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Tear osteopontin levels in patients with allergic conjunctival diseases

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Abstract *Background:* Recent reports have revealed the importance of several cytokines in chronic conjunctival allergic diseases (ACD) such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). Osteopontin (OPN) is a noncollagenous adhesive matrix protein that is expressed by activated macrophages. There has been considerable interest in the potential role of OPN in monocyte infiltration at sites of inflammation. We measured OPN level in tears using ELISA, to determine whether the level of this cytokine is elevated in ACD. *Methods:* The level of OPN in tears was measured by ELISA using samples from patients with VKC, AKC or allergic conjunctivitis (AC) and from normal subjects. The level of OPN in tears was compared with the clinical severity of ACD and serum level of total IgE. *Results:* The level of OPN in tears in AKC patients was significantly higher than that in AC and normal controls. Tear level of OPN in patients with VKC was also significantly elevated compared to those with AC and to normal controls. The clinical severity of ACD correlated significantly with the level of OPN. However, no correlation was observed between tear OPN level and serum level of total IgE. *Conclusions:* These results indicate that OPN plays an important pathophysiological role in severe ocular allergic conditions and that an elevated level of OPN in tear fluid reflects the local clinical status of ocular allergy, which may be an example of tissue remodeling.

Introduction

The pathogenesis of allergic conjunctival diseases (ACD) is not fully understood. However, recent progress in immunological research has revealed the importance of several cytokines in ocular allergy. IL-4 is increased in the culture supernatants of samples obtained by brush collection in patients with allergic conjunctivitis [10]. A previous study showed a significantly elevated mean level of IL-4 in the tears of patients with AC compared with normal controls, and levels in patients with vernal keratoconjunctivitis (VKC) and giant papillary conjunctivitis were also significantly higher than in those with AC and in normal controls [9]. Increased transcripts of IL-4 and IL-13 were detected by the reverse transcription (RT)-PCR method in samples from AC patients [11]. We have recently reported that the tear IL-4 level in patients with atopic keratoconjunctivitis (AKC) was significantly higher than in those with VKC or AC and in controls, and tear IL-5 levels in patients with diseases associated with proliferative lesions, VKC and AKC were higher than in those with AC and in normal controls [25].

Osteopontin (OPN) is a noncollagenous adhesive matrix protein, normally found in bone and on epithelial surfaces, that contains the arginine–glycine–aspartate (RGD) binding motif common to many extracellular matrix proteins [4, 18, 20]. OPN is mainly expressed by activated macrophages and is also expressed by helper T lymphocytes (Th), especially Th1, after activation [17]. In vitro, OPN promotes integrin- and CD44-mediated

cell adhesion and chemotaxis in monocytes and B lymphocytes [16, 26, 27]. OPN is known to induce chemotaxis of endothelial cells, macrophages and T lymphocytes, which favors the persistence of chronic inflammation [6, 7, 12]. OPN is also a tumor-associated protein secreted by many tumor cells in culture, and is detected in human plasma [21, 23]. OPN has also been detected in a variety of human body fluids including blood, urine, and milk [7, 12]. Considerable interest has been expressed in the potential role of OPN in monocyte infiltration at sites of inflammation. Since OPN binds strongly to macrophages, and subcutaneous injection of OPN has induced prominent monocyte infiltration in mice [22], it has been believed that OPN is a key cytokine that sets the stage for efficient immune responses through differential regulation of cytokine expression by macrophages [1]. The functional role and contribution of OPN to the development of granulomatous diseases such as sarcoidosis has been reported [5, 6, 19]. Expression of OPN has been identified in the retina, and OPN-like immunoreactivity is present in the ganglion cells of rats [14]. OPN also induces corneal herpes simplex infection in mice [1]. However, the function of OPN in allergic abnormalities, including ocular allergy, is unknown.

AKC, which is also one of the ocular allergic diseases, is a bilateral, chronic hypersensitivity disease of the ocular surface seen in association with systemic atopic dermatitis (AD), characterized by lesions of the conjunctiva and cornea that are variable in severity. The anterior ocular findings of AKC have a wide spectrum, and a subset of patients has the same signs as those of AC. However, in some cases corneal complications such as scarring, neovascularization, and plaque formation often result in compromised vision. VKC is a recurrent seasonal disease of childhood, characterized by severe inflammation. It is most commonly seen in male patients and, unlike the severe group with AKC, tends to resolve spontaneously after several years. To our knowledge, there is no published study on levels of OPN in tears in patients with VKC, AKC and AC.

In the present study, we used a sensitive antigen capture enzyme-linked immunoassay (ELISA) for tear OPN to determine whether this cytokine exists free in the tears in ACD, and whether the level of this cytokine is higher in patients with these diseases than in other groups of patients and in normal controls. We also investigated the correlation between the level of OPN in tears and the ocular clinical severity of ACD.

Materials and methods

We studied 49 patients with VKC, AKC and AC who presented to the Department of Ophthalmology, Yokohama City University Hospital, Yokohama, Japan, between February and July 1997. The investigation adhered to the principles of the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from all patients before sample collection. Tear samples were obtained from 5 male and 2 female VKC patients with a mean age of 13.6 (range 11–17) years, from 15 male and 13 female AKC patients with a mean age of 23.6 (range 8–64) years, and from 6 male and 8 female AC patients with a mean age of 24.7 (range 8–42) years. VKC, AKC and AC were diagnosed according to the guidelines proposed previously for the diagnosis and treatment of conjunctivitis [2]. VKC was diagnosed by slit-lamp examination, showing typical cobblestone excrescences (giant papillary conjunctivitis of the upper palpebral conjunctiva more than 1 mm in size), perilimbal gelatinous change and corneal lesions, such as stromal lipid deposition and pannus formation. Out of seven patients with VKC, four (57%) had allergic rhinitis (AR) and two (29%) had bronchial asthma (BA). AKC patients had AD and conjunctival and/or corneal involvement. The patients with AKC met the diagnostic criteria of AD proposed by Hanifin and Rajka [13]. Out of 28 patients with AKC, 18 (64%) had AR and 5 (18%) had BA. Patients with AC who showed seasonal or perennial symptoms, such as ocular itching and tearing without proliferative lesions, had CAP-RAST or skin-prick tests which were positive to one or several allergens including grass pollen. Eight patients (57%) had AR, but no patient had BA among the patients with AC. None of the patients was receiving systemic or local steroids or anti-allergic drugs at the time of sample collection. To avoid the influence of systemic treatment, a blood sample was obtained after suspension for 7 days of oral administration of anti-histaminergic agents, anti-allergic agents, theophylline, β stimulants and corticosteroids. Tear samples from 13 normal individuals (5 male, 8 female: age range 17–50 years: mean 26.5) served as controls. Serum total IgE level was measured in serum samples from ACD patients by radioimmunoassay (Ohtsuka Bioassay, Tokushima, Japan).

A volume of tears exceeding 40 µl was collected with a plastic tube without the use of saline solution as described in a previous report [24]. In summary, a micro-hematocrit tube (NRIS micro-hematocrit tube, Herlev, Denmark) was introduced into the inferior tear meniscus. Tears were expelled into the plastic tube by using a bulb attached to one end of the tube. This process was performed two or three times at 4- to 6-min intervals. In bilateral cases, the tear sample was collected from the eye clinically more severely affected. All samples were stored at –70°C until assay and used within 2 h after thawing, followed by centrifugation. The tears were diluted 10-fold, and the tear level of OPN was determined using a two-epitome "sandwich" enzyme-linked immunosorbent assay (ELISA) kit (Human Osteopontin EIA kit, IBL, Fujioka, Japan). The procedures recommended by the manufacturer were followed without modification, and optical absorbance values were read on a micro-ELISA autoreader (model MR 580; Dynatech, Cambridge, Mass.) at 490 nm. All samples were tested in duplicate, and the tear level of OPN was calculated by comparing the mean absorption of duplicate samples with that of the standard curve. The detection limit for OPN was 5 ng/ml. All samples were coded and read blind in the assay.

The clinical severity of keratoconjunctival lesions was scored according to the clinical scoring method reported previously [15] with modification as shown in Table 1. In summary, conjunctival, corneal and limbal lesions were each graded as mild: 1, moderate: 2, or severe: 3, and the total of the three scores was determined as the clinical score in each case. The proliferative spectrum of ACD, VKC and AKC with proliferative lesions was grouped into three forms (conjunctival, mixed and limbal) according to the clinical findings.

Non-parametric analysis was conducted. The Mann–Whitney test was used to identify differences between patient groups and normal controls. Spearman's rank correlation test was used to establish the significance of the correlation between paired groups. A level of $P<0.05$ was accepted as statistically significant.

Table 1 Grading of clinical severity of allergic conjunctival diseases

	Conjunctival lesion	Corneal lesion	Limbal lesion
Mild: 1	Small papillae localized in the fornix, smaller than 0.5 mm in diameter	Superficial punctate keratitis distributed in less than half of the cornea	Mild injection of gelatinous lesion distributed in less than 120° of the limbus
Moderate: 2	Moderate-sized (0.5–1.0 mm in diameter) papillae located in the whole upper tarsal conjunctiva or localized giant papillae (more than 1.0 mm in diameter)	Superficial punctate keratitis distributed equally or in more than half of the cornea	Injected or gelatinous lesion without Trantas' dots distributed in 120° to 240°
Severe: 3	Giant papillae covering whole upper tarsal conjunctiva	Corneal ulcer or plaque with or without pannus formation	Injected or gelatinous lesion of any size with Trantas' dots or those without Trantas' dots larger than 240°

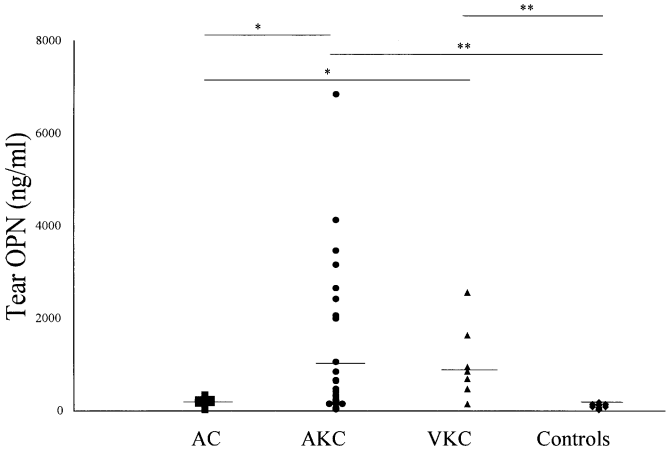

Fig. 1 Tear osteopontin (OPN) levels in patients with AC, AKC, VKC and normal controls. Each horizontal line represents the mean OPN level. Significance of difference between patient groups and controls or among patient groups: $^{**}P<0.001$, $^{*}P<0.01$

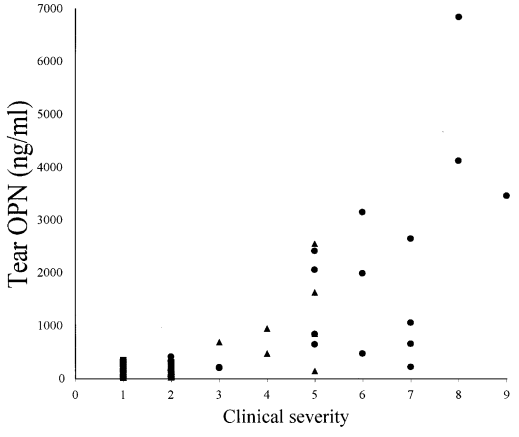

Fig. 2 Correlation between tear OPN level and clinical severity of ACD. A significant correlation was found between OPN level and clinical severity of ACD (rank correlation coefficient =0.735, P=0.0081). ■ AC, ● AKC, ▲ VKC, ◆ controls

Results

The level of OPN in tears for patients with VKC was 1037±808 ng/ml; in AKC patients, 1182±1623 ng/ml; and in AC patients, 160±113 ng/ml. That in normal controls was 103±52.4 ng/ml (Fig. 1). The level of OPN in tears for AKC patients was significantly higher than for AC patients and normal controls (P<0.01 and P<0.001, respectively). The level of OPN in tears for patients with VKC was also significantly elevated compared to that for AC patients and normal controls (P<0.01 and P<0.001, respectively).

As shown in Fig. 2, the clinical severity of ACD correlated significantly with the level of OPN (P<0.01). OPN showed high correlation with clinical severity (correlation coefficient 0.735). In this analysis, the three subgroups of ACD patients (AC, AKC and VKC) were deemed a single group. Although the level of OPN in tears in the mixed form of AKC and VKC (12 cases) (2264±1917 ng/ml) was higher than that in the conjunc-

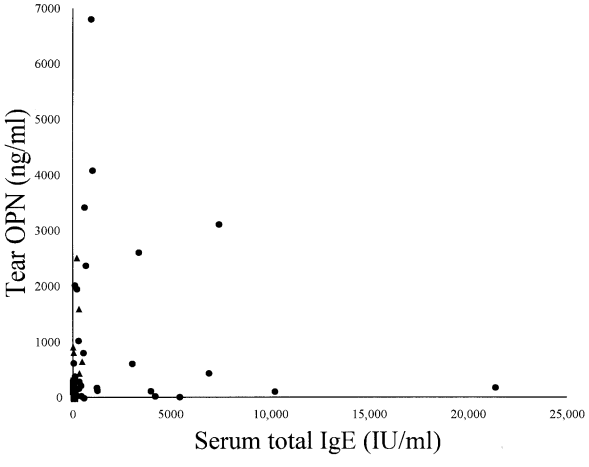

Fig. 3 Correlation between tear OPN level and serum total IgE level in ACD. No correlation was observed between OPN level and serum level of IgE (rank correlation coefficient =0.178, P=0.217). ■ AC, ● AKC, ▲ VKC

tival form of AKC and VKC (9 cases) (1183± 901 ng/ml), the difference did not reach significance.

Serum level of total IgE was measured to analyze the correlation between the level of OPN in tears and a systemic allergic parameter. Tear OPN level did not correlate with serum level of total IgE (Fig. 3).

Discussion

The present study demonstrated that ACD patients produce a considerable amount of OPN in tears. However, the source of OPN in tears is unclear from our present results. As mentioned above, OPN is expressed mainly by macrophages, but is also expressed by activated Th, especially Th1 [17]. We have recently demonstrated that patients with ACD produce detectable levels of Th1 type cytokines, IFN-γ and IL-2, in tears [25]. Although there are no known endogenous conjunctival cells that secrete IFN-γ or IL-2 either spontaneously or when activated, the most likely source of secretion of IFN-γ and IL-2, and a possible candidate for OPN production, is the small number of $CD3^+$ T cells seen in conjunctival follicles [11].

It was also observed that the clinical severity of ACD correlated significantly with the level of OPN (Fig. 2). However, no significant correlation was found in the present study between serum total IgE level and the level of OPN in tears (Fig. 3). Therefore, the elevated level of OPN in tears does not simply reflect the systemic severity of chronic allergic inflammation. There is a probable explanation for the elevated level of OPN in tears in the severe spectrum of ACD. We have reported an elevation of the level of IL-5 in tears for patient groups with proliferative keratoconjunctival lesions, i.e., VKC and AKC, and tear IL-5 could possibly be used as a marker to evaluate the clinical status of proliferative ocular allergic diseases or differentiate them from non-proliferative ocular allergic diseases such as AC [25]. Histopathological study has demonstrated that the conjunctival stromal inflammatory infiltrate in VKC and in the severe type of AKC is mainly composed of mononuclear cells, eosinophils and mast cells, and this histopathological characteristic may cause the elevation of the levels of IL-5 in tears observed in AKC and VKC [25]. It has been pointed out that OPN can function as a potent cytokine to induce granulomas composed of eosinophils and macrophages, such as eosinophilic granulomas (histiocytosis X) and Wegener's granulomatosis [6], but there has been no report that OPN is produced by eosinophils in vitro. However, it has been pointed out that mast cells and OPN play an important role in the intense inflammatory reaction followed by abnormal tissue repair, in other words tissue remodeling [8]. Proliferative lesions of the severe spectrum of ACD, such as giant papilla or corneal ulcer, have been recently recognized as an example of tissue remodeling characterized by the abundant infiltration of fibroblasts and extracellular matrix [3], leading to the hypothesis that elevated tear OPN reflects the results of tissue remodeling in severe cases of ACD. Further study is needed to clarify the pathophysiological role of OPN in allergic disorders including bronchial asthma, AD and ACD.

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