Expression of CD23/CD21 and CD40/CD40 ligand in vernal keratoconjunctivitis

AHMED M. ABU EL-ASRAR,
RASHED A. FATANI,
LUC MISSOTTEN,
KAREL GEBOES

Abstract

Purpose The overproduction of immunoglobulin E (IgE) antibodies is associated with vernal keratoconjunctivitis (VKC). CD23/CD21 and CD40/CD40 ligand (CD40L) interactions have been proposed to be involved in the regulation of IgE synthesis. The purpose of the present study was to investigate the presence and distribution of CD23, CD21, CD40 and CD40L in the conjunctiva from patients with active VKC.

Methods Conjunctival biopsy specimens from 8 subjects with active VKC and 6 control subjects were studied. We used immunohistochemical techniques and a panel of monoclonal antibodies (mAbs) directed against CD23, CD21, CD40 and CD40L. In addition, a panel of mAbs were used to characterise the composition of the inflammatory infiltrate.

Results In the normal conjunctiva, basal epithelial cells and vascular endothelial cells in the upper substantia propria showed a constitutive very weak immunoreactivity for CD40. The immunoreactivity for CD23, CD21 and CD40L was absent. In VKC specimens, the stromal inflammatory infiltrate was organised as a diffuse infiltrate and as small lymphoid follicles consisting of CD20⁺ B lymphocytes intermingled with smaller numbers of CD3⁺ T lymphocytes, and CD68⁺ monocytes/macrophages. Lymphocytes in the centre of the lymphoid follicles showed CD23 and CD21 immunoreactivity. CD40 immunoreactivity in epithelial cells and vascular endothelial cells was stronger in VKC specimens than in control eyes. Furthermore, the majority of mononuclear cells, including T and B lymphocytes, showed immunoreactivity for CD40. CD40L immunoreactivity was not detected.

Conclusion B lymphocytes in the lymphoid follicles expressing CD23, CD21 and CD40 are activated and might be precursors of IgE-producing B cells. These results suggest that the conjunctiva might contribute to IgE synthesis.

Key words Allergy, CD21, CD23, CD40, IgE, Vernal keratoconjunctivitis

Vernal keratoconjunctivitis (VKC) is an allergic, chronic seasonally exacerbated bilateral external ocular inflammation. The disease is characterised by recurrent symptoms of severe itching, photophobia, lacrimation and discharge. The overproduction of immunoglobulin E (IgE) antibodies in response to allergens forms the basis of allergic disease. Triggering of IgE-bound mast cells by allergens leads to the release of vasoactive mediators which produce clinical symptoms.¹ Increased levels of specific IgE were detected in tears from patients with VKC.^{2,3} The conjunctival tissue of patients with active VKC contained mast cells expressing membranous IgE, dendritic cells bearing IgE, and few IgE⁺ plasma cells. In addition, lymphocytes in the stromal lymphoid follicles expressed IgE. Eosinophils, basophils, B lymphocytes, plasma cells, CD4⁺ T lymphocytes expressing T-helper 2 (TH2)-type cytokines, monocytes/macrophages and dendritic cells were also found in the inflammatory infiltrate.⁴⁻¹⁰

Induction of IgE synthesis in human B cells requires two signals. The first is interleukin (IL)-4 or IL-13, which induce IgE isotype switching. The second signal is provided by a physical interaction between B cells and activated T cells, involving a number of surface and adhesion molecules. So far, several ligand pairs, such as CD40/CD40 ligand (CD40L) and CD21/CD23, have been proposed to be involved in the regulation of IgE synthesis.^{11,12} Amongst the numerous pairs of surface adhesion molecules, the CD21/CD23 pair seems to play a key role in the generation of IgE.¹¹

Although basic cellular and molecular requirements for the induction and synthesis of IgE have been extensively investigated, the mechanisms involved in the control of IgE production in VKC have not been elucidated. Because lymphoid follicles formed of B lymphocytes expressing IgE were constantly seen in the stromal inflammatory infiltrate in VKC, we hypothesised that these lymphoid

A.M. Abu El-Asrar
R.A. Fatani
Department of
Ophthalmology
College of Medicine
King Saud University
Riyadh, Saudi Arabia

L. Missotten
Department of
Ophthalmology
University Hospital St Rafael
University of Leuven
Leuven, Belgium

K. Geboes
Laboratory of
Histochemistry and
Cytochemistry
University Hospital St Rafael
University of Leuven
Leuven, Belgium

Dr Ahmed M. Abu El-Asrar,
MD, PhD ✉
Department of
Ophthalmology
King Abdulaziz University
Hospital
Airport Road
PO Box 245
Riyadh 11411, Saudi Arabia
Fax: +966 1 477 5741
e-mail:
abuasarar@KSU.edu.sa

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Table 1. Monoclonal antibodies used in this study

Antibody (clone – isotype)	Working dilution	Source ^a
CD23 (MHM6 – IgG1)	1:100	Dako
CD21 (IF8 – IgG1)	1:10	Dako
CD40 (MAB89 – IgG1)	1:100	Immunotech
CD40L (TRAP1 – IgG1)	1:10	Immunotech
CD20 (L26 – IgG2a)	1:100	Dako
CD3 (UCHT1 – IgG1)	1:400	Dako
CD68 (KP1 – IgG1)	1:1000	Dako
Anti-tryptase (AA1 – IgG1)	1:50	Dako
Anti-eosinophil peroxidase (Ab-1) (25.5 – IgG2a)	1:1000	Calbiochem

^aLocation of manufacturers: Dako, Glostrup, Denmark; Immunotech, Marseille, France; Calbiochem, Cambridge, MA, USA.

follicles are the sites of local IgE production. Given the critical role CD40/CD40L and CD21/CD23 interactions may play in human IgE regulation, *in situ* expression of CD40, CD40L, CD21 and CD23 was assessed in conjunctival biopsy specimens from patients with active VKC.

Patients and methods

Patients

Eight consecutive patients with active VKC seen at the outpatient clinic of King Abdulaziz University Hospital were included in the study. All the patients were male and their ages ranged from 10 to 17 years, with a mean of 12 years. The symptoms mentioned by all the patients were itching, redness, photophobia and tearing. Each patient underwent complete ophthalmic examination, and the corneal and conjunctival changes were noted and recorded. All patients had the limbal form of the disease characterised by broad gelatinous infiltrates of the limbus. Nasal or temporal limbal conjunctival biopsy specimens were obtained from each patient. None of the patients was on topical therapy before obtaining the biopsy. In addition, six limbal conjunctival biopsy specimens were obtained from the same areas in patients undergoing strabismus surgery without obvious inflammation and served as controls. The controls were from the same age group.

Immunohistochemical staining

The conjunctival biopsy specimens were immediately snap-frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Miles Laboratories, IN, USA) and maintained at –80 °C until use. For immunohistochemistry, 5 µm serially cut cryostat sections were dried overnight at room temperature, fixed in absolute acetone for 10 min, then treated with 2% hydrogen peroxide in methanol for 3 min to block endogenous peroxidase activity, and stained with a three-step avidin-biotinylated peroxidase-labelled complex procedure. Rehydrated slides were incubated for 30 min with the mouse monoclonal antibodies listed in Table 1 which were diluted to an optimum concentration. The sections were then incubated with the biotinylated secondary antibody and reacted with the

avidin-biotinylated peroxidase complex (Dakopatts, Copenhagen, Denmark). All incubations were carried out for 30 min at room temperature, then washed in three changes of phosphate-buffered saline at pH 7.2 for 15 min. The reaction product was visualised by incubation for 10 min in 0.05 M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethyl-carbazole and 0.01% hydrogen peroxide, resulting in bright red immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol. Control slides were treated in an identical manner, except that an irrelevant IgG mouse monoclonal antibody was used in the first step or the primary antibody was omitted. Positive controls were previously positive-staining tissue.

Results

There was no staining in the negative control slides (Fig. 1). In normal conjunctiva, a very weak focal membranous immunoreactivity for CD40 was detected on the basal epithelial cells, and on vascular endothelial cells in the upper substantia propria (Fig. 2). There was no immunoreactivity for CD23, CD21 and CD40L.

All VKC specimens showed similar findings. The epithelium was infiltrated by CD3⁺ T lymphocytes, CD68⁺ monocytes/macrophages, tryptase-positive mast cells and peroxidase-positive eosinophils. The underlying substantia propria showed a heavy inflammatory infiltrate that was concentrated just beneath the epithelium. The stromal inflammatory infiltrate was organised as a diffuse infiltrate and as small lymphoid follicles. The lymphoid follicles were composed of CD20⁺ B lymphocytes intermingled with smaller numbers of CD3⁺ T lymphocytes and a few CD68⁺ monocytes/macrophages. A ring of CD3⁺ T lymphocytes was arranged around the CD20⁺ lymphoid follicles (Fig. 3). The diffuse infiltrate was mixed in composition and consisted of CD3⁺ T lymphocytes, CD68⁺ monocytes/macrophages, tryptase-positive mast cells and peroxidase-positive eosinophils. Very few CD20⁺ B lymphocytes were detected in the diffuse infiltrate outside the lymphoid follicles.

Fig. 1. Vernal keratoconjunctivitis. Control slide, treated identically to the experimental slides except for the omission of the primary antibody, showing no labelling.

CD40 immunoreactivity on epithelial cells and vascular endothelial cells was stronger in VKC than in control eyes (Fig. 4). Furthermore, the majority of mononuclear cells in the diffuse stromal infiltrate, and lymphocytes in the lymphoid follicles, showed immunoreactivity for CD40 (Fig. 4). CD40 immunoreactivity was noted on both T and B lymphocytes in serial sections. CD40L staining was not detected. Membranous CD23 and CD21 immunoreactivity was detected only on lymphocytes in the centre of the lymphoid follicles in serial sections (Fig. 3). There was no staining for CD23 and CD21 outside the lymphoid follicles.

Discussion

In the normal conjunctiva, immunoreactivity for CD21 and CD23 was virtually non-existent. In contrast, CD21 and CD23 immunoreactivity was only detected on lymphocytes present in the lymphoid follicles in VKC. CD23, a low-affinity receptor for human IgE,^{13,14} is a type II membrane 45 kDa glycoprotein containing four highly conserved and two partially conserved cysteines with C-type lectins.¹⁵ Recently, it was shown that CD21, a 140 kDa membrane glycoprotein, is a ligand for CD23,^{16,17} CD21 and CD23 can be considered as a pair of

Fig. 2. Immunohistochemical staining for CD40 of conjunctiva from a normal control subject showing weak immunoreactivity in the basal epithelial cells and on vascular endothelial cells in the upper substantia propria.

adhesion molecules that play a crucial role in the regulation of IgE synthesis *in vitro* and *in vivo*.^{16,18–22} CD23–CD21 pairing controls IgE synthesis in an isotype-specific manner, has a role in T cell/B cell interaction which is required for IgE production in allergic individuals,^{11,12} can stimulate B cell growth and proliferation, promotes the survival of germinal centre B cells, and helps germinal centre B cells to differentiate into plasma cells.^{23–25} Several studies have demonstrated that CD23 is also involved in IgE-dependent presentation of antigen to T cells. In this way, antigen may be processed on CD23-bearing B cells by means of binding to IgE and later be presented to T cells resulting in activation and further expansion of allergen-specific TH2 cells.^{26,27}

Previous studies have clearly shown that peripheral blood lymphocytes from atopic patients with asthma and allergic rhinitis express more CD23 than those from control subjects.^{28–32} A significant positive correlation was found between the percentage of CD23⁺ lymphocytes and serum levels of total and specific IgE.^{29,30} In addition, Avunduk *et al.*³³ showed the presence of increased numbers of CD23-positive cells in tears from patients with chronic allergic conjunctivitis. Moreover, several studies have reported that the serum soluble CD23 (sCD23) levels are elevated in atopic patients with high IgE protein levels, and in patients with asthma and allergic rhinitis.^{31,32,34} Furthermore, lymphocytes expressing CD23 were increased in the skin from the patients with atopic dermatitis.³⁵ In the present study, CD23 and CD21 immunoreactivity was not detected on epithelial cells, which is in agreement with the findings of Yoshida *et al.*³⁶ in the conjunctival epithelium from normal controls and from patients with atopic dermatitis. On the other hand, other studies, using immunofluorescence staining of impression cytology specimens, reported CD23 expression by conjunctival epithelial cells from patients with chronic conjunctivitis, dry eye syndrome and after long-term treatment for glaucoma,^{37,38} and CD21 expression by normal conjunctival epithelium.³⁹ These differences might be explained by differences in techniques.

Fig. 3. Vernal keratoconjunctivitis. Serial sections illustrating immunohistochemical stainings for anti-CD20 (top left), anti-CD3 (top right), anti-CD23 (bottom left) and anti-CD21 (bottom right).

Fig. 4. Vernal keratoconjunctivitis. Immunohistochemical staining for CD40. Left: Epithelial cells (arrow) and vascular endothelium (arrowheads) express CD40. Right: Infiltrating mononuclear cells express CD40 (arrows).

The CD40 antigen is a 48 kDa type I transmembrane glycoprotein that belongs to the nerve growth factor (NGF)/tumour necrosis factor (TNF) receptor superfamily. Its ligand (CD40L) is a 39 kDa type II transmembrane glycoprotein displaying significant sequence homology to TNF.^{40,41} In the normal conjunctiva, CD40 was primarily confined to a low level of surface expression on the basal epithelial cells and vascular endothelial cells in the upper substantia propria. Our observations are in line with previous studies demonstrating the constitutive CD40 expression on cultured normal epithelial cells derived from skin (keratinocytes),^{42–44} and on resting vascular endothelial cells in culture.⁴⁵ In healthy human skin, very weak CD40 expression was noted on the basal and suprabasal layers of epidermis and on the vascular endothelium.^{42,43,45} Thymic epithelium^{46,47} and conjunctival epithelium⁴⁸ have also been shown to express CD40. Compared with normal conjunctiva, the conjunctiva from patients with VKC showed strong membranous CD40 immunoreactivity in the epithelial layer and on vascular endothelial cells. Furthermore, mononuclear cells, including T and B cells infiltrating the conjunctiva, expressed CD40. Importantly, the CD40 immunoreactivity co-localised with the enhanced expression of intercellular adhesion molecule-1 (ICAM-1) on epithelial cells and ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on vascular endothelial cells in VKC.⁸ Similarly, a significant expression of CD40 on keratinocytes as well as on the dermal mononuclear infiltrate and endothelial cells was a constant feature of inflammatory skin diseases.^{42,45} Increased expression of CD40 by conjunctival epithelial cells from patients with dry eye and chronic conjunctivitis was also reported.⁴⁸

CD40 is expressed by B cells, monocytes, dendritic cells and T cells. In recent years, the CD40 antigen has emerged as playing a key role in T-cell-dependent activation, proliferation and differentiation of B

lymphocytes.^{40,41} Furthermore, triggering of CD40 plus IL-4 or IL-13 induced IgE synthesis *in vitro* by highly purified B cells in the absence of accessory cells.^{40, 41,49} It is believed that the CD40/CD40L pair is likely to be a major co-signal required for the CD23/CD21 pairing effect on IgE synthesis.²²

CD40L is mainly expressed by activated CD4⁺ T cells.^{40,41} In the present study, CD40L was apparently not expressed by inflammatory cells present in the lymphoid aggregates or in the diffuse inflammatory infiltrate. A possible explanation is that the period during which T cells express CD40L in the presence of B cell CD40 is very short,⁵⁰ and that the protein is rapidly and transiently upregulated following CD4⁺ T cell activation via the CD3 complex.⁴⁰ Because CD40L was not expressed, it is tempting to speculate that alternative CD40 ligands may exist.⁴⁷ In this context, 4-1BB, another member of the TNF-receptor family, binds both a TNF-like molecule and extracellular matrix proteins.^{51,52}

Although the expression and function of CD40 on lymphoid cells has been well studied, the significance of CD40 on non-lymphoid cells such as epithelial cells and endothelial cells is not well characterised. Ligation of CD40 on thymic epithelial cells results in induced secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) in a process that requires the presence of IL-1 and interferon gamma (IFN- γ).⁴⁶ CD40 triggering induces enhanced secretion of IL-6, IL-8 and TNF- α by cultured keratinocytes,^{42–44} and enhances ICAM-1 upregulation on IFN- γ stimulated keratinocytes.⁴²

Signalling through CD40 on human vascular endothelial cells results in enhanced expression of ICAM-1, E-selectin and VCAM-1, which in turn facilitates leucocyte binding to endothelial cells.⁴⁵ These data suggest a role for CD40 in the regulation of inflammatory responses at mucosal sites. Our findings of enhanced CD40 immunoreactivity on conjunctival epithelial and vascular endothelial cells and its presence on mononuclear inflammatory cells demonstrates an *in vivo* association of this receptor with VKC.

In vitro studies demonstrated conspicuous homotypic aggregation of B cells before the production of IgE by activated B cells. Stimulation of resting B cells with IL-4, and CD40 mAb which mimics the effect of CD40L, results in extensive cellular aggregation and stimulates IgE production. This aggregation is predominantly mediated by ICAM-1/lymphocyte function associated antigen-1 (LFA-1) and CD23/CD21 adhesion molecules.^{18,49} Our results indicate that the *in vivo* counterpart of this *in vitro* response consists of the presence of lymphoid follicles in VKC formed of B lymphocytes, and fewer T lymphocytes. Lymphocytes in these follicles express CD23, CD21, CD40, ICAM-1 and LFA-1, and the majority of T lymphocytes in these follicles express CD4 marker.⁸ The expression of CD23 and CD21 by lymphocytes in the lymphoid follicles in VKC could allow CD4⁺ T cells to interact with B cells, a process known to be required for IgE synthesis.^{11,12} In addition, B cell-B cell homotypic interaction could lead to IgE production in a T cell independent system, since B cells expressed both CD23 and CD21 molecules.²² B cell-B cell interaction through ICAM-1 and LFA-1 was also found to regulate IgE synthesis.⁴⁹ The data presented here therefore show that the conjunctival microenvironment in VKC is able to support the formation of lymphoid follicles and give clues as to where B cell maturation and differentiation, and IgE production, could take place.

The CD23 molecule is positively and negatively regulated by cytokines which increase or decrease IgE production, respectively. IL-4 and IL-13 are known to induce IgE production by normal B cells by causing IgE isotype switching and to induce CD23 expression on B cells.^{28,53,54} On the contrary, the TH1-type cytokine, IFN- γ , inhibits the expression of CD23 on B cells and IgE production.^{28,53} The cytokine regulation of CD40 expression is dependent on the cell type. On cultured thymic epithelial cells and endothelial cells IFN- γ , IL-1 α , IL-1 β and TNF- α increased CD40 expression.^{45,46} IFN- γ and TNF- α increased the expression of CD40 by cultured conjunctival epithelial cells.⁴⁸ IFN- γ , IL-4 and IL-13 increased CD40 expression on B cells.^{55,56} CD40 expression on monocytes can be upregulated with IFN- γ , IL-3 and GM-CSF.⁵⁷ Possible sources for these regulatory cytokines in VKC are TH2 cells, mast cells, basophils and macrophages which are present in the inflammatory infiltrate.⁴⁻¹⁰

In conclusion, expression of CD23, CD21 and CD40 on lymphocytes in the lymphoid follicles indicates their activation, and that the B cells present in the lymphoid

follicles might be precursors of IgE-producing B cells in VKC. The presence of TH2-positive lymphocytes, mast cells and basophils as potentially IL-4- and IL-13-producing cells, as well as the expression of CD23, CD21 and CD40 and the increased number of B cells in VKC, provide all elements currently known to allow local IgE production in the conjunctiva. These results are consistent with our hypothesis that the lymphoid follicles may be the site of local IgE production in VKC conjunctiva. A greater understanding of the key players in IgE regulation will increase our chances of providing better medication in the future.

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