

Tear Levels and Activity of Matrix Metalloproteinase (MMP)-1 and MMP-9 in Vernal Keratoconjunctivitis

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PURPOSE. To study levels and activity of matrix metalloproteinase (MMP)-1 and -9 and their tissue inhibitor (TIMP-1) in tears of patients with vernal keratoconjunctivitis (VKC), with and without severe corneal damage.

METHODS. Tear samples were obtained from 16 patients with active VKC and 10 normal control subjects, after clinical evaluation and tear cytology. Tear levels of pro-MMP-1, pro-MMP-9, and TIMP-1 were measured by enzyme-linked immunosorbent assay (ELISA). Collagenase and gelatinase activity were measured in tears by MMP activity assays. Immunohistochemistry was performed on a fragment of superficial keratectomy from two vernal corneal ulcers.

RESULTS. Tear levels of pro-MMP-1 and pro-MMP-9 were significantly increased in patients with VKC compared with control subjects ($P < 0.001$). MMP-1/TIMP-1 and MMP-9/TIMP-1 molar ratios were significantly increased ($P < 0.001$) in VKC. MMP-1 and MMP-9 activities were significantly increased in VKC tears compared with control samples ($P < 0.005$). MMP-9 activity correlated significantly with corneal involvement and giant papillae formation. Immunohistochemistry showed positive staining for MMP-9, fibronectin, and eosinophil cationic protein (ECP) on the superficial corneal stroma of the ulcer bed, but no inflammatory cells.

CONCLUSIONS. Increased levels and activity of MMP-1 and -9 and an imbalance between MMPs and TIMP may be involved in the pathogenesis of VKC. (*Invest Ophthalmol Vis Sci.* 2003;44:3052-3058) DOI:10.1167/iovs.02-0766

Chronic severe forms of allergic conjunctivitis, in particular vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), are characterized by persistent conjunctival inflammation involving intense eosinophil infiltration, increased numbers of mast cells, basophils, neutrophils, macrophages, and type 2 T helper (Th2) lymphocytes.¹⁻³ Even in symptom-free patients, VKC is characterized by architectural remodeling of the conjunctival tissues due to an excess of extracellular matrix (ECM) deposition, subepithelial fibrosis, chronic cellular infiltrate, and epithelial thickening. These remodeling signs are more evident during active symptomatic phases. Corneal complications and plaque formation induced by the toxic effect of eosinophil products may also relate to the modification of ECM metabolism.

The matrix metalloproteinases (MMPs) degrade all components of the extracellular matrix.⁴ These enzymes are implicated in many critical physiological and pathologic processes, including development, wound healing, angiogenesis, cancer, and inflammation.⁵ They are broadly divided into four groups according to substrate specificity: collagenases, gelatinases, stromelysins, and the membrane-type MMPs. They are mainly synthesized by connective tissue cells, granulocytes, and monocyte macrophages. MMPs are inhibited by the naturally occurring proteins, tissue inhibitors of MMPs (TIMP), and by the broad-spectrum inhibitor, $\alpha 2$ -macroglobulin. The functional activity of MMPs is controlled by the equilibrium between levels of activated MMPs and free TIMPs that is essential for activating proteolysis and tissue invasion. MMP-1 typically degrades collagens I, III, and V, which are the main conjunctival and corneal collagens, whereas gelatinase B (MMP-9) typically degrades the basement membrane constituent, collagen IV, and other matrix proteins. MMP-9 has also been reported to play a crucial role in the migration of inflammatory cells through basement membranes⁶ and thus may be involved in eosinophil migration during allergic inflammation.⁷ Increased levels of MMP-9 and TIMP-1 have been found in bronchoalveolar lavage fluids and sputum of patients with asthma.⁸⁻⁹ The ECM remodeling and resultant airflow obstruction in asthma are thought to be caused in part by an imbalance between MMP-9 and its inhibitor.

Collagens and growth factors in VKC tissues¹⁰ and cytokines in tears have been shown to be increased and may stimulate fibroblast proliferation and collagen production.¹¹ It is not yet clear whether degradation of collagen or other ECM components is also involved in VKC.

The purposes of the present study were: (1) to identify the presence and the activity of MMP-1 and -9 and TIMP-1 in tears of patients with VKC, with and without corneal involvement and giant papillae formation; (2) to determine whether the concentration of these enzymes can be correlated with the severity of the clinical condition; and (3) to localize MMP-9 in rarely obtained VKC corneal ulcer biopsy specimens.

MATERIALS AND METHODS

Patients and Tear Samples

Sixteen consecutive patients with VKC (mean age, 11.2 ± 4.9 years; range, 7-22) and 10 normal subjects were included in the study. Patients with VKC (12 male and 4 female) were in an active disease phase and had been free of treatment for at least 3 days. The control group was age matched (mean age, 16.5 ± 5 ; range, 10-24) and included six males and four females. No subject in the control group used contact lenses or had any inflammatory signs and symptoms. The research adhered to the tenets of the Declaration of Helsinki. A written informed consent was obtained from all subjects or their parents before tears were collected. Diagnosis of VKC was based on clinical history and evaluation of signs and symptoms. Patients with VKC were tested for skin test reactivity and the presence of specific IgE in serum for common environmental allergens (CAP-System; Pharmacia & Upjohn, Uppsala, Sweden). A subjective clinical score (0-10) was given to

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TABLE 1. Demographic and Clinical Data of 16 Patients with VKC

Patient	Gender/Age	Tarsal/Limbal	Serum IgE (kU/L)	Allergen* (CAP-FEIA)	Signs (sum score)	Symptoms (sum score)	Cornea (score)	% Tear Eosinophils
1	F/10	T	4280	G,D	9	12	1	71
2	M/14	L	217	—	8	9	1	30
3	F/10	T	20	—	8	11	2	30
4	M/22	T	4744	G,P,B,D	12	9	1	50
5	M/7	L	442	—	5	9	0	65
6	M/9	T	1203	G,P,D	11	15	4	45
7	M/8	T	2300	P,D	14	15	4	50
8	M/8	L	279	G	5	4	0	NE
9	M/8	T	321	—	7	9	2	NE
10	M/7	L	219	—	7	8	0	40
11	F/11	L	188	—	7	8	0	60
12	M/9	T	23	—	6	8	0	55
13	F/20	L	51	—	6	8	0	45
14	M/18	T	199	G	7	9	2	32
15	M/8	T	1500	D,P	13	15	4	45
16	M/7	L	539	—	3	4	0	38

NE, not examined.

* G, *graminaceae*; P, *parietariae*; B, *betullae*; D, *dermadophagoides*.

each patient by one of the investigators (AL), to evaluate the overall severity of the disease. Clinical scores (0–4) for each ocular symptom (itching, tearing, photophobia, and foreign body sensation) and each sign (conjunctival erythema, mucous discharge, papillae, limbal infiltrates, and corneal epithelial disease) were assigned at the time of the visit. In particular, corneal disease was scored as follows: 1+, fine superficial epithelial defects; 2+, confluent epithelial defects; 3+, oval corneal ulcers; and 4+, oval corneal ulcers with plaque. Papillae were scored as follows: 1+, mild hyperemic scattered papillae; 2+, moderate hyperemic swollen papillae 1 mm size; 3+, at least two to three giant papillae more than 1 mm size; and 4+, giant papillae covering the superior tarsal plate.

Tear samples were collected from the outer canthus with a micro-capillary tube and centrifuged for 10 minutes at 1000 rpm to separate the cells from the tear fluid. Tear volume ranged from 30 to 200 μ L. Supernatants were stored at -80°C until assaying. Cell pellets were resuspended in 20 μ L aliquots, and the percentage of eosinophils, neutrophils, and lymphocytes present in five microscopic fields of 0.15 mm^2 were counted on precolored slides (Testsimplet; Roche Molecular Biochemicals, Mannheim, Germany) using a microscope at high-power (400 $\times$) magnification (Carl Zeiss Mediatech, Oberkochen, Germany).

In two of these young patients (AF, UT), the corneal plaque was surgically removed under general anesthesia. A written informed consent was obtained from the parents. A superficial keratectomy was performed, scraping off the plaque with a knife. Plaque debris were snap frozen with OCT in liquid nitrogen and maintained at -70°C for additional immunohistochemistry (IHC).

Measurement of MMP and TIMP-1 in Tears

Human pro-MMP-1, pro-MMP-9, and TIMP-1 were measured in duplicate using their respective enzyme-linked immunoassays (ELISA) kits (Chemicon International, Temecula, CA), according to the manufacturer's instructions. The sensitivity of the assays was as follows: pro-MMP-1, 0.11 ng/mL; pro-MMP-9, 3.1 ng/mL; and TIMP-1, 1.2 ng/mL.

MMP Activity Assay in Tears

MMP activity in culture medium was measured with the MMP-1 and -9 assay systems (Chemicon) according to the manufacturer's instructions. Tears were not activated. These colorimetric assays capture MMPs present in culture medium and measure their activity in cleaving a modified prodetection enzyme along with the subsequent cleavage of its chromogenic peptide substrate. Relative gelatinase activity in tear samples was obtained by measuring optical density (OD) at 450 nm on

a microplate reader. Data are expressed as relative activity compared with standard MMP activity (OD units).

Immunohistochemistry

Serial 5- μ m-thick cryosections were cut on an ultramicrotome, mounted on gelatin-covered slides, fixed in acetone, and processed for IHC. The following anti-human monoclonal antibodies (mAbs) were used: anti-fibronectin 1:100 (Sigma-Aldrich, St. Louis, MO), anti-MMP-9 1:100 (Chemicon) which recognizes both latent and activated forms, and anti-eosinophil cationic protein (anti-ECP) 1:100 (clone EG2; Pharmacia & Upjohn).

Briefly, for all mAb, unspecific binding was blocked by the addition of serum from the same animal species. The slides were washed twice in Tris-buffered saline (TBS: 10 mM Tris and 150 mM NaCl [pH 7.4]) and the appropriately diluted anti-human antibodies were placed on each tissue section and incubated in a moist chamber for 60 minutes. After the primary incubation, slides were washed in TBS, incubated for 30 minutes with the secondary antibodies, and treated with an alkaline phosphatase complex (APAAP; Dako, Glostrup, Denmark). The reaction was developed with fast red solution and counterstained with Mayer hematoxylin.

Two corneas from the Veneto Eye Bank were used as the control. Negative control specimens were incubated without the primary antibody. Tissue sections of placenta for fibronectin and sections of lymphoid tissue for cytokines and inflammatory cell markers were the positive control specimens.

The positive red reaction was analyzed in the VKC and normal specimens. It was classified as very intense (+++), intense (++), slight (+), or absent (–). A 10 $\times$ 10-mm grid was used with a microscope (Carl Zeiss Mediatech) for each assessment, and all quantifications were performed in a masked fashion.

Statistics

Differences between MMP and TIMP levels in VKC and normal subjects were analyzed with the Mann-Whitney test. Results are given as the mean $\pm$ standard deviation (SD). The Spearman rank correlation was also calculated to identify relationships between parameters. Statistical significance was set at $P \leq 0.05$.

RESULTS

Patients

The mean ages of patients with VKC and normal control subjects were similar. Of the 16 patients with VKC, 9 had the

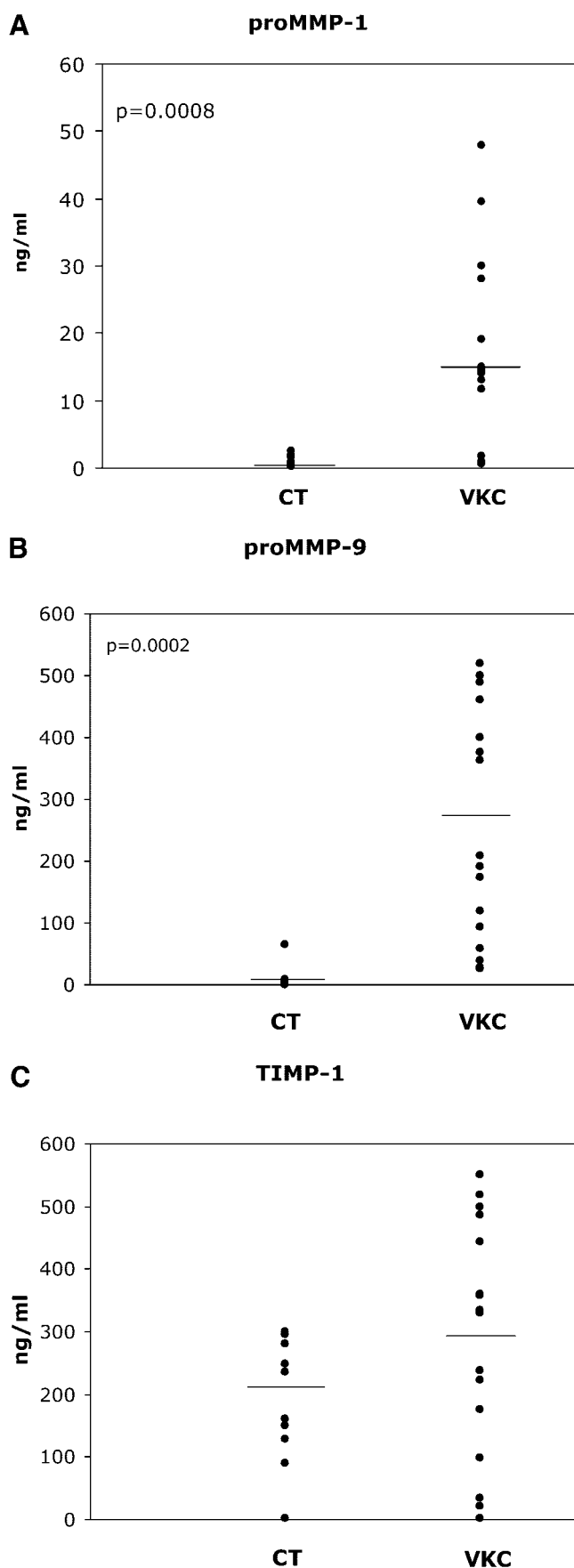


FIGURE 1. Concentrations of pro-MMP-1, pro-MMP-9 and TIMP-1 in tears of patients with VKC and normal subjects (CT).

tarsal form of the disease and 7 the limbal form. Five of the patients with tarsal disease had corneal complications: two with plaques, one with an ulcer with a translucent base, and two with only a transparent base. Three other patients had fine epithelial keratopathy. Table 1 summarizes the demographic and clinical data of patients with VKC. Tarsal VKC had a significantly higher mean clinical score of signs and symptoms compared with that of limbal disease ($P < 0.01$).

Tear cytology of normal subjects showed only a few neutrophils in two samples. In all VKC samples, tear cytology showed numerous leukocytes with a prevalence of eosinophils. Percentages of eosinophils ($48.7\% \pm 11\%$), neutrophils ($26.6\% \pm 13\%$), and mononuclear cells ($25\% \pm 10\%$) in VKC tear samples were significantly increased, compared with those of control samples (0%, $P < 0.0001$; $20\% \pm 42\%$, $P < 0.01$; $10\% \pm 31\%$, $P < 0.001$, respectively).

MMP-1 and -9 and TIMP-1 in Tears

In tears of normal subjects, pro-MMP-1 was very low or absent (mean concentration, 0.5 ± 0.2 ng/mL), whereas in tears of patients with VKC, it was significantly increased (16.6 ± 13.8 ng/mL, $P = 0.0008$; Fig. 1A). Concentrations were similar in tarsal and limbal VKC.

Pro-MMP-9 levels (Fig. 1B) were also significantly increased in tears of patients with VKC (253 ± 186 ng/mL) compared with those of normal subjects (10.5 ± 0.2 ng/mL; $P = 0.0002$), but were similar in tarsal and limbal VKC.

TIMP-1 levels in tears were increased in patients with VKC (291 ± 185 ng/mL); however, this difference was not statistically significant compared with normal subjects (217 ± 80 ng/mL; Fig. 1C).

The molar ratios of pro-MMP-1/TIMP-1 and pro-MMP-9/TIMP-1 in normal subjects were very low (0.001 ± 0.0002 and 0.01 ± 0.024 , respectively) with a predominance of inhibitor. Both of these ratios were significantly increased in VKC tear samples compared with the control samples (0.41 ± 0.048 , $P < 0.0001$; 1.1 ± 3.1 , $P < 0.002$, respectively; Fig. 2).

MMP-1 and -9 Activity

To integrate the ELISA results, quantitative MMP-1 and -9 activity were assessed in tear fluid. Mean tear MMP-1 activity in patients with VKC ($n = 12$; 76.85 ± 51 OD units) was significantly higher than in normal subjects ($n = 7$; 5.4 ± 12 OD units; $P = 0.0007$) and similar in tarsal or limbal disease.

Mean MMP-9 activity was significantly increased in patients with VKC ($n = 14$; 0.954 ± 1.1 OD units) compared with normal subjects ($n = 8$; 0.03 ± 0.05 OD units; $P = 0.0004$). The difference between MMP-9 activity in tarsal and limbal VKC approached statistical significance (1.47 ± 1.36 OD units vs. 0.26 ± 0.16 OD units; $P = 0.052$).

Correlation between MMP and Clinical Parameters

In patients with VKC, pro-MMP-1 levels correlated significantly with the sum score of signs and symptoms ($\rho = 0.53$; $P = 0.04$), but not with corneal and giant papillae scores or tear cell counts.

Pro-MMP-9 tear levels were significantly correlated with eosinophil count ($\rho = 0.65$; $P < 0.02$; Fig. 3A) and with the overall subjective clinical score of the disease ($\rho = 0.36$; $P < 0.03$; Fig. 3B), but not with the total score of symptoms and signs, corneal involvement, or the giant papillae score.

TIMP-1 levels in tears and pro-MMP/TIMP ratios did not correlate with clinical parameters or cell count.

As expected, MMP-1 activity correlated significantly with the pro-MMP-1/TIMP-1 molar ratio ($\rho = 0.62$; $P = 0.039$). Quantitative MMP-9 activity in tears correlated significantly

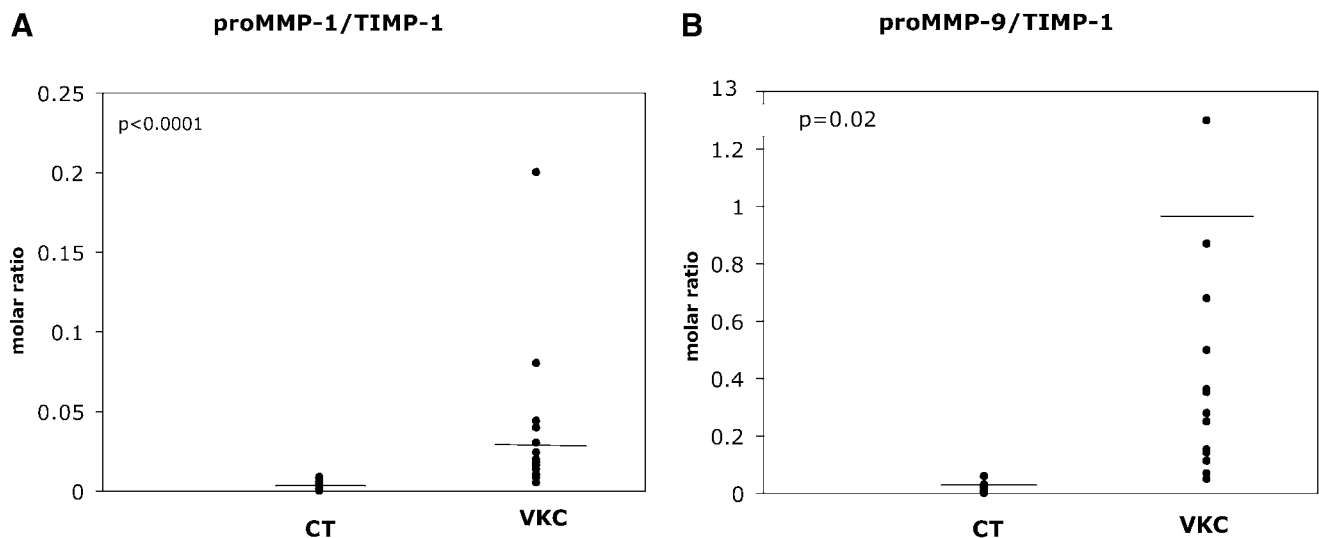


FIGURE 2. Molar ratios of pro-MMP-1/TIMP-1 and pro-MMP-9/TIMP-1 in tears of patients with VKC and normal subjects (CT).

with the levels of pro-MMP-9 in tears ($\rho = 0.54$; $P = 0.011$). In patients with VKC, MMP-9 activity correlated with corneal involvement ($\rho = 0.815$; $P = 0.003$; Fig. 3C), giant papillae clinical score ($\rho = 0.58$; $P = 0.037$), and the sum score of symptoms ($\rho = 0.55$; $P = 0.05$).

Immunohistochemistry

Only a few sections were generated from the small amount of the corneal tissue available. Hematoxylin staining showed no inflammatory cells in the corneal stroma. Corneal epithelial cells were absent. The superficial corneal stroma was covered with uniformly deposited fibronectin (+++) (Fig. 4A). The superficial stroma also stained positively (++) for MMP-9 (Fig. 4B) and slightly (+) for the eosinophil product, ECP (Fig. 4C), compared with negative controls. Normal corneas were negative for fibronectin, MMP-9 (Fig. 4D) and ECP on both the epithelium and stroma.

DISCUSSION

It is well known that chronic allergic inflammation of the eye can cause functional alterations due to corneal complications, changes of the conjunctival and lid margin anatomy, and tear film quality and function. Corneal complications include superficial keratopathy and corneal ulcers of varied severity often leading to corneal scarring. Structural changes of the conjunctiva in VKC include epithelial and basal membrane thickening, mucus metaplasia, and subepithelial fibrosis. Quantitative and qualitative changes of the conjunctival ECM have been reported¹⁰ to result in the formation of giant papillae, diffuse fibrosis, and scars. Collagen deposition consists mostly of type I and III collagens,¹² localized under the epithelium, forming either a dense layer rich in fibrillar collagen or fibrovascular structures that sustain the giant papillae. These remodeling changes suggest that an altered homeostasis of the ECM leads to increased collagen deposition. Conjunctival and corneal remodeling are accompanied by degradation of ECM in addition to synthesis and deposition of new matrix. The results of the present study corroborate this, because not only were actual MMP-1 and -9 tear levels found to be increased in patients with VKC compared with those in normal subjects, but also their activity was significantly increased in tears of patients with active VKC. In comparison with normal subjects, and considering the possible presence of other enzymes, con-

centrations of the MMPs exceeded those of their inhibitor TIMP-1. The altered balance between MMPs and their inhibitor in active inflammatory VKC suggest that degradation of ECM is necessary before or together with collagen deposition. It is also notable that MMP-9 activity was found to be higher in tarsal versus limbal VKC, where the remodeling process is decidedly more evident.

The higher levels of MMP-9 versus -1 suggest that gelatinase B may be involved in more than just tissue remodeling. MMP-9 activity correlated significantly with multiple clinical parameters: corneal involvement, giant papillae formation, signs, and symptoms.

Corneal complications are presumed to be the effect of epitheliotoxins released by activated eosinophils.¹³ These latter cells are captured by epithelial cell-expressed adhesion molecules¹⁴ and are recalled by chemokines such as eotaxin.^{15,16} Eosinophils and one of their products, major basic protein, have been localized in corneal ulcers of two patients with VKC.¹⁷ However, in the present study, no inflammatory cells were found in the superficial corneal stroma of the ulcer base, which was covered by fibronectin. It is interesting that the corneal stroma stained positively for both MMP-9 and the eosinophil activation product ECP, suggesting that activated eosinophils, slightly adherent to the corneal surface, may be responsible for the corneal damage. Furthermore, pro-MMP-9 tear levels correlated with eosinophil count, and MMP-9 activity, but not pro-MMP-9 levels, correlated with the severity of corneal involvement. MMP-9 is the major known proteinase expressed by eosinophils,^{7,18,19} and an increased immunostaining for gelatinase B has been recently identified in eosinophils of patients with VKC.²⁰ These findings indicate that eosinophils may be the origin of the MMP-9 levels found in the present study. It could be speculated that activated MMP-9 is involved in the pathogenesis of corneal damage. Clinical corneal complications in all their phases and degrees of severity are probably the result of both the initial toxic damage and the remodeling process of the epithelium, the Bowman's membrane, and the corneal stroma. Corneal ulcers in VKC rarely reach the deep stroma or cause perforation, probably because of coexpression of inhibitory factors such as TIMP-1. The failure to re-epithelialize corneal shield ulcers or plaques may also be related to overexpression of MMPs by resident corneal cells, as described in a model of corneal chemical injury.²¹

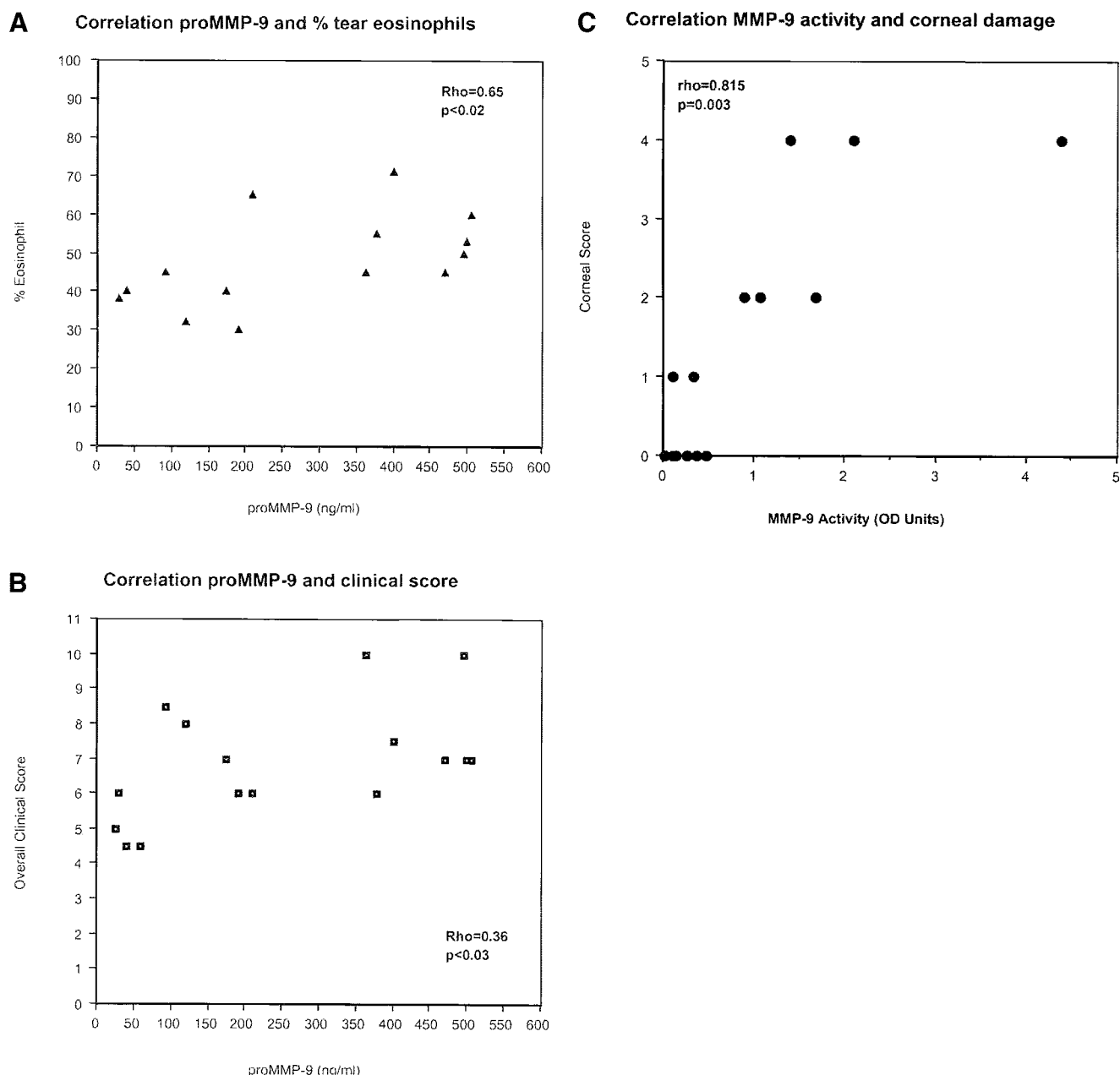


FIGURE 3. Correlation between tear pro-MMP-9 levels and the percent of tear eosinophils (A), pro-MMP-9 tear levels and total score for disease severity (0 to 10) (B), and MMP-9 activity and corneal clinical score (C), by the Spearman rank correlation test.

In VKC and other allergic diseases, the number of circulating eosinophils and their markers for activation are increased in serum and tears.^{22,23} The passage of eosinophils from the bone marrow to the blood vessels and from the blood vessels to the tissues involves many steps, including eosinopoiesis, chemokinesis, chemotaxis, expression and activation of eosinophil and endothelial adhesion molecules, and transmigration through the endothelial wall.²⁴ MMP-9 is implicated in some of these processes. In fact, lymphocytes, neutrophils, and eosinophils use MMP-9 for their basement membrane transmigration.⁶ In allergic eye diseases, eosinophils are frequently found in the epithelium and tears, suggesting that similar steps are involved in eosinophil transmigration through the stroma, basal membrane, and epithelial cells. The recently reported increased levels of eotaxin-1²⁵ and eotaxin-2 in tears²⁶ and increased expression of CC chemokines and their receptors in

conjunctival tissues^{27,28} are findings possibly involved in this process. Eotaxin may also promote eosinophil transmigration through the activation of the plasminogen-plasmin system.²⁹ Eosinophils may use two sources of MMP-9⁶: preformed, which is rapidly released on cellular activation, and de novo synthesized, which is implicated in chronic inflammation. Thus, the presently reported increase in MMP levels and activity may be critical for inflammatory cell transmigration through the conjunctiva.

Activation of pro-MMP-9 is achieved by clipping the amino terminal containing a critical cysteine residue responsible for latency by a mechanism known as the "cysteine switch."³⁰ Stromelysin-1 (MMP-3) and gelatinase A (MMP-2) may catalyze the conversion of pro-MMP-9 into the active form,^{31,32} thus joining in the cascade of plasmin and plasminogen activators.³³ Increased concentrations and activity of corneal epithelial cell-

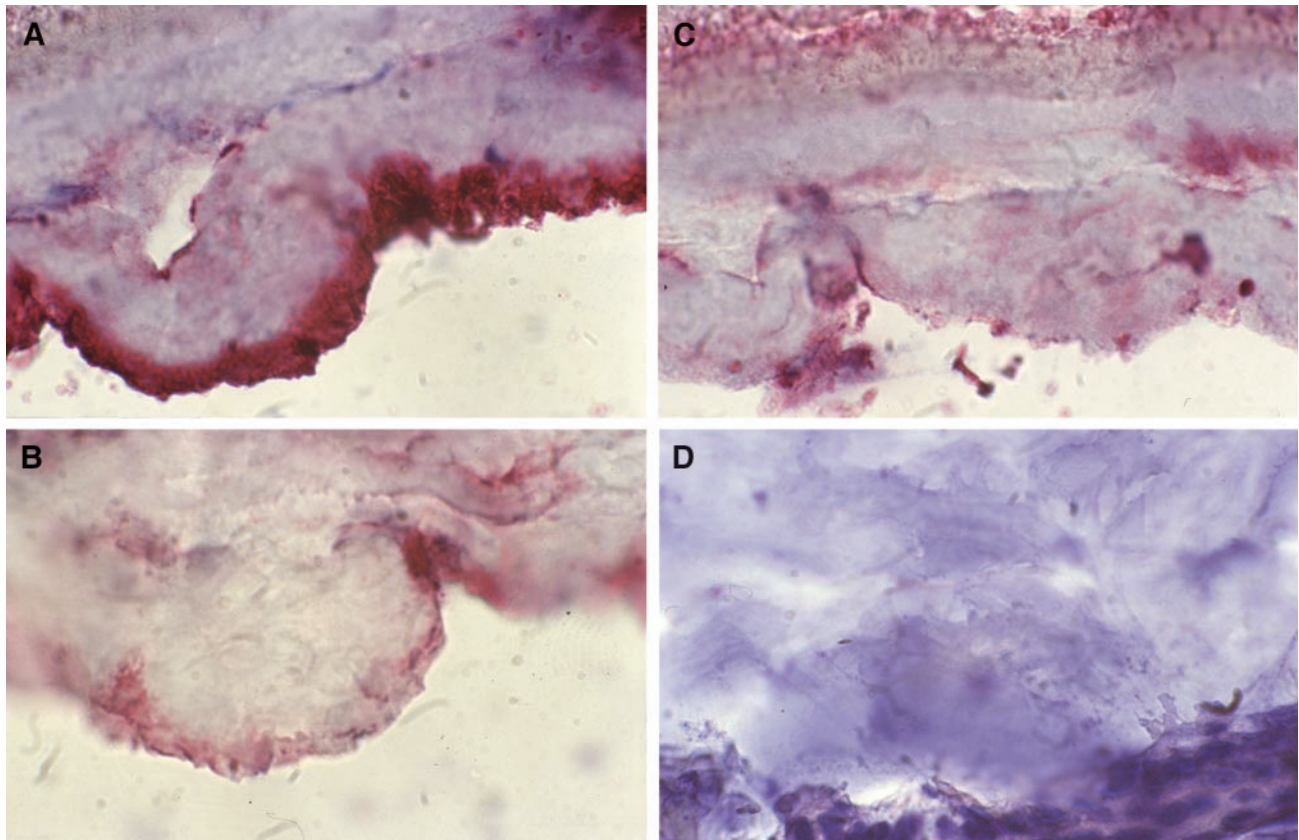


FIGURE 4. IHC of the superficial corneal stroma from a VKC corneal ulcer. (A) Positive staining for fibronectin on the superficial stroma, (B) positive staining for ECP, (C) positive staining for MMP-9, (D) negative staining for MMP-9 in normal cornea. Original magnification, $\times 100$.

derived MMP-9 and TIMP-1 were found in tears of patients with ocular rosacea.^{34,35} In this last study, MMP-3 alone was sufficient to activate MMP-9 on the ocular surface. Future studies are planned to determine whether similar mechanisms are involved in the reaction that characterizes VKC.

Active MMP-9 catalyzes the posttranslational activation of interleukin (IL)-1 β ,³⁶ and tumor necrosis factor (TNF)- α ,³⁷ potentiates IL-8,²⁹ processes chemokines,^{38,39} and degrades serine protease inhibitors such as α -1 antitrypsin.⁴⁰ In addition to the Th2-associated cytokines, IL-1 and TNF α ,¹¹ several chemokines^{25–27} have been reported to be increased in VKC. The serine proteases, tryptase, and chymase, are typically stored and released by activated mast cells during allergic reactions,^{41,42} It may then be speculated that MMPs have proinflammatory as well as proteolytic activities on specific ECM substrates.

Increased tear levels of MMP-1 may also be related to mast cell activation. In fact, the expression of collagenase-1 by human mast cells in both inflamed and normal tissues, and by a human mast cell line has been reported recently, suggesting a critical role for MMP-1 in cell invasion and migration into sites of inflammation.⁴³

MMP enzymes are inactivated by specific inhibitors, such as TIMP-1, or by nonspecific inhibitors, such as α 2-macroglobulin. In these VKC tear samples, TIMP-1 levels were not as increased as those of pro-MMPs, resulting in an imbalance between protease and inhibitor and, consequently, a significant increase in the MMP/TIMP ratio. That MMP activity was much greater in tears of patients with VKC than in those of control subjects further confirms that MMPs are increasingly released, activated, and not fully inactivated in this disease.

In conclusion, greater levels and activity of MMP correlated with clinical findings in patients with VKC, suggesting that

proteases are involved in allergic inflammation. An imbalance between MMPs and their inhibitors may facilitate inflammatory cell transmigration, tissue remodeling, and corneal damage in this disease.

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