European Journal of Ophthalmology / Vol. 13 no. 7, 2003 / pp. 606-610

Tumor necrosis factor-alpha (TNF-α) in seasonal allergic conjunctivitis and vernal keratoconjunctivitis

A. LEONARDI[1,2], P. BRUN[3], M. TAVOLATO[1], M. PLEBANI[2], G. ABATANGELO[3], A.G. SECCHI[1]

[1]Department of Neuroscience, Ophthalmology and Ocular Inflammation Unit
[2]Department of Laboratory Medicine
[3]Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova - Italy

PURPOSE. *To quantify the presence of the proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) in allergic conjunctivitis.*
MATERIALS AND METHODS. *Tears and peripheral blood samples were collected from patients with seasonal allergic conjunctivitis (SAC, n=6), vernal keratoconjunctivitis (VKC, n=12), and normal subjects (CT, n=12). From an additional six nonactive allergic patients, tears were collected before and after specific conjunctival allergen challenge (CAC). Upper tarsal conjunctival biopsies were obtained from five CT and five VKC patients. TNF-α in tears was measured by enzyme-linked immunoassay and identified in tissues by immunohistochemistry.*
RESULTS. *Tear TNF-α levels in VKC patients were significantly increased compared to CT (p=0.03), and were significantly correlated with the severity of the disease. No differences were found between SAC and CT tear samples. TNF-α serum levels were higher in VKC than CT; however, this difference was not statistically significant. After CAC, tear TNF-α levels were found increased in only one of six patients. In VKC tissues, TNF-α positive cells were significantly increased compared to CT (p=0.03).*
CONCLUSIONS. *TNF-α may have a significant role in severe forms of allergic conjunctivitis. (Eur J Ophthalmol 2003; 13: 606-10)*

KEY WORDS. *TNF-α, Mast cell, Vernal keratoconjunctivitis, Conjunctival allergen challenge*

Accepted: April 7, 2003

INTRODUCTION

Seasonal allergic conjunctivitis (SAC) is a self-limiting allergic disease associated with environmental exposure to allergens and not complicated by serious ocular inflammation and corneal involvement. Conversely, vernal keratoconjunctivitis (VKC) is a severe, chronic, and recurrent ocular inflammation that affects children and young adults, usually living in warm climates (1). Ocular allergic diseases are characterized by immunoglobulin E (IgE)–mast cell activation and a subsequent cascade of mediators, conjunctival inflammation with a prevalence of eosinophils, and an increased number of mast cells, and the presence of T-helper 2 (Th2) lymphocytes. All of these features are greatly pronounced in VKC. Among the cellular mediators and cytokines implicated in the development of allergic inflammation, tumor necrosis factor alpha (TNF-α) is one powerful proinflammatory cytokine thought to be involved in the pathogenesis of

1120-6721/606-05$02.50/0

asthma (2). Conjunctival mast cells *in vitro* have been shown to express mRNA for TNF-α and to release TNF-α protein after challenge with anti-IgE antibodies (3). Furthermore, TNF-α was found to be localized in conjunctival mast cells by immunohistochemistry (4); thus, it may be released after mast cell activation. However, the tear levels of TNF-α in patients with active SAC and VKC or after conjunctival allergen challenge have only been measured sporadically.

The aim of this study was to identify the presence of TNF-α in active allergic conjunctivitis, VKC, and the early phase of allergic reaction induced by the specific allergen challenge.

MATERIALS AND METHODS

Tear and peripheral blood samples were obtained from 12 patients with active VKC (10 male, 2 female; mean [± SD] age 11±4 years), 6 patients with active SAC (5 male, 1 female; age 32±6 years), and 12 normal subjects (8 male, 6 female; age 16±5 years) as a control group (CT). Tears were obtained from six additional male (age 38±8 years) nonactive allergic patients before and within 5 to 10 minutes after specific bilateral conjunctival provocation with grass pollen standardized extracts (Neo Abello', Spain) according to the allergen challenge standardized procedure (5). After informed consent was obtained, upper tarsal conjunctival biopsies were taken under local anesthesia from five patients with active tarsal VKC and from five normal nonatopic subjects who underwent surgery for strabismus or chalazion. Diagnosis of VKC and SAC was based on the patient's history, results of skin prick tests, the presence of itching, and, for patients with VKC, the characteristic signs of giant papillae on the upper tarsal plate or the gelatinous infiltrates and Horner-Trantas dots on the limbus. At the time of the visit for tear or tissue collection, an overall clinical estimation of disease activity (score 0 to 4) was assigned without considering permanent changes also present in the nonactive phase (i.e., the size of the papillae): 0 = non-active disease; 1 = minimal signs and symptoms; 2 = mild signs and subjectively supportable symptoms; 3 = severe signs and uncomfortable symptoms; 4 = very severe signs and symptoms. Tears (40 to 100 ml) were collected with a capillary tube, centrifuged at 800 x g for 10 minutes, and stored at –20 °C. Active patients did not receive any treatment for at least 3 days before the visit.

An enzyme-linked immunoassay was used to measure TNF-α (T Cell Diagnostics, Cambridge, MA). The sensitivity of the assay was 2 pg/ml. Samples were analyzed in duplicate.

For immunohistochemistry, cryosections were mounted on gelatin-covered slides, fixed in acetone, and incubated with the following monoclonal antibodies: anti-TNF-α 1:20 (Serotec, Oxford, UK), antitryptase 1:50 (AA-1, Dako, Denmark), anti-CD4 1:50 (Dako), antieosinophil cationic protein 1:50 (ECP; EG2, Pharmacia & Upjohn, Sweden), and anti-CD68 1:50 (Dako). After the primary incubation, slides were washed in TBS, incubated for 30 minutes with the secondary antibodies, and then treated with alkaline phosphatase complex (APAAP) (Dako). The reaction was developed with fast red solution and counterstained with Mayer hematoxylin.

Data were analyzed using the Mann-Whitney *U*-test. Results are illustrated as mean ± standard deviation (SD). The correlation between cytokine tear levels and clinical score was analyzed with the Spearman rank correlation test. For statistical significance, the assigned p value was ≤ 0.05.

RESULTS

Tear TNF-α levels were increased in 6 of 12 patients with VKC (30±76.6 pg/ml) compared to undetectable levels in the control group (Tab. I). This difference was statistically significant (p=0.03). TNF-α levels were also found to be significantly correlated with the clinical severity of VKC (p=0.04). This cytokine was not found in tear samples from patients with active SAC.

Serum TNF-α was increased only in three patients with VKC (Tab. I), while levels were below assay limits in the SAC and control groups. These differences were not statistically significant. No correlations were found between serum TNF-α levels in VKC and the clinical severity of the disease.

In the six patients challenged with allergen, tear levels of TNF-α were undetectable at baseline. Although all the patients showed a similar clinical conjunctival reaction, TNF-α was increased after challenge (108.6 pg/ml) in only one sample.

Immunohistochemistry demonstrated that the con-

TABLE I - TNF-α LEVELS IN PATIENTS WITH VERNAL KERATOCONJUNCTIVITIS

Sex/age, years	Tear TNF-α, pg/ml	Serum TNF-α, pg/ml	Clinical score (0 to 4)
M/8	31.7	0	2
M/8	0	96	4
F/9	15.6	0	3
M/18	0	0	2
M/10	0	31	3
M/11	3	0	2
M/17	0	0	2
M/17	270	0	2
M/7	4.7	0	3
F/7	36	0	4
M/14	0	19	2
M/9	0	0	2
Mean±SD	30±76	12.1±28	

TNF = Tumor necrosis factor

TABLE II - IMMUNOHISTOCHEMISTRY IN VKC TISSUES

Tissues	Mast cells	Eosinophils	Lymphocytes	Macrophages
VKC	64.8±20	184.9±63	258.0±76	154.9±35
CT	29.6±14	0.2±0.3	27.8±8	51.3 ±99
p Value	0.05	<0.01	<0.01	<0.01

VKC = Vernal keratoconjunctivitis; CT = Control tissues

junctival epithelium of patients with VKC and controls was TNF-α negative. In the conjunctival stroma, the number of cells positive for TNF-α was significantly higher in VKC compared to control tissues (46.5±12 cells/mm^2 vs 5.5±1.3 cells/mm^2; p=0.03) (Fig. 1). No extracellular staining for TNF-α was found in either VKC or normal subjects. The numbers of mast cells, lymphocytes (CD4+), eosinophils (EG2+), and macrophages (CD68+) were all significantly increased in patients with VKC compared to the control group (Tab. II).

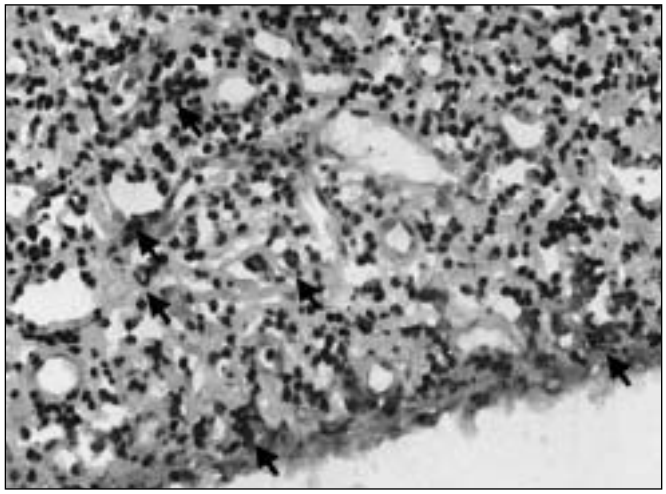

Fig. 1 - *Immunohistochemistry of conjunctival tissue from a patient with vernal keratoconjunctivitis. Scattered tumor necrosis factor-α–positive cells (arrows) can be found within the intense cellular infiltrate both in the deep stroma and in the subepithelium. Original magnification x 400.*

DISCUSSION

A relevant role of TNF-α has been suggested in the pathogenesis of both airway allergic inflammation and acquired bronchial hyperresponsiveness (2). Levels of

TNF-α have been found to increase in sputum of patients during acute allergic asthmatic episodes (6), yet tear TNF-α has been identified in only a few patients with VKC (7). In the present study, levels of TNF-α were found to be significantly increased only in tears of patients with VKC, but not in SAC or normal subjects. Compared to SAC, VKC is a more severe ocular inflammation characterized by an intense mixed cellular infiltrate with a prevalence of eosinophils, mast cells, and Th2 lymphocytes. An increased number of TNF-α positive cells was also found in VKC compared to control tissues. The morphology and distribution of cells positive for this cytokine suggest that the numerous mast cells in VKC are one possible source of TNF-α. TNF-α has been shown in a previous study to be produced by stimulated conjunctival-derived mast cells *in vitro* (3) and to be localized in mast cells of patients with allergic conjunctivitis (4). However, in the present study, high levels of TNF-α were found in only one of six patients after conjunctival allergen challenge and in none of the SAC patients. The findings of the present study are in striking contrast to those reported by Vesuloma et al in 1999 (8). In this latter study, very high TNF-α tear levels (1310 pg/ml) were found at baseline and increased even more after challenge in patients with SAC (1479 pg/ml). Because patients with signs and symptoms were eliminated from both studies by exclusion criteria, differences in assaying and tear sampling are the only possible explanations for this great discrepancy.

Nevertheless, in a clinically severe condition such as VKC, where the number of mast cells is increased, the mast cell subtype ratio is modified, and other inflammatory cell types are involved and activated, levels of TNF-α were significantly increased. The high variability observed among samples reflects the great variability in severity among patients with VKC. In addition, differences in tear flow or tear reflex among patients with VKC may have diluted TNF-α in tear fluid, contributing to the high variability in detectable tear levels. Correlation of TNF-α with the sum clinical score suggests that expression and release of this cytokine may be an aggravating factor in the pathogenesis of severe allergic ocular diseases.

These quantitative results clearly demonstrated the local production of TNF-α, but not its cell source. Mast cells, lymphocytes, macrophages, eosinophils, and epithelial cells are all possible sources (9, 10), and increased cytokine tear levels may be a result of multiple cell type activation. Being a powerful cytokine, TNF-α augments inflammatory cell influx into the mucosa by upregulating the expression of adhesion molecules on endothelial cells and subsequent recruitment and activation of inflammatory cells (11). Furthermore, TNF-α was shown to upregulate conjunctival mast cell surface receptors and cell-bound IgE (12), thereby increasing the number of FcεRI receptors on mast cells, findings that implicate TNF-α in the pathogenesis of conjunctival hypersensitivity. TNF-α has also been shown to be involved in eosinophil chemiotaxis by increasing the expression of adhesion molecule-1 and RANTES on epithelial cells (3, 13), and eotaxin in corneal (14) and conjunctival fibroblasts (15).

In conclusion, increased levels of TNF-α were found only in active VKC, suggesting a relevant role in the pathogenesis of severe chronic allergic conjunctivitis. Although it is premature to describe TNF-α as a mediator of severe allergic diseases, the dramatic beneficial results with anti-TNF-α monoclonal antibodies in the treatment of autoimmune diseases indicates that blocking a key inflammatory mediator may downregulate the entire immunologic reaction. Further studies on TNF-α may clarify an as yet unconfirmed role in other forms of allergic conjunctivitis as well.

Reprint requests to:
Andrea Leonardi, MD
Department of Neuroscience,
Ophthalmology and Ocular
Inflammation Unit
University of Padova
via Giustiniani 2
35128 Padova, Italy
andrea.leonardi@unipd.it

REFERENCES

1. Abelson MB, Schaefer K. Conjunctivitis of allergic origin: immunologic mechanisms and current approaches to therapy. Surv Ophthalmol 1993; 38 (suppl): S115-32.
2. Shah A, Church MK, Holgate ST. Tumor necrosis factor alfa: a potential mediator of asthma. Clin Exp Allergy 1995; 25: 1038-44.
3. Cook EB, Stahl JL, Miller ST, et al. Isolation of human conjunctival mast cells and epithelial cells: tumor necrosis factor-a from mast cells affects intercellular adhesion molecule 1 expression on epithelial cells. Invest Ophthalmol Vis Sci 1998; 39: 336-43.
4. MacLeod JDA, Anderson DF, Baddeley SM, Holgate ST, McGill JI, Roche WR. Immunolocalization of cytokines to mast cells in normal and allergic conjunctiva. Clin Exp Allergy 1997; 27: 1328-34.
5. Abelson MB, Chambers WA, Smith LM. Conjunctival allergen challenge. A clinical approach to studying allergic conjunctivitis. Arch Ophthalmol 1990; 108: 84-8.
6. Konno S, Gonokami Y, Kurokawa M, et al. Cytokine concentrations in sputum of asthmatic patients. Int Arch Allergy Immunol 1996; 109: 73-8.
7. Leonardi A, Borghesan F, DePaoli M, Plebani M, Secchi AG. Procollagens and inflammatory cytokine concentrations in tarsal and limbal vernal keratoconjunctivitis. Exp Eye Res 1998; 67: 105-12.
8. Vesaluoma M, Rosenberg ME, Teppo AM, Groenhagen-Riska C, Haahtela, Tervo T. Tumor necrosis factor alpha (TNFα) in tears of atopic patients after conjunctival challenge. Clin Exp Allergy 1999; 29: 537-42.
9. Finotto S, Ohno I, Marshall JS, et al. TNF-alpha production by eosinophils in upper airways inflammation (nasal polyposis). J Immunol 1994; 153: 2278-89.
10. Gamache DA, Dimitrijevich SD, Weimer LK, et al. Secretion of proinflammatory cytokines by human conjunctival epithelial cells. Ocul Immunol Inflamm 1997; 5: 117-28.
11. Pober JS, Gimbrone MA Jr, Lapierre LA, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol 1986; 137: 1893-6.
12. Stahl JL, Cook EB, Graziano FM, Barney NP. Human conjunctival mast cells. Expression of FceRI, c-kit, ICAM-1 and IgE. Arch Ophthalmol 1999; 117: 493-7.
13. Fukagawa K, Saito H, Tsubota, et al. RANTES production in a conjunctival epithelial cell line. Cornea 1997; 15: 564-70.
14. Kumagai N, Fukuda K, Ishimura Y, Nishida T. Synergistic induction of eotaxin expression in human keratocytes by TNF-α and IL-4 or IL-13. Invest Ophthalmol Vis Sci 2000; 41: 1448-53.
15. Leonardi A, Jose PJ, Zhan H, Calder VL. Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis. Ophthalmology 2003; 110: 487-92.

This paper has been selected to appear on the EJOWEB page free of charge
www.eur-j-ophthalmol.com/freearticle/index.htm