Association of Hemopexin in Tear Film and Conjunctival Macrophages With Vernal Keratoconjunctivitis

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Objective: Vernal keratoconjunctivitis (VKC) is a chronic allergic inflammatory disease with unclear etiology and pathogenesis. We investigated the tear film proteome of patients with VKC to understand the pathologic characteristics of VKC.

Methods: Tear samples were collected from healthy volunteers and patients with VKC. Electrophoresis was performed to display the tear proteomic profiles according to VKC severity. The identities of differentially expressed proteins were analyzed by mass spectrometry and quantified by enzyme-linked immunosorbent assay. Immunohistochemical examination of normal lacrimal tissues from mice showed that hemopexin was not expressed in any lacrimal apparatus. Under systemic and topical sensitization and challenge using hemopexin in mice, the affected eye had mild to moderate bead discharge, chemosis, and edema with excessive macrophage infiltration and conjunctival necrosis.

Conclusion: An association exists between tear hemopexin and the development and pathologic effects of VKC.

Clinical Relevance: Increased hemopexin may have a role in the development of VKC.


ERNAL KERATOCONJUNCTIVITIS (VKC) is a unique chronic ocular allergic inflammatory disease that mainly occurs in children in their first decade of life. Boys are more frequently affected than girls. European countries have an estimated prevalence of 3.2 cases per 10,000 people. A Japanese epidemiologic study showed that VKC accounted for 4% of all ocular allergies with a low mean age of patients and severe clinical presentation. Patients with VKC have pathologic anterior segment changes in the eyelids, conjunctiva, and cornea. Among different types of allergic conjunctivitis, VKC usually has early onset and high clinical morbidity. Some patients develop severe corneal complications with permanent visual impairment. Therefore, VKC is one of the most severe subtypes of ocular allergy.

The diagnosis of VKC is mainly based on signs and symptoms. Clinical signs of VKC vary. The classic signs include giant papillae on the palpebral conjunctiva, Trantas dots on the limbus, and corneal complications such as punctate epitheliopathy and shield ulcers. The other less-specific signs include mucus discharge, hyperemia, and chemosis, which are common in other forms of chronic conjunctivitis. Symptoms in most patients with VKC include itching, tearing, redness, and photophobia; these are similar to symptoms of acute allergic conjunctivitis. Some patients also show a characteristic climatic pattern, particularly in the spring, as in other forms of allergic conjunctivitis.

To date, there is no specific laboratory test suitable for VKC diagnosis and monitoring. Ancillary tests, such as skin prick and serum allergosorbent, can be useful for diagnosis and treatment. Vernal keratoconjunctivitis has mixed features and stages of acute and chronic ocular inflammatory conditions, and T cell–mediated responses and IgE-mediated hypersensitivity reactions are both involved. Studies from Asian regions have revealed an increase in serum IgE and
Table 1. Clinical Scoring System for Patients With Vernal Keratoconjunctivitis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Symptom</strong></td>
<td></td>
</tr>
<tr>
<td>Itchiness</td>
<td>None</td>
</tr>
<tr>
<td>Discharge</td>
<td>None</td>
</tr>
<tr>
<td>Pain</td>
<td>None</td>
</tr>
<tr>
<td>Tearing</td>
<td>None</td>
</tr>
<tr>
<td>Photophobia</td>
<td>None</td>
</tr>
<tr>
<td><strong>Sign</strong></td>
<td></td>
</tr>
<tr>
<td>Hyperemia</td>
<td>None</td>
</tr>
<tr>
<td>Trantas dots</td>
<td>0</td>
</tr>
<tr>
<td>Punctate keratitis</td>
<td>None</td>
</tr>
<tr>
<td>Papillary hypertrophy</td>
<td>None</td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Clinical severity</strong></td>
<td>None</td>
</tr>
</tbody>
</table>

According to disease severity, all patients underwent clinical assessment that included a modified scoring system based on their primary symptoms and signs. Briefly, for symptoms, a visual analog scale was developed with scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) used to assess symptoms of (1) itchiness, (2) discharge, (3) pain, (4) tearing, and (5) photophobia. For clinical signs, each patient was assessed by a qualified ophthalmologist, using the same visual analog scale, for (1) severity of hyperemia, (2) number of Trantas dots, (3) area of punctate keratitis, and (4) size of papillary hypertrophy in each affected eye (Table 1). The sum of the scores of clinical symptoms and signs was calculated, and the overall severity of VKC was classified as mild (≤ 9), moderate (10-18), and severe (> 18).

## TEAR COLLECTION

Tears were collected with a disposable glass capillary micropipette (Drummond Scientific, Broomall, Pennsylvania) designed for tear film collection. Each micropipette, 12 cm long and 1 mm wide, was calibrated with a mark at 10 µL to estimate the volume of tear film collected and was polished at the collection end. During tear collection, the patients with VKC and the control participants were positioned at a slit lamp. The slit beam was narrowed to avoid bright illumination and reflex tearing. With the patient’s lower lid gently everted, the operator gently placed the micropipette laterally at an angle into the inferior fornix cul-de-sac. Contact between the tear meniscus and the micropipette tip facilitated tear collection via capillary action. All tear samples were obtained within a single 5-minute collection, without topical anesthetics. All tear-collection procedures were performed by the same clinical ophthalmologist (J.C.F.P.). Any maneuvers that caused irritation to the conjunctiva during tear collection were recorded, and those specimens were discarded. All collected tears were recorded, and those specimens were discarded. All collected tears were...
were immediately transferred to a 0.2-mL centrifuge tube by rapid centrifugation, and supernatants were stored at −80°C until analysis.

TEAR PROTEOMIC STUDY

Tear film proteomic profiling was analyzed by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE; Bio-Rad Laboratories, Hercules, California) to display the differential proteome of all participants and then by matrix-assisted laser desorption ionization–time of flight mass spectrometry to sequence the indexed peptides for protein identification, as previously described.10,11 As in the previous study,10 tear samples from the same group of patients with VKC and control participants were age matched. Tear samples of the patients with VKC were further divided according to clinical severity. Based on the total clinical score, the 2-DPAGE images of mild, moderate, and severe VKC were grouped and compared. Each gel image was acquired by an imaging densitometer (GS-700; Bio-Rad Laboratories) with proprietary software (Quantity One; Bio-Rad Laboratories) and further subjected to imaging analysis by 2-dimensional densitometry software (PDQuest; Bio-Rad Laboratories) to match and analyze the intensities of each protein spot on the gels. The differentially expressed protein spots were excised from the gel, using a gel slicer, and subjected to mass spectrophotometry with a proteomics analyzer (ABI4700; Applied Biosystems Inc, Carlsbad, California) for protein identification. The classification of VKC clinical severity was masked.

QUANTIFICATION AND REGRESSION ANALYSIS

To validate the proteomic results, a commercial sandwich enzyme-linked immunosorbent assay kit (GenWay Biotech, San Diego, California) with specific antibodies against hemopexin was used for quantitative analysis, as reported in a previous study.10 Concentrations of human hemopexin from the same group of normal and VKC tear samples collected from that cohort study10 were measured and compared. In the current study, we first performed correlation analysis to determine the relationship between tear hemopexin and clinical severity by Spearman correlation analysis. Stepwise linear regression analyses were then performed, with the hemopexin concentrations in tear samples as dependent variables, to determine the relative effect of clinical signs and symptoms of VKC. Continuous variables, such as age, disease duration, and clinical sign and symptom scores, were entered without transformation. Categorical variables, such as sex, positive ancillary test results, atopic history, and disease recurrence, were first transformed into dummy variables. Statistical analyses were performed using SPSS for Windows software, version 13.0 (SPSS, Inc, Chicago, Illinois). A P value <.05 was considered significant.

IMPRESSION CYTOLOGY AND IMMUNOCYTOCHEMISTRY

To further characterize hemopexin in pathologic changes associated with VKC, impression cytology on the ocular surface of patients with VKC from a separate cohort was performed and compared with the ocular surface of healthy participants. Informed consent was obtained from all participants. With topical anesthesia, small pieces of sterile cellulose acetate filter paper were applied on the ocular surface so that the most superficial layer of the ocular surface from the temporal, inferior, and nasal regions in each eye was obtained and subjected to standard hematoxylin-eosin staining, to immunocytochemistry using specific antibodies against hemopexin (ABR Affinity BioReagents, Golden, Colorado), as well as to further inflammatory cell characterization.

ALLERGIC CONJUNCTIVITIS MODELS IN MICE

To study the expression of hemopexin in the normal eye, healthy adult inbred Institute for Cancer Research mice (Jackson Laboratory, Bar Harbor, Maine) were humanely killed. Whole eyeballs, with the eyelids and lacrimal apparatus structures, were excised, fixed in 4% paraformaldehyde and embedded in paraffin wax for sagittal sectioning and immunohistochemical staining for hemopexin.

To further investigate the pathologic roles of hemopexin in VKC, an allergic conjunctivitis mouse model, according to the protocol of Nakamura et al, was used to study the anatomic and cellular responses on the ocular surface.12 Animal ethics approval was obtained from The Chinese University of Hong Kong, and all animal care was carried out according to the institutional guidelines. Briefly, 8-week-old Institute for Cancer Research mice were randomized and systemically sensitized by an intraperitoneal injection of 0.1 mL of 10-µg/mL recombinant hemopexin protein (R&D Systems, Minneapolis, Minnesota) or vehicle (sterile phosphate-buffered saline solution) on alternate days for 2 weeks (day 0 to day 14). Short ragweed (SRW) pollen extracts (Greer Laboratories, Inc, Lenoir, North Carolina), at 100 µg/mL in 0.1 mL, were used for comparison because SRW has been used for genetic and immunologic studies in an allergic conjunctivitis model.11 The sensitization period was followed by administration of high-titer topical eye-drops of recombinant hemopexin protein (100 µg/mL in 0.02 mL) or SRW (400 µg/mL in 0.02 mL) to the left eye once daily for a week (day 13 to day 21) until the appearance of mild or moderate clinical symptoms. The right eye was administered normal saline as a control in the same animal. To avoid crossover, the mice were maintained in a lateral position during topical administration, and the volume was controlled to 5 µL in each drop 4 times during 5 minutes. Drop administration was completed in one eye and then in the other eye 30 minutes later. The experimental challenge of higher titers of hemopexin or SRW was performed on day 24. All animals were assessed by masked observers. Both the behavior of mice in the cage and specific clinical symptoms in each eye, such as tearing and discharge, conjunctival edema and redness, and lid edema and redness, were assessed. To evaluate the inflammatory cell infiltration in the conjunctiva during the late-phase reaction, whole eye tissues were excised 24 hours after the final challenge (day 25), fixed in 4% paraformaldehyde and embedded in paraffin wax for sagittal sectioning, standard histologic staining, and immunohistochemical analysis, as described in the “Impression Cytology and Immunocytochemistry” subsection. Particular attention was paid to anatomic changes in the cornea, conjunctival complications, and eyelid inflammation.

RESULTS

HEMOPEXIN LEVEL IN VKC TEAR PROTEOME AND ITS ASSOCIATION WITH VKC CLINICAL SEVERITY

As previously reported, 14 tear samples from 7 patients with VKC and 7 healthy controls were collected for initial tear proteomic analyses. According to the total clinical sign and symptom score, VKC tear samples were classified as severe (n = 2), moderate (n = 3), and mild (n = 2) and then were matched with control tear samples by age (±1 year) (Table 2). Figure 1 shows the representa-
tive protein separation of tear samples by 2D-PAGE from patients with various clinical severities of VKC (Figure 1A). Although there was some variation in samples of mild VKC, the overall protein spot intensity of ID 3743 in 2D-PAGE gel was significantly higher in VKC tear samples than in control samples (Figure 1B and Table 2). Subsequent protein identification by matrix-assisted laser desorption ionization–time of flight confirmed that hemopexin was differentially expressed between VKC tear samples and control tear samples.

Human hemopexin enzyme-linked immunosorbent assay quantitative kit (GenWay) was used for validation and quantification in tear samples from patients with VKC and control participants recruited in another cohort study. A total of 29 tear samples from 14 mild, 12 moderate, and 3 severe VKC cases were collected. Eight normal tear samples from individuals with no ocular diseases were collected as controls (Table 3). A scatterplot showed a strong correlation between the hemopexin concentrations in tear samples and clinical severity with a regression coefficient of $r = 0.85 \ (P < .001)$, suggesting a significant association of tear hemopexin concentrations with VKC clinical severity (Figure 2). Hemopexin concentrations increased from 21.9-fold to 105.5-fold for mild to severe VKC. In regression analysis, tear hemopexin concentrations were a significant determinant of total clinical sign and symptom scores ($P < .001$) but were independent of patient age, sex, disease duration, ancillary test results, atopic history, and disease recurrence (all $P > .05$). The adjusted $R^2$ value for significant variables was 0.48. In an additional stepwise multiple regression analysis, only clinical sign scores accounted for significant variance in tear hemopexin, with an adjusted $R^2$ of 0.26, suggesting a relationship with pathologic changes in the ocular surface.

HEMOPEXIN-INDUCED PATHOLOGIC CHANGES IN MICE

To determine the origin of hemopexin in the lacrimal system, an immunohistochemical study using a hemopexin-specific antibody on the lacrimal apparatus structures of normal mouse eyelids, including the meibomian glands, lacrimal glands, and goblet cells, was performed (Figure 4, 3 mice). No obvious hemopexin was detected in any tissues or cells. Thus, hemopexin is not usually found in healthy eyes, and hemopexin detected in the normal tear sample is not from tear-secreting structures. Few positive stained leukocytes were identified in the connective tissue of the eyelids of healthy animals.

In the allergic conjunctivitis animal model, we used recombinant human hemopexin (R&D Systems) in 10-µg/mL intraperitoneal injections and 100-µg/mL eye-drops for systemic sensitization ($n=5$) or SRW pollen extracts in 100-µg/mL intraperitoneal injections and 400-µg/mL eye-drops for topical sensitization ($n=5$). After a final challenge, both hemopexin- and SRW-treated animals developed mild to moderate irritation and crouching behavioral changes, together with increased tearing meniscus. On photographic examination, the hemopexin group showed more obvious small-grain to large-bead discharge in the lid margin, moderate to severe fornix-based chemosis, and mild to moderate lid edema in the affected eyes when compared with the SRW group.
(Figure 5A). The control eyes in both the hemopexin and SRW groups had no such morphologic changes. On detailed microscopic examination, severe conjunctival inflammation, corneal punctuate epitheliopathy, shield ulcer, stromal edema, and neutrophil and eosinophil infiltrations were observed in all animals of the hemopexin group, but not in animals of the SRW group. Notably, a predominance of necrotic conjunctival epithelial cells and macrophages was found only in the hemopexin-sensitized and challenged eyes (Figure 5B).
COMMENT

Hemopexin levels are elevated in VKC tear samples. This trend could be the result of a macrophage inflammatory process or hemopexin secretion in association with the allergic response and pathologic ocular surface changes of VKC.

Hemopexin belongs to the family of acute-phase proteins and is secreted after an inflammatory event. Hemopexin is a type II acute-phase reactant glycoprotein that serves as a scavenger and transporter of toxic plasma heme to protect against oxidative damage resulting from the catalytic activity of hemolysis or rhabdomyolysis. Hemopexin can also inhibit the toxic effects of heme on retinal epithelial cells. Hemopexin has been shown to have antioxidant properties by decomposing lipid peroxides, which occurs when a lipid molecule is attacked by a reactive oxygen species and undergoes oxidation, resulting in cell damage. In the presence of hemopexin, hemin-stimulated lipid peroxidation is inhibited.

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Hemopexin is mainly expressed in the liver and has a high affinity for heme with an equimolar ratio. After heme binding, hemopexin undergoes a conformational change for interaction with a specific receptor, expressed mainly on the hepatocyte membrane, and is then internalized for further catabolism in the cytosol. In addition to the liver, hemopexin is expressed in the central nervous system, retina, and peripheral nerves. Studies of mRNA have revealed that apo-hemopexin is found in the retina and is likely synthesized by neural retinal cells, including ganglion cells and photoreceptors. Because the blood-retinal barrier precludes the release of the heme-hemopexin complex, it is possible that the neuroretina has its own degradation mechanisms. Hemopexin can also inhibit the toxic effects of heme on retinal epithelial cells.
Hemopexin can bind and transport extracellular heme released by hemolysis, inflammation, and trauma. The heme-hemopexin complex can also bind nitric acid. Production of hemopexin locally maintains the integrity of the blood-retinal barrier against radical oxygen intermediates.

To our knowledge, there have been no studies demonstrating the presence of hemopexin in tears. We have shown for the first time a correlation between the elevation of hemopexin concentrations in tears and the clinical severity of VKC. Linear regression analysis demonstrated the significant correlation shown in Figure 2. With increased clinical severity, there were more corneal complications and more pathologic changes on the conjunctiva and limbus. Elevated concentrations of hemopexin in tears could be attributed to further breakdown of the ocular surface, increased vascular permeability from the circulation, or both of these mechanisms. The correlation of hemopexin with clinical severity could be a potential biomarker for disease diagnosis and monitoring.

Tears are produced from various sources, such as acinar cells of lacrimal glands, goblet cells, and meibomian glands. Further localization of the source of hemopexin led us to conduct immunohistochemical studies in animals. The negative results of hemopexin staining in lacrimal glands, meibomian glands, and goblet cells indicate that hemopexin may not be released from tear-secreting structures. The positive results of macrophage hemopexin immunostaining in the connective tissue of eyelids in healthy animals (Figure 3H) and the ocular surface of patients with VKC (Figure 4) suggest a possible systemic source of hemopexin. Similarly, various antimicrobial tear proteins are promptly delivered by circulating neutrophils to sites of microbial invasion. In particular, lactoferrin is an iron-scavenging transferrin protein that has antimicrobial activity via its high affinity with iron and can modulate the ability and aggregation of bacteria and inhibit both bacteria and viruses. The recruitment and release of hemopexin in the tear film from circulating neutrophils require further characterization.

Figure 4. Hemopexin in impression cytology in patients with vernal keratoconjunctivitis (VKC). High hemopexin immunoreactivity was detected in conjunctival epithelial cells and macrophages (red arrowheads) (B and D), but not in neutrophils (black arrowhead) (D), in VKC samples (original magnification ×100). The inset shows positive staining of CD163 antibody specific for macrophages (original magnification ×100). No hemopexin immunoreactivity was detected in control samples (A and C).
Hemopexin sensitization and challenge in the development of vernal keratoconjunctivitis (VKC) in mice. A, Gross macroscopic photographs show small-grain discharge on the ocular surface, moderate to severe fornix-based conjunctival chemosis, and mild to moderate lid edema in hemopexin-treated and short ragweed (SRW) pollen extracts–treated left eyes (red arrowheads). No macroscopic changes were observed in vehicle-treated right eyes in the SRW group (black arrowheads), and only mild eyelid edema and small-grain discharge were noted in the hemopexin group (red arrowheads). B, Microscopic examination shows disruption of cornea epithelium (black arrowheads), conjunctival infiltrates of neutrophils, eosinophils, and macrophages (red arrowheads) in hemopexin-sensitized and hemopexin-challenged eyes and stromal edema (gray arrowhead) (original magnification ×10).

We have also used impression cytology to demonstrate the presence of hemopexin in immunocytochemical studies on the conjunctiva of patients with VKC. Hemopexin is found in the cytoplasm of conjunctival epithelial cells and in macrophages in impression cytology samples (Figure 4). Furthermore, animal models of chronic allergic keratoconjunctivitis confirmed the pathologic role of hemopexin in VKC, as hemopexin can induce ocular pathologic changes in patients with VKC and mediate excessive macrophage infiltration and necrosis in affected conjunctiva (Figure 5). In our challenge test in mice, hemopexin reproduced similar conjunctival changes as in humans, but such pathologic changes were not observed in SRW-induced allergic keratoconjunctivitis. To test the specificity of hemopexin in VKC, tear hemopexin concentrations in other types of conjunctivitis can be measured in future studies. At this stage, it is still unclear whether increased hemopexin and macrophages in conjunctival epithelium are a primary or secondary result of the disease. Further detailed experiments, including cellular and molecular studies, are necessary to determine the source of hemopexin and how it interacts with other inflammatory proteins. Although the late-phase reactions of hemopexin on the ocular surface of animals may not reflect the local immune reaction of hemopexin in humans with VKC, the hemopexin sensitization study confirmed its direct allergic effects on the ocular surface and furthered our understanding of the pathogenesis and corneal complications of VKC.

Most tissues need protection from iron- and heme-mediated oxidative damage. The liver, for example, expresses both transferrin and hemopexin as extracellular protection against toxic radical oxygen intermediate–inducing effects of iron and heme, respectively. With the presence of the blood-retinal barrier, these protective proteins may be too slow and the levels may be too low to prevent damage. Thus, local production of hemopexin and haptoglobin by the photoreceptors and ganglion cells is necessary. Baumann and Gaudieuggested that production of such acute-phase proteins is mediated via IL-1 and IL-6 in rat hepatocytes. On the ocular surface, a similar mechanism is possible for protection against iron- and heme-mediated toxic effects. There could be local production and an increase from the circulation during inflammation. Increases of IL-1, IL-4, and IL-5 in tear film have been found in other studies and in our current tear proteomic profiling; IL-4 was also increased in tears in VKC. The cytokine IL-4 induces differentiation of naive helper T cells to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4. Understanding how hemopexin interacts with these interleukins at the ocular surface is important for understanding the pathogenesis of VKC.

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