Activation of Matrix Metalloproteinase-8 by Membrane Type 1-MMP and Their Expression in Human Tears after Photorefractive Keratectomy

Juba M. Holopainen,1 Jukka A. O. Moilanen,1 Timo Sorsa,2,3 Marjo Kivelä-Rajamäki,2,3 Taina Tervahartiala,2,5 Minna H. Vesaluoma,1 and Timo M. T. Tervo1

PURPOSE. Matrix metalloproteinases (MMPs) play a central role in the wound-healing process. The objective of this study was to identify and characterize the levels and molecular forms of human tear fluid collagenase-2 (MMP-8) and membrane type 1-MMP (MT1-MMP or MMP-14) in patients who had undergone excimer laser photorefractive keratectomy (PRK) and in healthy subjects. Whether MT1-MMP activates pro-MMP-8 was also determined.

METHODS. Tear fluid samples were collected with scaled and blunted microcapillaries from healthy control subjects and, on the second postoperative day, from patients who had undergone PRK. Time and the volume collected were registered. Molecular forms and levels of pro and active MMP-8 and MT1-MMP in these samples were determined by Western immunoblot analysis, quantitated by computer scanning. The concentration of MMP-8 was also determined by immunofluorescence assay. The conversion of pure human polymorphonuclear neutrophil (PMN) pro-MMP-8 to the active form by the catalytic domain of MT1-MMP was studied by Western immunoblot analysis.

RESULTS. The tear fluid flow was increased after PRK. Tear fluid flow–corrected excretion of MMP-8 was significantly higher in PRK-treated patients, as assessed by immunofluorescence assay and quantitative Western immunoblot analysis. The major MMP-8 species detected in tears of both PRK-treated patients and healthy control subjects represented latent and active 75- and 65-kDa highly glycosylated MMP-8 isoforms. The less-glycosylated 45- to 55-kDa MMP-8 isoform was not detectable. Tear fluid flow–corrected secretion of MT1-MMP was significantly higher in PRK-treated patients. Soluble 80-kDa MT1-MMP immunoreactivities were detected in tears of both healthy control subjects and PRK-treated patients, and may represent a complex captured by tissue inhibitor of metalloproteinase (TIMP)-2. Human PMN pro-MMP-8 was converted to the active form by MT1-MMP, and TIMP-2 prevented this activation.

CONCLUSIONS. Corneal renewal eventually occurs at a high rate and is affected by the rate of corneal collagen and other matrix protein breakdown. Accordingly, tear fluid MMP-8 and MT1-MMP levels were shown to be constantly high in normal subjects. With PRK, a fast wound-healing process was associated with even higher MMP-8 and MT1-MMP levels and their activation. The results suggest a role for a MMP-8 and MT1-MMP network in the corneal wound-healing cascade. Furthermore, MT1-MMP (MMP-14) seems to activate pro-MMP-8. (Invest Ophthalmol Vis Sci. 2003;44:2550–2556) DOI:10.1167/ iovs.02-1190

Intense ultraviolet radiation at 193 nm is used for the correction of refractive errors by excimer laser. Photorefractive keratectomy (PRK) is based on remodeling of the corneal curvature by photoablation of the Bowman’s layer and the anterior corneal stroma.1 Cell migration, mitosis, and differentiation as well as reconstitution of adhesive structures between the epithelium and the stroma are essential for corneal epithelial wound healing.2,5 After PRK, epithelialization of the ablated area usually lasts from 2 to 3 days. It is followed by epithelial restoration that continues for weeks. The wounded epithelial area is first covered by migration of activated epithelial cells from the wound edge.4 The normal stratified epithelium is reduced to two to three layers of epithelial cells at the wound margin, whereas a single layer of flattened cells forms the leading edge. In the former type, the cells are thought to form and break down temporary focal contacts with extracellular matrix (ECM) proteins, such as fibronectin.6 Human tears are known to contain components of proteolytic systems, such as plasminogen activator inhibitors, α2-antiplasmin, α2-macroglobulin, albumin, and α2-antitrypsin, as well as matrix metalloproteinases.7,7–11

Matrix metalloproteinases (MMPs; i.e., collagenases, gelatinases, matrilysins, stromelysins, and membrane-type MMPs) are important in the regulation of the migrating epithelial cells.12 Furthermore, MMPs are expressed at relatively low levels in normal conditions and are usually upregulated when degradation is required.13 Matsubara et al.14 found that a 170-kDa gelatinase was present in the epithelial and stromal tissue adjacent to the migrating edge of a scrape wound until the closure of the epithelial defect. Furthermore, 72-kDa (MMP-2) and 92-kDa (MMP-9) gelatinases have been detected in the migrating epithelium of excimer-ablated rat corneas but not in the untreated control.15 In humans, upregulation of MMP-2 and 9 has been observed in tears after PRK (Vesaluoma MH, unpublished data, 2000) and, of these, MMP-9 may be especially important in processing the basement membrane.15 Taken together, the evidence shows that MMPs are involved in the initial stromal degradation as well as the ultimate ECM remodel-
Collagenase-2 (MMP-8) belongs to the collagenase subfamily of MMPs and is a potential initiator of interstitial collagenolysis at the inflammatory sites. The molecular mass of MMP-8 varies between 50 and 85 kDa, reflecting a different degree of glycosylation and whether the enzyme is present in latent or activated form. MMP-8 is stored in subcellular-specific granules of circulating mature human peripheral blood polymorphonuclear neutrophils (PMNs), and the rate of degradation mainly regulates its activity. However, it has recently been shown that several lines of non-PMN lineage cells, such as fibroblasts, plasma cells, endothelial, bronchial, and gingival sulcular epithelial cells, express MMP-8 mRNA and the protein both in vitro and in vivo. 

**Materials and Methods**

**Patients and Control Subjects**

The present study was performed according to the provisions of the Declaration of Helsinki and was approved by the ethics review committee of Helsinki University Eye Hospital. Informed consent was obtained from each patient. Twenty-four and 15 patients (Table 1) were studied for the presence and levels of collagenase-2 (MMP-8) and MT1-MMP, respectively, on the second day after PRK. The levels of MMP-8 and MT1-MMP were compared with those in 21 and 10 healthy control subjects, respectively (Table 1). Clinical investigation before PRK or in the control subjects showed no sign of ocular inflammation or allergy. The PRK-treated patients were advised to stop wearing contact lenses 2 weeks before the operation.

**Tear Collection**

Tear fluid samples (minimum 4 μL) were collected with 10-μL micropipettes (Blaubrand Intramark; Brand GmBH, Wertheim, Germany) from the lower conjunctival sac. Tears were immediately transferred into 1.5-mL microcentrifuge tubes (Eppendorf, Fremont, CA), placed on dry ice, and kept at −70°C until analyzed. The capillary method used for collecting tears contained several possible sources of error, although performed with special attention to the technique. In brief, both the residual tears of the conjunctival fornix and the newly secreted tears were collected. The tear fluid flow in the capillary was thus considerably higher than the actual tear fluid secretion rate. Also, reflex tearing was easily stimulated during the collection of tear fluid. Most patients were not familiar with tear fluid collection, and in spite of the gentle aspiration technique used with fire-polished and blunted capillaries, some of them reported sensing the presence of a foreign body during tear collection. Despite the inaccuracy of the capillary tube method, we consider it a suitable technique for collecting tears to study the release of various proteins in tear fluid, once the limitations of the technique are realized.

**Photorefractive Keratectomy**

PRK was performed after surgical abrasion of the epithelium with a Beaver eye blade (BD Biosciences, Franklin Lakes, NJ). PRKs, 6 mm wide and of various ablation depths (15–77 μm), were performed without nitrogen blow, using an excimer laser (Star S2; VisX Co, Sunnyvale, CA, with accompanying software ver. 4.02).

**Western Immunoblot Analysis**

The molecular forms and levels of MMP-8 and MT1-MMP were analyzed by Western immunoblot analysis in nonreducing conditions with a rabbit polyclonal anti-human MMP-8–specific antibody (IgG fraction) and a polyclonal antibody specific for MT1-MMP (0.4 μg/mL; Biogenesis Ltd., Poole, UK), as described previously. After electrophoresis, the proteins in the gel were electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After the unoccupied sites were blocked with gelatin, the membrane was first reacted with the primary antibody (1:1500) and then with alkaline phosphatase-conjugated secondary antibody. Immunoreactive proteins were visualized by nitroblue tetrazolium (NBT; Sigma, Poole, UK) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Sigma). An imaging densitometer (Model GS-700; Bio-Rad, using Analyst software) was used for quantitation. The specificity of the anti-human MMP-8 antibody has been described elsewhere.

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>PRK Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>34.0 ± 10.0</td>
<td>36.0 ± 12.7</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>9/12</td>
<td>11/13</td>
</tr>
<tr>
<td>Ablation depth (°C)</td>
<td>41.7 ± 17.5</td>
<td>33.7 ± 12.7</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>32.4 ± 10.0</td>
<td>36.0 ± 12.4</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/3</td>
<td>7/6</td>
</tr>
<tr>
<td>Ablation depth (°C)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

This work was undertaken to study the levels and molecular forms of MMP-8 and MT1-MMP in tears of healthy subjects in comparison with patients who have undergone PRK. Furthermore, we present evidence that, in vitro, MT1-MMP activated pro-MMP-8, thus representing a novel cascade to potentiate the breakdown of the ECM component during corneal wound healing.

**Materials and Methods**

**Patients and Control Subjects**

The present study was performed according to the provisions of the Declaration of Helsinki and was approved by the ethics review committee of Helsinki University Eye Hospital. Informed consent was obtained from each patient. Twenty-four and 15 patients (Table 1) were studied for the presence and levels of collagenase-2 (MMP-8) and MT1-MMP, respectively, on the second day after PRK. The levels of MMP-8 and MT1-MMP were compared with those in 21 and 10 healthy control subjects, respectively (Table 1). Clinical investigation before PRK or in the control subjects showed no sign of ocular inflammation or allergy. The PRK-treated patients were advised to stop wearing contact lenses 2 weeks before the operation.
MMP-8 Immunofluorometric Assay

MMP-8 levels were also determined by immunofluorescence assay. The monoclonal MMP-8–specific antibodies 8707 and 8706 were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled with europium-chelate, as described previously. The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM CaCl₂, 50 μM ZnCl₂, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 μg/L diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for 1 hour, followed by incubation for 1 hour with tracer antibody. Enhancement solution was added, and after 5 minutes, fluorescence was measured with a fluorometer (model 1234 Delta Research Fluorometer; Wallac, Turku, Finland). Pro-MMP-8 was purified from human neutrophil (PMN) extracts. The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8.

Conversion of PMN Pro-MMP-8 by MT1-MMP

Conversion of human pro-MMP-8 by MT1-MMP was studied as follows. Human pro-MMP-8 (500 ng) was incubated with 50 ng of the catalytic domain of MT1-MMP (Invitek, Berlin, Germany) at 37 °C for 60 minutes. The pro-MMP-8 was also incubated with tissue inhibitor of metalloproteinase (TIMP)-2 (Chemicon, Temecula, CA) in activated MT1-MMP (molar ratio 1:1) and p-aminophenylmercuric acid (APMA). The reaction was terminated by heating at 100 °C for 5 minutes. Conversion of pro-MMP-8 was assessed by 10% SDS-PAGE and Western blot analysis with the use of a polyclonal antibody specific for MMP-8.

Statistical Analysis

All results are expressed as the mean ± SD. The densitometric and immunofluorescence assay data were analyzed by use of the Mann-Whitney test. P ≤ 0.05 was considered statistically significant.

RESULTS

Tear Fluid Flow, Immunoreactivity, and Excretion-Corrected Levels of Pro- and Active MMP-8 Species

All surgically treated eyes showed at least some reflex tearing 2 days after PRK, and all patients showed an epithelial defect of approximately 1 to 3 mm in diameter. Accordingly, tear fluid flow was found to be significantly higher in PRK-treated patients than in control subjects, 35.3 ± 10.4 and 4.4 ± 0.8 μL/min, respectively (P ≤ 0.001; Fig. 1A). The molecular species of MMP-8 were analyzed by Western immunoblot analysis. Two immunoreactive species were detected at approximately 75 and 65 kDa, most likely representing the latent and activated forms of the highly glycosylated MMP-8. The levels of pro-MMP-8 in the tear fluid samples of patients who underwent PRK and normal subjects are presented in Figure 1B. MMP-8 was detected in all samples and, unexpectedly, the levels of the MMP-8 species of PRK-treated patients and normal control subjects did not differ statistically, 1.6 ± 0.20 U/μL versus 1.4 ± 0.15 U/μL (P = 0.387; Fig. 1B). This finding probably reflects the dilution effect of excess tearing on pro-MMP-8 levels. Thus, tear fluid flow–corrected pro-MMP-8 release was found to be significantly higher in PRK eyes than in control eyes (60.2 ± 100.0 U/min vs. 5.7 ± 4.7 U/min, P = 0.001; Fig. 1C).

The major MMP-8 species detected in both PRK and healthy control tears represent active and latent forms of highly glycosylated 65- and 75-kDa MMP-8 isoforms. The less-glycosylated 45- to 55-kDa isoforms were not observed (Fig. 2).

We then proceeded to study the levels of active MMP-8 species. The levels of active MMP-8 were found to be the same in control and PRK-treated subjects 4.7 ± 0.44 U/μL versus 5.2 ± 0.20 U/μL (P = 0.539; Fig. 3A). However, according to
results in the immunofluorescence assay, MMP-8 concentrations were significantly higher in patients who underwent PRK than in control subjects (51 ± 56.4 vs. 10.1 ± 20.8 μg/L, P = 0.036). Tear fluid flow–corrected active MMP-8 concentration was significantly higher in surgically treated patients than in healthy control subjects (179.9 ± 275.4 U/min vs. 22.3 ± 21.8 U/min, P = 0.001; Fig. 3B).

The Western blot analysis also revealed one additional immunoreactive species of high-molecular-mass (>111 kDa) MMP-8 complex, which probably represents an MMP-8 species trapped by endogenous inhibitors (TIMPs or α2-macroglobulin) and/or MMP-8 dimers in one PRK-treated patient. In this regard, we also analyzed tears of three patients with corneal erosion. The high-molecular-weight species were also found in all these samples (data not shown).

**Excretion-Corrected Levels and Molecular Forms of MT1-MMP in Tear Fluid**

Western blot analysis revealed detectable levels of MT1-MMP in all tear samples of control subjects and PRK-treated patients. The molecular mass of MT1-MMP was approximately 85 kDa (Fig. 4) and may represent soluble 65-kDa MT1-MMP captured by 21-kDa TIMP-2 after in vivo activation or shedding. In line with this, our unpublished data show that TIMP-2 concentrations were elevated in tears of patients who underwent PRK. The tear fluid flow was significantly higher in patients after PRK, than in control subjects (P = 0.001; Fig. 5A). MT1-MMP concentration was significantly higher in control subjects than in patients who underwent PRK (P = 0.026; Fig. 5B), but tear fluid flow–corrected excretion of MT1-MMP was higher in PRK-treated patients than in control subjects 32.3 ± 12.3 U/min vs. 2.5 ± 0.5 U/min (P = 0.003; Fig 5C).

**Conversion of Pro-MMP-8 by MT1-MMP**

In light of the current findings, it seemed possible that the activity of MMP-8 might also be regulated by MT1-MMP, as has been reported for MMP-2 and -13.33,37 Accordingly, we then studied in vitro whether pro-MMP-8 is susceptible to conversion by MT1-MMP to the active form. Western blot analysis with anti-MMP-8 demonstrated 75- and 65-kDa immunoreactive bands that corresponded well to the calculated pro and active forms of human PMN MMP-8 (Fig. 6). The active MT1-MMP catalytic domain induced the 75-kDa PMN pro-MMP-8 conversion into the 60- to 65-kDa form, and TIMP-2 prevented the conversion (Fig. 6).

**DISCUSSION**

Cytokines, growth factors, and other tear components, or their receptors are likely to contribute to a complex network of metabolic cascades needed for the maintenance of a normal ocular surface and for corneal wound healing after injury.7,5,49–51 The avascular cornea provides a good model to study wound healing. However, the behavior of the known
modulators, such as interleukin-1 and MMPs, seems to be very similar to other tissues. Accordingly, many known components and their receptors are likely to be constitutively produced, and hence available during the various stages of the wound-healing response. The network of plasmin and MMPs seems to be largely responsible for the proteolytic cleaning phase of corneal wound healing.

MMP-8 plays a role in the tissue-destructive inflammatory diseases, and activation of MMP-8 has been demonstrated in chronic bronchiectasis, cystic fibrosis, asthma, rheumatoid arthritis, and periodontitis. In bronchiectasis, asthma, cystic fibrosis, and periodontitis, MMP-8 has been reported to reflect the severity and activity of the disease. Furthermore, expression of MMP-8 is also upregulated in wound healing and tissue remodeling. Overexpression of MMP-8 has been attributed to nonhealing chronic ulcers suggesting that corneal regulation of the activity of MMP-8 is important. It has been shown recently that epithelial cells produce MMP-8 in ulcerative keratolysis induced by topical nonsteroidal anti-inflammatory drugs. MMP-8 was assumed to either trigger or exacerbate the pathologic effect of this condition. In this study, synthesis of MMP-8 and its activation were elevated in human tear fluid in response to PRK. This finding corresponds to other body fluids associated with inflammatory disorders, such as bronchoalveolar fluid in patients with bronchiectasis and asthma. Furthermore, gingival crevicular fluid of patients with periodontal disease show high MMP-8 levels. Both PRK and healthy control tear fluids contained predominantly 65- and 75-kDa MMP-8 immunoreactivity, evidently representing active and latent forms, respectively, of highly glycosylated PMN-type MMP-8. Less-glycosylated non-PMN-type MMP-8 isoforms were not detectable. The finding that MMP-8 was present in large quantities in human tears was not unexpected, considering the need for rapid turnover rate of the matrix in the cornea after corneal wounds. Accordingly, type I collagen is a major structural component of the corneal stroma, and the precise arrangement of type I collagen fibrils is mandatory for stromal clarity and good visual acuity. Furthermore, after PRK in rats, dramatic increases in collagen I and III mRNAs have been observed recently. The presence of active forms of MMP-8 and MT1-MMP may serve as a reservoir for the rapid initiation of matrix turnover, which may be needed to initiate the wound-healing response.

collagenolytic activity is needed during wound healing. Collagenase (MMP)-I is probably not expressed by the injured corneal cells. The corneal tissue seems to remain in the reconstruction phase for an unusually long period, up to 1 year after wounding. Concomitantly, the cornea synthesizes new collagen. The precise arrangement of newly formed ECM components and remodeling necessitates collagenase activity. This was observed in our PRK-treated patients on the second postoperative day and suggests that tear fluid MMP-8 levels reflect dynamic changes in the corneal tissues during wound healing. Because whole human corneas are not

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933225/)

**Figure 5.** (A) Tear fluid flow, (B) concentration of MT1-MMP in tear fluid, and (C) tear fluid flow–corrected release of MT1-MMP in control subjects and patients after PRK.

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933225/)

**Figure 6.** Activation of human PMN pro-MMP-8 by human MT1-MMP. Lane a: pro-MMP-8; lane b: pro-MMP-8 treated with 1 mM APMA (60 minutes at 37°C); lane c: pro-MMP-8 treated with the catalytic domain of MT1-MMP (60 minutes at 37°C); lane d: as in lane c but MT1-MMP pretreated with TIMP-2 (molar ratio 1:1 for 60 minutes at 37°C). Molecular masses of pro-75-kDa and the active (65–kDa) forms of human PMN MMP-8 are indicated.
available after PRK, we focused on analyzing human tear samples. The present tear fluid results should be amended, however, by immunohistochemical staining. In this regard we received one fresh cornea from a patient with keratoconus and performed immunohistochemical staining of MMP-8 on this sample. The results were similar to those published earlier.21,22

The most intense MMP-8 immunoreaction was located in the corneal epithelial cells (data not shown). Inflamed human brachial and gingival sulcular epithelial cells have also been shown to express MMP-8 protein and its mRNA in bronchiectasia, asthma, and periodontitis in vivo.21,23,24

MT1-MMP is a known activator of progelatinase A (MMP-2)25 and procollagenes-3 (MMP-13)26 and contributes to the MMP network needed during corneal wound healing. Furthermore, MT1-MMP shows activity against a number of ECM components, including type I and III collagens. The MT1-MMP levels were found to be elevated in human tear fluid. Our present data on both PRK and healthy control tear fluids strongly suggests that MT1-MMP exists in soluble form in vivo, in addition to the generally recognized membrane-associated form. In accordance with our findings, cultured human mesangial cells and breast cancer cells have been shown to express and release soluble 65-kDa active MT1-MMP.60–62 Furthermore, human periodontitis-affected gingival crevicular fluid, induced sputum, and bronchoalveolar lavage fluid of patients with asthma or bronchiectasis have all been shown to contain elevated levels of soluble MT1-MMP.21,25,24 Evidently, the detected tear fluid 85-kDa MT1-MMP immunoreactivity represents the active form of soluble MT1-MMP (65 kDa), possibly captured by TIMP-2 (21 kDa). In rats, elevated levels of TIMP-2 were observed after PRK.63 Furthermore, we have found elevated TIMP-2 levels in tear fluid after PRK (Vesalouma MH, unpublished data, 2000). The present results further demonstrate that MT1-MMP can also activate MMP-8 in vitro and that thus activation of MMP-8 by MT1-MMP could also take place in vivo in tear fluid. Taken together, these results suggest that soluble MT1-MMP can regulate corneal wound healing by activating collagenolytic cascades and by cleaving the ECM components.

The tear fluid changes observed in the present study correspond well with the expression of other MMPs in the various wound models of animal cornea.55,64 Our study provides the first evidence that the collagenolytic MMP-8 and MT1-MMP shows dynamic changes after PRK in humans. Other MMPs, such as noncollagenolytic MMP-9, have already been reported to be involved in degradation of the epithelial basement membrane and initiation of formation of a corneal ulcer and to show prolonged expression in cases of failure of reepithelialization in rat cornea.14,64 It also seems feasible that MMP inhibitors capable of downregulation of both MMP-8 and MT1-MMP18,65–68 may prevent the formation of stromal haze.

Both MMP-8 and MT1-MMP were shown to be common components of human tears and probably participate in the wound-healing cascade by acting in concert with other modulators to restore normal corneal tissue. Accordingly, the expression of these two collagenolytic MMPs was elevated in tears after PRK. Furthermore, MT1-MMP activated MMP-8 in vitro, which suggests that similar activation may take place in vivo. These MMPs may serve as optional and adjunctive diagnostic tools14 for monitoring and evaluation of the integrity and function of corneal wound healing.

References


