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

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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:

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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.
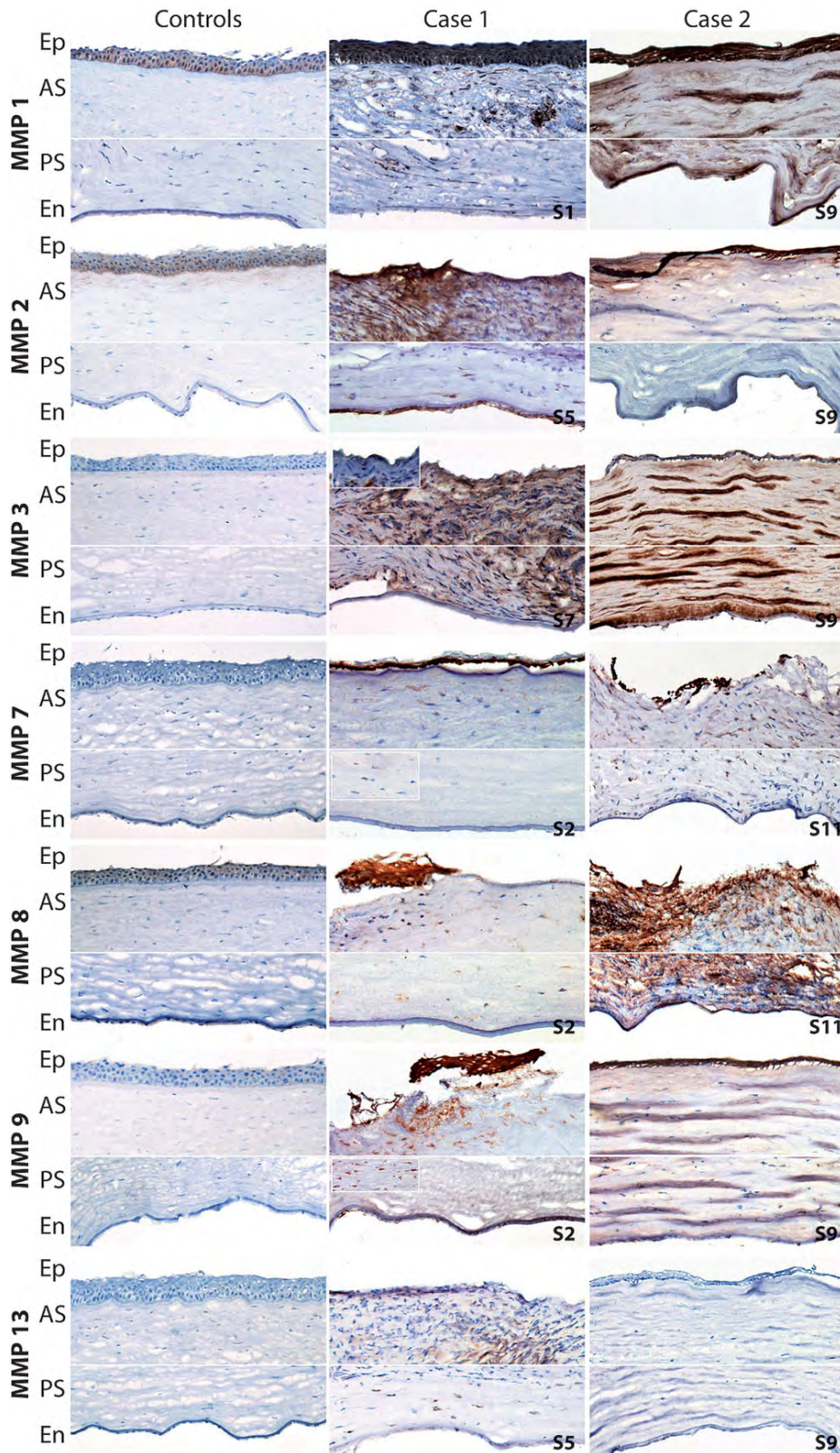
Figure 1. Immunohistochemical localization of matrix metalloproteinases in melted and control corneas. Immunohistochemical localization of MMP 1, 2, 3, 7, 8, 9, and 13 in representative images of melted corneal specimens obtained from patients with pSS (cases 1 and 2) and from control corneas. Original 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
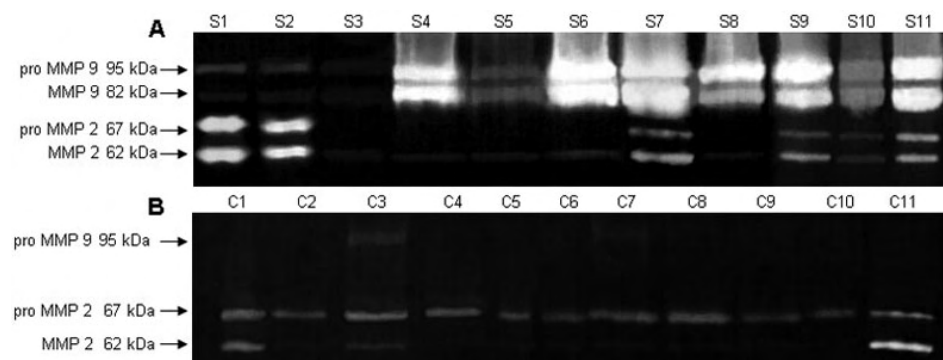
Figure 2. Gelatin zymography of matrix metalloproteinases 2 and 9 in melted and control corneas. Melted specimens **(A)** S1 and S2 showed extremely high levels, and specimens S7, S9, and S11 considerable levels, of both the proenzyme (67 KDa band) and the active form of MMP 2 (62 kDa band). Levels of 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.
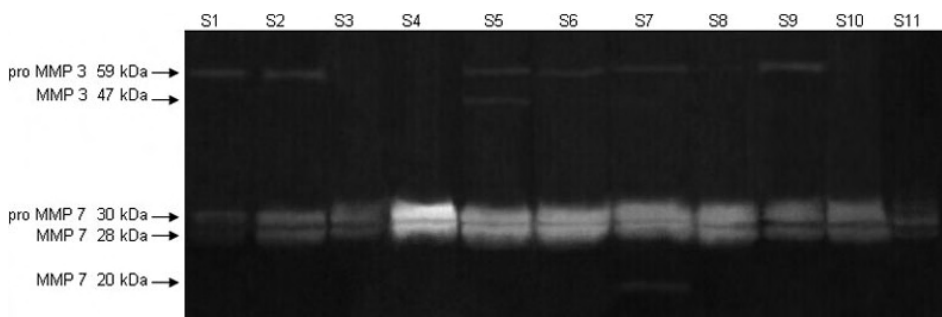
Figure 3. Casein zymography of matrix metalloproteinases 3 and 7 in melted specimens. Very slight bands of only the MMP 3 proform (59 kDa) were detected in five melted specimens (S1, S2, S6, S7, and S9) and of both the proform and active MMP 3 (47 kDa) in one specimen (S5). High levels of MMP 7 proenzyme (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

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.

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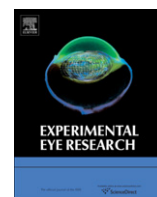
Role of matrix metalloproteinases in recurrent corneal melting[☆]

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ABSTRACT

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.

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).

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.

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,

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.
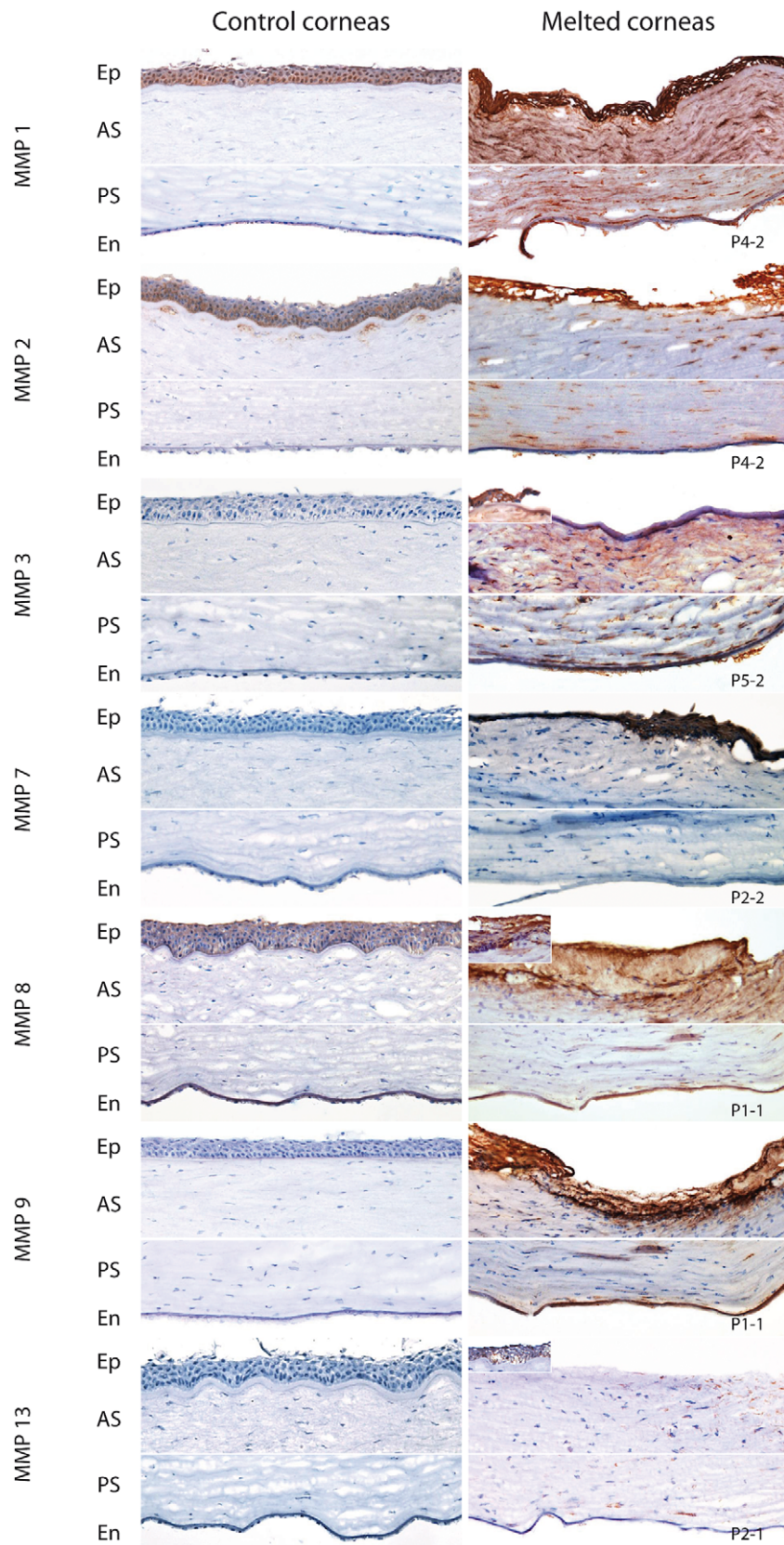
Fig. 1. Immunostaining of MMP-1, -2, -3, -7, -8, -9 and 13 in representative samples of melted and control corneas. The photomicrographs of melted corneas stained for MMP-3, -8 and -13 have insets showing the epithelium of the same specimen. Ep = epithelium, AS = anterior stroma, PS = posterior stroma, En = endothelium.
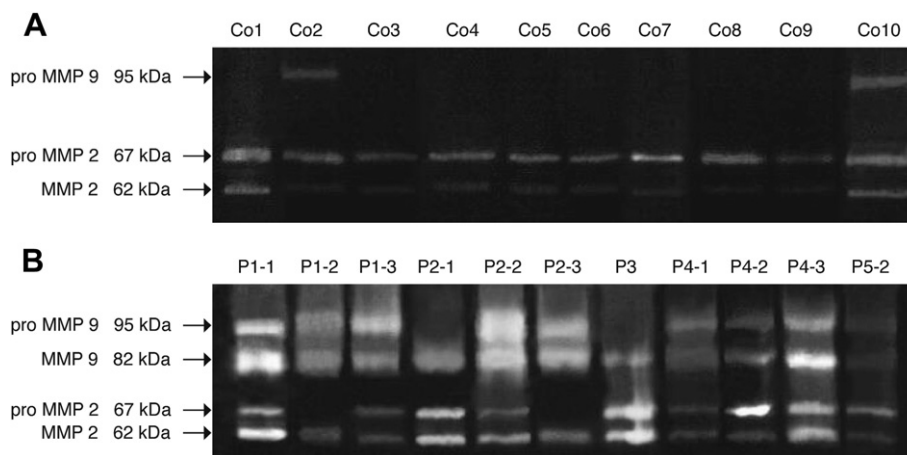
Fig. 2. Detection of MMP-2 and -9 activity using gelatin zymography in control corneas (A) and melted grafts (B). Prominent bands and faint bands of both the partly cleaved form of MMP-2 (67 kDa) and the active enzyme (band at 62 kDa) were detected in five (P1-1, P2-1, P2-2, P3, P4-3) and three (P1-3, 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
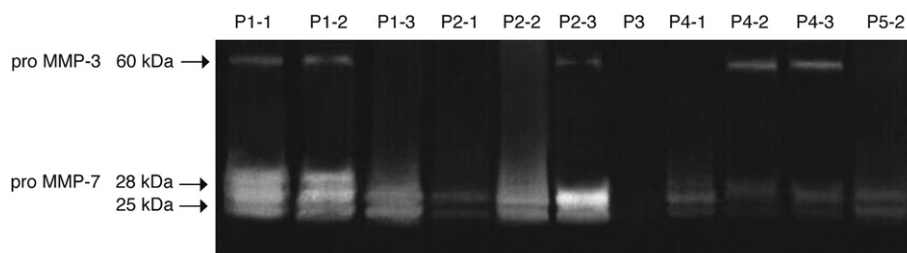

Fig. 3. Casein zymography in melted corneal grafts. Low levels of the MMP-3 proenzyme (60 kDa) were observed in five melted corneal samples (P1-1, P1-2, P2-3, P4-2, P4-3). Dense bands in five (P1-1, P1-2, P1-3, P2-2, P2-3) and faint bands in five (P2-1, P4-1, P4-2, P4-3, P5-2) melted corneas migrating 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.
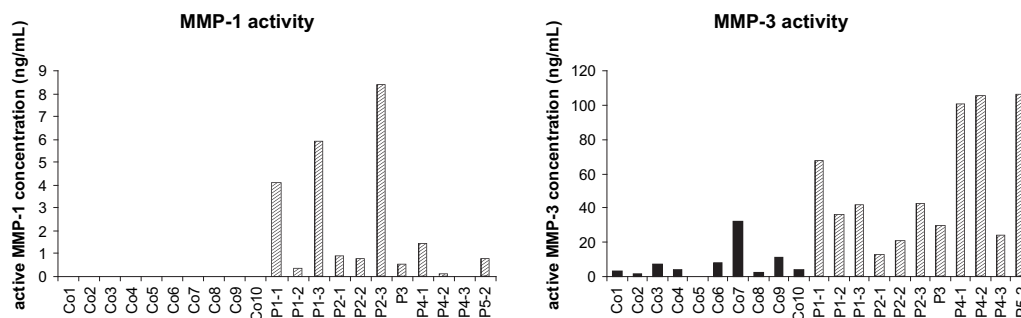
Fig. 4. The concentrations of MMP-1 and MMP-3 in control (Co) and melted corneas (P) determined by activity assays.

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

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