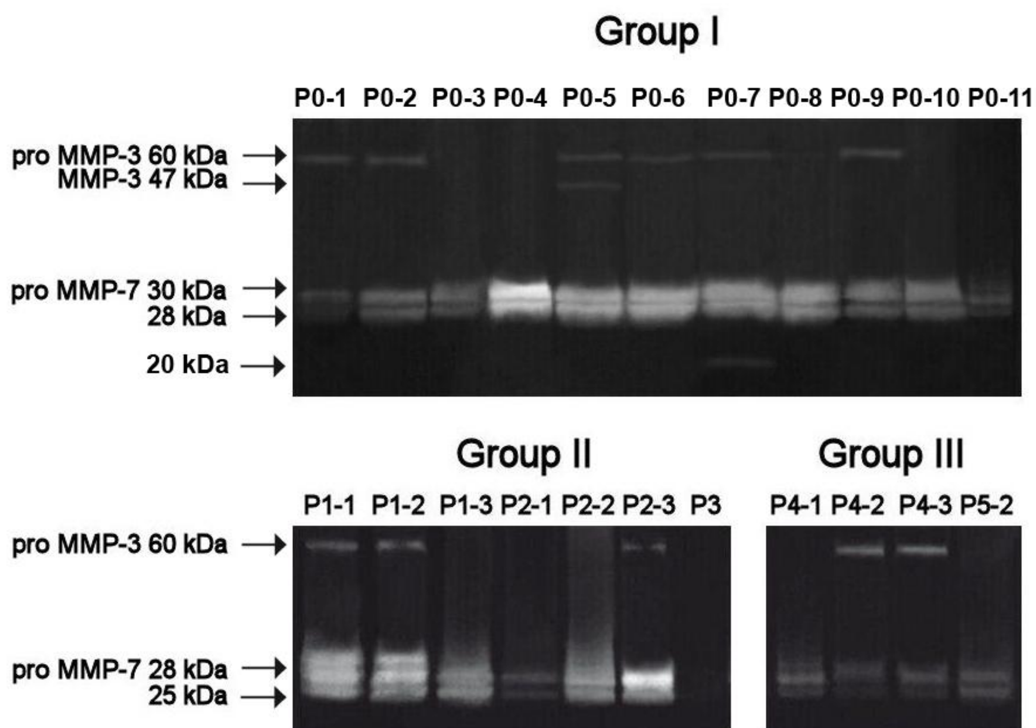
Casein zymography revealed neither the proenzyme nor the active enzyme of MMP-3 or -7 in any of the control specimens.

Negligible levels of the proform of MMP-3 were found in five melted specimens (P0-1, P0-2, P0-6, P0-7, and P0-9) of group I. Both forms, the proform and active MMP-3, were detected only in sample P0-5. Dense bands and faint bands 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) were observed, respectively. A very low level of the active MMP-7 was found in specimen P0-7 only (Fig. 9).

In group II, faint bands of the MMP-3 proenzyme in three melted corneal samples (P1-1, P1-2, and P2-3) were detected. High levels of the inactive MMP-7 forms (proenzyme cleavage products) were found in five (P1-1, P1-2, P1-3, P2-2, P2-3) melted corneas, whereas sample P2-1 exhibited faint bands of these two MMP-7 forms (Fig. 9).

Two specimens (P4-2 and P4-3) from group III displayed faint bands of the MMP-3 proenzyme. Faint bands corresponding to the inactive intermediate cleavage products of MMP-7 were shown in all tested melted samples (P4-1, P4-2, P4-3, and P5-2) (Fig. 9).

Figure 9. Casein zymography in melted specimens of patients from groups I, II and III. Detection of both forms of MMP-3 (the proenzyme - 60 kDa, and the active MMP-3 - 47 kDa) as well as several forms of MMP-7 (the proenzyme - 30 kDa, MMP-7 intermediate cleavage products - 25 and 28 kDa, and the active MMP-7 - 20 kDa) in the melted specimens.



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

No activity was detected in the control specimens. In group I, the active form of MMP-1 was found in eight of ten melted corneas (concentration range, 0.08 – 0.7 ng/ml; p=0.0011). Significantly higher MMP-1 activity was also shown in all melted corneas from group II (range, 0.35 – 8.38 ng/ml) and three of four tested pathologic samples from group III compared to the controls (range, 0.14 – 1,46 ng/ml; p-value < 0.001), (Fig. 10). Specimens P0-3 and P5-1 couldn't be evaluated due to the limited amount 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 ng/ml, p-value < 0.001) than in the controls, ten of which displayed low levels of the active MMP-3 (range, 0-32.4 ng/ml) (Fig. 11). Group I was not included in the MMP-3 activity assay evaluation due to the insufficient size of the sample.

Figure 10. The concentrations of active MMP-1 in control (C) and melted corneas from all three groups determined by MMP-1 activity assay.

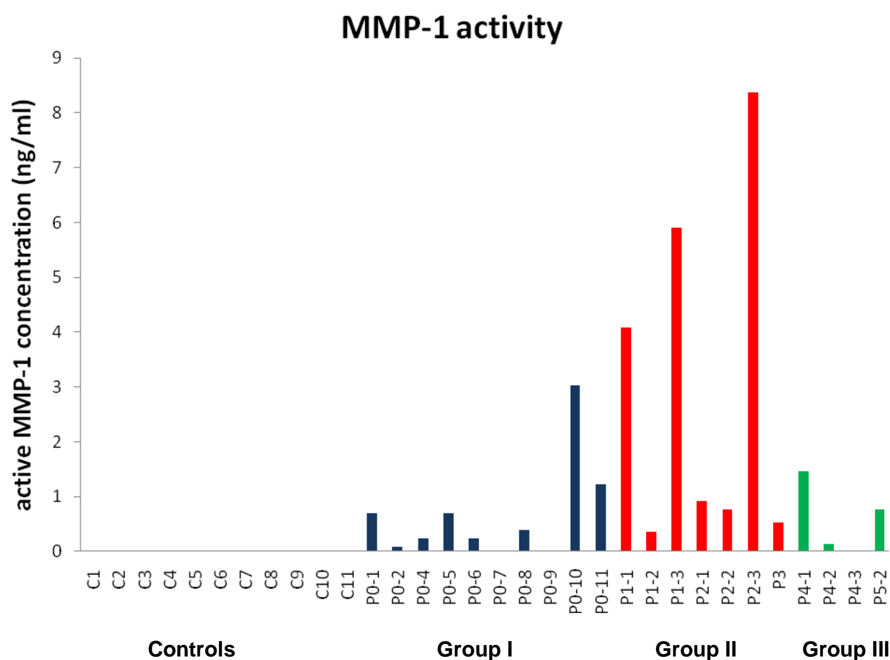
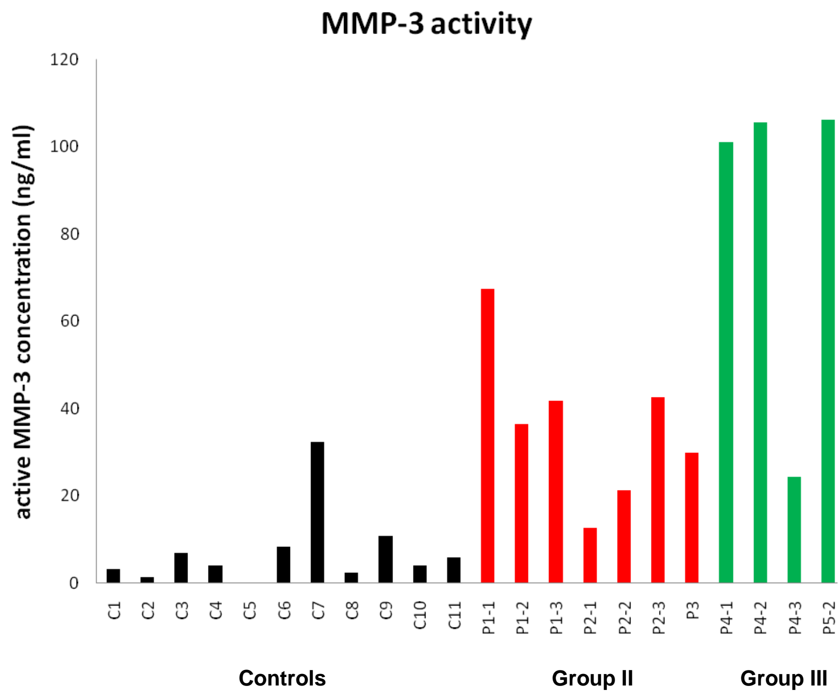


Figure 11. The concentrations of MMP-3 in control (C) and melted corneas from groups II and III determined by activity assays.



5 DISCUSSION

5.1 Highlights of the Work Presented

In this study three groups of patients are presented: those suffering from pSS (group I); from RA (group II); and patients with various underlying pathologies (group III), who underwent rapidly progressive recurrent corneal melting despite all available treatment. We obtained 23 tissue specimens in total from all seven patients investigated. Five samples were melted native corneas and 18 were corneal grafts or regrafts which had failed due to corneal melting. **Our findings clearly demonstrate markedly elevated levels of MMP-1, -2, -3, -7, -8 and -9 as well as a higher activity of MMP-1, -2, -3, -7, and -9, in melted corneas, compared to the control tissue.** These results strongly support the hypothesis that **MMPs are responsible for, or strongly contribute to, the pathological process of corneal melting.**

5.2 Matrix Metalloproteinases in the 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 [99, 174, 179, 181], the exact mechanism of corneal melting and MMP participation in this process haven't been satisfactorily described yet. In this work, which summarizes results about different MMP expression and activity in healthy and melted corneas, we have attempted to illustrate how individual MMPs could contribute to the corneal melting process in each corneal layer.

5.2.0 *Loss of the Epithelium and Degradation of the Basement Membrane*

The process of keratolysis usually precedes epithelial defect formation (see above), which is followed subsequently by the epithelial basement membrane and stromal degradation [207]. The failure to re-epithelialize, the delay in basement membrane repair and the degradation of stromal components are thought to be caused by excessive proteolytic activity in the cornea [118], for which MMPs are in all likelihood responsible [99, 118, 174, 179]. Gelatinases may play an important role in the first step of this process. Their presence in melted tissue was found by us as well as by others [99, 179]. Both the strong activity and immunostaining 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 [207]. According to the same hypothesis [207], which was examined on a mouse thermal burn model of corneal melting, MMP-2 synthesis is delayed and limited to a short period in the corneal melting process because it is involved in the attempts of the melted tissue to regenerate. This could explain the variability in MMP-2 activity detected by us in our melted specimens, which might have been obtained at different stages of the melting. This theory is supported by discrepancies among several studies reporting on MMP-2 expression in ulcerated corneas. Some studies showed its over-expression [9, 169, 179] whereas another detected only a weak presence of MMP-2 around a few keratocytes [99].

The epithelial basement membrane during corneal melting may also be degraded by other MMPs, as with MMP-3 and -7. Both of these enzymes are able to cleave components of the basement membrane (type IV collagen, procollagens, collagen cross-links, fibronectin, laminin) [137, 149]. Previously detected strong immunostaining of MMP-3 in the melted graft of a patient after photorefractive keratectomy treated with NSAIDs [179] is in compliance with our results as we detected a high level of this stromelysin in both the epithelium and the stroma in most of our melted specimens. Moreover, we proved a significant increase in MMP-3 activity in groups II and III compared to the control corneas by an MMP-3 activity assay and the presence of active MMP-3 in one specimen of group I by casein zymography. Unfortunately, there was not enough material available to perform a more sensitive MMP-3 activity assay on specimens in group I. Although we have shown high MMP-3 activity in groups II and III, we were not able to detect the active form of this enzyme using zymography in these specimens. This fact could be explained by the different sensitivity of both methods because the highest concentration of active MMP-3 measured by the activity assay (106.2 ng/ml) doesn't reach the detection limit of casein zymography for this enzyme (400 ng/ml) [208]. Discrepancies between the MMP-3 activity results (high levels detected by activity assay and no signal observed by zymography) as well as differences between MMP-3 activity and the immunostaining may be caused by the possible cross-reactivity of MMP-3 with MMP-10. Although there is no comment in the manufacturer's datasheet, these two MMPs have almost identical substrate specificities [137], and therefore the MMP-3 activity assay may recognize both the active MMP-3 and -10. Other more general reasons that could explain the observed variability in MMP expression are discussed below.

To the best of our knowledge, the 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 [156] in almost all the specimens of all three groups.

5.2.1 *Degradation of Stromal Extracellular matrix and Rupture of the Cornea*

As collagens, especially collagen types I and III, are the main ECM elements composing the Bowman's layer and corneal stroma [17], the participation of collagenases in corneal melting is expected. Our study confirmed a previously detected high level of MMP-1 in the melted corneas [174] and, moreover, demonstrated a statistically significant higher MMP-1 activity. However, the concentration of active MMP-1 in the melted specimens was lower than had been expected based on the 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 [209, 210]. Perceptible differences in immunostaining for MMP-8, a neutrophil collagenase, were shown in the stroma of all melted specimens. As MMP-8 is produced by neutrophils, the distribution of which in corneal stroma may vary among melted corneas, the level of MMP-8 could also differ among these specimens [88, 99, 137, 188]. The presence of this collagenase in the corneal stroma in areas of leukocyte infiltration was found in some of our specimens from group I, where neutrophils were detected using an anti-CD66b antibody (data not shown) which had been described previously in ulcerative keratolysis [99].

As far as we know, the presence of MMP-13 has not been previously tested in melted corneas. 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 [17], than it does types I and III [211]. These two collagen types are more likely cleaved by MMP-8 and -1, respectively (Tab. 1) [137].

Gelatinases represent another group of collagenolytic enzymes. From collagens present in corneal stroma, they are able to degrade collagen type V, VI and denatured collagens [18, 149, 162]. Moreover, MMP-2 is capable of cleaving native collagen types I and III in a similar manner to the collagenases, though with weaker activity [140, 150]. It also digests the stromal core protein decorin [161]. Our study demonstrated strong expression of both gelatinases (MMP-2 and -9) in the stroma and high levels of active MMP-9 in almost all tested specimens from all three groups.

Last but not least, the stromelysin-1 (MMP-3) and matrilysin (MMP-7), both of which we have shown to be present in the stroma of almost all specimens from all three groups,

could contribute to degradation of the corneal stroma. The stroma is also composed of proteoglycans, which are effectively degraded by MMP-3 and -7 [14, 149, 212]. These two enzymes also digest laminin and fibronectin which is expressed in the stroma around keratocytes [7, 149]. Moreover, MMP-3 cleaves procollagen peptides collagen type V as well as activating other MMPs [137, 139, 149].

Finally, once the stroma is completely lost, a descemetocele is formed, and the integrity of the whole cornea is disrupted. 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 [27, 137].

5.3 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 detected level and activity of individual MMPs 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 heterogenity in the expression of MMPs showing local variations (dependent on the centre of the lesion) within individual specimens. As our specimens were cut into two halves for subsequent use in immunohistochemical staining and the activity assay, any variations found in the results could reflect the heterogenity of MMP distribution or the distance from the central melting point. 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 months. Different levels of MMP expression can therefore explain the very different speeds 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 an increased expression of some unique combination of MMPs in samples from particular groups.

Discrepancies in results from immunostaining and the activity of some MMPs could be explained by another reason which lies in 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 respect 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 their quantitative differences between samples. Therefore, the staining results do not necessarily have to correlate with the results from the activity assay or zymography.

5.4 Future Possible Treatment of Corneal Melting

Although the present management of keratolysis includes several surgical techniques (see above) used according to the stage of this destructive process [102, 107], some beneficial effects of immunosuppressive agents have been reported [101, 213, 214]. However, in severe cases of keratolysis the treatment is rather unsuccessful [100, 106, 122, 174].

None of the disease-modifying therapies used in our patients were effective in the regulation of MMP production and keratolysis. According to our results, novel treatment strategies intent on inhibiting MMP activity or expression (especially MMP-1 and MMP-9 due to their massive overexpression in all melted specimens) should be considered in similar cases. Agents inhibiting MMP activity such as recombinant TIMPs or another synthetic matrix metalloproteinase inhibitor (Galardin), were successfully used previously to treat ulcerated rabbit corneas [104, 215]. 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 [120, 216, 217]. From agents known to inhibit MMP expression, TNF-α antagonist infliximab has been shown to have a beneficial effect in the treatment of progressive rheumatoid arthritis-associated corneal ulceration [218]. Infliximab inhibits TNF-α which is known to activate MMP production [137, 185, 218]. Finally, an alternative way to manage keratolysis could be focused on the recovery and strengthening of the stromal collagen structure by collagen cross-linking. Corneal cross-linking is used as standard to treat initial stages of keratoconus; however, some success in the treatment of several cases of refractive corneal melting has already been reported [219, 220].

5.5 Conclusions and Future Work

Taken together, our results have confirmed the important role of MMPs in noninfectious corneal melting and **extend the knowledge of their participation in this destructive process**.

Our work represents a comprehensive study **evaluating the main representatives (MMP-1, -8, -13; MMP-2, -9; MMP-3 and MMP-7) of four MMP subfamilies (collagenases, gelatinases, stromelysins and matrilysins) on a relatively large set of pathologic samples, which is rare due to the infrequency of keratolysis,** and shows that it is not only a few MMPs but a **wide spectrum of these enzymes which contribute to this destructive disease.**

We have defined which members of the large MMP family participate in corneal melting and in which corneal layer they mainly take effect. We have confirmed the results of other studies supposing the role of gelatinases (MMP-2 and-9), collagenases (MMP-1 and -8) and stromelysin MMP-3 [99, 174, 179-181] in corneal melting, but, moreover, **this is the first study describing the presence of matrilysin MMP-7 and the negligible level of collagenase MMP-13 in melted corneas [205, 206].**

To the best of our knowledge, we are the first to determine the activity of MMP-1, -3 and -7 in melted corneal specimens [205, 206].

Although we used specimens from patients with different underlying diseases in whom the exact trigger mechanisms of corneal melting may have varied, **the final progress of corneal degradation concerning MMP expression and activation was shown to be more or less similar in all patient specimens**.

Although we have shown 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 both corneal melting and the maintenance of homeostasis in normal cornea, but which have still not been studied. MMP-10 (stromelysin-2) is a good candidate for our future work to be focused on these topics, both because its presence in normal corneas was found in our first experiments and because of its similarity to MMP-3 and similar substrate specifity, which probably caused discrepancies in the MMP-3 activity results as well as differences between MMP-3 activity and the immunostaining.

Despite this research, the disease-initiating mechanisms are still unclear and need to be clarified. Our future work will mainly be focused on the elucidating of the 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 another point in our future plans.

6 LIST OF ABBREVIATIONS

aminophenylmercuric acetate	APMA
antigen presenting cells	APC
bicinchoninic acid	BCA
ethylenediaminetetraacetic acid	EDTA
extracellular matrix	ECM
Fas ligand	FasL
interleukin 1	IL-1
keratoconjunctivitis sicca	KCS
major histocompatibility complex	MHC
matrix metalloproteinases	MMPs
membrane-type MMPs	MT-MMPs
mitogen-activated protein kinases	MAPKs
nonsteroidal anti-inflammatory drugs	NSAIDs
ocular cicatricial pemphigoid	OCP
Optimal Cutting Temperature Compound	OCT
phosphate-buffered saline	PBS
polymorphonuclear neutrophils	PMN
primary Sjögren's syndrome	pSS
proenzyme form of MMP	proMMP
rheumatoid arthritis	RA
SDS-polyacrylamide gel electrophoresis	SDS-PAGE
sodium dodecyl sulfate	SDS
T-helper cell type 1	Th1
tissue inhibitors of metalloproteinases	TIMPs
transforming growth factor beta	TGF-β
tumor necrosis factor alpha	TNF-α

7 REFERENCES

1. Gospodarowicz D, Greenburg G, Birdwell CR. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res* 1978;38:4155-4171.
2. O'Sullivan F, Clynes M. Limbal stem cells, a review of their identification and culture for clinical use. *Cytotechnology* 2007;53:101-106.
3. Nishida T. *Cornea*. second edition ed. Philadephia, Edinburgh, London, New york, Oxford, St Luis. Sydney, Toronto: Elsevier Inc.; 2005:1409.
4. Mishima S. Corneal thickness. *Surv Ophthalmol* 1968;13:57-96.
5. Dua HS, Gomes JA, Singh A. Corneal epithelial wound healing. *Br J Ophthalmol* 1994;78:401-408.
6. Suzuki K, Saito J, Yanai R, et al. Cell-matrix and cell-cell interactions during corneal epithelial wound healing. *Prog Retin Eye Res* 2003;22:113-133.
7. 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.
8. Lee YC, Wang IJ, Hu FR, Kao WW. Immunohistochemical study of subepithelial haze after phototherapeutic keratectomy. *J Refract Surg* 2001;17:334-341.
9. 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.
10. Saika S, Ohnishi Y, Ooshima A, Liu CY, Kao WW. Epithelial repair: roles of extracellular matrix. *Cornea* 2002;21:S23-29.
11. Sta Iglesia DD, Stepp MA. Disruption of the basement membrane after corneal debridement. *Invest Ophthalmol Vis Sci* 2000;41:1045-1053.
12. Zieske JD. Extracellular matrix and wound healing. *Curr Opin Ophthalmol* 2001;12:237-241.
13. Willoughby CE, Ponzin D, Ferrari S, Lobo A, Landau K, Y. O. Anatomy and physiology of the human eye: effects of mucopolysaccharidoses disease on structure and function – a review. *Clinical and Experimental Ophthalmology* 2010;2-11.
14. 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.
15. Beuerman RW, Pedroza L. Ultrastructure of the human cornea. *Microsc Res Tech* 1996;33:320-335.
16. Wilson SE, Hong JW. Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. *Cornea* 2000;19:417-420.
17. 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.

18. 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.

19. Sakai LY, Keene DR, Morris NP, Burgeson RE. Type VII collagen is a major structural component of anchoring fibrils. *J Cell Biol* 1986;103:1577-1586.

20. Otori T. Electrolyte content of the rabbit corneal stroma. *Exp Eye Res* 1967;6:356-367.

21. Polack FM, Smelser GK. The persistence of isotopically labeled cells in corneal grafts. *Proc Soc Exp Biol Med* 1962;110:60-61.

22. Kao WW, Liu CY. Roles of lumican and keratocan on corneal transparency. *Glycoconj J* 2002;19:275-285.

23. Giraud JP, Pouliquen Y, Offret G, Payrau P. Statistical morphometric studies in normal human and rabbit corneal stroma. *Exp Eye Res* 1975;21:221-229.

24. Komai Y, Ushiki T. The three-dimensional organization of collagen fibrils in the human cornea and sclera. *Invest Ophthalmol Vis Sci* 1991;32:2244-2258.

25. Smelser GK, Polack FM, Ozanics V. Persistence of donor collagen in corneal transplants. *Exp Eye Res* 1965;4:349-354.

26. Scott JE, Bosworth TR. A comparative biochemical and ultrastructural study of proteoglycan-collagen interactions in corneal stroma. Functional and metabolic implications. *Biochem J* 1990;270:491-497.

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

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

29. Neufeld AH, Jumblatt MM, Matkin ED, Raymond GM. Maintenance of corneal endothelial cell shape by prostaglandin E2: effects of EGF and indomethacin. *Invest Ophthalmol Vis Sci* 1986;27:1437-1442.

30. Rodrigues MM, Sun TT, Krachmer J, Newsome D. Epithelialization of the corneal endothelium in posterior polymorphous dystrophy. *Invest Ophthalmol Vis Sci* 1980;19:832-835.

31. Joyce NC. Cell cycle status in human corneal endothelium. *Exp Eye Res* 2005;81:629-638.

32. Kay EP, Smith RE, Nimni ME. Basement membrane collagen synthesis by rabbit corneal endothelial cells in culture. Evidence for an alpha chain derived from a larger biosynthetic precursor. *J Biol Chem* 1982;257:7116-7121.

33. Laing RA, Chiba K, Tsubota K, Oak SS. Metabolic and morphologic changes in the corneal endothelium. The effects of potassium cyanide, iodoacetamide, and ouabain. *Invest Ophthalmol Vis Sci* 1992;33:3315-3324.

34. Watsky MA, McCartney MD, McLaughlin BJ, Edelhauser HF. Corneal endothelial junctions and the effect of ouabain. *Invest Ophthalmol Vis Sci* 1990;31:933-941.

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

36. Hull DS, Green K, Boyd M, Wynn HR. Corneal endothelium bicarbonate transport and the effect of carbonic anhydrase inhibitors on endothelial permeability and fluxes and corneal thickness. *Invest Ophthalmol Vis Sci* 1977;16:883-892.

37. Riley MV, Winkler BS, Peters MI, Czajkowski CA. Relationship between fluid transport and in situ inhibition of Na(+)-K+ adenosine triphosphatase in corneal endothelium. *Invest Ophthalmol Vis Sci* 1994;35:560-567.

38. Marfurt CF, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. *Exp Eye Res* 90:478-492.

39. Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res* 2003;76:521-542.

40. Beebe DC. Maintaining transparency: a review of the developmental physiology and pathophysiology of two avascular tissues. *Semin Cell Dev Biol* 2008;19:125-133.

41. Sack RA, Beaton A, Sathe S, Morris C, Willcox M, Bogart B. Towards a closed eye model of the pre-ocular tear layer. *Prog Retin Eye Res* 2000;19:649-668.

42. Sack RA, Tan KO, Tan A. Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid. *Invest Ophthalmol Vis Sci* 1992;33:626-640.

43. Forstot SL, Kaufman HE. Corneal transplantation. *Annu Rev Med* 1977;28:21-35.

44. Hippel AV. Eine Methode der Hornhauttransplantation. *Graefes Arch Ophthalmol* 1888;34:108-130.

45. Zirm EK. Eine erfolgreiche totale Keratoplastik (A successful total keratoplasty). 1906. *Refract Corneal Surg* 1989;5:258-261.

46. Report of the organ transplant panel. Corneal transplantation. Council on Scientific Affairs. *Jama* 1988;259:719-722.

47. Arentsen JJ. Corneal transplant allograft reaction: possible predisposing factors. *Trans Am Ophthalmol Soc* 1983;81:361-402.

48. Ing JJ, Ing HH, Nelson LR, Hodge DO, Bourne WM. Ten-year postoperative results of penetrating keratoplasty. *Ophthalmology* 1998;105:1855-1865.

49. Price FW, Jr., Whitson WE, Marks RG. Graft survival in four common groups of patients undergoing penetrating keratoplasty. *Ophthalmology* 1991;98:322-328.

50. Thompson RW, Jr., Price MO, Bowers PJ, Price FW, Jr. Long-term graft survival after penetrating keratoplasty. *Ophthalmology* 2003;110:1396-1402.

51. Verdier DD. *Penetrating Keratoplasty*. second ed. Philadephia: Elsevier Mosby; 2005:2087.

52. Beauregard C, Huq SO, Barabino S, Zhang Q, Kazlauskas A, Dana MR. Keratocyte apoptosis and failure of corneal allografts. *Transplantation* 2006;81:1577-1582.

53. Foulks GN, Sanfilippo F. Beneficial effects of histocompatibility in high-risk corneal transplantation. *Am J Ophthalmol* 1982;94:622-629.

54. Joseph A, Raj D, Shanmuganathan V, Powell RJ, Dua HS. Tacrolimus immunosuppression in high-risk corneal grafts. *Br J Ophthalmol* 2007;91:51-55.

55. Batchelor JR, Casey TA, Werb A, et al. HLA matching and corneal grafting. *Lancet* 1976;1:551-554.

56. Boisjoly HM, Tourigny R, Bazin R, et al. Risk factors of corneal graft failure. *Ophthalmology* 1993;100:1728-1735.

57. Inoue K, Amano S, Oshika T, Sawa M, Tsuru T. A 10-year review of penetrating keratoplasty. *Jpn J Ophthalmol* 2000;44:139-145.

58. 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.

59. Volker-Dieben HJ, D'Amaro J, Kok-van Alphen CC. Hierarchy of prognostic factors for corneal allograft survival. *Aust N Z J Ophthalmol* 1987;15:11-18.

60. Aggarwal RK. Deep lamellar keratoplasty--an alternative to penetrating keratoplasty. *Br J Ophthalmol* 1997;81:178-179.

61. 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.

62. Price MO, Thompson RW, Jr., Price FW, Jr. Risk factors for various causes of failure in initial corneal grafts. *Arch Ophthalmol* 2003;121:1087-1092.

63. Sharif KW, Casey TA. Penetrating keratoplasty for keratoconus: complications and long-term success. *Br J Ophthalmol* 1991;75:142-146.

64. Shi W, Gao H, Wang T, Xie L. Combined penetrating keratoplasty and keratolimbal allograft transplantation in comparison with corneoscleral transplantation in the treatment of severe eye burns. *Clin Experiment Ophthalmol* 2008;36:501-507.

65. Tuft SJ, Gregory WM, Davison CR. Bilateral penetrating keratoplasty for keratoconus. *Ophthalmology* 1995;102:462-468.

66. McColgan K. Corneal transplant surgery. *J Perioper Pract* 2009;19:51-54.

67. Leccisotti A. Air-assisted manual deep anterior lamellar keratoplasty for treatment of herpetic corneal scars. *Cornea* 2009;28:728-731.

68. Park KA, Ki CS, Chung ES, Chung TY. Deep anterior lamellar keratoplasty in Korean patients with Avellino dystrophy. *Cornea* 2007;26:1132-1135.

69. Salouti R, Hosseini H, Eghtedari M, Khalili MR. Deep anterior lamellar keratoplasty with melles technique for granular corneal dystrophy. *Cornea* 2009;28:140-143.

70. Huang T, Wang Y, Ji J, Gao N, Chen J. Deep lamellar endothelial keratoplasty for iridocorneal endothelial syndrome in phakic eyes. *Arch Ophthalmol* 2009;127:33-36.

71. Klintworth GK. Corneal dystrophies. *Orphanet J Rare Dis* 2009;4:7.

72. Mashor RS, Kaiserman I, Kumar NL, Sansanayudh W, Rootman DS. Deep Lamellar Endothelial Keratoplasty Up to 5-Year Follow-up. *Ophthalmology*.

73. Hamrah P, Dana MR. Corneal antigen-presenting cells. *Chem Immunol Allergy* 2007;92:58-70.

74. Mayer WJ, Irschick UM, Moser P, et al. Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers. *Invest Ophthalmol Vis Sci* 2007;48:4459-4467.

75. Niederkorn JY. The immune privilege of corneal grafts. *J Leukoc Biol* 2003;74:167-171.

76. Niederkorn JY. Anterior chamber-associated immune deviation. *Chem Immunol* 1999;73:59-71.

77. Niederkorn JY. Immunology and immunomodulation of corneal transplantation. *Int Rev Immunol* 2002;21:173-196.

78. Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270:1189-1192.

79. Hamrah P, Djalilian, A.R., Stulting, R.D. *Immunologically High-Risk Penetrating Keratoplasty*. second ed. Philadephia: Elsevier Mosby; 2005:2087.

80. Streilein JW. New thoughts on the immunology of corneal transplantation. *Eye (Lond)* 2003;17:943-948.

81. MacEwen CJ, Khan ZU, Anderson E, MacEwen CG. Corneal re-graft: indications and outcome. *Ophthalmic Surg* 1988;19:706-712.

82. 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.

83. Flynn TH, Ohbayashi M, Ikeda Y, Ono SJ, Larkin DF. Effect of allergic conjunctival inflammation on the allogeneic response to donor cornea. *Invest Ophthalmol Vis Sci* 2007;48:4044-4049.

84. Polack FM. The Effect Of Ocular Inflammation On Corneal Grafts. *Am J Ophthalmol* 1965;60:259-269.

85. Lafferty KJ, Prowse SJ, Simeonovic CJ, Warren HS. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Annu Rev Immunol* 1983;1:143-173.

86. Dana MR, Qian Y, Hamrah P. Twenty-five-year panorama of corneal immunology: emerging concepts in the immunopathogenesis of microbial keratitis, peripheral ulcerative keratitis, and corneal transplant rejection. *Cornea* 2000;19:625-643.

87. Streilein JW. Immunobiology and immunopathology of corneal transplantation. *Chem Immunol* 1999;73:186-206.

88. Kuffova L, Holan V, Lumsden L, Forrester JV, Filipec M. Cell subpopulations in failed human corneal grafts. *Br J Ophthalmol* 1999;83:1364-1369.

89. Reinhard T, Bocking A, Pomjanski N, Sundmacher R. Immune cells in the anterior chamber of patients with immune reactions after penetrating keratoplasty. *Cornea* 2002;21:56-61.

90. Alldredge OC, Krachmer JH. Clinical types of corneal transplant rejection. Their manifestations, frequency, preoperative correlates, and treatment. *Arch Ophthalmol* 1981;99:599-604.

91. Bartels MC, Otten HG, van Gelderen BE, Van der Lelij A. Influence of HLA-A, HLA-B, and HLA-DR matching on rejection of random corneal grafts using corneal tissue for retrospective DNA HLA typing. *Br J Ophthalmol* 2001;85:1341-1346.

92. Khaireddin R, Wachtlin J, Hopfenmuller W, Hoffmann F. HLA-A, HLA-B and HLA-DR matching reduces the rate of corneal allograft rejection. *Graefes Arch Clin Exp Ophthalmol* 2003;241:1020-1028.

93. The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative

Corneal Transplantation Studies Research Group. *Arch Ophthalmol* 1992;110:1392-1403.

94. Dickey JB, Cassidy EM, Bouchard CS. Periocular FK-506 delays allograft rejection in rat penetrating keratoplasty. *Cornea* 1993;12:204-207.

95. Hill JC. Systemic cyclosporine in high-risk keratoplasty. Short- versus long-term therapy. *Ophthalmology* 1994;101:128-133.

96. Reis A, Reinhard T, Voiculescu A, et al. Mycophenolate mofetil versus cyclosporin A in high risk keratoplasty patients: a prospectively randomised clinical trial. *Br J Ophthalmol* 1999;83:1268-1271.

97. Dana MR, Yamada J, Streilein JW. Topical interleukin 1 receptor antagonist promotes corneal transplant survival. *Transplantation* 1997;63:1501-1507.

98. Guymer RH, Mandel TE. Immunosuppression using a monoclonal antibody to ICAM-1 in murine allotransplantation. *Transplant Proc* 1992;24:218-219.

99. 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.

100. Palay DA, Stulting RD, Waring GO, 3rd, Wilson LA. Penetrating keratoplasty in patients with rheumatoid arthritis. *Ophthalmology* 1992;99:622-627.

101. Malik R, Culinane AB, Tole DM, Cook SD. Rheumatoid keratolysis: a series of 40 eyes. *Eur J Ophthalmol* 2006;16:791-797.

102. 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.

103. Pleyer U, Bertelmann E, Rieck P, Hartmann C. Outcome of penetrating keratoplasty in rheumatoid arthritis. *Ophthalmologica* 2002;216:249-255.

104. 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.

105. 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.

106. 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.

107. Donzis PB, Mondino BJ. Management of noninfectious corneal ulcers. *Surv Ophthalmol* 1987;32:94-110.

108. 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.

109. 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.

110. 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.

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

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

113. Norina TJ, Raihan S, Bakiah S, Ezanee M, Liza-Sharmini AT, Wan Hazzabah WH. Microbial keratitis: aetiological diagnosis and clinical features in patients admitted to Hospital Universiti Sains Malaysia. *Singapore Med J* 2008;49:67-71.

114. Peng CH, Cheng CK, Chang CK, Chen YL. Multiresistant enterococci: a rare cause of complicated corneal ulcer and review of the literature. *Can J Ophthalmol* 2009;44:214-215.

115. Srinivasan M, Lalitha P, Mahalakshmi R, et al. Corticosteroids for bacterial corneal ulcers. *Br J Ophthalmol* 2009;93:198-202.

116. Lifshitz T, Levy J. Rhodotorula rubra keratitis and melting after repeated penetrating keratoplasty. *Eur J Ophthalmol* 2005;15:135-137.

117. Tilak R, Singh A, Maurya OP, Chandra A, Tilak V, Gulati AK. Mycotic keratitis in India: a five-year retrospective study. *J Infect Dev Ctries* 4:171-174.

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

119. Kuckelkorn R, Schrage N, Keller G, Redbrake C. Emergency treatment of chemical and thermal eye burns. *Acta Ophthalmol Scand* 2002;80:4-10.

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

121. Gnadinger MC, Itoi M, Slansky HH, Dohlman CH. The role of collagenase in the alkali-burned cornea. *Am J Ophthalmol* 1969;68:478-483.

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

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

124. McIntosh E. The cost of rheumatoid arthritis. *Br J Rheumatol* 1996;35:781-790.

125. 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.

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

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

128. 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.

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

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

131. 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.

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

133. 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.

134. 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.

135. Ormerod LD, Fong LP, Foster CS. Corneal infection in mucosal scarring disorders and Sjogren's syndrome. *Am J Ophthalmol* 1988;105:512-518.

136. Pfister RR, Murphy GE. Corneal ulceration and perforation associated with Sjogren's syndrome. *Arch Ophthalmol* 1980;98:89-94.

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

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

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

140. 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.

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

142. Salowe SP, Marcy AI, Cuca GC, et al. Characterization of zinc-binding sites in human stromelysin-1: stoichiometry of the catalytic domain and identification of a cysteine ligand in the proenzyme. *Biochemistry* 1992;31:4535-4540.

143. Bode W, Reinemer P, Huber R, Kleine T, Schnierer S, Tschesche H. The X-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity. *Embo J* 1994;13:1263-1269.

144. 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.

145. 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.

146. 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.

147. 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.

148. 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.

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

150. Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med* 2008;29:290-308.

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

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

153. Xie B, Laouar A, Huberman E. Autocrine regulation of macrophage differentiation and 92-kDa gelatinase production by tumor necrosis factor-alpha via alpha5 beta1 integrin in HL-60 cells. *J Biol Chem* 1998;273:11583-11588.

154. Brooks PC, Stromblad S, Sanders LC, et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 1996;85:683-693.

155. Dumin JA, Dickeson SK, Stricker TP, et al. Pro-collagenase-1 (matrix metalloproteinase-1) binds the alpha(2)beta(1) integrin upon release from keratinocytes migrating on type I collagen. *J Biol Chem* 2001;276:29368-29374.

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

157. Santavicca M, Noel A, Angliker H, et al. Characterization of structural determinants and molecular mechanisms involved in pro-stromelysin-3 activation by 4-aminophenylmercuric acetate and furin-type convertases. *Biochem J* 1996;315 (Pt 3):953-958.

158. Mann K, Jander R, Korsching E, Kuhn K, Rauterberg J. The primary structure of a triple-helical domain of collagen type VIII from bovine Descemet's membrane. *FEBS Lett* 1990;273:168-172.

159. 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.

160. 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.

161. 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.

162. 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.

163. 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.

164. Curry TE, Jr., Osteen KG. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev* 2003;24:428-465.

165. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int J Dev Biol* 2004;48:411-424.

166. Rundhaug JE. Matrix metalloproteinases and angiogenesis. *J Cell Mol Med* 2005;9:267-285.

167. Brown D, Chwa M, Escobar M, Kenney MC. Characterization of the major matrix degrading metalloproteinase of human corneal stroma. Evidence for an enzyme/inhibitor complex. *Exp Eye Res* 1991;52:5-16.

168. Fini ME, Girard MT. Expression of collagenolytic/gelatinolytic metalloproteinases by normal cornea. *Invest Ophthalmol Vis Sci* 1990;31:1779-1788.

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

170. 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.

171. Yamagami S, Ebihara N, Usui T, Yokoo S, Amano S. Bone marrow-derived cells in normal human corneal stroma. *Arch Ophthalmol* 2006;124:62-69.

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

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

174. 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.

175. Dushku N, John MK, Schultz GS, Reid TW. Pterygia pathogenesis: corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells. *Arch Ophthalmol* 2001;119:695-706.

176. Fournie PR, Gordon GM, Dawson DG, Edelhauser HF, Fini ME. Correlations of long-term matrix metalloproteinase localization in human corneas after successful laser-assisted in situ keratomileusis with minor complications at the flap margin. *Arch Ophthalmol* 2008;126:162-170.

177. Maguen E, Zorapapel NC, Zieske JD, et al. Extracellular matrix and matrix metalloproteinase changes in human corneas after complicated laser-assisted in situ keratomileusis (LASIK). *Cornea* 2002;21:95-100.

178. Saghizadeh M, Brown DJ, Castellon R, et al. Overexpression of matrix metalloproteinase-10 and matrix metalloproteinase-3 in human diabetic corneas: a possible mechanism of basement membrane and integrin alterations. *Am J Pathol* 2001;158:723-734.

179. 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.

180. 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.

181. 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.

182. 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.

183. 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.

184. 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.

185. 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.

186. 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.

187. Guidera AC, Luchs JI, Udell IJ. Keratitis, ulceration, and perforation associated with topical nonsteroidal anti-inflammatory drugs. *Ophthalmology* 2001;108:936-944.

188. 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.

189. Flach AJ. Corneal melts associated with topically applied nonsteroidal anti-inflammatory drugs. *Trans Am Ophthalmol Soc* 2001;99:205-210; discussion 210-202.

190. Reviglio VE, Rana TS, Li QJ, Ashraf MF, Daly MK, O'Brien TP. Effects of topical nonsteroidal antiinflammatory drugs on the expression of matrix metalloproteinases in the cornea. *J Cataract Refract Surg* 2003;29:989-997.

191. Tomas-Barberan S, Fagerholm P. Influence of topical treatment on epithelial wound healing and pain in the early postoperative period following photorefractive keratectomy. *Acta Ophthalmol Scand* 1999;77:135-138.

192. Takahashi N, Miyakoshi M, Taka N, Kurihara T, Fujikawa K. [Cytotoxic effects of diclofenac sodium]. *Nippon Ganka Gakkai Zasshi* 1993;97:145-149.

193. Shimazaki J, Saito H, Yang HY, Toda I, Fujishima H, Tsubota K. Persistent epithelial defect following penetrating keratoplasty: an adverse effect of diclofenac eyedrops. *Cornea* 1995;14:623-627.

194. Berman M, Leary R, Gage J. Evidence for a role of the plasminogen activator--plasmin system in corneal ulceration. *Invest Ophthalmol Vis Sci* 1980;19:1204-1221.

195. Twining SS, Hatchell DL, Hyndiuk RA, Nassif KF. Acid proteases and histologic correlations in experimental ulceration in vitamin A deficient rabbit corneas. *Invest Ophthalmol Vis Sci* 1985;26:31-44.

196. Lijnen HR, Collen D. Tissue-type plasminogen activator. *Ann Biol Clin (Paris)* 1987;45:198-201.

197. Lijnen HR. Plasmin and matrix metalloproteinases in vascular remodeling. *Thromb Haemost* 2001;86:324-333.

198. Baggiolini M, Schnyder J, Bretz U, Dewald B, Ruch W. Cellular mechanisms of proteinase release from inflammatory cells and the degradation of extracellular proteins. *Ciba Found Symp* 1979;105-121.

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

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

201. Vizoso FJ, Gonzalez LO, Corte MD, et al. Study of matrix metalloproteinases and their inhibitors in breast cancer. *Br J Cancer* 2007;96:903-911.

202. Weiss A, Goldman S, Shalev E. The matrix metalloproteinases (MMPS) in the decidua and fetal membranes. *Front Biosci* 2007;12:649-659.

203. 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.

204. 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.

205. 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.

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

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

208. 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.

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

210. 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.

211. Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem* 1996;271:1544-1550.

212. 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.

213. Kenyon KR. Decision-making in the therapy of external eye disease: noninfected corneal ulcers. *Ophthalmology* 1982;89:44-51.

214. Phillips K, Arffa R, Cintron C, et al. Effects of prednisolone and medroxyprogesterone on corneal wound healing, ulceration, and neovascularization. *Arch Ophthalmol* 1983;101:640-643.

215. 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.

216. 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.

217. 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.

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

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

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

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 AND PRESENTATIONS

8.1 List of Author’s Publications

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 (IF 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 (IF 0.835)

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. Chapter in the book: Cell Apoptosis Research. Scott R. Erlich (editor), New York, Nova Science Publishers, 2007, 203-212.

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)

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 (IF 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 (IF 2.651)

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 List of Author's Presentations

Kristýna Juklová, Martin Filipec and Kateřina Jirsová. Repair of corneal endothelium after mechanical damage under tissue culture and hypothermic conditions. *Sjezd Evropské asociace očních tkáňových bank (EEBA)* Halle, Německo, 14.-16. 1. 2005. (poster)

Kristýna Juklová, Kateřina Jirsová, Martin Filipec. Zvýšená exprese matrix metaloproteináz 2 a 9 při keratolýze lidské rohovky. *XIV. Výroční sjezd České oftalmologické společnosti* Plzeň, 15.-17.6. 2006 (oral prezentation)

Kristýna Juklová, Martin Filipec and Kateřina Jirsová. Increased expression of matrix metalloproteinases 1, 2 and 9 in human corneal grafts during corneal meeting. *European association for Vision and Eye Research (EVER) 2006* Vilamoura, Portugal, 4. – 7.10. 2006 (poster)

Kristýna Juklová and Kateřina Jirsová. High expression of Matrix Metalloproteinases in Rheumatoid Corneal Melting. European *association for Vision and Eye Research (EVER) 2007* Portorož, Slovenia, 3. – 6.10. 2007 (poster)

Kristýna Juklová and Kateřina Jirsová. Role of Matrix Metalloproteinases in a Process of Corneal Melting Affecting Corneal Grafts of Rheumatoid Arthritis Patients. *The Association for Research in Vision and Ophthalmology (ARVO) 2008* 27.4.2008 – 1.5.2008 (poster)

Kristýna Juklová a Kateřina Jirsová. Keratolýza lidské rohovky je doprovázena vysokou expresí matrix metaloproteináz. *XVI. Výroční sjezd České oftalmologické společnosti* Špindlerův Mlýn, 25.– 27. 9. 2008 (oral prezentation)

Kristýna Brejchová, Amanda Vernon, Julie T. Daniels a Kateřina Jirsová. Kultivace a viabilita limbálních kmenových buněk a imunogenicita limbální tkáně v závislosti na typu a délce uchování rohovkových terčů dárců. *XVII. Výroční sjezd České oftalmologické společnosti* Praha, 1.-3.10.2009 (oral prezentation)

9 APPENDED PUBLICATIONS

Molecular Vision 2009; 15:2364-2372 <http://www.molvis.org/molvis/v15/a253>
Received 24 August 2009 | Accepted 1 November 2009 | Published 14 November 2009 © 2009 Molecular Vision

Matrix metalloproteinases in recurrent corneal melting associated with primary Sjörgen's syndrome

Kristyna Brejchova,[1] Petra Liskova,[1] Enkela Hrdlickova,[2] Martin Filipec,[2,3] Katerina Jirsova[1,4]

[1]Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and Charles University in Prague, 1st Faculty of Medicine, Prague, Czech Republic; [2]Department of Ophthalmology, General Teaching Hospital and Charles University in Prague, 1st Faculty of Medicine, Prague, Czech Republic; [3]Lexum European Eye Clinic, Czech Republic; [4]Ocular Tissue Bank, General Teaching Hospital and Charles University in Prague, 1st Faculty of Medicine, Prague, Czech Republic

Purpose: To investigate the contribution of matrix metalloproteinases (MMPs) to recurrent corneal melting in keratoconjunctivitis sicca associated with primary Sjörgen's syndrome (pSS).
Methods: One native melted cornea and ten melted corneal grafts from two patients with severe pSS were used. The presence of MMPs (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 were measured using an activity assay. Eleven unaffected corneas served as controls.
Results: The average values of the staining intensity revealed very intense MMP 1, intense MMP 2, 7, and 9 and moderate MMP 3 and 8 positivity, in the corneal epithelium of melted corneas. Intense MMP 1 and 9 staining, moderate MMP 2, 3, and 8 staining, and weak MMP 7 staining were found in the anterior stroma. The posterior stroma revealed intense MMP 1, moderate MMP 3 and 9, and weak MMP 2, 7, and 8 positivity. Immunostaining of the endothelium was moderate for MMP 9 and weak for MMP 1, 2, 3, 7, and 8. MMP 13 was negative in all but four melted specimens, where weak-to-moderate staining was found in the epithelium and stroma. Control corneas revealed moderate MMP 1 and 2 and weak MMP 8 staining in the epithelium, weak MMP 2 staining in the anterior stroma, and weak MMP 1 and 8 staining in the endothelium. Significantly elevated MMP 1 activity and extremely elevated MMP 9 activity were found in most of the tested pathological specimens, compared to healthy controls, where no activity of the two enzymes was present. Markedly elevated MMP 2 activity was found in 2 of 11 specimens, compared to normal tissue. The inactive form of MMP 3 was detected in half of the tested specimens, and inactive MMP 7 in all melted corneas. Active MMP 3 and 7 were found in one melted sample. Neither of these MMPs was found in any of the control specimens.
Conclusions: The increased expression and elevated activity of a wide range of MMPs in melted cornea samples from two patients diagnosed with pSS confirm that these enzymes contribute to the tissue destruction, leading to serious consequences such as corneal perforation and loss of vision.

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with an estimated prevalence of only about 0.5%. It is characterized by the destruction of the lachrymal and salivary glands, resulting in keratoconjunctivitis sicca syndrome (KCS) and xerostomia [1-3]. There is lymphocytic infiltration in the exocrine glands and the production of various autoantibodies [1,4,5]. Extraglandular systemic manifestations may involve several tissues and organs [3,6-8]. The ethiopathogenesis of pSS is complex; environmental factors are thought to trigger inflammation in individuals with a genetic predisposition, but the exact underlying cause remains unknown [2,5].

Most patients do not exhibit severe ocular complications. Those that need to attend ophthalmology clinics have been found to suffer, in addition to dry eye, from bacterial keratitis, pannus formation, and sterile corneal melting [9-11].

Corneal melting (keratolysis) is a rare, occasionally recurrent condition. It is characterized by the development of epithelial defects and the gradual reduction of stromal components, which may lead to descemetocele formation and subsequent perforation of the cornea [12-15]. Less than 20 cases of sterile corneal melting or corneal ulcers in association with pSS have been described in the literature [11,16,17]. Although the exact mechanism of corneal melting has not been elucidated, it is often linked to the overexpression of matrix metalloproteinases (MMPs), which are considered mainly responsible for the destruction and consequent loss of the extracellular matrix (ECM) [18-21]. Most of these endopeptidases are synthesized as inactive proenzymes that are activated by proteolytic cleavage [22,23]. On the basis of substrate specificity, sequence similarity, and domain organization, MMPs are classified into six groups:

Correspondence to: Kristyna Brejchova, Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, General Teaching Hospital and Charles University in Prague, First Faculty of Medicine, Ke Karlovu 2, Prague 2, 12808, Czech Republic; Phone: 00 420 224 967 139; FAX: 00 420 224 962 338; email: kjuklova@yahoo.com

collagenases, gelatinases, stromelysins, matrilysins, membrane type MMPs, and others. In the cornea, MMPs can be produced by keratocytes, epithelial cells, monocytes, and macrophages [20,24-27].

In this study, seven MMPs representing four main groups of these endopeptidases were investigated in extremely severe cases of KCS associated with pSS. These included collagenases (MMPs 1, 8, 13) capable of cleaving collagen types I, II, and III [23]; gelatinases (MMP 2 and 9) capable of degrading collagen types IV, V, and VI, as well as decorin, fibronectin, and laminin [28-30]; and stromelysins (MMP 3) and matrilysins (MMP 7), which have similar substrates—type IV collagen, procollagens, collagen cross-links, fibronectin, and laminin [22,23]. We report recurrent corneal melting in two patients with severe pSS and its relation to the activity of major MMPs.

METHODS

The study adhered to the tenets set out in the Declaration of Helsinki. Local Ethics Committee approval was granted. All melted explants were obtained from the Department of Ophthalmology, General Teaching Hospital and the 1st Faculty of Medicine, Charles University, in Prague.

Case report 1: A 77-year-old patient was diagnosed with pSS elsewhere at the age of 71. She tested positive for anti-SS-A/Ro, anti-SS-B/La, and antinuclear antibodies. No extraglandular manifestations were noted. Systemic immunosuppression administered to the patient included various combinations of cyclophosphamide, prednisolon, azathioprin, methylprednisolon, cyclosporine A, and mycophenolate mofetil. Upon first examination in our Department of Ophthalmology in 2002 at age 74, the patient presented with bilateral severe dry eye syndrome. In the course of three years, she suffered from numerous episodes of corneal melting, requiring a number of surgical procedures. In the right eye, seven penetrating keratoplasties were performed (6 grafts were used as specimens S2, S3, S4, S6, S7, and S8), along with a number of amniotic membrane transplants, tarsorrhaphies, and other surgeries aimed at improving the condition of the ocular surface and preventing progressive tissue melting. In the left eye, the patient underwent four penetrating keratoplasties (her native cornea was used as specimen S1 and one graft as specimen S5), as well as other, similar, surgeries to the right eye. Despite all the measures undertaken, her condition could not be controlled, and it led to bilateral blindness.

Case report 2: In case 2, the symptoms related to pSS started at the age of 46 when swollen salivary glands, xerostomia, and severe dry eye symptoms were noted by the patient. Histopathology from a labial salivary gland biopsy sample showed focal sialadenitis that was consistent with a diagnosis of pSS. The patient tested positive for rheumatoid factor, antinuclear antibodies, and Scl70 antibodies. Subsequently she also developed arthralgias. Systemic immunosuppression therapy was started at the age of 50. Initially, the patient was treated with oral prednisolon; later, various combinations of methotrexate, cyclophosphamide, and methylprednisolon were added. Upon first ocular examination at age 46, she had signs of moderate dry eye syndrome in both eyes. She gradually developed severe dry eye syndrome bilaterally. When the patient reached the age of 58, the first signs of peripheral ulcerative keratitis were observed in the right eye, followed three years later in the left eye. She rapidly developed corneal thinning, and underwent her first keratoplasty in the right eye at the age of 59 (specimen S9), followed by numerous other procedures due to complications related to melting of the graft, including two penetrating keratoplasties (specimens S10 and S11). At the last follow up visit, her visual acuity was full light projection in the right eye and hand movements with full projection of light in the left eye.

Specimen preparation: Eleven melted corneal specimens of the two patients were processed within three h after surgery. 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). The mean time from the donor's deaths to enucleation was 15 h, and the mean time from death to tissue freezing was 17 h. 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 (Christine Gröpl, Tulln, Austria). Before the activity assessment, the specimens were thawed, were 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 $ZnCl_2$), and underwent protein extraction for 2 days at 4 °C. Next, they were centrifuged for 30 min at 10,000× g; the supernatants were removed and frozen at -20 °C.

Indirect enzyme immunohistochemistry: Cryosections (7 µm thick) from each of the control and melted specimens were placed on gelatin-coated glass slides (four per slide), fixed with cold acetone for 10 min, rinsed in phosphate-buffered saline (PBS), and incubated for 30 min in 3% hydrogen peroxide in PBS. After washing in PBS, the specimens were blocked for 30 min with 2.5% bovine serum albumin in PBS. Then the slides were incubated for 1 h at room temperature with the primary antibodies listed in Table 1. Subsequently, the slides were washed in PBS, and the secondary antibodies (polyclonal rabbit anti-mouse IgG and swine anti-rabbit IgG conjugated with biotin, 1:200; DakoCytomation, Glostrup, Denmark) were applied for 1 h. After rinsing in PBS (three times for 5 min each), streptavidin/HRP (1:250; DakoCytomation, Glostrup, Denmark) was added for 30 min. Finally, the slides were developed with 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in PBS, counterstained with Harris hematoxylin,

Table 1. Matrix metalloproteinase detecting antibodies used for indirect immunohistochemistry.

Antibody	Catalogue number	Concentration	Company
Polyclonal rabbit anti-human MMP 1	AB8105	1:300	Chemicon Intl. Inc.
Monoclonal mouse anti-human MMP 2	MAB13431	1:350	Chemicon Intl. Inc.
Polyclonal rabbit anti-human MMP 3	29576	1:50	AnaSpec Inc., San Jose, CA
Monoclonal mouse anti-human MMP 7	MAB13414	1:150	Chemicon Intl. Inc.
Polyclonal rabbit anti-human MMP 8	AB8115	1:300	Chemicon Intl. Inc.
Monoclonal mouse anti-human MMP 9	MAB3309	1:150	Chemicon Intl. Inc.
Monoclonal mouse anti-human MMP 13	MAB13424	1:50	Chemicon Intl. Inc.

and mounted with Eukit (Fluka, Buchs, Switzerland). Negative control specimens (primary antibody omitted) were included on each slide. Samples of human placenta (MMP 1, 2, 3, and 7) and breast carcinoma (MMP 7, 8, 9, and 13) were used as positive controls [31,32]. 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.

Gelatin and casein substrate zymography: All specimens (native cellular protein quantity, 8.5 µg) were treated with sample buffer (1.5% sodium dodecyl sulfate [SDS], 15% glycerol, and 0.005% bromphenol blue). Gelatin and casein zymography were performed for the detection of MMP 2 and 9, and MMP 3 and 7, respectively, using 10% polyacrylamide gel containing 0.1% gelatin (AppliChem GmbH, Darmstadt, Germany) and 12% gel copolymerized with 0.09% β-casein (Sigma-Aldrich, St. Louis, MO). In brief, 20 µl of each specimen were loaded onto the gels, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 200 V at 4 °C for gelatin zymography, and at 20 mA at 4 °C for casein zymography. After electrophoresis, the gels were twice rinsed in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS, then incubated in 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 to allow the proteinases to digest their substrates. The gels were stained for 1 h at room temperature in 0.5% Coomassie brilliant blue R-250 (Serva Electrophoresis, Heidelberg, Germany) in 40% methanol and 10% acetic acid, 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.

Matrix metalloproteinase 1 activity assays: The concentrations of the active forms of MMP 1 were determined using a commercial kit (Amersham matrix metalloproteinase-1 Biotrak Activity assay system, Amersham Biosciences, Amersham, UK) according to the manufacturer's protocol. The values of the color reaction of the assays were read at 405 nm in a SUNRISE ELISA Reader (Tecan Trading AG, Männedorf, Switzerland). The activity of MMP 1 in ten samples was determined by interpolation from the standard curve. The activity assay could not be performed in S3, due to the lack of sufficient specimens.

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 statistical significance.

RESULTS

Localization of individual matrix metalloproteinases in melted and control specimens: All control corneas exhibited regular morphology, with a five- to six-layered epithelium. Severe damage was observed in most of the pathological specimens, ranging up to the complete absence of the epithelial layers and, in some specimens, a partly dissolved Bowmann's layer and a partly dissolved edge of the anterior stroma in the area of the lesions.

The levels of staining of antibodies against particular MMPs in control samples were averaged. No prominent differences in MMP staining were found among the individual control specimens for any of the MMPs tested. 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 adjacent to Bowman's layer, in approximately one-sixth of the anterior stroma. Weak staining was also found for MMP 1 and 8 in the endothelium of the control specimens. Immunostaining for MMP 3, 7, 9, and 13 was completely negative in all layers of all control corneas (Figure 1).

The staining intensity of different MMPs in melted specimens is summarized in Table 2. Immunohistochemical staining of both the control and melted specimens is shown in Figure 1. In the melted specimens, stronger staining for MMP 9 was found in the epithelial fragments of all tested corneas, if they were not destroyed. It was also found for MMP 1, 2, 3, and 7 in almost all melted corneas, compared to the controls. Increased staining for MMP 1 and 9 was found in the whole stroma in all tested corneas, and for MMP 2, 3, 7, and 8 in almost all melted grafts, compared to the controls. Positivity staining for MMP 2, 3, and 7 was detected in the endothelium of a few melted specimens, while staining for MMP 9 was observed in all melted grafts. MMP 13 revealed only a weak-to-moderate staining pattern in the epithelial fragments and in the stroma of four specimens and in the endothelium of one specimen.

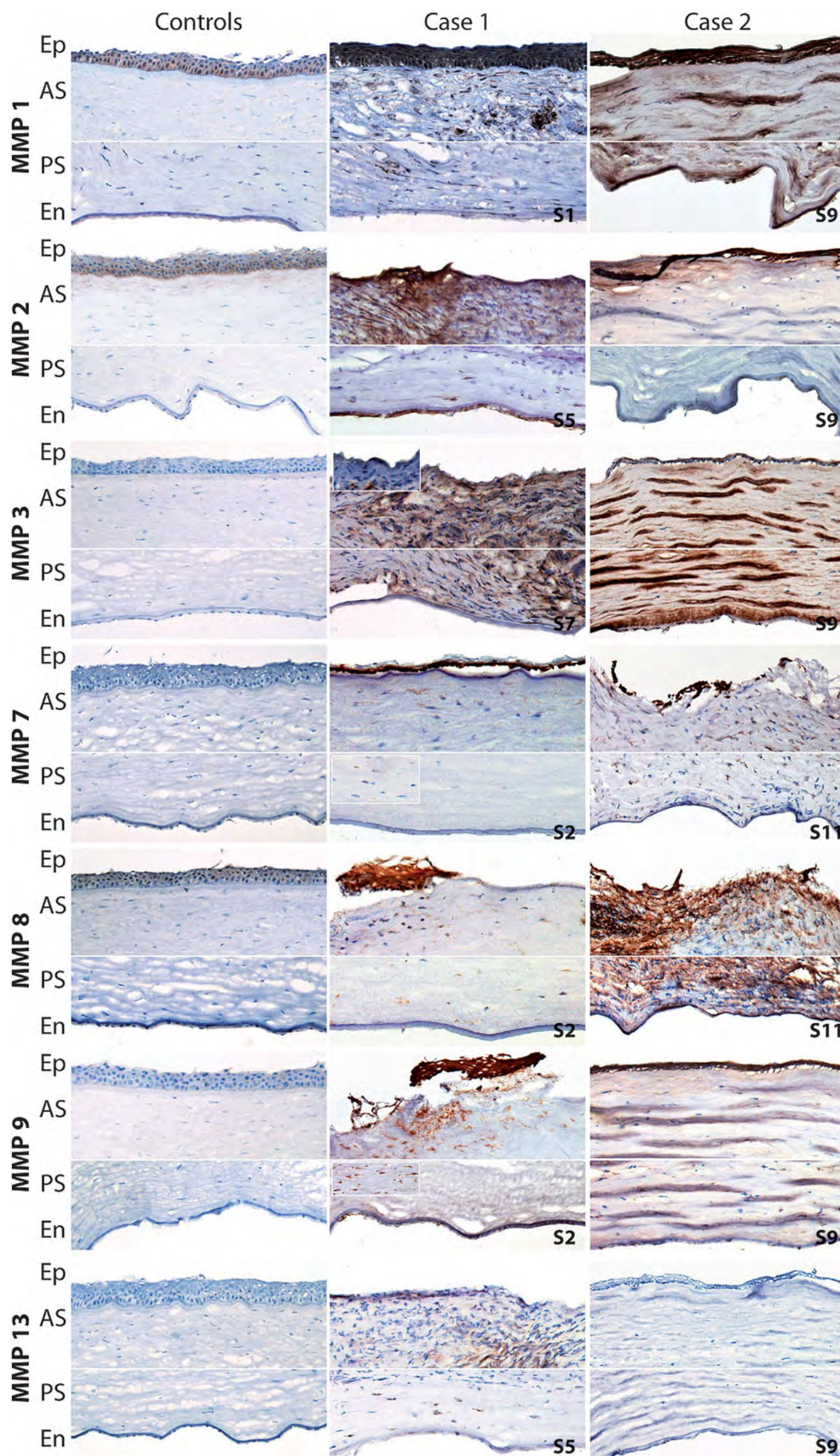


Figure 1. Immunohistochemical localization of matrix metalloproteinases in melted and control corneas. Immunohistochemical localization of MMP 1, 2, 3, 7, 8, 9, and 13 in representative images of melted corneal specimens obtained from patients with pSS (cases 1 and 2) and from control corneas. Original magnification, 100×. Ep = epithelium, AS = anterior stroma, PS = posterior stroma, En = endothelium.

Detection of metalloproteinase activity:

Gelatin and casein zymography—Using gelatin zymography, 2 of the 11 melted specimens (S1 and S2) displayed extremely high levels of both the proenzyme and active form of MMP 2, compared to the control corneas.

Table 2. The immunohistochemical localization of individual MMPs in the corneal specimens obtained from two patients with pSS (S1-8 and S9-11 respectively) and the average values of immunohistochemical staining in all melted specimens (S) and controls (C).

		Sample												
MMP	**Corneal layer**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S**	**C**
MMP 1	Ep	4	4	D	D	D	2	D	4	4	D	D	4	2
	AS	3	4	3	2	3	1	4	4	4	2	3	3	0
	PS	2	3	2	2	4	2	3	4	4	1	3	3	0
	En	0	3	D	D	0	0	D	D	2	D	D	1	1
MMP 2	Ep	3	3	D	D	D	4	3	2	4	D	D	3	2
	AS	4	2	2	0	4	2	2	3	3	2	3	2	1
	PS	3	2	1	0	1	0	0	3	0	2	1	1	0
	En	2	1	0	0	3	2	0	D	1	0	0	1	0
MMP 3	Ep	2	2	D	D	D	2	0	2	3	2	3	2	0
	AS	2	2	1	0	2	1	4	2	4	0	2	2	0
	PS	1	0	2	0	2	1	4	2	4	0	2	2	0
	En	D	0	0	0	1	0	D	D	2	D	D	1	0
MMP 7	Ep	0	4	D	D	D	4	3	4	0	D	4	3	0
	AS	0	1	2	0	3	2	1	3	0	0	2	1	0
	PS	0	1	2	0	3	1	1	3	0	0	2	1	0
	En	D	D	D	0	2	1	D	D	0	D	D	1	0
MMP 8	Ep	2	4	D	D	D	1	0	2	2	D	D	2	1
	AS	2	1	1	2	3	2	0	2	1	2	4	2	0
	PS	0	1	0	2	3	1	0	2	1	1	4	1	0
	En	D	D	0	D	2	0	0	D	2	0	D	1	1
MMP 9	Ep	2	4	D	D	D	1	2	2	4	D	3	3	0
	AS	3	3	1	3	4	4	2	3	1	2	3	3	0
	PS	1	3	0	1	2	3	2	3	1	1	4	2	0
	En	4	2	D	1	2	1	D	D	1	D	0	2	0
MMP 13	Ep	0	0	D	D	D	1	0	1	0	D	D	0	0
	AS	0	0	1	0	2	1	0	1	0	0	0	0	0
	PS	0	0	1	0	2	0	0	1	0	0	0	0	0
	En	D	0	0	0	1	0	D	D	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. D = destroyed tissue. Ep = epithelium, AS = anterior stroma, PS = posterior stroma and En = endothelium.

Levels of MMP 9 proenzyme and the active form were extremely high in seven (S4, S6-S11) melted specimens and prominent in one (S5). Three melted specimens (S1-S3) revealed faint bands for both forms of MMP 9, and two controls did so for MMP 9 proenzyme only (Figure 2A).

Casein zymography revealed neither the proenzyme nor the active enzyme of MMP 3 or 7 in any of the control specimens. Negligible levels of the proform of MMP 3 were found in five melted specimens (S1, S2, S6, S7, and S9). Both forms, the proform and active MMP 3, were detected in one sample (S5) only. Nine melted corneas (S2-S10) revealed high levels of MMP 7 proenzyme and its intermediate cleavage product. Two samples (S1 and S11) revealed low levels. Very low levels of active MMP 7 were found in one specimen (S7) only (Figure 3).

Matrix metalloproteinase 1 activity assay—The active form of MMP 1 was found in eight of ten melted corneas at concentrations ranging from 0.08 to 3.03 ng/ml (p=0.0011). No activity was detected in the control specimens (Table 3).

DISCUSSION

In this study, we present two cases with pSS undergoing rapidly progressive recurrent corneal melting despite all available treatment, including immunosuppression. We obtained two series of tissue specimens from the eyes of one repeatedly grafted patient: six consecutive ones from the right eye, and two from the left . We also obtained one series of three consecutive tissue samples from the right eye of another patient with pSS. Our findings clearly demonstrated the increased presence of MMP 1, 2, 3, 7, 8, and 9, as well as higher activity of MMP 1, 2, 3, 7, and 9, in the pathological pSS specimens, compared to the control tissue.

To the best of our knowledge, this is the first time that these enzymes have been studied in corneal melting associated with pSS. Previously, differences in corneal MMP expression were detected in patients with keratolysis associated with rheumatoid arthritis [21], an autoimmune disorder that has some overlapping clinical features with pSS, and in patients with melted corneas after cataract surgery and photorefractive keratectomy, both of which are treated with diclophenac [18,19,33]. Our study demonstrated a statistically

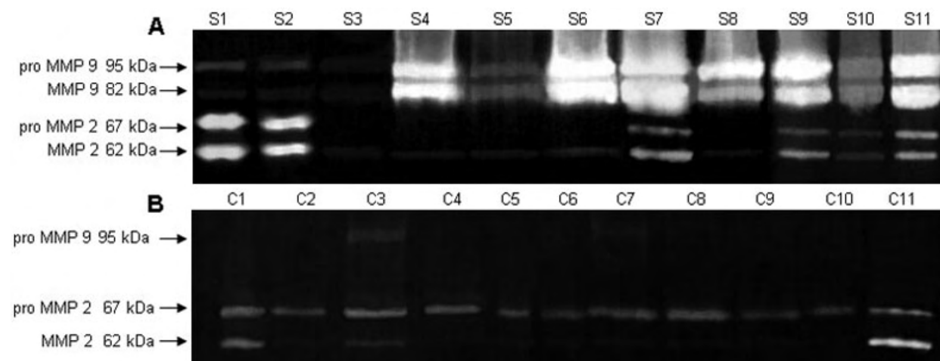


Figure 2. Gelatin zymography of matrix metalloproteinases 2 and 9 in melted and control corneas. Melted specimens **(A)** S1 and S2 showed extremely high levels, and specimens S7, S9, and S11 considerable levels, of both the proenzyme (67 KDa band) and the active form of MMP 2 (62 kDa band). Levels of MMP 9 proenzyme (95 kDa band) and the active form (82 kDa band) were extremely high in S4 and S6-S11, and prominent in S5. Weak bands for both MMP 9 forms were found in specimens S1-S3. In control samples **(B)**, a moderate level of the MMP 2 proenzyme was present in all specimens, whereas the active form of MMP 2 was either not present or very faint, except in samples C1, C3, and C11. As for MMP 9 proenzyme, only C3 and C7 showed faint bands.

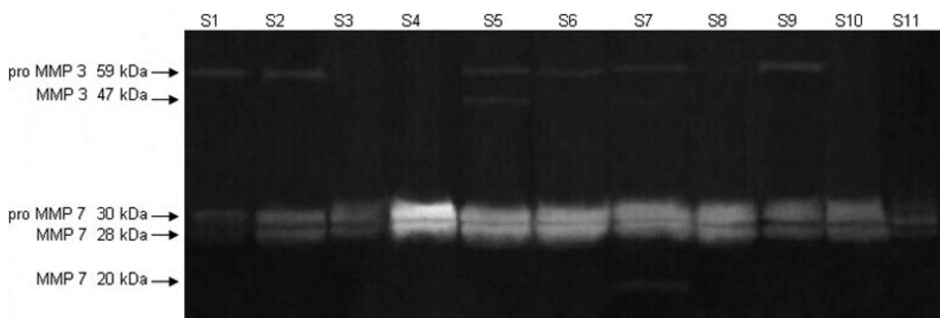


Figure 3. Casein zymography of matrix metalloproteinases 3 and 7 in melted specimens. Very slight bands of only the MMP 3 proform (59 kDa) were detected in five melted specimens (S1, S2, S6, S7, and S9) and of both the proform and active MMP 3 (47 kDa) in one specimen (S5). High levels of MMP 7 proenzyme (30 kDa) and its intermediate cleavage product (28 kDa) were detected in nine melted corneas, S2-S10, and weak bands of these two MMP 7 inactive forms were found in two melted specimens (S1 and S11). A very slight band of active MMP 7 (20 kDa) was found in one specimen only (S7).

significant higher activity of MMP 1 and a high expression of MMP 9 in the corneal epithelium and stroma. Both these results conform to the immunostaining results of others [18, 19,21,33]. As for MMP 2, its overexpression in both the epithelium and stroma, as well as its higher activity, have been reported in other investigations [18,33]. However, one study showed only a weak presence of MMP 2 in the stroma of a patient who had undergone cataract surgery and had been treated with diclophenac [19]. Our results also demonstrated variability in the MMP 2 expression in melted corneas, since only 2 of the 11 specimens revealed a considerable increase in its activity, compared to the controls. One possible explanation for this phenomenon is that MMP 2 activity is limited to a short period in the melting process, unlike the other MMPs. This hypothesis is supported by delays in the activity increase of this enzyme in corneas after alkali burn, suggesting its role to be in the regeneration and remodeling of the corneal ECM, rather than in the degradation process [34, 35].

A marked increase of MMP 3 in the epithelium and stroma of melted grafts has been detected previously, in the stroma of a patient after photorefractive keratectomy treated with topical diclofenac [18]. Although we confirmed the presence of MMP 3 in melted corneas as well, we were not able to detect a prominent increase in its activity. This may be due to the low sensitivity of casein zymography [36]. Unfortunately, there was not enough material available to perform other, more sensitive methods of MMP 3 detection.

Our study is the first to demonstrate the presence and activity of MMP 7 in melted corneas. Additionally, casein zymography showed a large quantity of inactive MMP 7 in all tested specimens and active MMP 7 in one specimen. We suggest that MMP 7 is an important element in the degradation of the corneal basement membrane in corneal melting, as it

 © 2009 Molecular Vision

Table 3. MMP 1 Activity Assay. The concentrations of the active forms of MMP 1 in melted corneal tissue were determined by interpolation from the standard curve.

Specimen	Concentration of active MMP 1 (ng/ml)
S1	0.69
S2	0.08
S4	0.23
S5	0.7
S6	0.23
S7	0.0
S8	0.39
S9	0.0
S10	3.03
S11	1.22

was abundant in the corneal epithelium, especially in its basal layer.

Up to now, MMP 8 has been studied only in one melted cornea following cataract surgery, where it was found to be considerably increased in both the epithelium and stroma [19]. In our study, we found a weak-to-moderate presence of this enzyme in both the epithelium and stroma. We attribute such differences to the fact that the occurrence of MMP 8 in the stroma depends on the presence of neutrophils [22], the distribution of which may vary among melted corneas [19, 37,38].

We also found a weak increase in the presence of MMP 13 in the stroma of three samples. To the best of our knowledge, no other study has previously evaluated MMP 13 in melted corneas. The expression of MMP 13 has only been described in the epithelium of wounded corneas [39] and in the epithelium and stroma of corneas with keratoconus [40].

We did not observe any trend towards an increase or decrease of individual MMP expression over time, or of disease progression, in any of the consecutive patient samples. Instead, the combination of MMPs detected seemed to be completely different in each specimen. This could have a number of causes, such as the different stages of melting at which the explants were obtained. It may also be that the expression of MMPs showed local variations within individual specimens, depending on the distance from the central melting point. It should be noted, however, that the staining and activity of individual MMPs were similar for consecutive sections obtained from each specimen.

MMPs in patients suffering from primary SS have previously been studied in tears [41], saliva [42], and salivary glands [43,44]. It has been suggested that the activation of these enzymes is the key factor responsible for the corneal barrier disruption, as well as for the destruction of the salivary glands [42,44-46]. Given the characteristic features of pSS, there may be more than one mechanism leading to the induction of different MMPs. For example, lymphocytic infiltrates secrete pro-inflammatory cytokines [2] that are known to initiate MMP expression in various tissues via different pathways [22]. Up-regulated IL-1β, found in the tears of pSS patients [41], could also play a role in the expression of MMPs, especially MMP 9, via mitogen-activated protein kinase signaling pathways [47]. Finally, a mouse model of dry eye has shown that desiccation and hyperosmolar stress may lead to the induction of MMPs via the stimulation of proinflammatory cytokines [45,47]. We hypothesize that in advanced cases of pSS, such as in our patients, MMPs may be upregulated to such an extent that the epithelial barrier is substantially degraded, followed by the dissolution of its basement membrane (caused mainly by MMP 3, 7, and 9) and the gradual degradation of the stroma, involving MMP 1, 3, 7, 8, and 9. After the stroma is completely lost, a descemetocele is formed, and finally the integrity of the whole cornea is disrupted.

The fact that none of the disease-modifying therapies used in these patients was effective in decreasing MMP production and keratolysis suggests that different treatment strategies with anti-MMP therapies should be considered in similar cases, such as using recombinant tissue inhibitors of MMPs [48] or chemical inhibitors of MMPs. For example, the TNF-α antagonist infliximab has been shown to inhibit one of the activators of MMP production [49]. Direct inhibition of MMPs can be achieved by tetracyclines, medroxyprogesteron, or ion-chelating agents such as cysteine or thylenediaminetetraacetic acid [50-53]. Finally, an alternative approach in keratolysis treatment could be focused on the recovery and strengthening of the collagen structure by collagen cross-linking [54].

Our study examined extremely severe cases of corneal melting associated with pSS, and has elucidated the participation of some MMPs in this destructive process. It confirmed that these enzymes play an important role in the severe degradation of corneal tissue leading to corneal perforation and loss of vision. Their involvement suggests that MMP inhibitors may play an important role in the treatment of this condition.

ACKNOWLEDGMENTS

This study was supported by the research project of the Czech Ministry of Education, Youth and Sports MSM0021620806/20610011.

REFERENCES

1. Fox PC. Autoimmune diseases and Sjogren's syndrome: an autoimmune exocrinopathy. Ann N Y Acad Sci 2007; 1098:15-21. [PMID: 17332090]
2. Garcia-Carrasco M, Fuentes-Alexandro S, Escarcega RO, Salgado G, Riebeling C, Cervera R. Pathophysiology of Sjogren's syndrome. Arch Med Res 2006; 37:921-32. [PMID: 17045106]
3. Rehman HU. Sjogren's syndrome. Yonsei Med J 2003; 44:947-54. [PMID: 14703600]
4. Fox RI, Michelson P, Casiano CA, Hayashi J, Stern M. Sjogren's syndrome. Clin Dermatol 2000; 18:589-600. [PMID: 11134854]
5. Hansen A, Lipsky PE, Dorner T. Immunopathogenesis of primary Sjogren's syndrome: implications for disease management and therapy. Curr Opin Rheumatol 2005; 17:558-65. [PMID: 16093833]
6. Haga HJ, Peen E. A study of the arthritis pattern in primary Sjogren's syndrome. Clin Exp Rheumatol 2007; 25:88-91. [PMID: 17417996]
7. 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. [PMID: 12230633]
8. 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. [PMID: 2784865]
9. Ormerod LD, Fong LP, Foster CS. Corneal infection in mucosal scarring disorders and Sjogren's syndrome. Am J Ophthalmol 1988; 105:512-8. [PMID: 3369518]
10. Pfister RR, Murphy GE. Corneal ulceration and perforation associated with Sjogren's syndrome. Arch Ophthalmol 1980; 98:89-94. [PMID: 7352872]
11. Vivino FB, Minerva P, Huang CH, Orlin SE. Corneal melt as the initial presentation of primary Sjogren's syndrome. J Rheumatol 2001; 28:379-82. [PMID: 11246683]
12. Malik R, Culinane AB, Tole DM, Cook SD. Rheumatoid keratolysis: a series of 40 eyes. Eur J Ophthalmol 2006; 16:791-7. [PMID: 17191183]
13. Palay DA, Stulting RD, Waring GO 3rd, Wilson LA. Penetrating keratoplasty in patients with rheumatoid arthritis. Ophthalmology 1992; 99:622-7. [PMID: 1584581]
14. 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; 17:124-30. [PMID: 12759840]
15. Pleyer U, Bertelmann E, Rieck P, Hartmann C. Outcome of penetrating keratoplasty in rheumatoid arthritis. Ophthalmologica 2002; 216:249-55. [PMID: 12207127]
16. Petroutsos G, Paschides CA, Kitsos G, Skopouli FN, Psilas K. Sterile corneal ulcers in dry eye. Incidence and factors of occurrence. J Fr Ophtalmol 1992; 15:103-5. [PMID: 1640062]
17. Saripalli L, Harrington TM, Notz RG, Torretti D. Corneal melt in rheumatic disorders: effect of disease-modifying antirheumatic drugs on morbidity. J Clin Rheumatol 2005; 11:134-9. [PMID: 16357731]
18. Gabison EE, Chastang P, Menashi S, Mourah S, Doan S, Oster M, Mauviel A, Hoang-Xuan T. Late corneal perforation after photorefractive keratectomy associated with topical diclofenac: involvement of matrix metalloproteinases. Ophthalmology 2003; 110:1626-31. [PMID: 12917183]
19. 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; 108:656-9. [PMID: 11297478]
20. Ollivier FJ, Gilger BC, Barrie KP, Kallberg ME, Plummer CE, O'Reilly S, Gelatt KN, Brooks DE. Proteinases of the cornea and preocular tear film. Vet Ophthalmol 2007; 10:199-206. [PMID: 17565550]
21. 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 1995; 9:703-18. [PMID: 8849537]
22. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993; 4:197-250. [PMID: 8435466]
23. Cawston TE. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Pharmacol Ther 1996; 70:163-82. [PMID: 8888065]
24. Brown D, Chwa M, Escobar M, Kenney MC. Characterization of the major matrix degrading metalloproteinase of human corneal stroma. Evidence for an enzyme/inhibitor complex. Exp Eye Res 1991; 52:5-16. [PMID: 1868885]
25. Fini ME, Girard MT. Expression of collagenolytic/gelatinolytic metalloproteinases by normal cornea. Invest Ophthalmol Vis Sci 1990; 31:1779-88. [PMID: 2170294]
26. Webster NL, Crowe SM. Matrix metalloproteinases, their production by monocytes and macrophages and their potential role in HIV-related diseases. J Leukoc Biol 2006; 80:1052-66. [PMID: 16959898]
27. Yamagami S, Ebihara N, Usui T, Yokoo S, Amano S. Bone marrow-derived cells in normal human corneal stroma. Arch Ophthalmol 2006; 124:62-9. [PMID: 16401786]
28. 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; 154:1193-201. [PMID: 10233857]
29. 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; 322:809-14. [PMID: 9148753]
30. 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; 15:490-6. [PMID: 8862926]
31. Vizoso FJ, Gonzalez LO, Corte MD, Rodriguez JC, Vazquez J, Lamelas ML, Junquera S, Merino AM, Garcia-Muniz JL. Study of matrix metalloproteinases and their inhibitors in breast cancer. Br J Cancer 2007; 96:903-11. [PMID: 17342087]

32. Weiss A, Goldman S, Shalev E. The matrix metalloproteinases (MMPS) in the decidua and fetal membranes. Front Biosci 2007; 12:649-59. [PMID: 17127325]
33. Hargrave SL, Jung JC, Fini ME, Gelender H, Cather C, Guidera A, Udell I, Fisher S, Jester JV, Bowman RW, McCulley JP, Cavanagh HD. Possible role of the vitamin E solubilizer in topical diclofenac on matrix metalloproteinase expression in corneal melting: an analysis of postoperative keratolysis. Ophthalmology 2002; 109:343-50. [PMID: 11825822]
34. Matsubara M, Zieske JD, Fini ME. Mechanism of basement membrane dissolution preceding corneal ulceration. Invest Ophthalmol Vis Sci 1991; 32:3221-37. [PMID: 1660857]
35. Ye HQ, Azar DT. Expression of gelatinases A and B, and TIMPs 1 and 2 during corneal wound healing. Invest Ophthalmol Vis Sci 1998; 39:913-21. [PMID: 9579471]
36. 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. [PMID: 9009103]
37. Hsu JK, Johnston WT, Read RW, McDonnell PJ, Pangalinan R, Rao N, Smith RE. Histopathology of corneal melting associated with diclofenac use after refractive surgery. J Cataract Refract Surg 2003; 29:250-6. [PMID: 12648633]
38. Kuffova L, Holan V, Lumsden L, Forrester JV, Filipec M. Cell subpopulations in failed human corneal grafts. Br J Ophthalmol 1999; 83:1364-9. [PMID: 10574815]
39. Ye HQ, Maeda M, Yu FS, Azar DT. Differential expression of MT1-MMP (MMP-14) and collagenase III (MMP-13) genes in normal and wounded rat corneas. Invest Ophthalmol Vis Sci 2000; 41:2894-9. [PMID: 10967042]
40. Mackiewicz Z, Maatta M, Stenman M, Konttinen L, Tervo T, Konttinen YT. Collagenolytic proteinases in keratoconus. Cornea 2006; 25:603-10. [PMID: 16783151]
41. 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; 42:2283-92. [PMID: 11527941]
42. Asatsuma M, Ito S, Watanabe M, Takeishi H, Nomura S, Wada Y, Nakano M, Gejyo F, Igarashi A. Increase in the ratio of matrix metalloproteinase-9 to tissue inhibitor of metalloproteinase-1 in saliva from patients with primary Sjogren's syndrome. Clin Chim Acta 2004; 345:99-104. [PMID: 15193983]
43. Ito K, Funayama S, Hitomi Y, Nomura S, Katsura K, Saito M, Hayashi T, Kaneko N, Nohno K, Igarashi A. Proteome analysis of gelatin-bound salivary proteins in patients with primary Sjogren's syndrome: identification of matrix metalloproteinase-9. Clin Chim Acta 2009; 403:269-71. [PMID: 19302990]
44. Perez P, Kwon YJ, Alliende C, Leyton L, Aguilera S, Molina C, Labra C, Julio M, Leyton C, Gonzalez MJ. Increased acinar damage of salivary glands of patients with Sjogren's syndrome is paralleled by simultaneous imbalance of matrix metalloproteinase 3/tissue inhibitor of metalloproteinases 1 and matrix metalloproteinase 9/tissue inhibitor of metalloproteinases 1 ratios. Arthritis Rheum 2005; 52:2751-60. [PMID: 16142742]
45. 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; 47:3293-302. [PMID: 16877394]
46. Pflugfelder SC, Farley W, Luo L, Chen LZ, de Paiva CS, Olmos LC, Li DQ, Fini ME. Matrix metalloproteinase-9 knockout confers resistance to corneal epithelial barrier disruption in experimental dry eye. Am J Pathol 2005; 166:61-71. [PMID: 15632000]
47. 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; 45:4293-301. [PMID: 15557435]
48. 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; 35:677-84. [PMID: 8113019]
49. Thomas JW, Pflugfelder SC. Therapy of progressive rheumatoid arthritis-associated corneal ulceration with infliximab. Cornea 2005; 24:742-4. [PMID: 16015096]
50. Brown SI, Weller CA. Collagenase inhibitors in prevention of ulcers of alkali-burned cornea. Arch Ophthalmol 1970; 83:352-3. [PMID: 4313450]
51. 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; 9:157-64. [PMID: 16634928]
52. Perry HD, Golub LM. Systemic tetracyclines in the treatment of noninfected corneal ulcers: a case report and proposed new mechanism of action. Ann Ophthalmol 1985; 17:742-4. [PMID: 4091374]
53. Phillips K, Arffa R, Cintron C, Rose J, Miller D, Kublin CL, Kenyon KR. Effects of prednisolone and medroxyprogesterone on corneal wound healing, ulceration, and neovascularization. Arch Ophthalmol 1983; 101:640-3. [PMID: 6188447]
54. Koller T, Seiler T. Therapeutic cross-linking of the cornea using riboflavin/UVA. Klin Monatsbl Augenheilkd 2007; 224:700-6. [PMID: 17846959]

The print version of this article was created on 11 November 2009. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.

Author's personal copy

Experimental Eye Research 90 (2010) 583–590

Contents lists available at ScienceDirect

Experimental Eye Research

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

Role of matrix metalloproteinases in recurrent corneal melting☆

Kristyna Brejchova [a,*], Petra Liskova [a], Jitka Cejkova [b], Katerina Jirsova [a,c]

[a] Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, Charles University in Prague, First Faculty of Medicine and General Teaching Hospital Prague, Ke Karlovu 2, 12808 Prague 2, Czech Republic
[b] Department of Eye Histochemistry and Pharmacology, Institute of Experimental Medicine, Academy of Science of the Czech Republic, Videnska 1083, 14220 Prague 4, Czech Republic
[c] Ocular Tissue Bank, General Teaching Hospital and Charles University in Prague, First Faculty of Medicine, U nemocnice 2, 12808 Prague 2, Czech Republic

A R T I C L E I N F O

Article history:
Received 16 October 2009
Accepted in revised form 4 February 2010
Available online 11 February 2010

Keywords:
corneal melting
matrix metalloproteinases
extracellular matrix degradation
rheumatoid arthritis
ocular cicatricial pemphigoid

A B S T R A C T

The aim of this study was to compare the presence and activity of matrix metalloproteinases (MMPs) 1, 2, 3, 7, 8, 9 and 13 in human melted and cadaverous corneas. Twelve melted corneal specimens from three patients with rheumatoid arthritis, one patient with ocular cicatricial pemphigoid and one patient with melting attributed to spastic entropion and ten control corneal buttons were used. The presence of MMPs 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 and -9 was seen in the corneal epithelium and the anterior stroma of all, and for MMP-2, -3, -7 and -8 of almost all, melted corneas compared to the negative or slightly positive staining of the controls. The posterior stroma showed the presence of MMP-1, -2, -3 and -9 in almost all and of MMP-7 and -8 in half of all melted specimens. A markedly higher level of active MMP-2 was detected in six and active MMP-9 in all of eleven pathologic specimens compared to control specimens, using gelatin zymography. The proenzymes of MMP-3 and -7 and the MMP-7 intermediate cleavage product were detected only in melted corneas using casein zymography. Significantly increased MMP-1 and -3 activity was also found in the melted corneas using activity assays. The markedly increased immunostaining for MMP-1, -2, -3, -7, -8 and -9 as well as the elevated levels of the active forms of MMP-1, -2, -3 and -9 in melted corneal specimens from patients with various diagnoses suggest that although different stimuli may trigger the pathways that lead to the destruction of the extracellular matrix, these enzymes could play a subsequent role in this process.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Corneal melting (keratolysis) is a rare but recurrent and difficult-to-treat condition leading to corneal destruction. It is characterized by the development of epithelial defects and gradual stromal thinning due to the destruction and loss of the extracellular matrix (ECM), which may lead to descemetocele formation and subsequent perforation of the cornea (Malik et al., 2006; Palay et al., 1992; Perez et al., 2002; Pleyer et al., 2002).

Keratolysis could be associated with infectious (Barletta et al., 1996; McElvanney, 2003), non-infectious inflammatory (Bernauer et al., 1995; Donzis and Mondino, 1987), traumatic (Joseph et al., 2001; Liu et al., 2002), or trophic causes (Vajpayee et al., 2003), but may also arise for no apparent reason (Hagen et al., 1997). Non-infectious corneal melting has many different etiologies. It could occur as an isolated ocular problem; however, it is more often linked to autoimmune diseases such as rheumatoid arthritis (RA) and rarely to ocular cicatricial pemphigoid (OCP) (Bernauer et al., 1995; Malik et al., 2006; Solomon et al., 2002).

Management of corneal melting is difficult and has to be tailored to the individual patient. The application of tissue adhesives or amniotic membrane grafts may stabilize the anatomic integrity of the eye (Donzis and Mondino, 1987; Perez et al., 2002); however, in advanced stages of keratolysis, these approaches are frequently unsuccessful (Bernauer et al., 1995; Solomon et al., 2002). Finally, keratoplasty has to be performed in cases with imminent corneal perforation (Palay et al., 1992; Perez et al., 2002; Pleyer et al., 2002). Unfortunately, in most transplanted patients new grafts also recurrently fail by melting (Bernauer et al., 1995; Palay et al., 1992).

☆ Financial support: Ministry of Education, Youth and Sports of the Czech Republic MSM0021620806.

* Corresponding author. Tel.: +420 224 967 139; fax: +420 224 962 338.
E-mail address: kjuklova@yahoo.com (K. Brejchova).

0014-4835/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2010.02.002

Author's personal copy

Corneal melting is generally linked to the increased presence of matrix metalloproteinases (MMPs) (Gabison et al., 2003; Hargrave et al., 2002; O'Brien et al., 2001; Riley et al., 1995). MMPs are a family of endopeptidases capable of degrading various components of the extracellular matrix (Birkedal-Hansen et al., 1993). They are synthesized as inactive proenzymes, which are then activated by proteolytic cleavage (Cawston, 1996). On the basis of domain organization and substrate preference, MMPs are grouped into collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10), matrilysins (MMP-7, -26), membrane type MMPs (MT1–MT6-MMPs) and others (e.g., macrophage metalloelastase – MMP-12 or enamelysin – MMP-20) (Visse and Nagase, 2003). From among the MMP substrates that are present in the normal human cornea, collagenases are able to cleave fibrillar collagen types I and III present in the stroma (Cawston, 1996; Marshall et al., 1991a), while gelatinases can cleave especially basal membrane components (collagen types IV and VII, fibronectin and laminin) and stromal collagen types IV, V and VI, the core protein decorin and denatured collagens (Cawston, 1996; Funderburgh et al., 1998; Giannelli et al., 1999; Imai et al., 1997; Ljubimov et al., 1995; Marshall et al., 1991b; Myint et al., 1996). Stromelysins participate in the activation of various MMPs and together with matrilysins can cleave stromal and corneal basal membrane components (type IV collagen, procollagens, collagen cross-links, fibronectin, laminin) (Birkedal-Hansen et al., 1993; Cawston, 1996; Imai et al., 1995; Ljubimov et al., 1995).

The aim of this study was to determine the localization and activity of members of all the main groups of MMPs (MMP-1, -2, -3, -7, -8, -9 and -13) in order to identify their involvement in the recurrent keratolysis process.

2. Methods

2.1. Patients and specimens

The study adhered to the tenets of the Declaration of Helsinki. Twelve specimens from five patients (P1-5) obtained between October 2001 and December 2007 during penetrating keratoplasty (including regrafts) for corneal melting were examined. The mean age of the patients was 78 years (71–91 years). Patients 1–3 were diagnosed with RA, case 4 with OCP and idiopathic autoimmune hemolytic anemia. No systemic disorder was found in case 5. All patients were evaluated by rheumatologists, and the severity of RA was classified into four stages (http://www.wheelessonline.com). Patients with autoimmune disorders were administered systemic immunosuppressives. Except for patient 5, who underwent patch keratoplasty, full thickness grafts ranging from 7.75 to 8.50 mm in diameter were transplanted. Keratolysis occurred in all native corneas (specimens P2-1, P4-2, P5-2) and grafts (specimens P1-1, P1-2, P1-3, P2-2, P2-3, P3, P4-1, P4-3, P5-1) either centrally or paracentrally. The presence of accompanying keratoconjunctivitis sicca (KCS) was classified as mild, moderate or severe (Lemp, 2008). Eleven melted explants were obtained from the Department of Ophthalmology, General Teaching Hospital and 1st Faculty of Medicine, Charles University in Prague, Czech Republic and one from the Department of Ophthalmology, Sokolov Hospital, Czech Republic. Patient details are summarized in Table 1.

Ten donor corneal buttons (Co1–Co10, mean age 60 years, 16–84 years), not suitable for transplantation due to low endothelial cell density, were obtained from the Ocular Tissue Bank, General Teaching Hospital, Prague, and served as controls. The mean time from the donor's death to enucleation was 15 h and 23 min (time range 9 h 45 min–20 h 45 min), and the mean time from death to tissue freezing was 17 h 15 min (time range 11 h 45 min–21 h 55 min).

All specimens were dissected into two halves, snap-frozen in liquid nitrogen, and one half was embedded in Optimal Cutting Temperature Compound (OCT) (Christine Gröpl, Tulln, Austria). All samples were stored at −70 °C. Melted grafts were frozen within 3 h after surgery.

2.2. Indirect enzyme immunohistochemistry

Seven-μm-thick cryosections of each OCT-embedded control and melted specimen were fixed with cold acetone for 10 min and re-hydrated in phosphate buffered saline (PBS). Endogenous peroxidase was blocked by a 30 min incubation in 3% hydrogen peroxide in PBS. After washing in PBS, the specimens were blocked for 30 min with 2.5% bovine serum albumin in PBS. The sections were subsequently immunolabeled with the primary antibodies [monoclonal mouse anti-human MMP-2 (MAB13431), MMP-7 (MAB13414), MMP-9 (MAB3309), MMP-13 (MAB13424) and polyclonal rabbit anti-human MMP-1 (AB806), MMP-8 (AB8115); all from Millipore, Bedford, MA, USA; polyclonal rabbit anti-human MMP-3 (29576) AnaSpec, Fremont, CA, USA] for 1 h at room temperature. According to the manufacturers, each antibody is specific for a single MMP with no cross-reactivity with other MMPs. After washing in PBS, the secondary antibodies (polyclonal rabbit anti-mouse IgG and swine anti-rabbit IgG conjugated with biotin; DakoCytomation, Glostrup, Denmark) were applied for 1 h, then

Table 1
Clinical details of patients with recurrent corneal melting.

No	Age	Sex	Specimen	Eye	PK order in the eye	Autoimmune systemic disorder	Factors influencing the ocular surface	Comments
P1	71	M	P1-1	L	1	RA stages III–IV	Moderate KCS	Descemetocele formation
			P1-2	L	2			
			P1-3	L	3			
P2	77	F	P2-1	L	1	RA stage III	Severe KCS	Corneal perforation
			P2-2	L	3			Descemetocele formation
			P2-3	L	4			Descemetocele formation
P3	80	F	P3	R	2	RA stage IV	Severe KCS	
P4	81	F	P4-1	L	2	OCP stage I; idiopathic autoimmune hemolytic anemia	Severe KCS	Descemetocele formation
			P4-2	R	1			Corneal perforation
			P4-3	R	2			
P5	91	F	P5-1	R	2	None detected	Entropion	Lysis of previous patch keratoplasty and perforation of adjacent native cornea
			P5-2	R	3			Descemetocele formation next to the patch keratoplasty; excentric keratoplasty (6.0 mm in diameter) comprising mainly native cornea performed

RA = rheumatoid arthritis, OCP = ocular cicatricial pemphigoid, KCS = keratoconjunctivitis sicca, M = male, F = female, L = left, R = right, PK = penetrating keratoplasty.

Author's personal copy

the slides were rinsed in PBS again, followed by incubation with streptavidin/HRP (1:250, DakoCytomation) for 30 min. 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). One section on each slide, where the primary antibody was omitted, served as a negative control. The antibodies were verified using positive controls that are known to express MMPs: human placenta (MMP-1, -2, -3, -7) and breast carcinoma (MMP-7, -8, -9, -13) (Vizoso et al., 2007; Weiss et al., 2007). Samples were evaluated using an Olympus BX51 light microscope (Olympus Co., Tokyo, Japan) at a magnification of 100×. The intensity of the signal was evaluated separately in the epithelium, anterior and posterior stroma and endothelium. All experiments were performed in duplicate.

2.3. Preparation of samples for zymography and the activity assays

Eleven melted specimens (P1-1, P1-2, P1-3, P2-1, P2-2, P2-3, P3, P4-1, P4-2, P4-3, P5-2; Table 1) and ten control specimens (Co1–10) were processed as previously described (Vajtr et al., 2002). 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 1 nM $ZnCl_2$), then underwent protein extraction for two days. The samples were centrifuged for 30 min at 10 000 G, the supernatants removed and frozen at −20 °C. Specimen P5–1 was used only for immunohistochemistry due to the lack of material.

2.4. Gelatin and casein substrate zymography

Twelve μl of each specimen, adjusted to represent the same quantity of cellular protein (8.5 μg), was used for both gelatin and casein zymography. The specimens were treated with sample buffer [1.5% Sodium dodecyl sulfate (SDS), 15% glycerol, and 0.005% bromphenol blue] without boiling or reduction. Zymography was carried out as described previously (Mesa et al., 2006) 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) at 200 V or a 12% gel containing 0.09% casein (Sigma–Aldrich, St. Louis, MO, USA) at 20 mA for 90 min at 4 °C. The gels were twice soaked in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS, then 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 to allow proteinase digestion of their substrates. Gels were rinsed again in a mixture of 40% methanol and 10% acetic acid, stained with a solution of 0.5% Coomassie brilliant blue R-250 (Serva Electrophoresis, GmbH, Heidelberg, Germany) in 40% methanol and 10% acetic acid for 1 h, and destained with a 40% methanol and 10% acetic acid mixture. 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 buffer lacking calcium and containing 20 mM EDTA. Gels were incubated overnight at 37 °C and, after the incubation period, were stained and destained as described above.

2.5. MMP-1 and -3 activity assays

The concentrations of the active forms of MMP-1 and -3 were determined using commercial kits (Amersham matrix metalloproteinase-1 and Amersham matrix metalloproteinase-3, Biotrak Activity assay system, Biotrak, Amersham Biosciences, UK) according to the manufacturer's protocol and as described previously (Li et al., 2003). In brief, microplate wells coated with $F(ab')_2$ goat anti-mouse IgG were incubated with anti-MMP-3 antibody for 3 h at 37 °C, then rinsed with a wash solution four times. A commercially prepared anti-MMP-1-coated microplate was allowed to equilibrate to room temperature. One hundred μl each of pro-MMP-1 (0.78–12.5 ng/ml) or pro-MMP-3 (0.25–8 ng/ml) served as standards. One hundred μl of the tissue samples (diluted 1:10) and 100 μl of assay buffer as a blank were incubated at 4 °C overnight in microplate wells pre-coated with anti-MMP-1 or anti-MMP-3 antibody. To measure the total activity of MMP-1 or MMP-3 in the standards, bound pro-MMP-1 or pro-MMP-3 was activated with 50 μl of 0.025 mM or 100 μl of 1 mM *p*-aminophenylmercuric acetate in assay buffer, respectively. Fifty μl of assay buffer was added to each sample in which the endogenous level of active MMP-1 or -3 was measured. To activate MMP-3 proenzyme, a 30-min incubation at 37 °C was used, and all wells were washed four times. Detection reagent was added to each well of the MMP-1 or MMP-3 activity assay microplates and incubated at 37 °C for 4 or 4.5 h, respectively. Active MMP-1 or MMP-3 was evaluated based on the color change resulting from the cleavage of a chromogenic peptide substrate. The resultant color was read at 405 nm in an ELISA Reader (ELISA Reader SUNRISE, Tecan Trading AG, Männedorf, Switzerland). The activity of MMP-1 or MMP-3 in all samples was determined by interpolation from a standard curve. Due to the limited amount of sample material available, we performed these experiments only once.

2.6. 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 statistical significance.

3. Results

3.1. Detection of MMPs by indirect enzyme immunohistochemistry

Moderate and weak MMP-1 staining was present in the epithelium and endothelium of each control specimen, respectively. Increased MMP-1 staining was found in the epithelial fragments and the anterior stroma of all melted specimens compared to controls; the epithelium of one specimen was destroyed (P1-3). MMP-1 immunostaining was also elevated in the posterior stroma of nine (P1-1, P1-2, P2-1, P2-3, P3, P4-1, P4-2, P4-3, P5-2) and in the endothelium of four (P1-1, P2-1, P4-2 and P4-3) melted corneas.

Moderate staining for MMP-2 was present throughout the epithelium of all control corneas, and a weak signal was observed in the anterior stroma in the area adjacent to Bowman's layer. A stronger staining intensity was present in the epithelial fragments of seven (P2-1, P2-2, P2-3, P3, P4-2, P4-3, P5-1), the posterior stroma of eight (P1-1, P1-2, P2-1, P2-3, P3, P4-2, P5-1, P5-2) and the endothelium of eight (P1-1, P2-1, P2-2, P2-3, P3, P4-2, P4-3, P5-1) melted corneas. Increased MMP-2 staining was found in the anterior stroma of all melted specimens as well, except for one (P4-1).

Immunostaining for MMP-3 was negative in all control corneas. In the pathologic samples, MMP-3 was detected in the epithelial fragments (P1-1, P2-1, P2-2, P2-3, P3, P4-1, P4-2, P4-3, P5-1, P5-2) of ten and the anterior stroma (P1-1, P1-2, P1-3, P2-1, P2-2, P3, P4-2, P4-3, P5-2) of nine melted specimens. Additionally, positivity was observed in the posterior stroma of seven (P1-1, P1-2, P2-1, P3, P4-2, P4-3, P5-2) and the endothelium of three melted specimens (P2-1, P4-3, P5-2).

MMP-7 was not found in any layer of any of the ten control specimens, whereas this enzyme was found in the epithelial fragments (P1-1, P2-1, P2-2, P2-3, P3, P4-1, P4-2, P5-1, P5-2) of nine, the anterior stroma (P1-1, P1-2, P2-2 P1-3, P2-1, P3, P4-2, P4-3, P5-1,

Author's personal copy

P5-2) of ten and the posterior stroma (P1-1, P2-1, P3, P4-2, P4-3, P5-2) of six melted corneas. The endothelium of two pathological samples (P2-1, P4-2) revealed weak immunostaining for MMP-7.

Weak MMP-8 staining was detected in the epithelium and the endothelium of all control specimens, while the other corneal areas were negative. A similar staining intensity in the epithelium was seen in four melted corneas (P2-2, P3, P4-1, P5-2), whereas the other specimens exhibited markedly elevated staining or were destroyed (P1-2, P1-3). MMP-8 was also present in the anterior stroma of nine (P1-1, P1-3, P2-1, P2-2, P3, P4-2, P4-3, P5-1, P5-2) and the posterior stroma of six (P1-1, P2-1, P3, P4-2, P4-3, P5-1) melted specimens. The endothelium was mostly negative or weakly positive except for one sample (P2-1), where slightly elevated MMP-8 expression was found.

MMP-9 was not found in any of the control specimens. The presence of this enzyme was detected in the epithelial fragments and the anterior stroma of all pathologic samples; the epithelium of one (P1-3) specimen and the epithelium and anterior stroma of another (P2-2) specimen were destroyed. MMP-9 expression was found in the posterior stroma of nine (P1-1, P1-2, P1-3, P2-1, P2-2, P3, P4-2, P4-3, P5-2) and in the endothelium of one (P2-1) melted cornea.

No positivity for MMP-13 was detected in any control or melted cornea except for a low level of this enzyme in the epithelial fragments, the posterior stroma and the endothelium of one (P2-1) and in the anterior stroma of three melted corneas (P1-1, P2-1, P4-3).

The immunolocalization of individual MMPs in control and pathologic specimens is shown in Fig. 1. Control specimens revealed little intra-individual or inter-individual variability in the staining intensity, while more pronounced differences were observed in the melted specimens. No immunostaining was present in any of the negative controls.

3.2. Detection of MMP activity: gelatin and casein zymography

In the patient group, markedly increased levels of both the partly cleaved form of MMP-2 (Stefansson et al., 1994) and the active form of MMP-2 were found in five (P1-1, P2-1, P2-2, P3, P4-3) melted corneas, while one sample (P4-2) revealed the increased expression of the proenzyme only (Fig. 2, B). Two pathologic samples (P1-2, P2-3) showed the presence of active MMP-2 only. In three melted corneas (P1-3, P4-1, P5-2) and all ten tested control corneas, a faint band of the partly cleaved proenzyme and no or a very faint band of the active form of MMP-2 was found.

High levels of both forms of MMP-9 were detected in eight (P1-1, P1-2, P1-3, P2-2, P2-3, P4-1, P4-2, P4-3) and weak levels in one (P5-2) melted specimen (Fig. 2B). Two melted corneas (P2-1, P3) revealed a prominent band of active MMP-9 only. Two controls exhibited a faint band of the MMP-9 proenzyme (Fig. 2A).

Casein zymography revealed faint bands of the MMP-3 proenzyme in five melted corneal samples (P1-1, P1-2, P2-3, P4-2, and P4-3). Dense bands migrating at 28 kDa and 25 kDa, corresponding to the proenzyme of MMP-7 and its intermediate cleavage product, were found in five (P1-1, P1-2, P1-3, P2-2, P2-3) melted corneas, whereas five melted samples (P2-1, P4-1, P4-2, P4-3, P5-2) exhibited faint bands of these two MMP-7 forms (Fig. 3). No MMP-3 or MMP-7 forms were found in any of the control specimens.

3.3. Detection of MMP activity: MMP-1 and MMP-3 activity assay

Significantly higher MMP-1 activity was shown in melted corneas compared to controls (p-value < 0.001). The active form of MMP-1 was found in ten of eleven melted corneas at levels ranging from 0.1 to 8.4 ng/ml; no activity was detected in control specimens (Fig. 4).

The concentration of active MMP-3 in the melted specimens was significantly higher (range, 12.6–106.22, p-value < 0.001) than in controls, nine of which displayed low levels of active MMP-3 (range, 0–32.4 ng/ml) (Fig. 4).

4. Discussion

In this study we found markedly elevated levels of MMP-1, -2, -3, -7, -8 and -9 in melted corneas obtained from a set of patients suffering from various underlying pathologies.

Non-infectious corneal melting usually precedes epithelial defect formation, which is followed in turn by the loss of the epithelial basement membrane and stromal degradation (Matsubara et al., 1991). The failure to re-epithelialize, the delay in basement membrane repair and the degradation of stromal components are thought to be caused by excessive proteolytic activity in the cornea (Fini et al., 1998). MMPs are believed to be mainly responsible for this process (Fini et al., 1998; Gabison et al., 2003; O'Brien et al., 2001; Riley et al., 1995).

In agreement with our results, gelatinases play an important role in corneal melting. These findings are supported by the results of other studies describing the expression of MMPs in corneal melting associated with RA (Smith et al., 2001) as well as with diclofenac treatment (Gabison et al., 2003; O'Brien et al., 2001). As was found earlier, MMP-9 participates in the degradation of the corneal basement membrane during the process of corneal melting in thermally burned corneas, whereas MMP-2 exhibits delayed synthesis; the involvement of MMP-2 in the attempts of the melted tissue to regenerate has been suggested (Matsubara et al., 1991). The variability in MMP-2 activity detected by us may therefore be explained by the different stages of melting present in the specimens used for analysis. The presence of both MMP-2 forms in control tissue, which has been found by other authors (Fini et al., 1992; Kenney et al., 1994) as well as by us, supports the hypothesis that MMP-2 may participate in the maintenance of ECM homeostasis (Matsubara et al., 1991). On the other hand, the presence of active MMP-2 in control tissue could also be related to the absence of inhibitors in the sample buffer that protect proteins against degradation by proteinases in the samples. Prolonged extraction of tissue in the absence of inhibitors could have contributed to the cleavage of MMPs by other enzymes, thus leading to the appearance of low levels of activated forms even in control tissue.

The degradation of the epithelial basement membrane during keratolysis could also be caused by MMP-3 and -7. In the present study, their elevated expression in the melted specimens is in agreement with an earlier report describing the presence of MMP-3 in corneal melting associated with diclofenac treatment (Gabison et al., 2003). Moreover, our results revealed for the first time a significant increase in MMP-3 activity compared to control corneas. The highest concentration of active MMP-3 measured by the activity assay (106.2 ng/ml) is much lower than the detection limit of casein zymography for this enzyme (400 ng/ml), thus explaining why we were not able to detect bands corresponding to active MMP-3 using casein zymography (Quesada et al., 1997). The observed differences between the MMP-3 activity results (very high activity levels) and both the histological (strong staining) and zymography results (no signal) could be caused by the possible cross-reactivity of MMP-3 with MMP-10. Although the manufacturer's datasheet does not address this possibility, these two MMPs have almost identical substrate specificities (Birkedal-Hansen et al., 1993), thus the MMP-3 activity assay may recognize active MMP-10 as well. Other, more general reasons that could be responsible for the observed variability in MMP expression are discussed below.

Author's personal copy

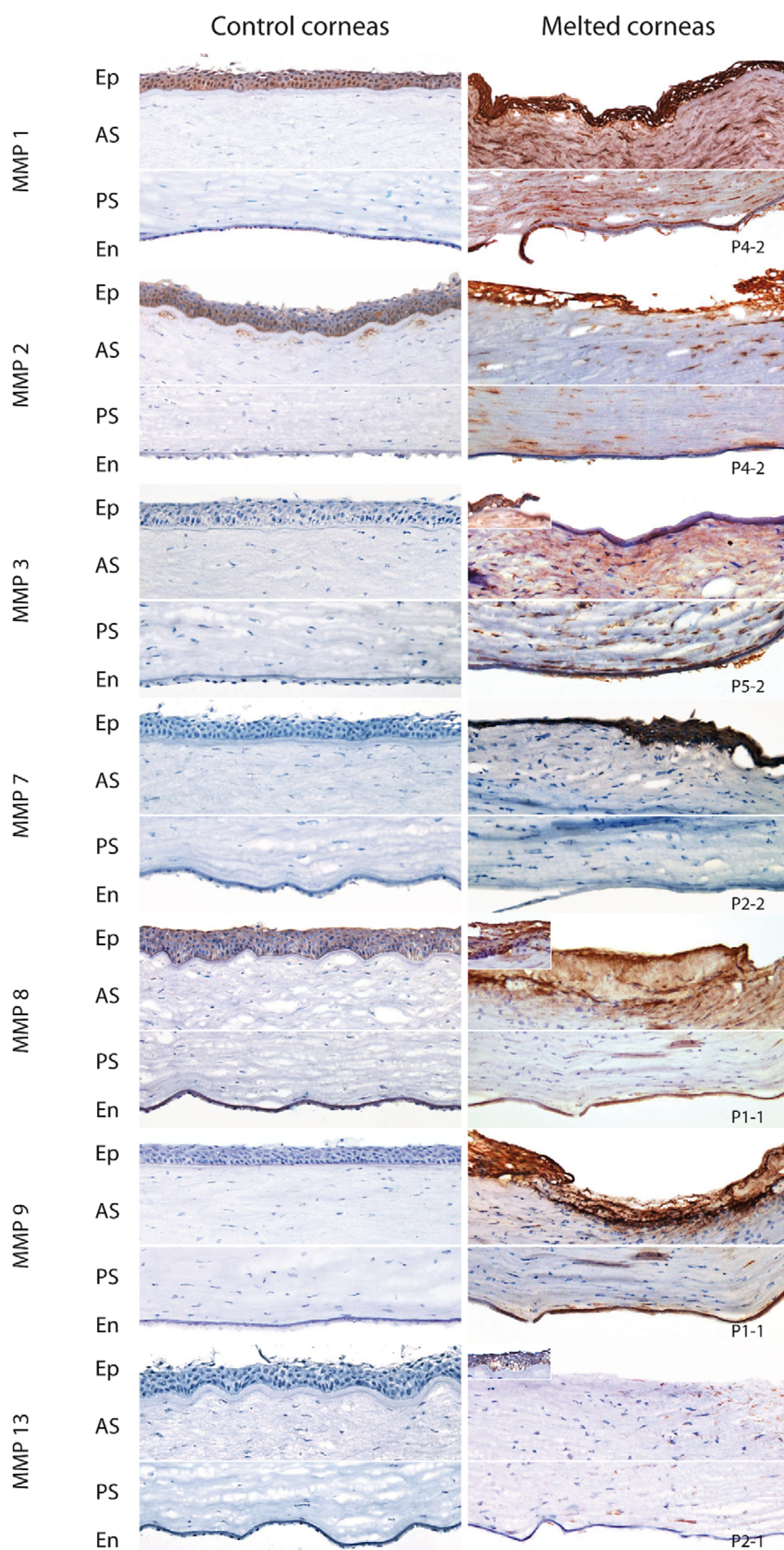


Fig. 1. Immunostaining of MMP-1, -2, -3, -7, -8, -9 and 13 in representative samples of melted and control corneas. The photomicrographs of melted corneas stained for MMP-3, -8 and -13 have insets showing the epithelium of the same specimen. Ep = epithelium, AS = anterior stroma, PS = posterior stroma, En = endothelium.

Author's personal copy

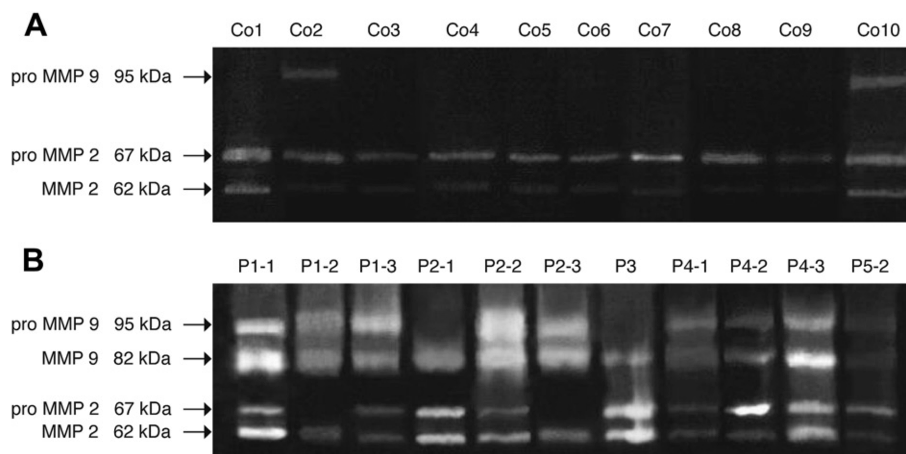


Fig. 2. Detection of MMP-2 and -9 activity using gelatin zymography in control corneas (A) and melted grafts (B). Prominent bands and faint bands of both the partly cleaved form of MMP-2 (67 kDa) and the active enzyme (band at 62 kDa) were detected in five (P1-1, P2-1, P2-2, P3, P4-3) and three (P1-3, P4-1, P5-2) melted corneas, respectively. Two samples showed the presence of the active form only (P1-2, P2-3), while the remaining specimen (P4-2) revealed a prominent band of the proenzyme and a faint band of active MMP-2. A faint band of partly cleaved MMP-2 and no or a very faint band of active MMP-2 were found in all control corneas. Markedly increased levels of both forms of MMP-9 (a 95-kDa band of the proenzyme and an 82-kDa band corresponding to the active form) or only the active form were seen in eight melted specimens (P1-1, P1-2, P1-3, P2-2, P2-3, P4-1, P4-2, P4-3) and two melted corneas (P2-1, P3), respectively. Weak bands of both forms were found in one specimen (P5-2). Two controls (Co2, Co10) exhibited a faint band at 95-kDa corresponding to MMP-9 proenzyme.

To the best of our knowledge, evidence for the expression of MMP-7 in melted corneas has not been previously shown by other researchers. Although we did not detect the active form of MMP-7, we found a large quantity of MMP-7 proenzyme and its partially activated form (Yu and Woessner, 2000).

As collagen types I and III are the main components of the corneal stroma (Marshall et al., 1991a), the participation of collagenases in its degradation is expected. We confirmed a high level of MMP-1 in the melted corneas (Riley et al., 1995). Moreover, we found significantly elevated MMP-1 activity. Surprisingly, the concentration of active MMP-1 in the melted tissues was rather low, which does not correlate with our immunostaining results. This could be due to the presence of some tissue inhibitors of matrix metalloproteinases or other proteins (α2-macroglobulin, etc.). The presence of MMP-1 in the normal corneal epithelium, at levels less than those seen in pathological specimens, has been reported by other researches as well (Mackiewicz et al., 2006; O'Brien et al., 2001). To the best of our knowledge, MMP-13 has also not been previously studied in melted corneas by other researchers. Surprisingly, we detected low levels of MMP-13 in only three melted corneas. This could be due to the fact that MMP-13 is more efficient at cleaving collagen type II, which is absent from the human cornea (Marshall et al., 1991a; Nakayasu et al., 1986), than types I and III (Knauper et al., 1996). Immunostaining for MMP-8, a neutrophil collagenase, was mostly located in the same area where neutrophils were detected using an anti-CD66b antibody (data not shown). The presence of this collagenase in the corneal stroma in areas of leukocyte infiltration was described previously in ulcerative keratolysis associated with diclofenac use (O'Brien et al., 2001).

We found some variability in the expression of individual MMPs in each specimen. This could be caused by a number of reasons such as the influence of the diverse underlying pathologies or the different stages of melting at which the explants were obtained. The expression of MMPs may also be inhomogeneous, showing local variations within individual specimens; our specimens were cut into two halves for subsequent use in immunohistochemical staining and the activity assay, thus any differences found could reflect the non-uniformity of MMP distribution. It should be noted, however, that the staining of individual MMPs was similar for consecutive sections obtained from each cornea. Another reason could lie in the different levels of the active and inactive forms of individual MMPs. The antibodies used for immunostaining do not distinguish between the proenzyme and active forms of the MMPs, unlike the activity assay. Based on the methods used, it is impossible to determine whether 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 their quantitative differences between samples.

To date, little is known about the mechanisms responsible for the induction of corneal melting. We agree with the suggestions of

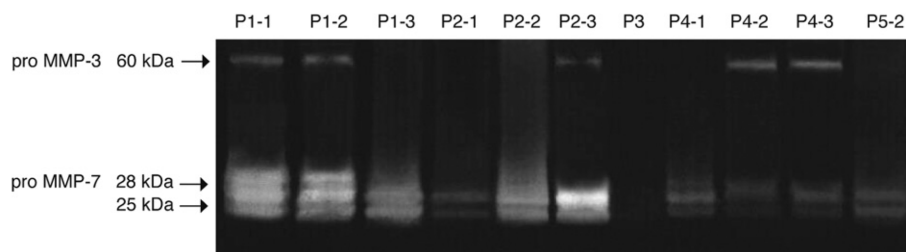


Fig. 3. Casein zymography in melted corneal grafts. Low levels of the MMP-3 proenzyme (60 kDa) were observed in five melted corneal samples (P1-1, P1-2, P2-3, P4-2, P4-3). Dense bands in five (P1-1, P1-2, P1-3, P2-2, P2-3) and faint bands in five (P2-1, P4-1, P4-2, P4-3, P5-2) melted corneas migrating at 28 kDa and 25 kDa, corresponding to the proenzyme of MMP-7 and its intermediate cleavage product, were detected. No MMP-3 or -7 proenzymes or active enzymes were found in any of the control specimens.

Author's personal copy

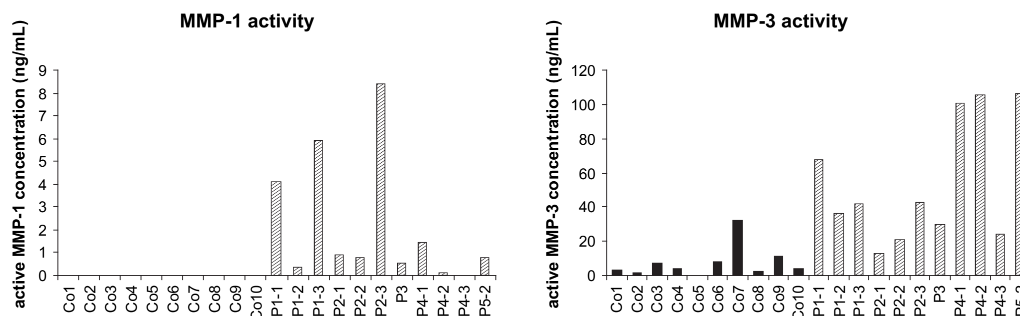


Fig. 4. The concentrations of MMP-1 and MMP-3 in control (Co) and melted corneas (P) determined by activity assays.

previous reports (Kervick et al., 1992; Palay et al., 1992; Riley et al., 1995) that corneal melting associated with autoimmune disorders could be induced by immunomodulatory mediators. Pro-inflammatory cytokines, which may enter the cornea from the tears via a damaged epithelial barrier, could stimulate the production of MMPs by stromal fibroblasts and attract an invasion of polymorphonuclear neutrophils and macrophages (Burrage et al., 2006; Kervick et al., 1992; Webster and Crowe, 2006).

Although our results support a crucial role for MMPs in corneal melting, plasminogen activator or some cathepsins may also participate in keratolysis. For example, plasminogen activator, which activates plasminogen into plasmin, has been shown to activate some MMPs as well in order to cleave some ECM components (Berman et al., 1980; Lijnen, 2003/2004; Twining et al., 1985).

Our results extend our knowledge about the participation of MMPs in corneal melting. Although we used specimens from patients with three different underlying causes, in whom the exact stimuli responsible for the initiation of corneal melting could vary, the final progress of ECM degradation in regards to MMP expression and activity was found to be almost similar in all patients/specimens.

Acknowledgement

This work was supported by the research project of the Czech Ministry of Education, Youth and Sports 0021620806/20610011.

References

Barletta, J.P., Angella, G., Balch, K.C., Dimova, H.G., Stern, G.A., Moser, M.T., van Setten, G.B., Schultz, G.S., 1996. Inhibition of pseudomonal ulceration in rabbit corneas by a synthetic matrix metalloproteinase inhibitor. Invest. Ophthalmol. Vis. Sci. 37, 20–28.
Berman, M., Leary, R., Gage, J., 1980. Evidence for a role of the plasminogen activator–plasmin system in corneal ulceration. Invest. Ophthalmol. Vis. Sci. 19, 1204–1221.
Bernauer, W., Ficker, L.A., Watson, P.G., Dart, J.K., 1995. The management of corneal perforations associated with rheumatoid arthritis. An analysis of 32 eyes. Ophthalmology 102, 1325–1337.
Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., Engler, J.A., 1993. Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med. 4, 197–250.
Burrage, P.S., Mix, K.S., Brinckerhoff, C.E., 2006. Matrix metalloproteinases: role in arthritis. Front Biosci. 11, 529–543.
Cawston, T.E., 1996. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Pharmacol. Ther. 70, 163–182.
Donzis, P.B., Mondino, B.J., 1987. Management of noninfectious corneal ulcers. Surv. Ophthalmol. 32, 94–110.
Fini, M.E., Yue, B.Y., Sugar, J., 1992. Collagenolytic/gelatinolytic metalloproteinases in normal and keratoconus corneas. Curr. Eye Res. 11, 849–862.
Fini, M.E., Cook, J.R., Mohan, R., 1998. Proteolytic mechanisms in corneal ulceration and repair. Arch. Dermatol. Res. 290 (Suppl.), S12–S23.
Funderburgh, J.L., Hevelone, N.D., Roth, M.R., Funderburgh, M.L., Rodrigues, M.R., Nirankari, V.S., Conrad, G.W., 1998. Decorin and biglycan of normal and pathologic human corneas. Invest. Ophthalmol. Vis. Sci. 39, 1957–1964.
Gabison, E.E., Chastang, P., Menashi, S., Mourah, S., Doan, S., Oster, M., Mauviel, A., Hoang-Xuan, T., 2003. Late corneal perforation after photorefractive keratectomy associated with topical diclofenac: involvement of matrix metalloproteinases. Ophthalmology 110, 1626–1631.
Giannelli, G., Pozzi, A., Stetler-Stevenson, W.G., Gardner, H.A., Quaranta, V., 1999. Expression of matrix metalloprotease-2-cleaved laminin-5 in breast remodeling stimulated by sex steroids. Am. J. Pathol. 154, 1193–1201.
Hagen, K.B., Waring 3rd, G.O., Johnson-Wint, B., 1997. Progressive nonulcerative paracentral keratolysis associated with elevated corneal metalloproteinases. Cornea 16, 486–492.
Hargrave, S.L., Jung, J.C., Fini, M.E., Gelender, H., Cather, C., Guidera, A., Udell, I., Fisher, S., Jester, J.V., Bowman, R.W., McCulley, J.P., Cavanagh, H.D., 2002. Possible role of the vitamin E solubilizer in topical diclofenac on matrix metalloproteinase expression in corneal melting: an analysis of postoperative keratolysis. Ophthalmology 109, 343–350.
Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M.D., Okada, Y., 1997. Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochem. J. 322 (Pt 3), 809–814.
Imai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N., Okada, Y., 1995. Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J. Biol. Chem. 270, 6691–6697.
Joseph, A., Dua, H.S., King, A.J., 2001. Failure of amniotic membrane transplantation in the treatment of acute ocular burns. Br. J. Ophthalmol. 85, 1065–1069.
Kenney, M.C., Chwa, M., Opbroek, A.J., Brown, D.J., 1994. Increased gelatinolytic activity in keratoconus keratocyte cultures. A correlation to an altered matrix metalloproteinase-2/tissue inhibitor of metalloproteinase ratio. Cornea 13, 114–124.
Kervick, G.N., Pflugfelder, S.C., Haimovici, R., Brown, H., Tozman, E., Yee, R., 1992. Paracentral rheumatoid corneal ulceration. Clinical features and cyclosporine therapy. Ophthalmology 99, 80–88.
Knauper, V., Lopez-Otin, C., Smith, B., Knight, G., Murphy, G., 1996. Biochemical characterization of human collagenase-3. J. Biol. Chem. 271, 1544–1550.
Lemp, M.A., 2008. Advances in understanding and managing dry eye disease. Am. J. Ophthalmol. 146, 350–356.
Li, D.Q., Shang, T.Y., Kim, H.S., Solomon, A., Lokeshwar, B.L., Pflugfelder, S.C., 2003. 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. 44, 2928–2936.
Lijnen, H.R., 2003/2004. Metalloproteinases in development and progression of vascular disease. Pathophysiol. Haemost. Thromb. 33, 275–281.
Liu, H., Zhang, W., Pan, Z., Wu, Y., 2002. Experimental study on the treatment of corneal melting after alkali burn with GM 6001. Zhonghua Yan Ke Za Zhi 38, 539–542.
Ljubimov, A.V., Burgeson, R.E., Butkowski, R.J., Michael, A.F., Sun, T.T., Kenney, M.C., 1995. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab. Invest. 72, 461–473.
Mackiewicz, Z., Maatta, M., Stenman, M., Konttinen, L., Tervo, T., Konttinen, Y.T., 2006. Collagenolytic proteinases in keratoconus. Cornea 25, 603–610.
Malik, R., Culinane, A.B., Tole, D.M., Cook, S.D., 2006. Rheumatoid keratolysis: a series of 40 eyes. Eur. J. Ophthalmol. 16, 791–797.
Marshall, G.E., Konstas, A.G., Lee, W.R., 1991a. Immunogold fine structural localization of extracellular matrix components in aged human cornea. I. Types I–IV collagen and laminin. Graefes Arch. Clin. Exp. Ophthalmol. 229, 157–163.
Marshall, G.E., Konstas, A.G., Lee, W.R., 1991b. Immunogold fine structural localization of extracellular matrix components in aged human cornea. II. Collagen types V and VI. Graefes Arch. Clin. Exp. Ophthalmol. 229, 164–171.
Matsubara, M., Zieske, J.D., Fini, M.E., 1991. Mechanism of basement membrane dissolution preceding corneal ulceration. Invest. Ophthalmol. Vis. Sci. 32, 3221–3237.
McElvanney, A.M., 2003. Doxycycline in the management of pseudomonas corneal melting: two case reports and a review of the literature. Eye Contact Lens 29, 258–261.
Mesa, C., Mirza, M., Mitsutake, N., Sartor, M., Medvedovic, M., Tomlinson, C., Knauf, J.A., Weber, G.F., Fagin, J.A., 2006. Conditional activation of RET/PTC3 and BRAFV600E in thyroid cells is associated with gene expression profiles that

Author's personal copy

predict a preferential role of BRAF in extracellular matrix remodeling. Cancer Res. 66, 6521–6529.

Myint, E., Brown, D.J., Ljubimov, A.V., Kyaw, M., Kenney, M.C., 1996. Cleavage of human corneal type VI collagen alpha 3 chain by matrix metalloproteinase-2. Cornea 15, 490–496.

Nakayasu, K., Tanaka, M., Konomi, H., Hayashi, T., 1986. Distribution of types I, II, III, IV and V collagen in normal and keratoconus corneas. Ophthalmic Res. 18, 1–10.

O'Brien, T.P., Li, Q.J., Sauerburger, F., Reviglio, V.E., Rana, T., Ashraf, M.F., 2001. The role of matrix metalloproteinases in ulcerative keratolysis associated with perioperative diclofenac use. Ophthalmology 108, 656–659.

Palay, D.A., Stulting, R.D., Waring 3rd, G.O., Wilson, L.A., 1992. Penetrating keratoplasty in patients with rheumatoid arthritis. Ophthalmology 99, 622–627.

Perez, V.L., Azar, D.T., Foster, C.S., 2002. Sterile corneal melting and necrotizing scleritis after cataract surgery in patients with rheumatoid arthritis and collagen vascular disease. Semin. Ophthalmol. 17, 124–130.

Pleyer, U., Bertelmann, E., Rieck, P., Hartmann, C., 2002. Outcome of penetrating keratoplasty in rheumatoid arthritis. Ophthalmologica 216, 249–255.

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

Riley, G.P., Harrall, R.L., Watson, P.G., Cawston, T.E., Hazleman, B.L., 1995. Collagenase (MMP-1) and TIMP-1 in destructive corneal disease associated with rheumatoid arthritis. Eye 9 (Pt 6), 703–718.

Smith, V.A., Rishmawi, H., Hussein, H., Easty, D.L., 2001. Tear film MMP accumulation and corneal disease. Br. J. Ophthalmol. 85, 147–153.

Solomon, A., Meller, D., Prabhasawat, P., John, T., Espana, E.M., Steuhl, K.P., Tseng, S. C., 2002. Amniotic membrane grafts for nontraumatic corneal perforations, descemetoceles, and deep ulcers. Ophthalmology 109, 694–703.

Stefansson, S., Aimes, R.T., Seward, N.B., Alexander, D.S., Quigley, J.P., 1994. Native TIMP-free 70 kDa progelatinase (MMP-2) secreted at elevated levels by RSV transformed fibroblasts. J. Cell. Physiol. 161, 419–428.

Twining, S.S., Hatchell, D.L., Hyndiuk, R.A., Nassif, K.F., 1985. Acid proteases and histologic correlations in experimental ulceration in vitamin A deficient rabbit corneas. Invest. Ophthalmol. Vis. Sci. 26, 31–44.

Vajpayee, R.B., Vanathi, M., Tandon, R., Sharma, N., Titiyal, J.S., 2003. Keratoplasty for keratomalacia in preschool children. Br. J. Ophthalmol. 87, 538–542.

Vajtr, D., Kukacka, J., Adámek, T., 2002. Findings in myocardial extracellular matrix in fatal poisoning with addictive substances. Soud Lek 47, 54–58. Czech.

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

Vizoso, F.J., Gonzalez, L.O., Corte, M.D., Rodriguez, J.C., Vazquez, J., Lamelas, M.L., Junquera, S., Merino, A.M., Garcia-Muniz, J.L., 2007. Study of matrix metalloproteinases and their inhibitors in breast cancer. Br. J. Cancer 96, 903–911.

Webster, N.L., Crowe, S.M., 2006. Matrix metalloproteinases, their production by monocytes and macrophages and their potential role in HIV-related diseases. J. Leukoc. Biol. 80, 1052–1066.

Weiss, A., Goldman, S., Shalev, E., 2007. The matrix metalloproteinases (MMPS) in the decidua and fetal membranes. Front Biosci. 12, 649–659.

Wheeless, C.R., Nunley, J.A., Urbaniak, J.R., 1996–2009. 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.

Yu, W.H., Woessner Jr., J.F., 2000. Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7). J. Biol. Chem. 275, 4183–4191.