

## Original article

# Cytokines, matrix metalloproteases, angiogenic and growth factors in tears of normal subjects and vernal keratoconjunctivitis patients

**Background:** To detect the presence of multiple mediators and growth factors in tears of vernal keratoconjunctivitis (VKC) patients with active disease using stationary phase antibody arrays.

**Methods:** Tears were collected from 12 normal subjects (CT) and 24 active VKC patients. Tears were centrifuged and successively probed using three microwell plate arrays specific for: (i) cytokines: interleukin (IL)-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, interferon- $\gamma$  and tumour necrosis factor- $\alpha$ ; (ii) growth factors: basic fibroblast growth factor (bFGF), platelet-derived growth factor, thrombopoietin, angiopoietin-2, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), keratocyte growth factor, tissue inhibitor of metalloprotease (TIMP)-1 and heparin-binding epithelial growth factor (HB-EGF) and (iii) matrix metalloprotease (MMP)-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1 and TIMP-2.

**Results:** Interleukin-8 signals were detected in all CT and highly detected in all VKC samples. The Th2-type cytokines, IL-4, IL-5 and IL-10 were detected only in tears of VKC patients. Signals for bFGF, HB-EGF, VEGF and HGF were detected in 41–87% of VKC samples and in few CT samples. Only TIMP-1 and TIMP-2 were found in all normal and patient tear samples, whereas MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10 were highly present in all VKC samples.

**Conclusions:** Stationary phase antibody array methodology was useful for the screening of various cytokines, growth factors and MMPs in tears. These analyses identified in tears of VKC patients previously unreported factors including MMP-3 and MMP-10 and multiple proteases, growth factors and cytokines, which may all play an important role in the pathogenesis of conjunctival inflammation.

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Key words: cytokines; matrix metalloprotease; microwell array; vernal keratoconjunctivitis.

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Vernal keratoconjunctivitis (VKC) is a severe ocular allergic disease that occurs predominantly in children and young adults (1). It is characterized by intense ocular symptomatology such as itching, photophobia, foreign body sensation, conjunctival hyperemia and mucous secretion, typically accompanied by giant papillae formation on the upper tarsal conjunctiva or in the limbal form, by limbal infiltrates and nodules, or both signs in the mixed form of the disease. Corneal involvement is common, characterized by punctate keratitis or sterile corneal ulcers, as a result of the epitheliotoxic effects of proteins and enzymes released by activated eosinophils (1). The disease is accompanied by a gamut of alterations involving structural cells and tissues such as conjunctival thickening, subepithelial fibrosis, mucous metaplasia, neovascularization and scarring (1). Many elements contribute to this dramatic response, including epithelial changes, connective tissue deposition, inflammatory cell infiltration and glandular hypertrophy.

Vernal keratoconjunctivitis is an immunoglobulin (Ig)E- and Th2-mediated disease in which only 50% of patients present a clear allergic sensitization (1–4). Clinical diagnosis is supported by the determination of specific allergic sensitization and the detection of eosinophils by tear cytology or conjunctival scrapings.

The concentration and distribution of inflammatory mediators or inhibitors in the tear fluid have been extensively studied in ocular allergy, finding an attempt to find a 'disease marker', to better understand the immune mechanisms involved in the ocular surface inflammation and to identify potential targets for therapeutic interventions (5–9).

Tear collection is a noninvasive diagnostic procedure but has an insurmountable limitation regarding the quantity of sample obtainable. Determination of tear mediator, cytokine or chemokine levels is not yet used for diagnosis, but only for the study of allergic physiopathology or for the evaluation of efficacy of anti-allergic

agents. Cytokines have been measured in tears individually using enzyme-linked immunosorbent assay (ELISA) techniques, albeit with great limitation as to what could be learned from one sample (6, 7). Advances in techniques have now allowed for multiple cytokine assaying. The 'multiplexed bead-based flow cytometry' allows the simultaneous measurement of various mediators in one sample of 10–20 µl of tear fluid (8–11). However, this technique is not yet widely available for clinical applications because of its high cost.

Membrane array characterization allows for the identification of up to 80 chemokines, cytokines and growth factors in one tear sample, and consequently, a more global picture of immunoregulation comes into focus (12). Dozens of cytokines, chemokines, growth factors, angiogenic modulators, enzymes and inhibitors can be identified in small tear samples using these proteomic techniques (12–14). Microwell plate antibody array is a relatively inexpensive technique that also can be applied to tear analysis (15).

The aims of this study were to detect the presence of multiple mediators, proteases, angiogenic and growth factors in tears of normal subjects (CT) and VKC patients with active disease using a previously modified (15) microwell plate antibody array. The present findings demonstrate the utility and limitations of this proteomic analysis, while providing further insight into the changes in the tear film protein profile associated with VKC.

## Materials and methods

### Subjects and tear collection

Written informed consent was obtained from all subjects or, in the case of minors, from their parents after explanation of the nature and the possible consequences of the study. The research followed the Tenets of the Declaration of Helsinki. Institutional Review Board approval was obtained. Twelve CT and 24 active VKC patients (16 tarsal and 8 limbal forms) were included in the study. Diagnosis of VKC was established according to the patient's history, the clinical signs and symptoms, skin test results, and determination of serum total and specific IgE (Table 1). Although often found in association with VKC, none of the patients had keratoconus. In all patients, a subjective clinical activity score (0–10) was given considering the overall severity of the disease (0 = no symptoms and no active signs; 10 = very severe symptoms and signs) at the time of sample collection. Additionally, an individual score for corneal involvement was given: 0 = clear cornea, 1 = local punctate keratitis, 2 = diffuse punctate keratitis; 3 = focal confluent de-epithelization; 4 = corneal shield ulcer. All patients and normal volunteers were free of medication for at least 5 days before sample collection; 20–50 µl of open eye tears were gently collected from the external canthus of the most affected eye using a capillary micropipette and avoiding the tear reflex as much as possible. Samples were placed in Eppendorf tubes, centrifuged at 160 *g* for 8 min and stored at -80°C. All samples were taken in the outpatient service during the morning, from 9 to 12 AM. Samples were successively probed using three microwell plate arrays.

Table 1. Demographic and clinical data of the 24 VKC patients

| Patient | Gender/age | Type of VKC | Clinical activity score (0/10) | Corneal clinical score (0/4) | Serum IgE (KU/l) | Serum-specific IgE/prick test | Associated allergic diseases |
|---------|------------|-------------|--------------------------------|------------------------------|------------------|-------------------------------|------------------------------|
| M-D     | M/10       | T           | 10                             | 4                            | 584              | D, G, P, Cat                  | R, E                         |
| V-T     | M/7        | T           | 10                             | 4                            | 59               | Neg                           | E                            |
| T-N     | M/16       | T           | 6                              | 0                            | 95               | Neg                           |                              |
| Z-G     | M/5        | T           | 6                              | 0                            | 35               | D                             | E                            |
| B-F     | M/15       | T           | 9                              | 4                            | 16               | Neg                           |                              |
| F-G     | M/8        | T           | 9                              | 3                            | 874              | D, G, P, Tr, C                | R, A, E                      |
| F-L     | M/9        | T           | 7                              | 1                            | 216              | D                             |                              |
| L-M     | M/37       | T           | 5                              | 0                            | 23               | Neg                           | R                            |
| P-C     | F/27       | T           | 7                              | 1                            | 42               | Neg                           |                              |
| S-C     | M/8        | T           | 8                              | 2                            | 1979             | G, D, P, C, Cat               | R, A                         |
| S-F     | F/38       | T           | 6                              | 1                            | 78               | Tr, C                         |                              |
| D-M     | M/26       | T           | 8                              | 0                            | 35               | D, G, P                       | A                            |
| A-W     | M/30       | T           | 5                              | 0                            | 23               | Neg                           |                              |
| S-S     | M/8        | T           | 7                              | 1                            | 334              | Neg                           |                              |
| C-M     | M/7        | T           | 7                              | 1                            | 86               | D, Alt                        |                              |
| D-S     | M/13       | T           | 5                              | 0                            | 75               | G                             |                              |
| G-G     | F/5        | L           | 5                              | 0                            | 100              | Neg                           |                              |
| M-A     | M/9        | L           | 6                              | 0                            | 8                | Neg                           | R                            |
| P-A     | M/11       | L           | 6                              | 0                            | 285              | D, G, P, Tr                   | R                            |
| B-M     | M/17       | L           | 8                              | 1                            | 204              | G, D, P, Tr                   |                              |
| T-D     | M/23       | L           | 7                              | 1                            | 360              | Neg                           |                              |
| G-E     | F/27       | L           | 5                              | 0                            | 65               | D, G, P                       | R                            |
| C-N     | M/5        | L           | 6                              | 1                            | 57               | Neg                           |                              |
| V-N     | M/14       | L           | 8                              | 1                            | 96               | Neg                           |                              |

F, female; M, male; L, limbal VKC; T, tarsal VKC; R, rhinitis; A, asthma; E, eczema; Neg, negative; D, dermatophagoides; G, graminaceae; P, parietariae; C, compositae; Alt, *alternaria alternata*; Tr, trees pollens; Cat, cat dander.

### Microwell plate array assays

Assays were carried out using a laboratory designed protocol, employing some of the contents of three commercially available antibody array kits: the Pierce SearchLight™ Th1/Th2 Cytokine Array, the Pierce SearchLight™ Human Angiogenic Array and the Pierce SearchLight™ Matrix Metalloprotease Array (Pierce, Rockford, IL, USA). The Th1/Th2 array is designed to simultaneously measure IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, interferon (IFN)γ and tumour necrosis factor (TNF)α using volumes of up to 40 µl biological fluids. The sensitivities of each of the assays are reported to be 0.2, 0.4, 0.2, 0.4, 0.2, 0.4, 7.8, 0.2 and 1.56 pg/ml, respectively. The matrix metalloprotease (MMP) array is designed to simultaneously measure MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13 and tissue inhibitor of metalloprotease (TIMP)-1 and TIMP-2. This assay targets epitopes common to both active and inactive forms of the MMPs. The sensitivities of each of the assays are reported to be 24, 7.8, 9.7, 24, 20, 2.4, 9.7, 8 and 1.2 pg/ml, respectively, based upon calibration with the supplied standards (proMMPs and TIMPs, personal communication from Pierce). The Angiogenic Array permits the simultaneous assaying of angiotensin-2 (ANG-2), vascular endothelial growth factor (VEGF), heparin-binding epithelial growth factor (HB-EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), hepatocyte growth factor (HGF), keratocyte growth factor (KGF), thrombopoietin (TPO)-2 and TIMP-1 using volumes of up to 40 µl biological fluids, with the sensitivities of each of the assays reported to be 4.9, 2.0, 1.8, 2.7, 1.0, 1.6, 1.0, 5.9 and 4.9 pg/ml, respectively.

All assays are based upon a classical dot sandwich ELISA protocol employing biotin-streptavidin-horseradish peroxidase amplification and chemiluminescence for detection. Samples were assayed separately using all the three arrays. Based upon previous studies (15), 50 µl of the synthetic blocking agent, MEGA BLOCK 3™ (Cell Associates, Inc. Pearland, TX, USA), were added to each sample for 1 h at room temperature. Depending on the assay, 2–20 µl of tear fluid, along with a serial dilution of the supplied recombinant protein standards in the MEGA BLOCK 3™ solution, were added in 50 µl volumes in duplicate, and whenever possible, in a 1 : 1 dilution to individual wells. After incubation at room temperature with agitation for 60 min, the residual tear fluid and the standard protein fluids were decanted and discarded. Wells were washed six times using the supplied wash buffer. Fifty microlitres of diluted biotinylated secondary antibodies were added to each well. After a 30 min-incubation at room temperature, the wells were further incubated for 30 min with 50 µl of the supplied streptavidin-peroxidase-linked reporter enzyme. To detect the bound complex, 50 µl of a freshly prepared solution of long-acting ChemiGlow™ (Alpha Innotech, San Leandro, CA, USA) were used. Wells were visualized using a Chemidoc XRS image station (Bio-Rad, Hercules, CA, USA). Imaging was carried out without binning for periods ranging from 1 min to as long as 1 h. The images of each well in the plate were visually examined for artifactual background chemiluminescence. Integration was carried out using Quantity one-4.5.0™ (Bio-Rad proprietary software, Bio-Rad, Hercules, CA, USA) with the background chemiluminescence subtracted.

### Statistics

Analyses of the frequencies of positive signals in tears of VKC and CT were evaluated and compared with the chi-squared test followed by the exact Fisher test using the SAS programme (SAS Institute, Cary, NC, USA). Correlations were calculated using Spearman's correlation coefficient. The minimal level of confidence at which the results were judged significant was  $P < 0.05$ .

### Results

In the Th1/Th2 array assay, tears from all CT (12/12) showed a modest positive signal to IL-8, but only 2/12 showed some signal for other cytokines. The Th2-type cytokines, IL-4, IL-5 and IL-13, were detectable only in VKC tears (Table 2, Fig. 1).

Analysis of tears from VKC patients showed that 11/24 samples were positive for IL-4 (eight tarsal, three limbal), 12/24 for IL-5 (eight tarsal, four limbal), 24/24 for IL-8, 10/24 for IL-10 (eight tarsal, two limbal), 7/24 for TNFα (five tarsal, two limbal), 5/24 for IL-12, IL-13 and IFNγ, and 3/24 for IL-2. Statistical analysis demonstrated that the frequencies of positive signal for IL-4 ( $P = 0.006$ ), IL-5 ( $P = 0.002$ ) and IL-10 ( $P = 0.05$ ) in tears of VKC patients were significantly greater than in CT samples.

The angiogenic and growth factor array assays were performed on all 24 VKC and 10 CT. All assays with the exception of the assay for ANG-2 produced standard curves consistent with the literature in the assay kit when a series of dilutions of recombinant protein standards was spiked into reflex tear fluid. The assay for ANG-2, however, exhibited an irregular pattern making this assay invalid. As such, assays were only carried out for the remaining eight proteins. All control tear samples were positive for TIMP-1; 4/10 controls were slightly positive for HGF and VEGF; 3/10 for bFGF and 2/10 for HB-EGF (Table 2). In VKC tears, a positive signal for TIMP-1 was seen in all patients; a positive signal for TPO in 10/24 (six tarsal, four limbal); for HGF in 21/24 (14 tarsal, 7 limbal); for bFGF in 10/24 (six tarsal, four limbal); for VEGF in 21/24 (14 tarsal, 7 limbal); for HB-EGF in 11/24 (seven tarsal, four limbal); and for KGF in 5/24 (three tarsal, two limbal). None of the sample was positive for PDGF-BB (Fig. 2). Statistical analyses demonstrated that the frequency of positive signals for TPO ( $P = 0.017$ ), HGF ( $P = 0.008$ ) and VEGF ( $P = 0.008$ ) were significantly higher in VKC compared with CT tears.

Because of the limited volume of samples, the microwell plate assay for MMPs was only performed with tears from 15 active VKC patients (10 tarsal, 5 limbal) and 8 CT tears. In all CT tears, TIMP-1 and TIMP-2 were strongly present. A weak signal for MMP-3 was found in 2/8 CT, for MMP-10 in 4/8 CT and for MMP-9 in 3/8 control tear samples (Table 2).

Strong signals for MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, TIMP-1 and TIMP-2 were found in all VKC samples (Fig. 3). The MMP-8 was strongly present in 10/15 (eight tarsal, two limbal) patients, but MMP-13 was not found in any sample. The strongest signals were for MMP-3, MMP-9 and MMP-10.

Statistical analyses demonstrated that the frequencies of positive signals for MMP-1, MMP-2 and MMP-3 (all  $P < 0.0001$ ), MMP-9, MMP-10 ( $P = 0.001$ ) and MMP-8 ( $P = 0.027$ ) were significantly greater in VKC than CT tears.

Table 2. Detection of cytokines, angiogenic factors and MMPs in tears of normal subjects and VKC patients

| Diagnosis | IL-2 | IL-4 | IL-5 | IL-8 | IL-10 | IL-12 | IL-13 | IFN $\gamma$ | TNF $\alpha$ | TIMP $\alpha$ | PDGF | TPO | KGF | HGF | FGF | VEGF | HB-EGF | MMP-1 | MMP-2 | MMP-3 | MMP-8 | MMP-9 | MMP-10 | MMP-13 | TIMP |
|-----------|------|------|------|------|-------|-------|-------|--------------|--------------|---------------|------|-----|-----|-----|-----|------|--------|-------|-------|-------|-------|-------|--------|--------|------|
| WKC T     | 0    | 0    | 0    | 0    | 0     |       |       |              | 0            | 0             |      |     | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     | 0    | 0    | 0    | 0    | 0     |       |       | 0            |              | 0             |      |     | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       | 0     | 0            |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       |       | 0            |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       |       | 0            |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       | 0     |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC T     |      | 0    | 0    | 0    | 0     |       | 0     | 0            |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC L     |      |      |      | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| WKC L     |      |      | 0    |      |       |       |       |              |              | 0             |      | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC L     |      |      | 0    | 0    |       |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC L     |      |      | 0    | 0    |       |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| WKC L     | 0    | 0    | 0    | 0    |       |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| Control   |      |      |      | 0    |       |       |       |              |              |               |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      |      | 0    |       |       |       |              |              | /             | /    | /   | /   | /   | /   | /    | /      | /     | /     | /     | /     | /     | /      | /      |      |
| Control   |      |      | 0    | 0    |       |       | 0     |              | 0            | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             | 0    | 0   | 0   | 0   | 0   | 0    | 0      | 0     | 0     | 0     | 0     | 0     | 0      | 0      |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |
| Control   |      |      | 0    | 0    |       |       |       |              |              | 0             |      |     |     |     |     |      |        |       |       |       |       |       |        |        |      |

IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; TIMP, tissue inhibitor of metalloproteinase; PDGF, platelet-derived growth factor; TPO, thrombopoietin; KGF, keratocyte growth factor; HGF, hepatocyte growth factor; HB-EGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; VKC T, tarsal VKC; VKC L, limbal VKC; o, positive signal; /, not evaluated; blank, negative signal.

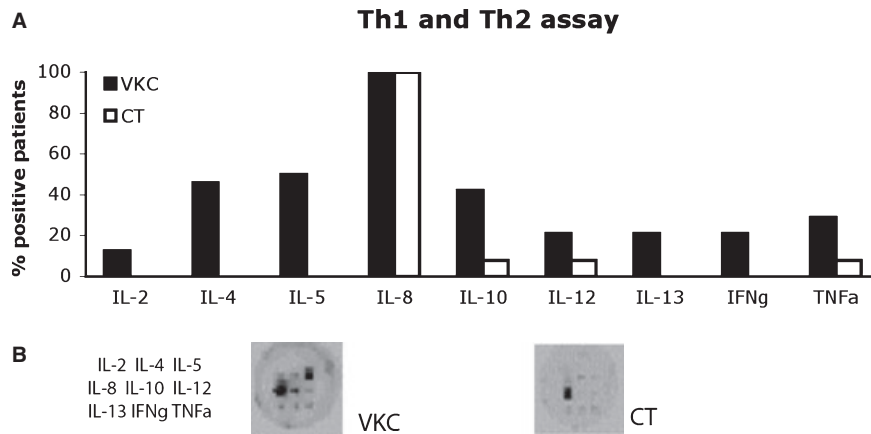


**Figure 1.** Percentage of VKC patients with positive signal in tears for nine different cytokines analyzed by the Th1/Th2 microwell array assay. All the VKC patients and CT showed positive signals for IL-8, whereas typical Th2-type cytokines, IL-4, IL-5 and IL-13 were expressed only in VKC (A). (B) The schematic position of the antibodies in each microwell and the results from a tear sample of one VKC patient with clear signal for IL-5, IL-8 and IL-10, and one CT with positive signal for IL-8 only.

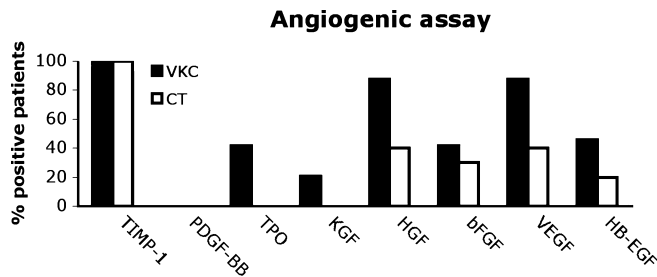


**Figure 2.** Percentage of VKC patients with positive signal in tears for angiogenic and growth factors analyzed by angiogenic microwell array assay. While TIMP-1 was present in all VKC and CT, TPO and KGF were expressed only in VKC patients. Vascular endothelial growth factor and HGF were present in 87.5% of VKC patients.

No correlations were found between the frequency of signals for any cytokine or other factor and the clinical activity of the disease or the presence of corneal ulcer. No

differences were identified between tarsal and limbal VKC concerning the pattern of cytokines and other factors in tears.

## Discussion

Tear samples are relatively easy to collect from VKC patients and provide valuable information on differential tear cytology and levels of mediators at the site of inflammation. The critical limitation is the volume of the tear sample, which ideally should be collected without stimulating the tear reflex. It is now possible to identify multiple mediators and cytokines or groups of cytokines with opposing or complementary functions using different methods. For example, using highly sensitive membrane-bound antibody technology, Sack detected 40 of 79 bioactive proteins in tears from CT (12). In ocular allergy, using the multiplexed flow cytometry assay,

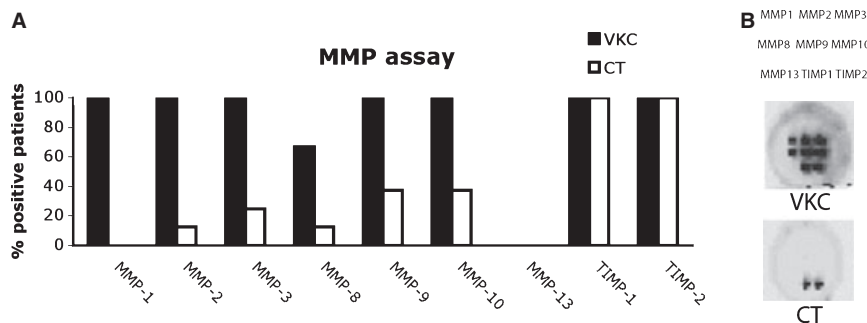


**Figure 3.** (A) Percentage of VKC patients with positive signal in tears for metalloproteases (MMPs) and their inhibitors (TIMP-1 and TIMP-2) analyzed by the MMP microwell array assay: MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10 were present in 100% of VKC. (B) The schematic position of the antibodies in each microwell and the results from a tear sample of one VKC patient with clear positive signals for MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, TIMP-1 and TIMP-2. In the CT, see the positive signal for TIMP-1 and TIMP-2 only.

significant differences between allergic and nonallergic patients have been shown for levels of  $\text{TNF}\alpha/\text{IFN}\gamma$ , IL-5/ $\text{IFN}\gamma$  and IL-5/IL-10 (8). In a recent study, IL-4, IL-5, IL-13 and  $\text{IFN}\gamma$  together with the pro-inflammatory cytokines, IL-1, IL-6 and  $\text{TNF}\alpha$  were significantly increased in tears collected from different forms of ocular allergic diseases, particularly in VKC (11). Although very sensitive, multiplex bead analysis of tear samples may not be always practical for use in a clinical setting. Improved well plate methodology, which will be detailed elsewhere, may provide a methodology suitable for clinical application. A comparison between the results of this semi-quantitative technique improved with densitometry of spot intensity, and the results from Multiplexed flow cytometry performed in the same tear samples is the focus of an ongoing study.

In the present study, a marked increase in the presence of many inflammatory mediators was demonstrated in active VKC *vs* control tears using a microwell plate protein array system. This relatively inexpensive technique has been extensively validated for direct nonquantitative assaying of serum, urine, tissue culture media and other biological fluids. Tears contain proteins, glycoproteins and proteases that can interfere with microwell-bound immunoglobulin, thereby reducing the efficiency of ELISA assays in general and making it extremely difficult to obtain quantitative data (15, 16). Thus, in preliminary assessments (15), a modified protocol was developed that reduced these matrix effects and allowed the semi-quantitative assaying of targeted proteins in tear samples. In this proteomic screening, the same sample was used for several small arrays, each retaining maximal signal-to-noise ratios (15).

We screened for the presence of 26 compounds (TIMP-1 is built into two arrays) in most VKC patients and CT using these microwell arrays. The majority of VKC patients showed a significantly greater production of Th2-type cytokines, in particular IL-4 and IL-5. These findings are consistent with previous reports from different laboratories (5–8, 11). However, looking at the overall results (Table 2), there are some patients with multiple Th1- and Th2-type cytokines and others with very few cytokines in tears. Using a different membrane array for 40 inflammatory mediators, Shoji and co-workers (17) recently reported strong signals for IL-6R and eotaxin, but variable signals for many of the same Th1/Th2 cytokines that were evaluated in the present study, with no statistically significant differences observed in their distribution between normal and six VKC tear samples. A low tear cytokine profile has also been reported in VKC patients in previous studies (5–11); these results may depend on tear reflex dilution, sample volume and timing, sensitivity of the assay, presence of interfering proteins in tear samples, or simply by individual differences in cytokine expression.

Results from the angiogenic and growth factor arrays showed significantly increased expressions of VEGF,

HGF and TPO in VKC compared with normal tears, with a relatively high number of patients expressing bFGF and HB-EGF. Growth factors are known to regulate the expression of integrins, collagens and extracellular matrix components. Expression of VEGF, transforming growth factor (TGF)- $\beta$ 1, bFGF and PDGF was previously shown in VKC conjunctival tissues by immunohistochemistry (18, 19). Epithelial cells, fibroblasts and inflammatory cells are possible sources of these factors in VKC (20, 21) similar to what is described in the respiratory tract during allergic inflammation (22). Interestingly, HGF, TPO and HB-EGF have not previously been detected in VKC tears. Hepatocyte growth factor has been shown to regulate epithelial and stromal corneal wound healing (23). Thrombopoietin plays an important role in the development of human mast cells from  $\text{CD34}^+$  bone marrow cells in concert with stem-cell factor, and provides direct evidence for differentiation into the mast cell lineage of human multi-potential bone marrow-derived progenitors (24). Heparin-binding epithelial growth factor is implicated in the EGF signalling pathway (25) and is involved in epithelial healing or overgrowth, as has been shown in the pathogenesis of pterygium (26).

Angiogenic and growth factor expression in pathological conditions is frequently associated with the expression of proteases. For example, VEGF modulates the expression of MMP-9 in asthma (27). Conversely, MMP-9 and MMP-3 are known to cleave latent growth factors, such as FGF or TGF- $\beta$ , from proteoglycan storage of bound sites (28). Matrix metalloproteases aid the extravasation and infiltration of leukocytes through limited proteolysis of basement membranes and matrix material (29). Their increased production and activation, or an imbalance between MMPs and their natural tissue inhibitors, TIMP, are all probably involved in the

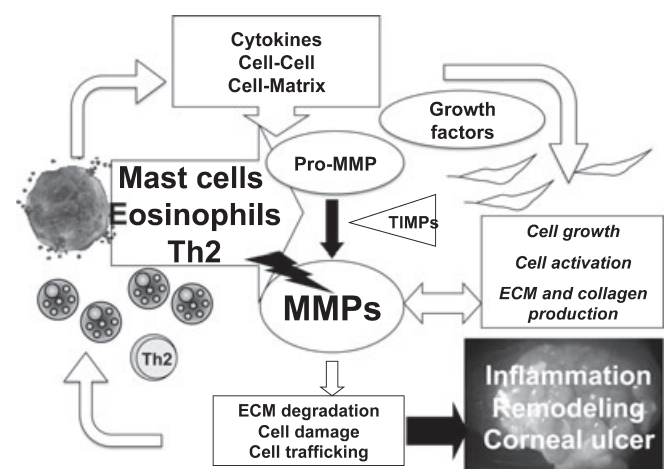


Figure 4. Schematic diagram illustrates the possible pathogenic relationships among cytokines, growth factors and proteases (MMP) in inducing inflammation, cell migration and cell growth, and tissue remodelling in chronic ocular allergy.

pathogenesis of conjunctival inflammation, remodelling and corneal changes in VKC (30, 31). In the present study, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-10 were highly expressed in all forms of active VKC, but in only a small number of control subjects. In contrast, while both TIMP-1 and TIMP-2 showed a stronger signal in VKC, they were also expressed in all control samples. Differential MMP expression has been shown in tissues from VKC, nasal polyps and asthmatic bronchi, suggesting that different patterns of MMP activation may result in different types of allergic tissue remodelling (32). Interestingly, increased expression of MMP-3 and MMP-10 has not previously been shown in VKC tears. The MMP-3 and MMP-10 are known to degrade proteoglycans, laminin, fibronectin and collagens and to activate the precursor forms of other MMPs, including MMP-1, MMP-2, MMP-8 and MMP-13 (33). The pro-inflammatory cytokines, IL-1 $\beta$  and TNF $\alpha$ , have been shown to up-regulate gelatinase B (MMP-9), collagenases (MMP-1 and MMP-13) and stromelysins (MMP-3, MMP-10 and MMP-11) in cultured human corneal epithelial cells (34). The Th2 type cytokines, IL-4

and IL-13, are known to modulate MMP-1 and TIMP-1 (31), and to inhibit MMP-3 expression in conjunctival fibroblasts (35). These data clearly indicate that MMPs are involved in the corneal epithelial damage and tissue remodelling processes that define chronic VKC pathology (Fig. 4).

In conclusion, microwell array assays are powerful tools for screening large numbers of trace proteins in tears. This analysis revealed previously undetected proteins that might participate in the pathogenesis of VKC, as well as demonstrating the profound changes in tear composition associated with prolonged allergic inflammation. This technology might be used to define tear mediator patterns specific to a single disease or disease phase, thus providing important diagnostic information for clinicians and researchers who study allergic diseases.

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