Gelatinase B in Vernal Keratoconjunctivitis
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Objectives: To investigate the expression of gelatinase B in the conjunctiva of patients with vernal keratoconjunctivitis (VKC) and the cellular source of this enzyme.

Methods: Conjunctival biopsy specimens from 12 patients with active VKC and 12 control subjects were studied using immunohistochemical techniques and a monoclonal antibody against gelatinase B. The phenotype of gelatinase B+ inflammatory cells was examined using double immunohistochemical analysis and monoclonal antibodies against eosinophil peroxidase or macrophage CD68. Quantitative zymography was used to compare the activity of gelatinase B in conjunctival biopsy specimens from 10 patients with active VKC and 7 control subjects.

Results: Gelatinase B was detected in a few polymorphonuclear cells in 8 control specimens. All VKC specimens showed gelatinase B immunoreactivity in the epithelial and stromal inflammatory infiltrate. Compared with control specimens, VKC specimens showed significantly more gelatinase B–positive cells (mean±SD, 40.8±29.9 vs 10.3±2.4; *P*<.02). Most gelatinase B–positive cells were eosinophils (90.2%±3.6%). Zymography revealed that gelatinase B levels in VKC specimens were significantly higher than the levels found in normal conjunctiva (3780.3±3541.0 vs 610.1±397.1 scanning units; *P*<.03).

Conclusions: These findings suggest overexpression of gelatinase B by eosinophils in VKC specimens and participation of gelatinase B in the pathologic changes in VKC.

Clinical Relevance: Control of the release and/or activation of gelatinase B in eosinophils may provide a new therapeutic strategy for treating VKC.


VERNAL keratoconjunctivitis (VKC) is an allergic chronic seasonally exacerbated bilateral external ocular inflammation that affects children and young adults, with a predominance in males. The disease is characterized by recurrent symptoms of severe itching, photophobia, lacrimation, and discharge. There are 3 forms of the disease: palpebral, limbal, and mixed. The classic sign of palpebral VKC is the giant papillae or cobblestones in the upper tarsal conjunctiva. The limbal form is characterized by gelatinous infiltrates of the limbus. Corneal findings are common and include punctate epithelial keratitis, epithelial erosions, corneal ulcers, and plaque formation.1,2 The typical histological features of VKC are the conjunctival infiltration by eosinophils, basophils, mast cells, B lymphocytes, plasma cells, CD4+ T lymphocytes expressing T-helper 2 (TH2)–type cytokines, and monocytes/macrophages, extracellular matrix hyperplasia, and remodeling caused by increased collagen deposition.3,9 The matrix metalloproteinases are recognized as key enzymes for normal extracellular matrix turnover and for the exaggerated extracellular matrix breakdown associated with pathologic conditions, including tumor cell invasion and metastasis, angiogenesis, inflammatory reactions, wound healing, and scar formation.10,11 The major members of this family include the following: collagenases, which degrade and denature fibrillar collagen types I, II, and III; gelatinases A and B (respectively, the 65-kd to 75-kd matrix metalloproteinase-2 and the 85-kd to 96-kd matrix metalloproteinase-9), which cleave denatured collagens (gelatins), collagen types IV, V, VII, and X, elastin, and fibronectin; and stromelysins, which degrade proteoglycans, laminin, fibronec- tin, type IV collagen, and the globular domains of other extracellular matrix macromolecules.12 More recently, membrane-type matrix metalloproteinase expressed on cell membranes is identified as a fourth category.13 Because of its unique and broad substrate specificity and its involvement in

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PATIENTS AND METHODS

IMMUNOHISTOCHEMICAL ANALYSIS

Twelve patients with active VKC seen at the outpatient clinic of King Abdulaziz University Hospital, Riyadh, Saudi Arabia, were included in the study. All the patients were males aged 7 to 17 years, with a mean age of 12 years. The symptoms mentioned by all the patients were itching, redness, photophobia, and tearing. Each patient underwent complete ophthalmic examination, and the corneal and conjunctival changes were noted and recorded. All patients had the limbal form of the disease characterized by broad gelatinous infiltrates of the limbus. Nasal or temporal limbal conjunctival biopsy specimens were obtained from each patient. None of the patients was receiving topical or systemic therapy before obtaining the biopsy. In addition, 12 limbal conjunctival biopsy specimens were obtained from the same areas from patients undergoing cataract extraction or strabismus surgery without obvious inflammation and served as controls. The controls were from the same age group. This study was approved by the Research Center, College of Medicine, King Saud University (Riyadh, Saudi Arabia), and the patients admitted to the study gave their informed consent.

The conjunctival biopsy specimens were immediately snap-frozen in optimum cutting temperature compound (Tissue-Tek; Miles Laboratories, Elk hart, Ind) and maintained at −80°C until use. For immunohistochemical analysis, 5-µm serially cut cryostat sections were dried overnight at room temperature, fixed in absolute acetone for 10 minutes, and stained with a 3-step avidin/biotin peroxidase–labeled complex procedure. Rehydrated slides were incubated for 30 minutes with gelatinase B–specific REGA-2D9 monoclonal antibody (1:30). The mouse monoclonal antibody REGA-2D9 was raised against natural human neutrophil gelatinase B. This implies that the antigen preparation was devoid of gelatinase A. The REGA-2D9 is an immunoglobulin (Ig)G1 subtype with a dissociation constant (Kd) value of 9.5 × 10⁻⁹ M, which implies extremely high specificity. This monoclonal was compared with other antibody preparations and found to be superior for immunohistochemical analysis. The secondary and tertiary reagents consisted of biotin-conjugated rabbit antimouse immunoglobulin and the avidin/biotin peroxidase–labeled complex, respectively (Dakopatts A/S,Copenhagen, Denmark). All incubations were carried out for 30 minutes at room temperature, then washed in 3 changes of phosphate-buffered isotonic sodium chloride solution at pH 7.2 for 15 minutes. The reaction product was visualized by incubation for 10 minutes in 0.05M acetate buffer at pH 4.9, containing 0.03% 3-amino-9-ethyl-carbazole (Sigma-Aldrich, Bornem, Belgium) and 0.01% hydrogen peroxide, resulting in bright red immunoreactive sites. The slides were faintly counterstained with Harris hematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol. Control slides were treated in an identical manner, except that an irrelevant IgG mouse monoclonal antibody was used in the first step, or the primary antibody was omitted.

Double Immunohistochemical Analysis

To examine the phenotype of gelatinase B–expressing inflammatory cells, cryostat sections were studied by double immunohistochemical analysis. Colocalization studies were performed in 4 VKC specimens, using mouse antihuman phenotype monoclonal antibodies CD68 (1:1000, macrophages) (Dakopatts A/S) or eosinophil peroxidase (Ab-1) (1:1000, eosinophils) (Oncogene Research Products, Cambridge, Mass) together with REGA-2D9 monoclonal antibody. After rinsing the slides with phosphate-buffered isotonic sodium chloride solution, they were incubated for 30 minutes with REGA-2D9 monoclonal antibody and rinsed again with phosphate-buffered isotonic sodium chloride solution. Subsequently, the sections were incubated for 30 minutes with peroxidase (EnVision+ system, mouse; DAKO Corporation, Carpinteria, Calif) and washed again with phosphate-buffered isotonic sodium chloride solution, and the reaction product was visualized by incubation for 10 minutes in 0.05 M acetate buffer at pH 4.9, containing 0.03% 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.01% hydrogen peroxide, resulting in red immunoreactive staining. Afterward, the sections were rinsed in phosphate-buffered isotonic sodium chloride solution, washed with distilled water, and incubated for 30 minutes with the secondary monoclonal antibody to determine cellular phenotype (CD68 or eosinophil peroxidase). After a wash with phosphate-buffered isotonic sodium chloride solution, the few polymorphonuclear cells located in the vascular lumens and in the perivascular areas in 8 of 12 specimens (Figure 1). All VKC specimens showed immunoreactivity with the monoclonal antibody against gelatinase B in the epithelial and stromal inflammatory infiltrate (Figure 2). In the stroma most of the positively stained cells were located just beneath the epithelium. The numbers of gelatinase B–positive cells in VKC specimens were significantly greater than the numbers found in control specimens (40.8 ± 29.9 vs 10.3 ± 2.4; P < .02).

Double immunohistochemical analysis to confirm the phenotype of gelatinase B–positive inflammatory cells showed that most inflammatory cells expressing gelatinase B were eosinophils (90.2% ± 3.6%, n=4) (Figure 3).

RESULTS

IMMUNOLOCALIZATION OF GELATINASE B

There was no staining in the negative control slides. In normal conjunctiva, gelatinase B was detected only in a

other chronic inflammatory diseases,¹⁴ we hypothesized that excessive expression of gelatinase B may play a role in extracellular matrix remodeling in VKC. To evaluate this hypothesis, we examined conjunctival specimens obtained from patients with VKC using immunohistochemical analysis and gelatin zymography. The findings in VKC were compared with the findings in the conjunctiva from normal individuals.

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sections were incubated for 30 minutes with a biotin-labeled rabbit antimonials antibody, followed by a monoclonal anti–biotin-alkaline phosphatase conjugate (Sigma-Aldrich). The blue reaction product was developed using 4-benzoylamino-2,5-diethoxybenzene-diazonium chloride (Fast Blue BB salt; Sigma-Aldrich) for 5 minutes.

**Quantitation**

Cells were counted in 5 representative microscopic fields. Counting was performed by 2 independent observers (A.M.A. and K.G.). One of them (K.G.) was unaware of the origin of the specimens. In case of disagreement, the results obtained by the blinded observer were used. We used an eye piece calibrated grid (original magnification ×25). With this magnification and calibration, we counted the cells present in an area of 0.155 × 0.155 mm. For the colocalization studies, cells expressing both gelatinase B and eosinophil peroxidase or CD68 were counted and expressed as a percentage of cells expressing gelatinase B.

**ZYMOGRAPHY**

Ten male patients aged 7 to 15 years (mean age, 9.5 years) with severe active VKC were included in the study. All patients had the palpebral form of the disease characterized by the presence of giant polygonal flat-topped cobblestone papillae affecting the upper palpebral conjunctiva. Upper palpebral conjunctival biopsy specimens were obtained from each patient after obtaining informed consent. None of the patients was receiving topical or systemic therapy before obtaining the biopsy. In addition, 7 upper palpebral conjunctival biopsy specimens were obtained from patients who served as controls and were in a similar age group undergoing strabismus surgery without obvious inflammation.

The conjunctival biopsy specimens were immediately snap-frozen in optimum cutting temperature compound (Tissue-Tek; Miles Laboratories) and maintained at −80°C until use. For gelatin zymography, frozen tissues were thawed and transferred into 50 µL of phosphate-buffered isotonic sodium chloride solution at a pH of 7.4, supplemented with 1% Triton x-100 (Sigma-Aldrich). This preparation was sonicated at 0°C for 3 minutes, and protein concentrations were determined on a fraction. For each specimen an equivalent amount of 100 µg protein was analyzed as described by Masure et al. Phosphorylase b (97 kd) was used as a standard protein. The localization of gelatinolytic enzymes and their molecular masses were derived by including protein-size standards on each gel and on the basis of the known migration of gelatinase B variants (monomer, dimer, and heterodimer with neutrophil gelatinase B–associated lipocalin) that were purified by Masure et al. Briefly, samples were examined by electrophoresis in 7.5% polyacrylamide gels that had been copolymerized with 0.1% (weight/volume [wt/vol]) gelatin (Sigma-Aldrich). Stacking gels contained 5% polyacrylamide. Electrophoresis was for 4°C for 16 hours at 90 V. The gels were then incubated in washing buffer (50 mM Tris-hydrochloride [Sigma-Aldrich], pH 7.5, 10 mM calcium chloride, 0.02% [w/v] sodium azide, 2.5% [v/v] Triton x-100) at room temperature, twice for 20 minutes, to remove sodium dodecyl sulfate and overnight in developing buffer (50 mM Tris-hydrochloride, pH 7.5, 10 mM calcium chloride, 0.02% [w/vol] sodium azide, 1% [vol/vol] Triton x-100) at 37°C. The gels were then stained in Coomassie brilliant blue R-250 and destained in methanol/acetic acid. The sites of gelatinase activity appeared as unstained bands on a blue background and were quantified by densitometry and zymolytic activities that were expressed as arbitrary laboratory scanning units. Densitometry was with a densitometric scanner (PDI, New York, NY), and the raw data were processed with Pharmacia Biotech software programs (LabScan Utility [version 2.00] and Image Master ID [version 2.0]; Pharmacia Biotech, Uppsala, Sweden). The linear range was between 200 and 7000 scanning units. Quantitation of gelatinase activity by zymography proved to be a sensitive nonisotopic detection method with a sensitivity in the picogram range.

**STATISTICAL ANALYSIS**

All data are presented as mean±SD. The Mann-Whitney U test was used to compare the mean numbers of gelatinase B–expressing inflammatory cells in VKC patients with controls. The t test was used to compare the mean gelatinase A and B levels in VKC patients vs controls. Logarithm transformation was used to reduce variances before applying the t test. The differences were considered significant at P<.05.

Gelatinase B values in VKC specimens were significantly higher than the values found in control specimens (control specimens, 610.1±397.1 scanning units; VKC specimens, 3780.3±3541.0 scanning units; P<.03).

Zymography and immunohistochemical analysis indicated increased activity and expression of gelatinase B in the conjunctiva from patients with VKC compared with control subjects. The up-regulation of gelatinase B in this study is consistent with previous studies that documented the increased expression of gelatinase B messenger RNA and protein in bronchial biopsy specimens from patients with severe asthma. The expression of gelatinase B was significantly higher in patients with severe active VKC than in control subjects (control subjects, 410.3±287.1 scanning units; VKC subjects, 2450.0±2192.3 scanning units; P=.03).

**COMMENT**

Smaller numbers of inflammatory cells expressing gelatinase B were monocytes/macrophages (10.9%±5.0%, n = 4) (Figure 4).

**ZYMOGRAPHY**

Constitutive gelatinase A was detected in 6 of 7 control specimens and in 6 of 10 VKC specimens (Figure 5). Gelatinase A values in VKC specimens were higher than the values found in control specimens, but the difference between the 2 groups was not statistically significant (control specimens, 210.5±114.1 scanning units; VKC specimens, 663.3±484.2 scanning units; P=.08).

Inducible gelatinase B was detected in all control specimens and in 8 of 10 VKC specimens (Figure 5).

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subjects with asthma.\textsuperscript{19,20} Furthermore, gelatinase B levels were increased in the bronchoalveolar lavage fluids\textsuperscript{21-23} and in the sputum\textsuperscript{24} of patients with asthma. The numbers of eosinophils were correlated with the degree of expression of gelatinase B in bronchial biopsy specimens,\textsuperscript{20} and gelatinase B activity strongly correlated with the numbers of eosinophils and neutrophils recovered in the bronchoalveolar lavage fluid.\textsuperscript{22} In the present study, gelatinase A levels did not vary significantly among conjunctival biopsy specimens from control subjects and those from subjects with VKC. Similarly, levels of gelatinase A levels were low or undetectable in bronchoalveolar lavage fluids from patients with asthma.\textsuperscript{23} In general, these observations agree with those of Paemen et al\textsuperscript{25} for constitutive gelatinase A distribution in cerebrospinal fluid of patients with a variety of short- and long-term inflammatory nervous system disorders. Gelatinase A was expressed equally in the cerebrospinal fluid of patients and control subjects. Human gelatinase A occurs constitutively in body fluids (serum, synovial fluid, cerebrospinal fluid) and cell culture supernatants.\textsuperscript{25} It is suggested that gelatinase A seems to be involved in some basal extracellular matrix turnover events, whereas gelatinase B seems to be involved in more acutely regulated events.\textsuperscript{26} In normal human and mouse macrophages and human macrophage cell lines, gelatinase A is constitutively produced in small but detectable quantities, and this production remains at the same level after macrophage stimulation. In contrast, unstimulated macrophages do not produce gelatinase B but secrete large amounts of this enzyme after appropriate stimulation by cytokines and endotoxin, for example.\textsuperscript{12,14,18,27}

Double immunohistochemical analysis showed that gelatinase B immunoreactivity was predominantly associated with eosinophils present in the inflammatory infiltrate in VKC specimens. Our observations are consistent with a previous report that most cells expressing gelatinase B messenger RNA in bronchial biopsy specimens from subjects with asthma were eosinophils.\textsuperscript{19} Previous studies indicate that circulating eosinophils\textsuperscript{28} and eosinophils in normal bronchial tissues\textsuperscript{29} do not express gelatinase B messenger RNA. Therefore, it is likely that eosinophils are triggered to synthesize gelatinase B in the conjunctiva from patients with VKC. Factors regulating the expression of gelatinase B by eosinophils in VKC are incompletely understood, however, certain cytokines may be involved. The TH2-derived cytokines interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor prolong eosinophil survival as well as activate these cells.\textsuperscript{29-32} Recently, Okada et al\textsuperscript{33} showed that IL-5, platelet-activating factor, or both increased release of gelatinase B by eosinophils in vitro. There is strong evidence that these TH2-type cytokines are centrally involved in the pathogenesis of VKC\textsuperscript{34} and other allergic

\textbf{Figure 1.} Immunohistochemical staining of conjunctiva from a normal control subject showing immunoreactivity in few polymorphonuclear cells (arrow) (gelatinase B, original magnification $\times$500).

\textbf{Figure 2.} Vernal keratoconjunctivitis. Immunohistochemical staining showing immunoreactivity in the epithelial (arrows) and stromal (arrowheads) inflammatory infiltrate (gelatinase B, original magnification $\times$300).
diseases.\textsuperscript{35,36} In addition, CC chemokines have been recognized to play an important role in eosinophil recruitment and activation.\textsuperscript{37} Recently, we have shown increased expression of the CC chemokines RANTES (regulated on activation, normal T cell expressed and secreted), eotaxin, and monocyte chemotactic protein-3 in the conjunctiva from patients with VKC.\textsuperscript{38}

The recruitment of eosinophils from the circulation to the site of inflammation is regarded as a key event in the development and maintenance of allergic inflammation. The molecular events involved in the infiltration of eosinophils through endothelium and the epithelium have been investigated intensively in vivo and in vitro systems, showing the involvement of adhesion molecules,\textsuperscript{39} cytokines,\textsuperscript{40} and chemokines.\textsuperscript{35,37} Since one of the main components of endothelial and epithelial basement membrane is type IV collagen, which is specifically cleaved by gelatinase B,\textsuperscript{41,42} eosinophil gelatinase B might permit the extravasation of eosinophils through the basement membrane zone underlying endothelial and epithelial cell layers. Recently, gelatinase B was reported in an in vitro system to play a crucial role in the transmigration of eosinophils through basement membrane components.\textsuperscript{31} Furthermore, Kumagai et al\textsuperscript{43} demonstrated that inhibitors of matrix metalloproteinases prevent the cellular infiltration and the induction of airway hyperresponsiveness in a murine model of allergic asthma. Increased expression of gelatinase B in VKC could also render basement membranes vulnerable and increase vascular permeability, facilitating conjunctival edema and the transmigration of inflammatory cells through the basement membrane. Indeed, the conjunctiva from patients with VKC is characterized by remarkable inflammation from the aspects of cellular infiltration and the expression of adhesion molecules, as indicated by our previous studies.\textsuperscript{3-5}

Eosinophils are now recognized to play a central role in the pathophysiologic characteristics of VKC. Strong basic cytotoxic proteins such as major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin are released from eosinophils and damage the conjunctival and corneal epithelium.\textsuperscript{44,45} More recently, eosinophils were recognized as a source of proinflammatory cytokines, which may act to perpetuate the local immune response.\textsuperscript{46} In addition, gelatinase B released from eosinophils may contribute to progressive breakdown of conjunctiva in VKC. Gelatinase B degrades denatured collagens (gelatin), collagen types IV, V, VII, and X, elastin, and fibronectin.\textsuperscript{41,42} This tissue destruction may be followed by remodeling of the conjunctiva with increased deposition of collagens. In a previous immunohistochemical study,
we demonstrated new collagen type V formation, increased deposition of basement membrane collagen IV, and fibril-forming interstitial collagen types I and III in the conjunctiva from patients with VKC. 9

In conclusion, our findings demonstrate a significant increased expression of gelatinase B in the conjunctiva from patients with VKC, suggesting its implication in inflammatory processes in VKC. The major cellular sources were eosinophils. Control of the release and/or activation of gelatinase B in eosinophils may be a new therapeutic strategy for VKC.

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Figure 5. A, Gelatin zymography of conjunctival biopsy specimens from normal control subjects and from patients with active vernal keratoconjunctivitis (VKC). The zymographies of these samples show the presence of both gelatinase A (MMP-2) and gelatinase B (MMP-9). The standard protein (ST) shows a 97 kd band. (The figure is a montage of several gels.) B, Gelatin zymographies (3 gels) of conjunctival biopsy specimens from normal control subjects (lanes indicated with C) and from patients with active VKC (lanes indicated with V). The zymographies of these samples show the presence of both gelatinase A (MMP-2) and gelatinase B (MMP-9). A protein standard showing a 97-kd band (lanes indicated with ST) was run on each gel. The lanes without indication show different kinds of laboratory samples, including samples with known amounts of gelatinases.

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