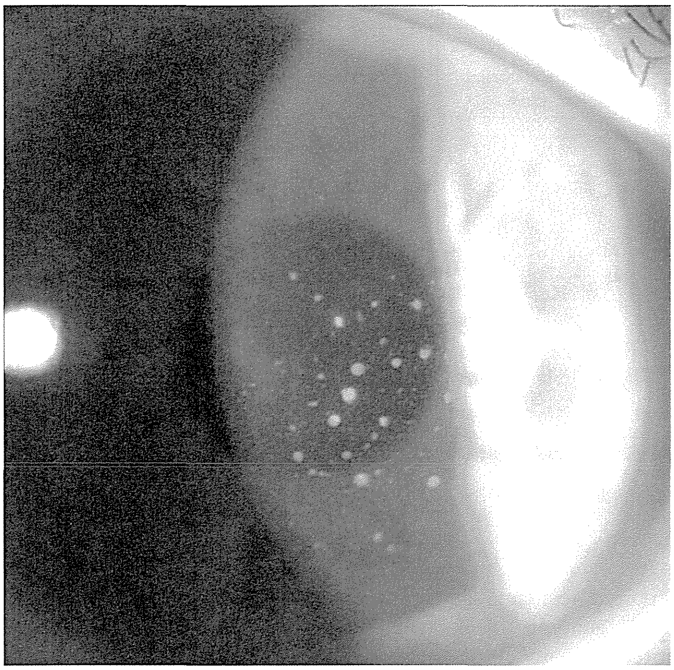

Figure 1 Case 4: Slit-lamp microscopy photo with cytomegalovirus-associated iridocyclitis. Mutton fat keratic precipitates with some pigmentation were scattered within the central area of the cornea. There was mild inflammation found within the anterior chamber.

anti-glaucoma agents effectively controlled the inflammation in the anterior segment of the eye as well as the high IOP.

Corneal endothelial cell loss

Specular microscopic examination revealed significant corneal endothelial cell loss (≥35%) in all 11 patients (table 2). Severe corneal endothelial cell loss larger than 70% was recorded in more than one-half of the endotheliitis group eyes. In contrast, this severe cell loss was observed in one of the seven patients with iridocyclitis.

There were several patients (cases 1, 8, 10 and 11; see table 2) with corneal endothelial cell counts <700 cells/mm^2. Among the patients, three cases had a low visual acuity between 0.3 and 0.6. However, one patient had a good visual acuity of 1.5.

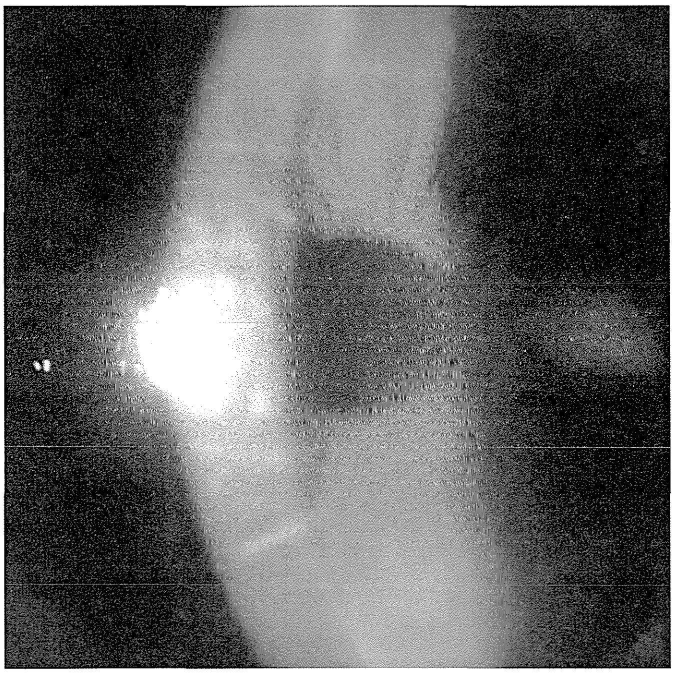

Figure 2 Case 8: Slit-lamp microscopy photo with cytomegalovirus-associated corneal endotheliitis. Diffuse corneal stromal oedema with folds in Descemet's membrane was observed.

PCR analysis of the aqueous humour samples

Multiplex PCR analyses confirmed the presence of CMV genomic DNA, but none of the other human herpes viruses (HSV-1, HSV-2, VZV, EBV, HHV-6, HHV-7 or HHV-8) in all 11 of the patients (table 2).

Quantitative real-time PCR detected significant viral loads of CMV genomic DNA in the aqueous humour of all 11 patients, with values ranging from 5.4×10^3 to 5.9×10^6 copies/ml (table 2). The mean values for the CMV viral load in the iridocyclitis and corneal endotheliitis groups were 9.4×10^5 and 1.2×10^6 copies/ml, respectively. The differences in CMV viral load between the two groups were not significant ($p=0.571$).

The corneal endothelial cell damage intensity was correlated to the CMV viral load in the aqueous humour. Results of the linear regression analysis demonstrated a positive correlation between the CMV viral load and the corneal endothelial cell loss (Spearman's correlation coefficient by rank test, $r=0.664$; $p=0.036$; figure 3).

However, there was no correlation between the interval from the disease onset to the aqueous sampling and the viral load in the aqueous humour (Spearman's correlation coefficient by rank test, $r=0.445$; $p=0.159$). Furthermore, the interval from the disease onset to the sampling was not correlated with the corneal endothelial cell damage intensity (Spearman's correlation coefficient by rank test, $r=0.373$; $p=0.239$). In addition, there was also no correlation between the viral load and many other ocular findings, such as cells and flare in the anterior chamber, types of KPs, gonioscopic findings, IOP and post-treatment BCVA.

DISCUSSION

The present study analysed ocular manifestations and CMV viral loads in the aqueous humour of patients with CMV-associated iridocyclitis and corneal endotheliitis. Our major findings included: (1) presence of significant corneal endothelial cell loss in both corneal endotheliitis and iridocyclitis tested eyes; and (2) a significant correlation between corneal endothelial cell loss and CMV viral load in the aqueous humour.

Even though it has been demonstrated that viral infections play a significant role in many inflammatory diseases, a qualitative PCR method that is capable of determining the pathological role of these viral infections has yet to be elucidated. If the presence of viral DNA in an affected disease site could be proven, the quantitative determination and correlation with the clinical manifestations of the viral infection could lead to a much deeper understanding of the role of the virus as a pathogenic disease candidate. For example, we have previously reported on two intraocular inflammatory disorders: one involving uveitis associated with human T-cell leukaemia virus type 1 (HTLV-1)[13 14] and the other involving anterior uveitis associated with VZV.[14] In HTLV-1 uveitis, a significantly higher HTLV-1 viral load was detected in the peripheral blood mononuclear cells of the patients compared with asymptomatic HTLV-1 carriers.[13] This viral load was significantly correlated with the vitreous inflammation of the disease.[14] In our report on anterior uveitis associated with VZV, we demonstrated there was a high VZV viral load within the patient's aqueous humour. Furthermore, there was a significant correlation between the viral load and the intensity of the iris atrophy in these patients.[15]

Table 2 Virological analysis and corneal endothelial cell findings in patients with CMV anterior uveitis

Case	Herpes virus DNA: CMV (copies/ml)	Herpes virus DNA: Others*	Endothelial cell count (cells/mm^2): Affected eye	Endothelial cell count (cells/mm^2): Fellow eye	Corneal endothelial cell loss (%)†	Post-treatment BCVA	Interval from onset to sampling (months)
1	2.3×10^5	-	642	2738	77	0.4	96
2	5.5×10^3	-	1633	2869	43	0.8	8
3	1.3×10^4	-	1695	2789	39	1.5	48
4	6.5×10^4	-	1618	3576	55	1.5	24
5	3.5×10^5	-	1445	2608	38	1.2	14
6	5.9×10^6	-	919	2288	45	1.2	16
7	5.4×10^3	-	2512	3917	60	1.2	6
8	1.0×10^6	-	573	2427	76	0.6	12
9	2.8×10^4	-	1427	2262	35	0.7	5
10	1.2×10^4	-	593	2092	72	0.3	4
11	3.6×10^6	-	620	2674	77	1.5	20

Using aqueous humour samples, genomic DNA of the human herpes viruses was measured by qualitative multiplex PCR and quantitative real-time PCR. Corneal endothelial cell count was examined by specular microscopy.

*Herpes viruses excluding CMV, ie herpes simplex virus type 1 and type 2, varicella zoster virus, Epstein–Barr virus, and human herpes virus types 6, 7 and 8.

†Corneal endothelial cell loss was calculated as described in the methods section.

BCVA, best-corrected visual acuity (decimal fraction); CMV, cytomegalovirus.

Although we found that there was a positive correlation between the corneal endothelial cell loss and the CMV viral load in the aqueous humour, there was no correlation between the viral load and many other ocular signs such as cells and flare in the anterior chamber, types of KPs, gonioscopic findings, IOP, post-treatment visual acuity and the interval from the disease onset to the aqueous sampling. These patients had been treated with topical corticosteroids (eg betamethasone) and anti-glaucoma agents (eg timolol and latanoprost) before they were referred to us by local ophthalmologists. These treatments are known to reduce the intensity of anterior uveitis, IOP and other ocular manifestations, but have no effect on recovering the corneal endothelial cell damage, because the corneal endothelial cell damage is barely reversible.

The cells and flare in the anterior chamber were mild in all 11 patients. A possible explanation why the intensity of the inflammatory reaction in the anterior chamber was so mild in this disease might be related to the involvement of the anterior chamber-associated immune deviation (ACAID).[16 17] In an experimental rabbit corneal endotheliitis model, eyes inoculated with inactivated HSV-1 prior to an active HSV-1 infection exhibited less severe inflammatory reactions and corneal endotheliitis. In addition, they also developed an immune deviation to HSV-1.[18] Although CMV-related ACAID has not been previously reported, real-time PCR in the present study demonstrated that CMV genomic DNA was present at high levels within the anterior chamber of the patients. Therefore, it may be that ACAID in response to CMV occurs in the eye, resulting in a relatively mild inflammatory reaction.

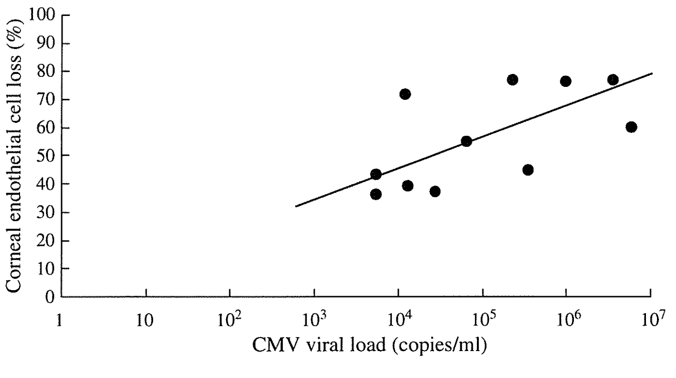

Figure 3 Correlation between cytomegalovirus (CMV) viral load and corneal endothelial cell damage. The CMV viral load was plotted on a logarithmic graph versus the corneal endothelial cell loss (%). The scatter plot shows significant correlation between the CMV viral load and the corneal endothelial cell loss (Spearman's correlation coefficient by rank test, r=0.664; p=0.036).

While our results showed CMV infection in the anterior segment of the eye caused inflammation and corneal endothelial cells loss in immunocompetent hosts, our study cannot answer many other questions. For example, why does CMV cause intraocular inflammation in immunocompetent hosts? Where does the CMV that is detected in the aqueous humour come from? And how is CMV able to cause inflammatory disorder only within the anterior segment of the eye? One possible explanation why our patients developed CMV anterior uveitis is that all our patients had been given topical corticosteroids for a long period of time. This may have contributed to induce local immunosuppressive condition in the anterior segment of the eye and resulted in reactivation of CMV.[8] Further clinical and experimental investigations are necessary to clarify these important questions.

In conclusion, significant corneal endothelial cell damage was detected in all CMV-associated iridocyclitis- and corneal endotheliitis-tested eyes. In addition, a significant correlation was found between corneal endothelial cell loss and the CMV viral load in the aqueous humour.

Competing interests None.

Ethics approval This study was conducted with the approval of the Institutional Ethics Committee of Tokyo Medical and Dental University.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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Expression of the interleukin-4 receptor α in human conjunctival epithelial cells

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Expression of the interleukin-4 receptor α in human conjunctival epithelial cells

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ABSTRACT

Aim To investigate the expression and function of interleukin-4 receptor α (*IL-4Rα*) in human conjunctival epithelial cells (HCjECs).

Methods The presence of *IL-4Rα* mRNA and protein was examined by reverse transcription (RT) PCR and immunohistology, respectively. Cell surface expression was examined by flow cytometry. The effects of interleukin (IL)-4 or IL-13 on the tyrosine phosphorylation of signal transducer and the activator of transcription 6 (STAT6) were evaluated by immunoblot analyses. The transcripts upregulated upon IL-4 stimulation were examined using GeneChip, and confirmed by quantitative RT-PCR.

Results *IL-4Rα* mRNA and protein were detected in human conjunctival epithelium. *IL-4Rα* protein was expressed on the cell surface of HCjECs. IL-4 and IL-13 induced tyrosine phosphorylation of STAT6. GeneChip analysis showed that nine transcripts were upregulated more than fourfold by IL-4 stimulation in the primary HCjECs from two individuals. Quantitative RT-PCR assay confirmed the upregulation of these transcripts: *lecithin retinol acyltransferase (LRAT), calpain (CAPN14), tumour necrosis factor alpha-induced protein 6 (TNFAIP6), RAS guanyl-releasing protein 1 (RASGRP1), endothelin receptor type A (EDNRA), hyaluronan synthase 3 (HAS3), cathepsin C (CTSC), carbonic anhydrase II (CA2)* and *cytokine-inducible SH2-containing protein (CISH)*.

Conclusions HCjECs expressed functioning *IL-4Rα*, and IL-4 stimulation induced the expression of several genes.

INTRODUCTION

Interleukin-4 receptor α (*IL-4Rα*), a component of the interleukin (IL)-4 and the IL-13 receptor, is essential for both IL-4 and IL-13 signalling. The type I IL-4 receptor is composed of two subunits, an α subunit (*IL-4Rα*), which binds IL-4 and transduces its growth-promoting and transcription-activating functions, and a γc subunit, common to several cytokine receptors, that amplifies signalling of *IL-4Rα*. The IL-13 receptor is composed of the *IL-4Rα* chain and the IL-13Rα1 chain.

IL-4R is representative of the candidate genes for allergic diseases such as atopy and asthma, which are biologically linked to T-helper type 2 (Th2) cytokine-driven inflammatory mechanisms.[1,2] Furthermore, we previously reported that IL-4R is also representative of the candidate genes for Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) with ocular complications.[3,4] Although there are many reports regarding *IL-4Rα* expression and function of human corneal fibroblasts[5–7] and human conjunctival fibroblasts,[8,9] there have been no reports of *IL-4Rα* expression in relation to the function of human ocular surface epithelial cells.

In this study, we examined the expression and function of IL-4R in the conjunctival epithelium of the human ocular surface.

MATERIALS AND METHODS

Human conjunctival epithelial cells

For reverse transcription (RT)-PCR, assay we obtained human conjunctival epithelial cells (HCjECs) from healthy volunteers by brush cytology using the previously described method.[10] Briefly, a tiny brush (Cytobrush S; Medscand AM, Malmo, Sweden) was used to scrape epithelial cells from the bulbar conjunctiva. For flow cytometric analysis, immunoblot analyses, GeneChip analysis and real-time quantitative PCR, primary HCjECs were harvested from conjunctival tissue obtained at conjunctivochalasis surgery and cultured using the previously described method.[10] Briefly, conjunctival tissues were washed and immersed for 1 h at 37°C in 1.2 U/ml purified dispase (Roche Diagnostic Ltd, Basel, Switzerland), and epithelial cells were detached, collected and cultured in low-calcium defined keratinocytes–serum free medium (SFM) with defined growth-promoting additives including insulin, epidermal growth factor, fibroblast growth factor and 1% antibiotic–antimycotic solution. By use of this method, the cell colonies usually became obvious within 3 to 4 days. After reaching 80% confluence in 7 to 10 days, the cultured HCjECs were used in subsequent procedures. When we confirmed the purity of the cells by immunohistochemical staining of vimentin, which is a marker of fibroblasts, vimentin-positive cells were scarcely found in the cultured HCjECs (Supplemental figure). For immunohistological analysis, we also used conjunctival tissue obtained at the time of conjunctivochalasis surgery. All donor specimens used in this study had no atopic history.

RT-PCR

We analysed HCjECs for *IL-4Rα* mRNA expression as described in our previous study.[11] Briefly, total RNA was isolated from HCjECs and human peripheral mononuclear cells using Trizol Reagent (Life Technologies, New York, USA) according to the manufacturer's instructions. For the RT reaction we used the SuperScript Preamplification kit (Invitrogen Corporation, Carlsbad, California, USA). PCR amplification was with DNA polymerase (cTaq; Toyobo Co., Ltd, Osaka, Japan); the conditions were 38 cycles at 95°C for 1 min, followed by 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, with final extension at 72°C for 2 min on a commercial PCR machine (GeneAmp; PE Applied Biosystems, Foster City, California, USA). The specific primers for IL-4R were 5′-GACCTGGAGCAACCCGTA TC-3′ and 5′-CATAGCACAACAGGCAGACG-3, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3. RNA integrity was assessed electrophoretically in ethidium bromide-stained 1.5% agarose gels.

Immunohistological study of *IL-4Rα* in human conjunctival sections

Serial sections of human conjunctiva were prepared from samples obtained at the time of conjunctivochalasis surgery. The sections were fixed for 30 min with methanol, and then incubated overnight in a moist chamber at 4°C with mouse anti-human *IL-4Rα* monoclonal antibody (mAb; R&D Systems, Inc., Minneapolis, Minnesota, USA) or isotype control mouse IgG2a (DakoCytomation, Kyoto, Japan), and then washed in PBS(−). Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Inc., Eugene, Oregon, USA) was applied for 1 h at room temperature; the slides were then washed, followed by the application of anti-fade mounting medium with Propidium iodide (PI) (Vectashield; Vector Laboratories, Inc., Burlingame, California, USA).

Flow cytometric analysis

Primary HCjECs were analysed for cell surface expression of *IL-4Rα* by flow cytometry as previously described.[11] Briefly, primary HCjECs were treated with 0.02% EDTA, and cells were incubated with mouse anti-human *IL-4Rα* monoclonal antibody (mAb; R&D Systems) or isotype control mouse IgG2a (DakoCytomation) for 30 min at 4°C. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes) was used as the secondary antibody. Stained cells were analysed with a FACS Calibur (Becton, Dickinson and Co., San Jose, California, USA); data were analysed using Cellquest software (Becton, Dickinson and Co.).

Immunoblot analysis of STAT6 phosphorylation

The tyrosine phosphorylation of the signal transducer and the activator of transcription 6 (STAT6) in the primary HCjECs was examined by immunoblot analysis. Primary HCjECs were incubated with 10 ng/ml IL-4 or IL-13 for various lengths of time, then washed twice and lysed by scraping in 0.2 ml of ice-cold CelLytic M Cell Lysis Reagent (Sigma-Aldrich Corp., St Louis, Missouri, USA).

Sodium dodecyl sulphate (SDS)—polyacrylamide gel electrophoresis was performed using the NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's instructions. The separated proteins were transferred to a polyvinylidene difluoride membrane using the iBlot Gel Transfer Device (Invitrogen) according to the manufacturer's instructions. The membrane was incubated in 5% skimmed milk with 0.1% Tween 20/TBS buffer for 1 h at room temperature for blocking, then subjected to immnoblot analysis with rabbit anti-phospho STAT6 or anti-STAT6 polyclonal antibodies (Cell Signalling Technology, Inc., Danvers, Massachusetts, USA) for first antibodies and HRP-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for second antibodies. For the detection of the proteins, ECL Plus Western Blotting Detection System (GE Healthcare) was used.

Gene expression analysis

Gene expression profiles were investigated using a high-density oligonucleotide probe array, GeneChip, of Human Genome U133 Plus 2.0 (Affymetrix, Inc., Santa Clara, California, USA). Total RNA was extracted by use of the Qiagen RNeasy kit (Qiagen, Valencia, California, USA). cRNA preparation and target hybridisation were done according to the Affymetrix GeneChip technical protocol. The DNA chips were scanned with a specially designed confocal scanner (GeneChip Scanner 3000; Affymetrix). Array data analysis done was with Affymetrix GeneChip operating software (GCOS) version 1.0 (Affymetrix); this software analyses image data and computes an intensity value for each probe cell. To quantitate RNA abundance, the average-difference values (ie, gene expression levels) representing the perfect match—mismatch for each gene-specific probe family were calculated, and the fold-changes in the average-difference values were determined according to Affymetrix algorithms and procedures.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems) according to previously described procedure.[11] The primers and probes were purchased from Applied Biosystems. The quantification data were normalised to the expression of the housekeeping gene *GAPDH*.

Data analysis

Data were expressed as mean±SE and evaluated by Student t test using the Excel program.

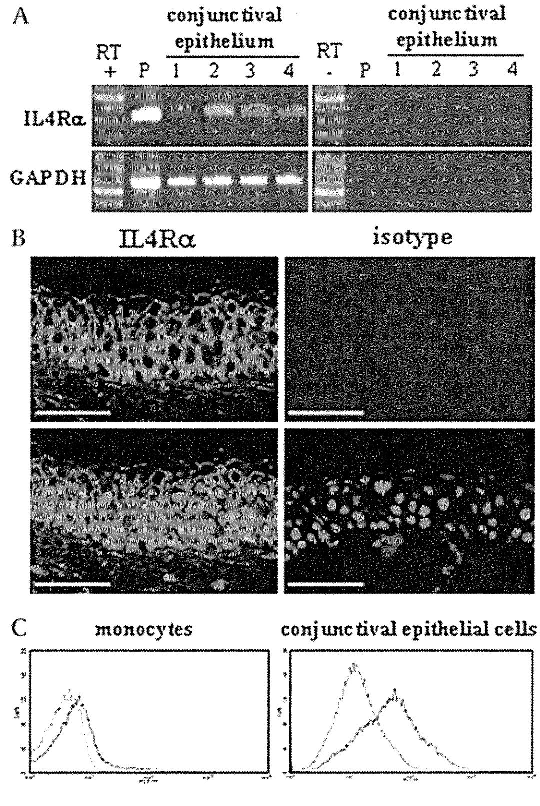

Figure 1 (A) Reverse transcription (RT) PCR analyses of the expression of *IL-4Rα*-specific mRNA in human conjunctival epithelium. As a positive control, mRNA isolated from human peripheral-blood mononuclear cells was subjected to RT-PCR (left column). RT− indicates data were obtained without RT (controls). (B) Immunohistological analysis for *IL-4Rα* in human conjunctival epithelium. Bound antibodies were visualised by Alexa Fluor 488 goat anti-mouse IgG, and the nuclei were visualised by PI staining. Each bar represents a length of 50 μm. (C) Flow cytometry analysis of cell surface expression of *IL-4Rα* in human conjunctival epithelium. The histogram data are representative of three separate experiments (solid line, *IL-4Rα* antibody; dotted line, isotype control).

RESULTS

Expression of *IL-4Rα* in human conjunctival epithelium

IL-4Rα-specific mRNA expression was present in HCjECs harvested by brush cytology from healthy volunteers who had no atopic history (figure 1A). This finding demonstrates that the *IL-4Rα* gene is constitutively expressed in normal HCjECs. We subjected conjunctival tissues to immunohistochemical study to determine the presence and localisation of *IL-4Rα* expression in stratified conjunctival epithelium. *IL-4Rα* protein was consistently and abundantly expressed at the cell membrane in human conjunctival epithelium and was located in cells from the basal to the superficial layer of the conjunctival epithelium (figure 1B). The *IL-4Rα* protein was also expressed at the cell surface by primary HCjECs harvested from conjunctival tissue at levels higher than comparable monocytes to lymphocytes (figure 1C2). Our results showed that *IL-4Rα* mRNA and protein were present in human conjunctival epithelium.

IL-4- and IL-13-induced phosphorylation of STAT6 in HCjECs

STAT proteins are activated by tyrosine phosphorylation in cells treated with cytokines. It is well known that STAT6 is activated with IL-4 or IL-13, which are ligands of *IL-4Rα*. To determine whether IL-4 and IL-13 activates STAT6 in HCjECs, we examined the effects of these cytokines on the tyrosine phosphorylation of STAT6. Primary HCjECs were incubated for various lengths of time with IL-4 (10 ng/ml) or IL-13 (10 ng/ml), then lysed and subjected to immunoblot analysis. Both IL-4 and IL-13 induced the tyrosine phosphorylation of STAT6 in a time-dependent manner (figure 2). These effects were apparent as early as 15 min after exposure to IL-4 or IL-13, and were most apparent after 60 min. Thus, these findings show that STAT6 protein is activated by IL-4 or IL-13 in HCjECs, suggesting that HCjECs express functioning *IL-4Rα*.

Upregulation of transcripts upon IL-4 stimulation

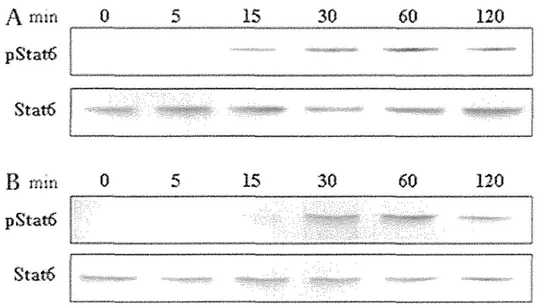

Figure 2 Effect of IL-4 (A) or IL-13 (B) on the tyrosine phosphorylation of STAT6 in human conjunctival epithelial cells. Data are representative of three independent experiments.

Moreover, to examine the function of *IL-4Rα*, we performed gene expression analysis of primary HCjECs from two individuals that were, or were not, cultured with 10 ng/ml IL-4 for 3 h using GeneChip. We found nine transcripts that were upregulated more than fourfold upon IL-4 stimulation in the primary HCjECs from two individuals (table 1). These transcripts were *lecithin retinol acyltransferase (LRAT), calpain (CAPN14), tumour necrosis factor alpha-induced protein 6 (TNFAIP6), RAS guanyl-releasing protein 1 (RASGRP1), endothelin receptor type A (EDNRA), hyaluronan synthase 3 (HAS3), cathepsin C (CTSC), carbonic anhydrase II (CA2)* and *cytokine-inducible SH2-containing protein (CISH)* (table 1). Quantitative RT-PCR assay confirmed the upregulation of these nine transcripts (figure 3).

DISCUSSION

To the best of our knowledge, this is the first study to document the expression and function of *IL-4Rα* on human conjunctival epithelium. Our results showed that *IL-4Rα* mRNA and protein were detected in human conjunctival epithelium and that *IL-4Rα* protein was expressed on the cell surface. IL-4 and IL-13, which are ligands of *IL-4Rα*, induced tyrosine phosphorylation of STAT6. Moreover, nine transcripts were confirmed to be upregulated upon IL-4 stimulation in primary HCjECs. Thus, HCjECs might express functioning *IL-4Rα*.

Others have reported that *IL-4Rα* was expressed on human corneal fibroblasts[5–7] and human conjunctival fibroblasts.[8 9] In human corneal fibroblasts, IL-4 stimulation with tumour necrosis factor (TNF)-α induced eotaxin and thymus- and activation-regulated chemokine (TARC) production.[9] In human conjunctival fibroblasts, IL-4 stimulation induced the production of extracellular matrix proteins such as collagen I, collagen III and fibronectin,[8] These reports suggest that fibroblasts play a central role in the induction and amplification of ocular allergic inflammation. On the other hand, it has been reported that IL-4 (with or without TNF-α) stimulation of human corneal epithelial cells did not induce the production of eotaxin or TARC.[9] We confirmed that IL-4 stimulation (with or without TNF-α) of HCjECs did not result in their induction (data not shown).

Our findings indicate that HCjECs express functioning *IL-4Rα* and that IL-4 stimulation could induce the expression of various

Table 1 Gene expression upregulated by IL-4 stimulation

		Ratio IL-4$^+$/IL-4$^-$	
Probe-set ID	**Gene name (gene symbol)**	**Case 1**	**Case 2**
220317_at	Lecithin retinol acyltransferase (LRAT)	52.0	5.7
1557321_a_at	Calpain 14 (CAPN14)	22.6	6.5
206026_s_at	Tumour necrosis factor, alpha-induced protein 6 (TNFAIP6)	13.0	7.0
205590_at	RAS guanyl-releasing protein 1 (RASGRP1)	9.2	5.3
204463_s_at	Endothelin receptor type A (EDRA)	7.5	4.0
204464_s_at	Endothelin receptor type A (EDRA)	7.0	4.6
223541_at	Hyaluronan synthase 3 (HAS3)	7.0	6.0
231234_at	Cathepsin C (CTSC)	6.1	6.5
209301_at	Carbonic anhydrase II (CAII)	5.7	10.6
225646_at	Cathepsin C (CTSC)	4.6	5.7
223377_x_at	Cytokine-inducible SH2-containing protein (CISH)	4.3	7.0

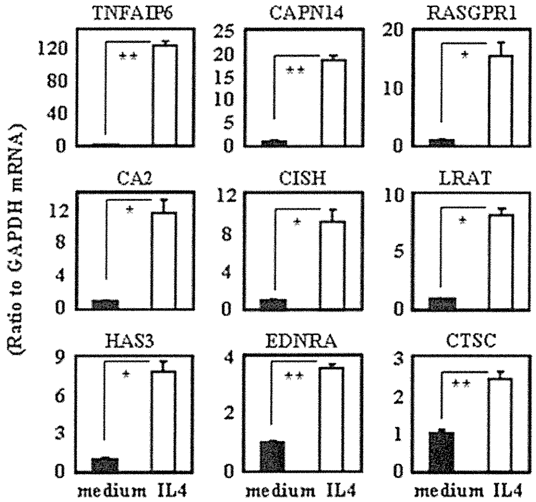

Figure 3 mRNA expression of the nine transcripts in human conjunctival epithelial cells exposed to 10 ng/ml IL-4 for 6 h. The quantification data were normalised to the expression of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. The y-axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show the mean±SEM from one experiment carried out in four wells per group (*p<0.01, **p<0.001).

genes such as *LRAT*, *CAPN14*, *TNFAIP6*, *RASGRP1*, *EDNRA*, *HAS3*, *CTSC*, *CA2* and *CISH*.

LRAT is one of the enzymes involved in vitamin A metabolism, and is reportedly upregulated by cellular differentiation in human keratinocytes.[12] RASGRP1 is one of the diacylglycerol/phorbol ester receptors, and its overexpression reportedly inhibits the expression of differentiation markers in keratinocytes.[13] EDNRA is one of the receptors of endothelin-1, and it reportedly induces epithelial—mesenchymal transition in alveolar epithelial cells.[14] HAS3 is one of the hyaluronan synthases, and hyaluronan reportedly controls epithelial proliferation and regeneration.[15] Thus, these four genes may be associated with processes related to epithelial differentiation or proliferation.

On the other hand, TNFAIP6 and CISH are anti-inflammatory molecules. TNFAIP6 is an anti-inflammatory protein present in bronchoalveolar lavage fluid from patients with asthma; its level is reportedly increased after allergen challenge.[16] CISH is one member of the suppressors of the cytokine signalling family of proteins, and is reportedly an important negative regulator for inflammatory cytokine signalling.[17]

CA2 is a one of the carbonic anhydrases and is thought to be related to the transport of fluids and ions.[18] CTSC, a member of the cathepsin family, is upregulated in bronchial biopsies tissues of asthma patients; however, its function remains unknown.[19] CAPN14 is a newly discovered member of the calpain family that functions as calcium-dependent cysteine proteases; its function is also not known at present.[20] Intriguingly, two of these nine transcripts, *TNFAIP6*[16] and *CTSC*,[19] have been reported to be upregulated in asthma patients. We speculate that their upregulation in bronchoalveolar lavage or biopsy tissues obtained from these asthma patients may be attributable to being derived from epithelial cells.

Although fibroblasts could produce chemokines such as eotaxin and TARC, and were able to augment allergic inflammation, it is possible that epithelial cells might suppress allergic inflammation via TNFAIP6 and CISH. Furthermore, conjunctival epithelial cells might contribute to the cellular differentiation or proliferation. Although the functions of *IL-4Rα* in conjunctival epithelial cells remain unclear, it is possible that epithelial cells might have different roles from fibroblasts in ocular surface inflammation. We previously showed that prostaglandin (PG) E receptor subtype EP3 is expressed on the ocular surface, and that the PGE_2—EP3 pathway in conjunctival epithelium acts as a negative regulator in allergic conjunctivitis.[21] Obviously, ocular surface epithelial cells play important roles in allergic inflammation, yet the role of conjunctival epithelial cells in allergic inflammation remains to be elucidated.

In summary, we demonstrated that HCjECs expressed functioning *IL-4Rα* and that IL-4 stimulation could induce the expression of various genes, for example, cellular differentiation-related molecule genes such as *LRAT*, *RASGRP1* and *EDNRA*, and anti-inflammatory molecule genes such as *TNFAIP6* and *CISH*.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Review Board at Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the tenets of the Declaration of Helsinki.

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Contribution of IPS-1 to polyI:C-induced cytokine production in conjunctival epithelial cells

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ABSTRACT

We previously demonstrated that ocular surface epithelium expressed TLR3 and that its ligand, polyI:C, stimulation induced the secretion of inflammatory cytokines and type I IFN. It was recently reported that RIG-I and MDA5 also recognize viral dsRNA mimicking polyI:C. In this study, we investigated whether RIG-I and/or MDA5 contribute to polyI:C-inducible responses in conjunctival epithelium. The expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelium was examined by RT-PCR and their up-regulation after polyI:C stimulation by quantitative RT-PCR and immunoblot analysis. Human conjunctival epithelial cells also expressed RIG-I, MDA5 and TLR3 mRNA and protein. The expression of RIG-I and MDA5, but not of TLR3, was markedly up-regulated upon polyI:C stimulation. We also examined the function of IPS-1 (an adaptor molecule common to RIG-I and/or MDA5) and TLR3 in conjunctival epithelium using IPS-1 KO and TLR3 KO mice. To analyze *in vivo* murine conjunctival epithelial cells, 10 μl of a 100 μg/ml polyI:C solution were delivered subconjunctivally and as eye drops, then conjunctival epithelial cells were subjected to gene expression analysis. We focused on 10 transcripts up-regulated in murine conjunctival epithelium upon polyI:C stimulation. *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2* and *Rtp4* were dominantly regulated by IPS-1, *Ccl5* by TLR3, and *Rsad2, Mx2* and *Cmpk2* were regulated by TLR3 and IPS-1. Our results showed that conjunctival epithelial cells express RIG-I and MDA5, and IPS-1, an adaptor molecule common to RIG-I and MDA5, contributes to polyI:C-inducible cytokine production in conjunctival epithelial cells.

1. Introduction

Innate immunity, the early host defense against microbes, is primarily studied in host immune-competent cells such as macrophages. The ability of cells to recognize pathogen-associated molecular patterns (PAMPs) depends on the expression of a family of Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [1]. Although TLRs detect PAMPs either on the cell surface or the lumen of intracellular vesicles such as endosomes or lysosomes, RLRs and NLRs are cytosolic detection systems for intracellular PAMPs. RLRs belong to the RNA helicase family that specifically detects RNA species derived from viruses in the cytoplasm; they coordinate anti-viral programs via the induction of type I interferon (IFN) [1].

Elsewhere we reported that human ocular surface epithelium expresses TLR3, which recognizes double-stranded (ds) RNA mimicking polyinosine–polycytidylic acid (polyI:C) (a synthetic dsRNA), and that polyI:C stimulation induces the secretion of inflammatory cytokines such as IL (interleukin)-6, IL-8, and type I IFN such as IFN-β [2,3]. Moreover, our gene expression analysis of primary human conjunctival epithelial cells (PHCjECs) using oligonucleotide microarrays to examine the comprehensive effects of stimulation by polyI:C determined that transcripts including *CXCL11, RIG-I, IL28A, CXCL10, CCL5, CCL4, MDA5, IL7R, TSLP, CCL20,* and *ICAM-1,* were up-regulated more than 10-fold by polyI:C stimulation.

Abbreviations: IFN, interferon; IPS-1, IFN-β promoter stimulator-1; PAMPs, pathogen-associated molecular patterns; TLRs, Toll-like receptors; RIG-I, retinoic acid-inducible gene-I; RLRs, retinoic acid-inducible gene-I-like receptors; NOD, nucleotide-binding oligomerization domain; NLRs, nucleotide-binding oligomerization domain-like receptors; polyI:C, polyinosinic:polycytidylic acid; PHCjECs, primary human conjunctival epithelial cells; CXCL, chemokine (C–X–C motif) ligand; CCL, chemokine (C–C motif) ligand; MDA5, melanoma-differentiation-associated gene 5; TSLP, thymic stromal lymphopoietin; ICAM-1, intercellular adhesion molecule-1; Rsad2, radical *S*-adenosyl methionine domain containing 2; Cmpk2, cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial; Mx, myxovirus (influenza virus) resistance; Ifi44, interferon-induced protein 44; Ifi203, interferon-activated gene 203; Iigp2, interferon-inducible GTPase 2; Rtp4, receptor transporter protein.

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This finding suggests that polyI:C stimulation could not only induce anti-viral innate immune responses, but that it could also regulate allergic reactions in PHCjECs [4,5].

The cytoplasmic helicase proteins, RIG-I (retinoic-acid-inducible protein I; also known as Ddx58) and MDA5 (melanoma-differentiation-associated gene 5; also known as Ifih1) have been reported to be implicated in the recognition of viral dsRNA. Our results show that new receptors that recognize dsRNA and polyI:C, RIG-I, and MDA5, are also expressed in PHCjECs [4,5].

Because, not only TLR3, but also RIG-I and MDA5 detect viral dsRNA, we examined the expression of RIG-I and MDA5 in human conjunctival epithelium, and investigated the function of IPS-1, an adaptor molecule common to RIG-I and/or MDA5, using IPS-1 KO mice to examine whether RIG-I and/or MDA5 contribute to polyI:C-inducible responses in conjunctival epithelium.

2. Materials and methods

2.1. Human conjunctival epithelial cells and PHCjECs

Our study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan; all experiments were conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients after they were given a detailed explanation of the purpose of the research and the experimental protocols.

For RT-PCR we obtained conjunctival epithelial cells from healthy volunteers by brush cytology using previously described methods [6]. For immunoblot analysis and quantitative RT-PCR, PHCjECs harvested from conjunctival tissue obtained at conjunctivochalasis surgery were cultured as described elsewhere [7]. Briefly, conjunctival tissues were washed and immersed for 1 h at 37 °C in 1.2 U ml^{-1} purified dispase (Roche Diagnostic Ltd., Basel, Switzerland), and epithelial cells were detached, collected, and cultured in low-calcium defined keratinocyte-SFM medium containing defined growth-promoting additives (Invitrogen, Carlsbad, CA) including insulin, epidermal growth factor, fibroblast growth factor, and 1% antibiotic–antimycotic solution. Using this method, cell colonies usually became visible within 3–4 days. After they reached 80% confluence in 7–10 days, the cultured PHCjECs were used in subsequent procedures.

2.2. RT-PCR assay

Total RNA was isolated from human conjunctival epithelial cells with TRIzol (Invitrogen) according to the manufacturer's instructions. For RT-PCR we employed the SuperScript™ preamplification system (Invitrogen). Amplification was with DNA polymerase (Takara, Shiga, Japan) for 35 cycles at 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min for human RIG-I, MDA5, and TLR3 (GeneAmp®; PE Applied Biosystems, Foster City, CA, USA). The primers were: (forward) 5′-CCTATGCAGCTCCGCCTCGC-3′ and (reverse) 5′-GCCACGGAACCAGCCTTCCT-3′ for human RIG-I; (forward) 5′-GCAAGAGCATCCCCGGAGCC-3′ and (reverse) 5′-TCGTGGCCCCTCCAACACCA-3′ for human MDA5; (forward) 5′-CGCCAACTTCACAAGGTA-3′ and (reverse) 5′-GGAAGCCAAGCAAAGGAA-3′ for human TLR3; and (forward) 5′-CCATCACCATCTTCCAGGAG-3′ and (reverse) 5′-CCTGCTTCACCACCTTCTTG-3 for human GAPDH. The integrity of RNA was electrophoretically confirmed on ethidium bromide-stained 1.5% agarose gels.

2.3. Immunoblot analysis of the protein expression of RIG-I, MDA5, and TLR3

Protein expression in PHCjECs was examined by immunoblot analysis using a previously described method [8]. The cells were incubated for various periods with 25 μg/ml polyI:C, washed twice, and then lysed by immersion in 0.2 ml ice-cold CelLytic M Cell Lysis reagent (Sigma–Aldrich Corp., St. Louis, MO). SDS-PAGE was on a NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's instructions. Separated proteins were transferred to a polyvinylidene difluoride membrane using the iBlot Gel Transfer device (Invitrogen) according to the manufacturer's instructions. The membrane was incubated in 5% skim milk with 0.1% Tween 20/TBS buffer for 1 h at room temperature for blocking. Immunoblot analysis was with rabbit anti-RIG-I or rabbit anti-MDA5 (Cell Signaling Technology, Inc., Danvers, MA) or rabbit anti-TLR3 antibodies (Abcam, Cambridge, MA) for first antibodies and with HRP-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for secondary antibodies. Protein detection was with the ECL Plus Western blotting Detection System (GE Healthcare).

2.4. Mice and reagents

C57BL/6 and Balb/c mice were purchased from CLEA (Tokyo, Japan); C57BL/6 background *IPS-1*$^{-/-}$ mice [9] and Balb/c background *TLR3*$^{-/-}$ mice [10] were a gift from Professor Akira. Wild-type C57BL/6 and Balb/c mice were the respective controls. All mice were used at 8–12 weeks of age; they were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan and all studies were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

PolyI:C was purchased from Invivogen (San Diego, CA). For the *in vivo* analysis of murine conjunctival epithelial cells we prepared a 100 μg/ml polyI:C solution in 50% VISCOAT® (Alcon Laboratories Ltd., Fort Worth, TX)/PBS to assure the *in vivo* stimulation by polyI:C of murine conjunctival epithelium. VISCOAT® is an ophthalmic viscosurgical product used in cataract surgery; it is a sterile, non-pyrogenic, viscoelastic solution of highly purified, non-inflammatory medium molecular-weight sodium chondroitin sulfate and sodium hyaluronate. PolyI:C solution (10 μl) was injected subcon junctivally and dropped into the eyes, respectively.

2.5. Quantitative reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from murine conjunctival epithelium and PHCjECs using the RNeasy Mini kit (QIAGEN, Valencia, CA)

Table 1
The primers for quantitative RT-PCR assay of murine samples.

Gene	Primer for quantitative RT-PCR: forward and reverse
Cxcl10	5′-TGAATCCGGAATCTAAGACCATCAA-3′ 5′-AGGACTAGCCATCCACTGGGTAAAG-3′
Mx1	5′-CTGAGATGACCCAGCACCTGAA-3′ 5′-CTCCAGGAACCAGCTGCACTTAC-3′
Rsad2	5′-TGGGGATGCTGGTGCCCACT-3′ 5′-ACCCCGGACCTGTGGCTGTT-3′
Ifi44	5′-TCGGTGCCAGCCTCGGTACA-3′ 5′-TGCAGGCACTTTTCTTGCTGCCA-3′
Iigp2	5′-TTCCCCACAGGACCCTGCCC-3′ 5′-AGCAACGGGGCAACCTGCTC-3′
Cmpk2	5′-CCTTGCGACCACCCGGACAC-3′ 5′-ATGCGCAGTCCACCTGCACC-3′
Ccl5	5′-CACCACTCCCTGCTGCTT-3′ 5′-ACACTTGGCGGTTCCTTC-3′
Rtp4	5′-GGGATGGAGGCAGCACCAGC-3′ 5′-CGTGCCTGGCCCTGCGATTT-3′
Ifi203	5′-TGTGAGAGAATTAAGGCACCAAGGAGA-3′ 5′-ACTTTCAACACCATCACTTGTTTGGGA-3′
Mx2	5′-GTGCGGCCCTGCATTGACCT-3′ 5′-GGCCACTCCAGACAGTGCTTCTAGT-3′

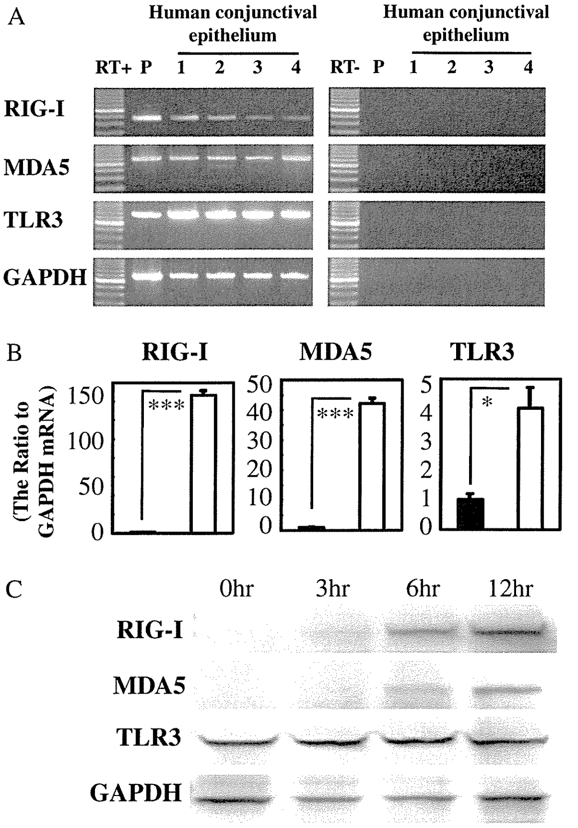

Fig. 1. Up-regulation of RIG-I, MDA5, and TLR3 expression in human conjunctival epithelial cells stimulated with polyI:C. (A) We examined the expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelial cells by RT-PCR assay. The positive control was mRNA isolated from human mononuclear cells. The expected length of PCR products for RIG-I (360 bp), MDA5 (601 bp), and TLR3 (689 bp) was obtained from the positive control and the human conjunctival epithelial cells. (B) We used quantitative RT-PCR assay to examine changes in the mRNA expression of the *RIG-I*, *MDA5*, and *TLR3* genes after the stimulation of PHCjECs with polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 3 wells per group. (C) Up-regulation by polyI:C stimulation of the protein expression of RIG-I and MDA5 in PHCjECs. Data are representative of 3 independent experiments.

according to the manufacturer's instructions. For the RT reaction we used the SuperScript™ Preamplification kit (Invitrogen). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers for the murine samples are shown in Table 1. The primers and probes for human RIG-I, MDA5, and TLR3 were purchased from Applied Biosystems. The results were analyzed with sequence detection software (Applied Biosystems). Quantification data were normalized to the expression of the housekeeping gene GAPDH.

3. Results

3.1. RT-PCR assay of the expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelial cells

The expected length of the PCR products for RIG-I (360 bp), MDA5 (601 bp), and TLR3 (689 bp) was obtained from the positive controls (mRNA isolated from human mononuclear cells) and from conjunctival epithelial cells, suggesting that human conjunctival epithelial cells express RIG-I, MDA5, and TLR3 mRNA (Fig. 1A). To confirm the specificity for the detection of RIG-I, MDA5, and TLR3 mRNA we isolated and sequenced the PCR products. The obtained sequences were identical to the human RIG-I, MDA5, and TLR3 mRNA sequence.

3.2. Up-regulation of the expression of RIG-I, MDA5 and TLR3 in polyI:C-stimulated human conjunctival epithelial cells

The expression of mRNA in*RIG-I, MDA5,* and *TLR3* genes was up-regulated by polyI:C stimulation (Fig. 1B). Up-regulation was particularly pronounced in *RIG-I* and *MDA5*. Immunoblot analysis of polyI:C-stimulated PHCjECs to examine changes in the protein expression of RIG-I, MDA5, and TLR3 showed marked up-regulation in RIG-I and MDA5 but not TLR3 (Fig. 1C).

3.3. Up-regulated transcripts in polyI:C-stimulated murine conjunctival epithelium

For gene expression analysis of polyI:C-stimulated murine conjunctival epithelium, polyI:C was delivered by eye drops and subconjunctival injection.

We selected the top 15 genes of the ratio of polyI:C-administered sample signal: vehicle-administered sample signal (data not shown) and focused on the 10 genes whose expression was confirmed to be up-regulated more than 5-fold in polyI:C-stimulated samples compared to unstimulated samples using quantitative RT-PCR (Fig. 2). They were *Rsad2* (radical *S*-adenosyl methionine domain containing 2), *Cxcl10* (chemokine (C–X–C motif) ligand 10), *Cmpk2* (cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial), *Mx1* (myxovirus (influenza virus) resistance 1), *Ifi44* (interferon-induced protein 44), *Mx2* (myxovirus (influenza virus) resistance 2), *Ifi203* (interferon-activated gene 203), *Iigp2* (interferon-inducible GTPase 2), *Rtp4* (receptor transporter protein 4), and *Ccl5* (chemokine (C–C motif) ligand 5).

3.4. Change in the expression of transcripts in murine polyI:C-stimulated conjunctival epithelium

Quantitative RT-PCR to compare the gene expression of the above 10 transcripts upon polyI:C stimulation showed that all but *Ccl5* were significantly down-regulated in the conjunctival epithelium of IPS-1 KO compared with wild-type mice (Fig. 3A).

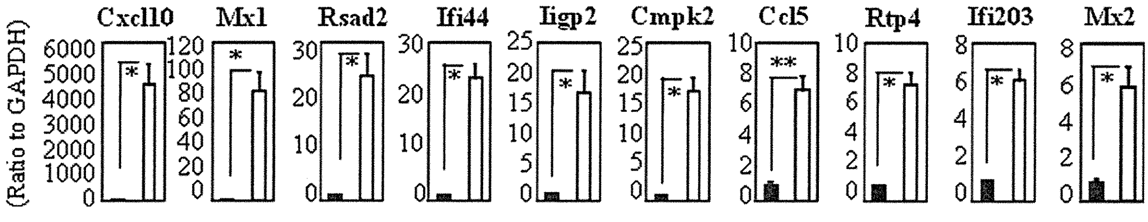

Fig. 2. Transcripts up-regulated in murine polyI:C-stimulated conjunctival epithelium. In murine conjunctival epithelium, the expression of mRNA in 10 transcripts up-regulated by 6-h stimulation with polyI:C was 5-fold higher than in unstimulated samples. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 3 mice per group ($^*p < 0.05$, $^{**}p < 0.005$).

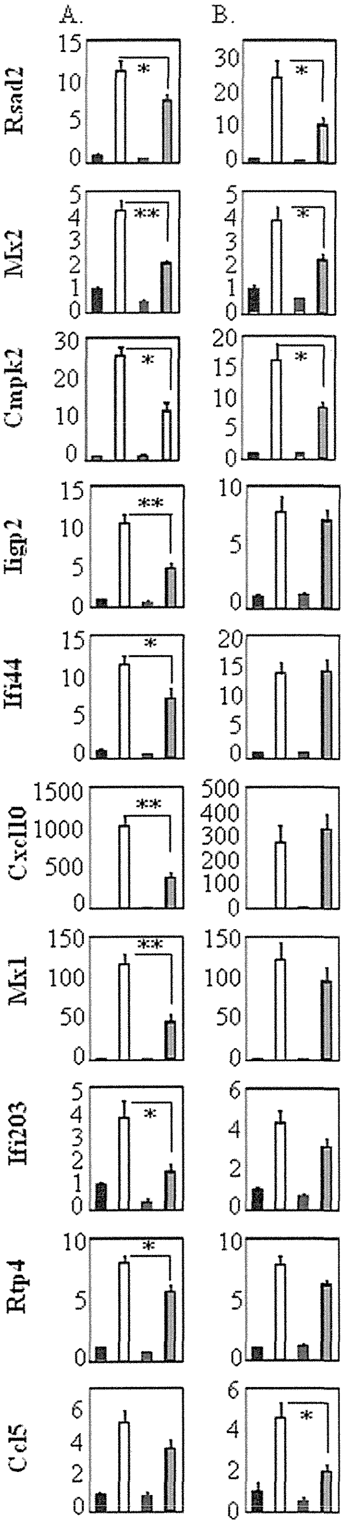

Fig. 3. Comparison of changes in the expression of transcripts induced by the stimulation of conjunctival epithelium with polyI:C in wild-type (C57BL/6)- and IPS-1 KO mice and in wild-type (Balb/c)- and TLR3 KO mice. Expression of mRNA in 10 transcripts up-regulated by 6-h stimulation of conjunctival epithelium with polyI:C in wild-type (C57BL/6)- and IPS-1 KO mice (A), and in wild-type (Balb/c)- and TLR3 KO mice (B). The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 6 mice per group ($^{*}p < 0.05$, $^{**}p < 0.005$).

PolyI:C could be recognized not only by RIG-I and MDA5, whose common adaptor molecule is IPS-1, but also by TLR3 whose adaptor molecule is TRIF. To compare the gene expression of the above 10 polyI:C-induced transcripts in wild-(Balb/c) and TLR3 KO mice, we performed quantitative RT-PCR. We found that *Rsad2, Mx2, Cmpk2*, and *Ccl5*, but not *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were significantly down-regulated in the conjunctival epithelium of TLR3 KO compared to wild-type mice (Fig. 3B).

Our results suggest that *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were dominantly regulated by RIG-I or/and MDA5, that *Ccl5* were dominantly regulated by TLR3, and that *Rsad2, Mx2*, and *Cmpk2* were regulated by both TLR3 and RIG-I or/and MDA5.

4. Discussion

Our results demonstrate that conjunctival epithelial cells express TLR3, RIG-I and MDA5, and that not only TLR3 but also RIG-I and/or MDA5 contribute to polyI:C-inducible immune responses because *Rsad2, Mx2, Cmpk2, Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were significantly down-regulated in conjunctival epithelial cells of IPS-1 knock-out compared to control mice. Moreover, *Rsad2, Mx2, Cmpk2*, and *Ccl5*, but not *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were also significantly down-regulated in the conjunctival epithelium of TLR3 KO compared to wild-type mice.

We posit that *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* are dominantly regulated by IPS-1 (RIG-I or/and MDA5), *Ccl5* by TLR3, and that *Rsad2, Mx2*, and *Cmpk2* are regulated by both TLR3 and IPS-1 (RIG-I or/and MDA5). Our studies indicate that IPS-1 signaling plays an important role in eliciting innate immune responses in conjunctival epithelial cells.

Although the TLR family detects PAMPs either on the cell surface or in the lumen of intracellular vesicles such as endosomes or lysosomes, recent studies have shown the existence of a cytosolic detection system for intracellular PAMPs [1]. These cytosolic PRRs include RLRs and NLRs. RLRs, which include RIG-I and MDA5, belong to the RNA helicase family that specifically detects RNA species derived from viruses in the cytoplasm; they coordinate anti-viral programs via the induction of type I IFN [1].

According to Lai et al. [11] following injury, TLR3 is required for part of the normal inflammatory response in the skin epithelium; TLR3-deficient mice produced significantly less IL-6 and TNF-α at the wound edge than wild-type controls. Torres et al. [12] reported that TLR3/TRIF-dependent pathway activation was required for the development of the dsRNA-induced exacerbation of allergic reactions; they were reduced in TRIF (−/−) mice. They also found that *in vivo* and *in vitro* treatment with dsRNA amplified airway epithelial cytokine production. Our findings document that *Ccl5* were dominantly regulated by TLR3, and that *Rsad2, Mx2*, and *Cmpk2* were regulated by TLR3 and IPS-1 (RIG-I or/and MDA5); *Rsad2, Mx2, Cmpk2*, and *Ccl5* were significantly down-regulated in conjunctival epithelium of TLR3 KO compared with wild-type mice. These observations document that TLR3 contributes to the production of cytokine and to inflammation.

In intestinal epithelial cells, the production and expression of polyI:C-inducible type I IFN were dependent on the activation of RIG-I and IPS-1 [13]. We found that *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were dominantly regulated by IPS-1 (RIG-I or/and MDA5), and that *Rsad2, Mx2*, and *Cmpk2* were regulated by both TLR3 and IPS-1 (RIG-I or/and MDA5); *Rsad2, Cxcl10, Mx1, Ifi44, Mx2, Cmpk2, Ifi203, Iigp2*, and *Rtp4* were significantly down-regulated in conjunctival epithelium of IPS-1 KO compared to wild-type mice.

Moreover, polyI:C incorporated by dendritic cells is accessible to both TLR3 and MDA5; they activate respective signaling pathways for the robust induction of cytokines [14]. Similarly, in conjunctival epithelial cells, polyI:C-inducible cytokine production requires signaling through both TLR3 and IPS-1 (RIG-I or/and MDA5).

Rsad2 is an interferon-inducible protein that inhibits many DNA and RNA viruses [15]. *Mx2* is also an interferon-regulated gene that selectively inhibits hanta virus replication [16]. *Cmpk2*, a pyrimidine nucleoside monophosphate kinase, is thought to be involved in macrophage activation and inflammatory responses [17].

In our study, *Rsad2*, and *Mx2*, which exert anti-viral actions, and *Cmpk2* involving in inflammatory responses, were regulated by TLR3 and IPS-1 (RIG-I or/and MDA5).

Iigp2 plays a cell-autonomous role in IFN-γ-mediated chlamydia inhibition [18] and *Ifi44* is associated with hepatitis C virus infection although its function is unknown [19]. *Cxcl10* is expressed primarily in response to a wide range of DNA and RNA viruses; it plays a role in the recruitment of leukocytes during inflammation [20]. Its expression is also increased in allergic diseases; it was elevated in the epidermis of patients with atopic dermatitis [21] and in the bronchoalveolar lavage fluid of patients with severe asthma [22]. *Mx1* is an interferon-regulated gene that selectively interferes with the multiplication of influenza viruses [23]. *Ifi203*, a member of the *Ifi-200* gene family, is induced by type I and type II interferons; it has been reported as a regulator of cell proliferation and differentiation, and plays a role in apoptotic and inflammatory processes [24]. *Rtp4* is a member of the receptor transport protein (RTP) family; it participates in the export of odorant and taste receptors [25]. We document that these 6 transcripts (*Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4*) are dominantly regulated by IPS-1 (RIG-I or/and MDA5).

Like *Cxcl10*, *Ccl5* is up-regulated in the presence of viral infection [26]; it is also up-regulated in the skin lesions of chronic atopic dermatitis [27]. Our study shows that *Ccl5*, which play a role in inflammation and allergy, were dominantly regulated by TLR3. Elsewhere we reported that TLR3 regulated the late-phase reaction of experimental allergic conjunctivitis in a mouse model; eosinophilic conjunctival inflammation was reduced in TLR3 KO mice and exacerbated in TLR3 transgenic mice [10]. These findings suggest that TLR3 can induce anti-viral innate immune responses and that it exerts other functions such as the regulation of allergic reactions. Others [28] reported that in the absence of viral infection TLR3 amplified immune responses during acute inflammatory processes, a phenomenon that may involve TLR3 stimulation by endogenous RNA from necrotic cells. Innate immunity can respond to endogenous molecules released by host cells as a result of necrosis, pathogen infection, damage, injury, and certain pathological conditions that are directly or indirectly recognized by TLRs, NLRs, RLRs, and by yet to be identified sensors [1]. It is possible that endogenous RNA from tissues or cells may stimulate not only TLR3 but also RIG-I or/and MDA5.

In summary, our results showed that conjunctival epithelial cells express RIG-I and MDA5, and IPS-1, an adaptor molecule common to RIG-I and MDA5, contributes to polyI:C-inducible cytokine production in conjunctival epithelial cells.

Acknowledgments

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Comment. Topical interferon was first described by Maskin[1] in 1994 as being effective in the treatment of ocular neoplasia. A limited number of cases in the literature also show the cytostatic effect of ATRA on ocular surface dysplasia.[4]

Our early experience with topical ATRA alone was consistent with early reports of effectiveness, with no response occurring in certain patients. Our early experience with topical interferon alfa-2b demonstrated a more consistent clinical response, and recent studies have documented an 80% treatment efficacy using topical interferon alfa-2b.[2] Mitomycin C and fluorouracil are alternative topical therapies for ocular surface dysplasia. However, interferon has fewer ocular adverse effects compared with these topical chemotherapeutic agents.[2] Retinoic acid is known to irritate the conjunctiva in higher doses.[4]

In our patient, neither ATRA nor interferon alfa-2b alone was effective in slowing growth of the ocular lesion. Longer treatment with interferon alfa-2b may have led to a better response. The rapid clinical response to the combined treatment with topical interferon alfa-2b and ATRA seems remarkable. However, previous studies have described the synergistic effects of interferon alfa-2b and ATRA in combination, both in vitro and in vivo. These same studies, although not of an eye or eye model, reported that ATRA can permit growth inhibition by interferons in interferon-unresponsive cells.[5]

Prospective studies with more patients and longer follow-up are needed to confirm the treatment efficacy and safety profile of this combination therapy as a well-tolerated alternative to topical mitomycin C and fluorouracil. Appropriate further studies may reveal a benefit for both dysplastic and neoplastic lesions.

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Prostaglandin E Receptor 4 Expression in Human Conjunctival Epithelium and Its Downregulation in Devastating Ocular Surface Inflammatory Disorders

Prostanoids are a group of lipid mediators that form in response to various stimuli, including prostaglandin (PG) D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2. There are 8 types of prostanoid receptors that are conserved in mammals ranging from mice to humans: the PGD receptor, 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor, the PGI receptor, and the thromboxane A receptor.[1] In regard to PGE receptor subtype EP4, it was reported that EP4 messenger RNA was present in the intestinal epithelium[2] and that EP4 maintained intestinal homeostasis and downregulated immune response.[3] Like the intestine, the ocular surface is also one of the mucosa that are in contact with commensal bacteria. In this study, we examined the expression of EP4 in human conjunctival epithelium and compared its expression between various ocular surface diseases.

Methods. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. For reverse transcription–polymerase chain reaction assay, we obtained human conjunctival epithelial cells from healthy volunteers by brush cytology using previously described methods.[4] The primers were (forward) 5′-TCA ACC ATG CCT ATT TCT ACA GCC ACT ACG-3′ and (reverse) 5′-AGG TCT CTG ATA TTC GCA AAG TCC TCA GTG-3′ for human *PTGER4* and (forward) 5′-CCA TCA CCA TCT TCC AGG AG-3′ and (reverse) 5′-CCT GCT TCA CCA CCT TCT TG-3′ for human *GAPDH*. For immunohistochemistry, we used nearly normal bulbar conjunctival tissues obtained during surgery for conjunctivochalasis as a control, and human conjunctival tissues were also prepared from samples obtained during surgery to reconstruct the ocular surface such as treatment for various ocular surface diseases including Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), ocular cicatricial pemphigoid (OCP), and pterygium. For EP4 staining, we used the rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan).

Results. The presence of *PTGER4* messenger RNA and EP4 protein in human conjunctival epithelium was examined by reverse transcription–polymerase chain reaction and immunohistological analysis, respectively. The *PTGER4* messenger RNA was detected in normal human conjunctival epithelium (**Figure**, A). The sequences obtained from these polymerase chain reaction products were identical to the human *PTGER4* complementary DNA sequence. The EP4 protein was also detected in the nearly normal conjunctival epithelium obtained from the patients with conjunctivochalasis (Figure, B). Next, we examined the conjunctival tissues with various ocular surface diseases. The EP4 protein was detected in conjunctival epithelium from patients with pterygium as well as in the conjunctival epithelium from

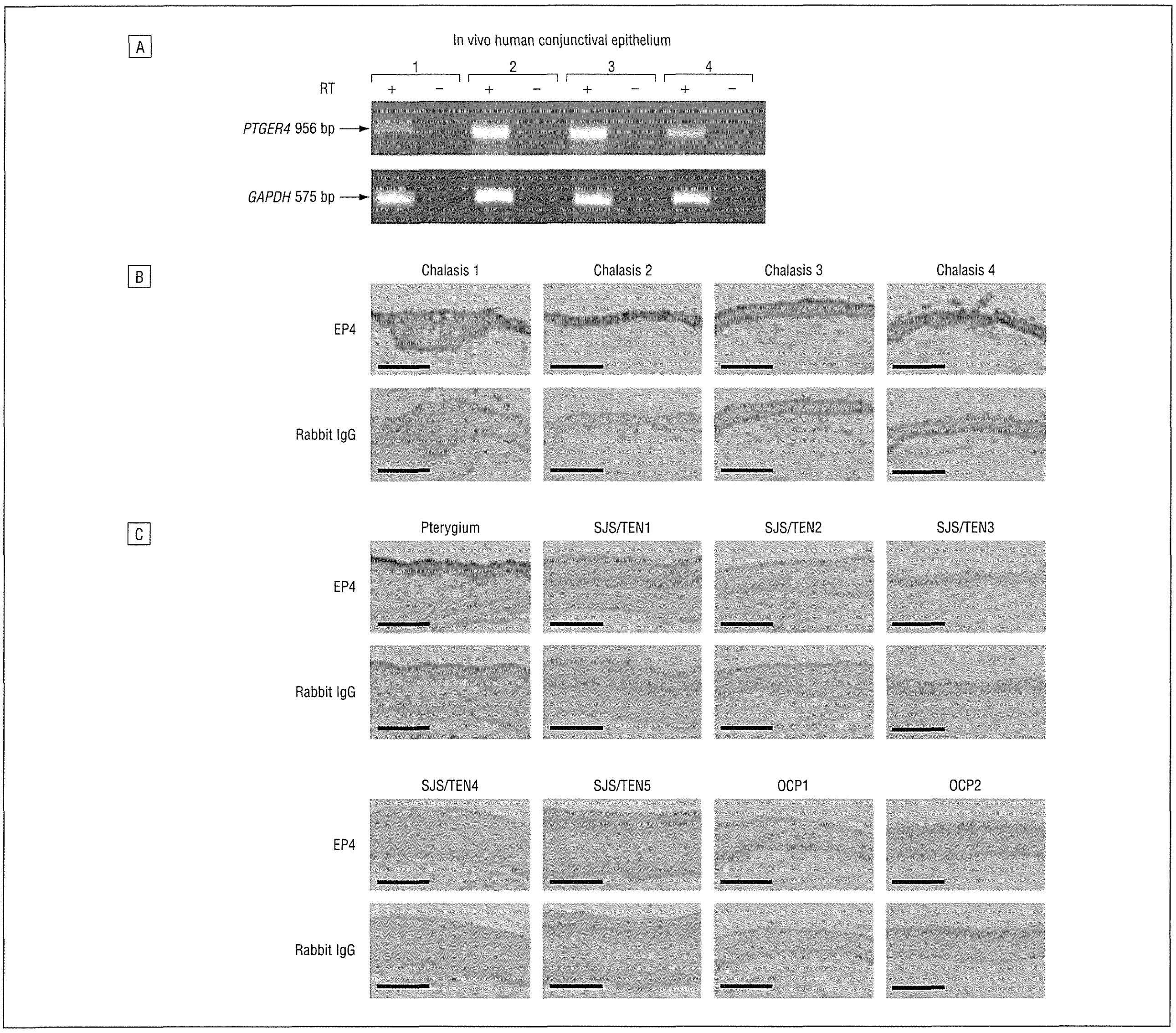

Figure. Polymerase chain reaction and immunohistological analysis results. A, Reverse transcription (RT)–polymerase chain reaction analyses of the expression of *PTGER4*-specific messenger RNA in human conjunctival epithelium derived from 4 volunteers. bp indicates base pairs. Immunohistological analysis for prostaglandin E receptor 4 (EP4) in human conjunctival epithelium in nearly normal conjunctival tissues with conjunctivochalasis (B) and in conjunctival tissues with various ocular surface diseases such as pterygium, Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), and ocular cicatricial pemphigoid (OCP) (C). Scale bars indicate 100 μm. For EP4 staining, we used the rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan) and Biotin-SP–conjugated AffiniPure F(ab′)2 fragment donkey antirabbit IgG (H + L) (Jackson Immuno Research, Baltimore, Maryland) as the secondary antibody; then, Vectastain ABC reagents (Vector Laboratories, Inc, Burlingame, California) were used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories, Inc) as a chromogenic substrate.

control patients with conjunctivochalasis. However, we did not detect EP4 immunoreactivity in the conjunctival epithelium from patients with SJS/TEN or OCP (Figure, C). Our results showed that EP4 is strongly downregulated in the conjunctival epithelium of tissues with devastating ocular surface disorders such as SJS/TEN and OCP, although it is usually expressed in human conjunctival epithelium.

Comment. To our knowledge, this is the first documentation regarding downregulation of EP4 expression in human conjunctival epithelium in tissues with devastating ocular surface inflammatory disorders, although there were reports of expression of EP receptors in ocular tissues.[5] Kabashima et al[3] reported that EP4 deficiency impaired mucosal barrier function and aggregation of neutrophils and lymphocytes in the colon and that administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis; they concluded that EP4 maintains intestinal homeostasis. On the other hand, Yao et al[6] recently reported that PGE_2 acts on its receptor EP4 on T cells and dendritic cells and promotes immune inflammation.

In human conjunctival tissues, the EP4 protein was detected in only epithelial cells but not infiltrating cells into subconjunctival tissues. Because there is mucosal inflammation on the ocular surface even in patients with chronic-phase SJS/TEN or OCP, we suspect that the downregulation of EP4 expression in conjunctival epithelium might be associated with the ocular surface inflammation in patients with SJS/TEN or OCP and that there is a possibility that EP4 in human normal conjunctival epithelium suppresses the ocular surface inflammation.

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Subconjunctival Mycetoma as an Unusual Cause of Tears With Black Deposits

Ocular mycosis is a rare condition that is usually related to ocular trauma, preexisting ocular disease, or immunocompromised states. We report a case of subconjunctival mycetoma secondary to *Exophiala dermatitidis* in a healthy middle-aged woman with recalcitrant ocular inflammation and black deposits in her tears.

Report of a Case. A 44-year-old woman had recurrent discharge from her right eye and black deposits in her tears for 2 years. Her symptoms persisted despite the use of topical antibiotics, steroids, and antihistamine. She was otherwise healthy and was not receiving any systemic or other topical medication. She denied any history of ocular trauma or surgery. She did not use contact lenses or eye makeup.

On examination, her general condition was excellent. Her visual acuity, intraocular pressure, and fundi were all normal. There was no eyelid swelling or erythema. On everting the right upper eyelid, some subconjunctival black deposits were noted (**Figure**, A). During biopsy, the conjunctiva was incised and multiple black, mulberry-like concretions extruded with mucoid discharge (Figure, B). Topical chloramphenicol, 0.5%, with dexamethasone sodium phosphate, 0.1%, eyedrops were prescribed postoperatively. Histopathological evaluation of these concretions showed large amounts of fungal hyphae (Figure, C and D) with chronic inflammation over the conjunctiva. The diagnosis was subconjunctival mycetoma. Initial culture results for fungal growth were negative, but further evaluation with 28S ribosomal RNA gene sequencing identified the causative organism as *E dermatitidis*. At subsequent follow-up visits, the patient had complete resolution of symptoms. Topical antifungal treatment was not given as she was asymptomatic and there was no recurrence of mycetoma at month 3 after débridement.

Comment. Tears with black deposits are extremely rare. In our case, we initially thought the black deposits were either foreign bodies or adrenochrome deposits, but they proved to be shedding from the subconjunctival mycetoma. Patients with tears with black deposits should therefore be evaluated for the presence of subconjunctival mycetoma. A similar clinical entity termed *melanodacryorrhea* (black tears) is caused by extraocular extension of uveal melanoma.[1]

In immunocompetent subjects, fungal infection can remain superficial and localized as illustrated in our case. Subconjunctival mycetoma has been reported after subtenon corticosteroid injection in an immunocompromised host[2] and in an immunocompetent woman with no risk factors, similar to our patient.[3] The *Exophiala* species are dematiaceous mold commonly recovered from soil, plants, water, and decaying wood materials. This strain of black yeasts has been reported to cause deep infection (especially in the lung), cutaneous infection involving skin and mucous membranes, and subcutaneous infection manifested as mycetoma.[4] *E dermatitidis* has been described as the causative agent in fungal keratitis that occurred after keratoplasty[5] and laser in situ keratomileusis,[6] but to our knowledge it has not been reported to cause subconjunctival mycetoma.

Treatments described for subconjunctival mycetoma are diverse, ranging from aggressive topical and systemic antifungal treatments following surgical intervention[2] to surgical débridement alone.[3] A study by Zeng et al[4] evaluated the activity of amphotericin B, itraconazole, voriconazole, and posaconazole against *E dermatitidis* and reported that all 4 antifungal agents have low minimum inhibitory concentrations (range, 0.03-0.5). However, data on correlation between in vitro and in vivo susceptibility are unavailable.

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REVIEW

Innate Immunity of the Ocular Surface

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Keywords: Inflammation, innate immunity, ocular surface epithelium, single-nucleotide polymorphism (SNP), Toll-like receptor (TLR)

Introduction

The ocular surface epithelium serves as the defensive front line of the innate immune system. While the detection of microbes is arguably the most important task of the immune system, an exaggerated host defense reaction to endogenous bacterial flora may initiate and perpetuate inflammatory mucosal responses.

The ocular surface epithelium can produce inflammatory cytokines such as interleukin (IL)-1α, tumor necrosis factor α, IL-6, and IL-8.[1] Therefore, ocular surface epithelium can theoretically respond to various pathogens, resulting in inflammation. At the same time, there are commensal bacteria on the ocular surface as well.[2] Although ocular surface epithelia are in constant contact with bacteria and bacterial products, a healthy ocular surface is not inflammatory. Our group has been engaged in studying pathogen recognition of the ocular surface epithelium under the assumption that it possesses a unique innate immune mechanism to regulate microbe-induced inflammation.

Recognition of Pathogen-Associated Molecular Patterns

The ability of cells to recognize pathogen-associated molecular patterns depends on the expression of a family of Toll-like receptors (TLRs).[3] The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon (IFN) α/β. For example, TLR2 recognizes lipoproteins and peptidoglycan (PGN), both components of the Gram-positive bacterial cell wall. In addition, TLR2 forms a heterodimer with either TLR1 or TLR6. TLR5 recognizes flagellin, a component of bacterial flagella. Both TLR7 and TLR8 recognize viral single-stranded RNA. TLR9 recognizes CpG DNA contained in both bacteria and viruses. TLR4 recognizes lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall. TLR3 recognizes viral double-stranded RNA.

Our group first examined whether human ocular surface epithelium expresses specific mRNA for TLRs 1–10. TLR-specific reverse transcription-polymerase chain reaction (RT-PCR) showed that mRNA from all but TLR8 was present in normal human corneal epithelium and that all TLR1–10-specific mRNA expression was present in the human conjunctival epithelium.[4,5]

One of the TLR2 ligands, PGN, is a major component of the Gram-positive bacteria cell wall. On the other hand, LPS, a TLR4 ligand, is a component of the Gram-negative bacteria cell wall. In providing an immune response, both PGN and LPS can induce the production of inflammatory cytokines such as IL-6 and IL-8. We stimulated cultured primary human corneal epithelial cells with the TLR4 ligand LPS and the TLR2 ligand PGN. However, the cells did not respond to either LPS or PGN.[6] Monocytes can phagocytose LPS intracellularly, but owing to their lack of phagocytotic activity corneal epithelial cells cannot. We therefore used the transfection agent DOTAP to force the introduction of LPS intracellularly into human corneal epithelial cells. However, even when LPS was present in the cytoplasm of the corneal epithelial cells, the cells did not respond to its introduction.[6] Yet, unlike our results, other groups report

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both TLR2 and TLR4 of the corneal epithelium of mammalian cornea responding to their ligands.[7,8]

TLR3 recognizes viral double-stranded RNA, which almost all viruses synthesize at the time of duplication. Since viral double-stranded RNA is mimicked by polyinosinic:polycytidylic acid (polyI:C), the ligand for TLR3, and we used polyI:C in our experiments to stimulate both human peripheral mononuclear cells and primary human corneal epithelial cells. In the human peripheral mononuclear cells, LPS but not polyI:C stimulation significantly increased the production of IL-6 and IL-8 (Fig. 1A). On the other hand, in human corneal epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8, whereas LPS treatment did not (Fig. 1A).[4] IFN-β is controlled by TLR3 signaling. Thus, IFN-β-specific mRNA was significantly elevated in the polyI:C-stimulated cells. However, and quite surprisingly, IFN-β-specific mRNA expression was markedly higher in human corneal epithelial cells than in human peripheral mononuclear cells (Fig. 1A).[4] PolyI:C stimulation also upregulated mRNA expression of the antiviral chemokine IFN-γ inducible protein 10 (IP-10), myxovirus resistance gene A, and 2′,5′-oligoadenylate synthetase.[9]

TLR5 recognizes flagellin, which is the bacteria flagella protein. Flagella are present mainly on Gram-negative bacteria. *Pseudomonas aeruginosa* contributes to the inflammatory response of the human corneal epithelium.[10] Ocular surface-related bacteria with flagella include *P. Aeruginosa* as a pathogen, and *Bacillus subtilis* as a nonpathogen. Third, we stimulated both human peripheral mononuclear cells and primary human corneal epithelial cells by various kinds of flagellin as the ligand of TLR5. In this experiment, flagellin derived from the ocular pathogenic *P. aeruginosa* and from the ocular nonpathogenic *B. subtilis* were used. Moreover, the intestinal, but not ocular, pathogen *Salmonella typhimurium* derived from flagellin was also used. All flagellin stimulation of the human peripheral mononuclear cells significantly increased the production of IL-6 and IL-8. On the other hand, only the ocular pathogenic *P. aeruginosa*-derived flagellin significantly induced the secretion of IL-6 and IL-8 in human corneal epithelial cells, while ocular nonpathogenic *B. subtilis*-derived flagellin and intestinal pathogenic *S. typhimurium*-derived flagellin did not induce any secretion at all.[11]

Another type of ocular surface epithelial cells, human conjunctival epithelial cells, did not secrete any IL-6 or IL-8 in response to LPS stimulation. They can secrete both IL-6 and IL-8 in response to the ocular pathogenic *P. aeruginosa*-derived flagellins only, but not to non-ocular pathogenic *S. typhimurium*-derived flagellins and ocular nonpathogenic *B. subtilis*-derived flagellins.[4]

Our immunohistochemical studies showed that TLR2, -3, and -4 proteins were located in cells ranging from the basal to the superficial layer of both the corneal and conjunctival epithelium.[1] TLR5 protein was consistently and abundantly expressed only at basal and wing sites in stratified corneal and conjunctival epithelium, indicating a spatially selective presence on the basolateral but not the apical side (Fig. 1B).[1,4,11]

Ocular surface epithelial cells respond to the flagellin derived from ocular-pathogenic bacteria through TLR5 and produce inflammatory cytokines. However, the superficial ocular surface epithelial cells do not express TLR5. Therefore, it is reasonable to speculate that TLR5 of ocular surface epithelium cannot function in a healthy ocular surface without any epithelial defect.[1,4,11]

In summary, immune competent cells such as macrophages may recognize various microbial components through TLRs, induce the inflammation, and then exclude the microbes. On the other hand, ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. The difference between macrophages and ocular surface epithelial cells might be due to the dissimilarity in the coexistence with commensal bacteria (Fig. 1C). The unique innate immune response of the ocular surface epithelium might contribute to its coexistence with commensal bacteria.[1]

Ocular Surface Inflammation

We also hypothesized that an abnormality in the proper innate immunity of the ocular surface may result in ocular surface inflammation.

IκBζ is induced by diverse pathogen-associated molecular patterns and regulates nuclear factor (NF)-κB activity.[12] Thus, IκBζ is important for TLR/IL-1 receptor signaling, which is essential for an innate immune response. IκBζ knockout (KO) mice expressly exhibit severe, spontaneous ocular surface inflammation accompanied by the eventual loss of almost all goblet cells.[13] Moreover, Balb/c-background IκBζ KO mice exhibit not only spontaneous ocular surface inflammation but also spontaneous perioral inflammation (Fig. 2).[14] IκBζ induced by diverse pathogen-associated molecular patterns regulates NF-κB activity, possibly to prevent excessive inflammation in the presence of bacterial components. The spontaneous ocular surface inflammation observed in IκBζ KO mice suggests that dysfunction/abnormality of innate immunity can play a role in ocular surface inflammation.[1]

We considered the possibility of an association between a disordered innate immune response and Stevens-Johnson syndrome (SJS), a severe ocular-surface inflammatory disease. Under the hypothesis of a disordered innate immune response in SJS, we performed gene expression analysis of monocytes, cells that are essential for innate immunity. We found differences in IL-4R gene expression; upon LPS stimulation, it was downregulated in SJS/toxic epidermal necrolysis (TEN) patients and slightly upregulated in the controls.[1] After culture for 1 h without LPS, the expression of IκBζ- and IL-1α-specific mRNA was lower in monocytes from SJS/TEN patients than in those from normal controls.[1] Our findings suggest that the reduced

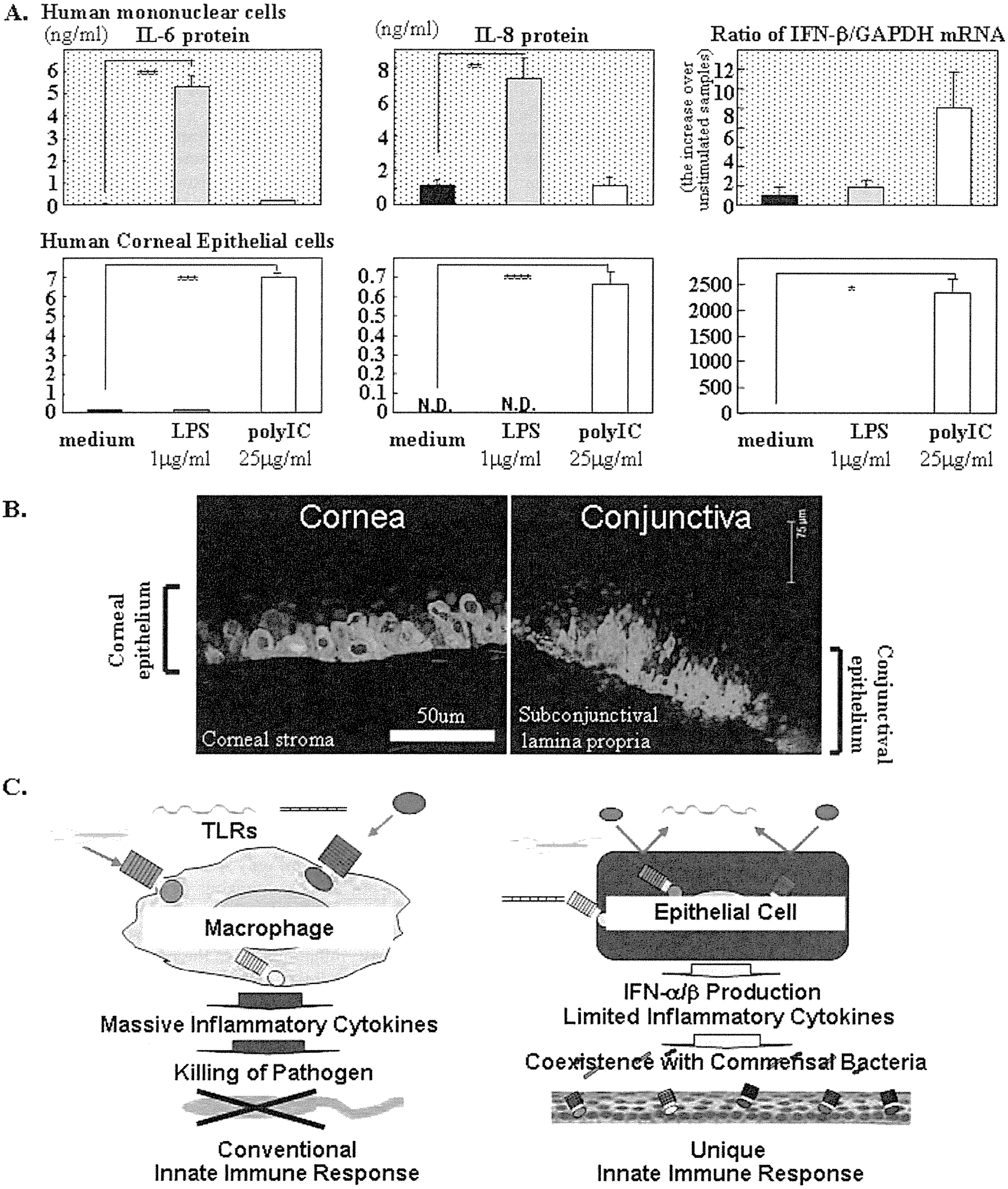

Figure 1. **A** In human peripheral mononuclear cells, lipopolysaccharide (*LPS*) but not polyinosinic:polycytidylic acid (*polyI:C*) stimulation significantly increased the production of interleukin (*IL*)-6 and IL-8. On the other hand, in human corneal epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8, while LPS treatment did not. Interferon (*IFN*)-β-specific mRNA expression was markedly higher in human corneal epithelial cells than in human peripheral mononuclear cells, although IFN-β-specific mRNA was significantly elevated in polyI:C-stimulated cells (modified with permission from M. Ueta[1,5]). **B** Human ocular surface epithelium expresses toll-like receptor (*TLR*) 5 protein. Bound antibodies were visualized by Alexa Fluor 488 donkey anti-mouse IgG and nuclei by PI staining. TLR5 was detected only at basal and wing sites of corneal and conjunctival epithelium, indicating its spatially selective presence on the basolateral but not the apical side (modified with permission from M. Ueta,[1] Kojima et al.,[4] and Hozono et al.[11]). **C** Immune competent cells such as macrophages could recognize various microbial components through TLRs and induce the inflammation and then exclude the microbes. On the other hand, ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. The difference between macrophages and ocular surface epithelial cells might be due to the dissimilarity in the coexistence with commensal bacteria. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.