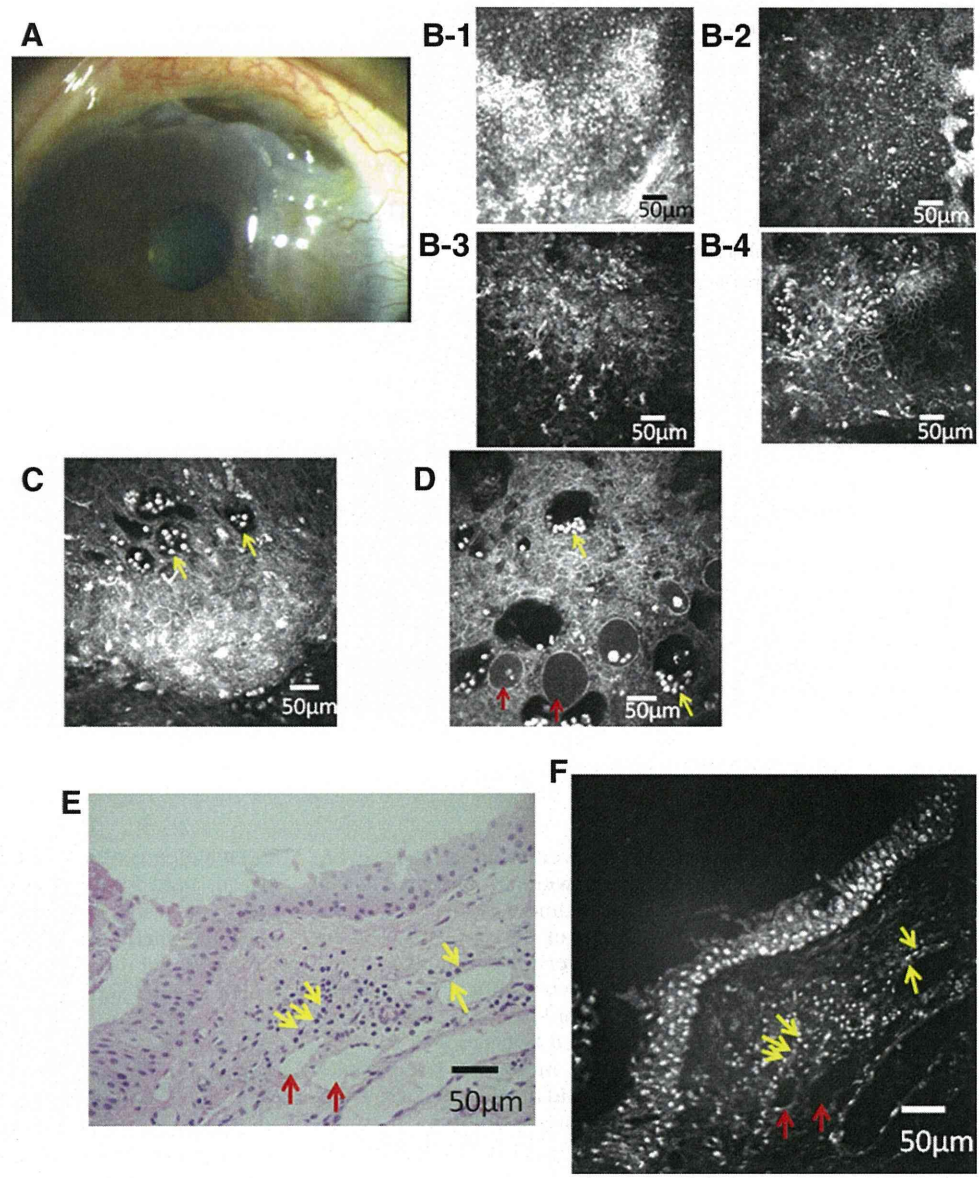

FIGURE 2. Slit lamp microscopy examination, in vivo laser scanning confocal microscopy, and histologic findings in the left eye of patient 4 (eye 5). (**A**) Corneal ulcer with 120° of limbal involvement. (**B**) Corneal findings by in vivo laser scanning confocal microscopy 1 day before corneal perforation (**B-1**, limbal side; **B-2**, central side; **B-3**, superior edge; **B-4**, inferior edge). (**C**) Dark cysts filled with inflammatory infiltrates (*yellow arrows*) in limbal subconjunctiva. (**D**) Fluid-filled cysts (*red arrows*) appeared, as well as dark cysts harboring polymorphs (*yellow arrows*). (**E**) Histologic findings in conjunctiva obtained by conjunctival excision (hematoxylin and eosin staining). (**F**) In vivo confocal microscopy finding in the same specimen shown in (**E**). Note that confocal microscopy effectively discerned features such as cysts (*red arrows*) and inflammatory cells (*yellow arrows*). Magnification: (**E**) ×100.

eration, senile furrow degeneration, ocular rosacea, and leukemia.[6] Pathologically, resected conjunctiva and limbal cornea specimens from subjects with Mooren's ulcer show a large number of plasma cells, lymphocytes, histiocytes, plasma cells, and macrophages.[2,19,20] Schaap et al.[21] reported that circulating autoantibodies in the IgG immunoglobulin class in human corneal epithelium were seen in the serum of patients with Mooren's ulcer. Brown et al.[22] demonstrated the presence of circulating antibodies in both the conjunctival and corneal epithelium in the sera of patients with Mooren's ulcer. Once the diagnosis is established, the only means of following up the course of the disease and/or the treatment responses is by a careful slit lamp examination and detection of circulating antibodies. Histopathologic specimens in patients undergoing conjunctival excision added to our understanding of the disease pathogenesis in Mooren's ulcers (Fig. 2).

The ICD of eyes at the enrollment visit, day 0, showed a strong correlation with the extent of limbal involvement with ulcers measured at the slit lamp microscopic examination (R^2 = 0.8199). Although conjunctival histopathologic alterations have been reported extensively, PubMed and MedLine searches using the key words "in vivo confocal microscopy" and "Mooren's ulcer" revealed no studies in the literature.

In vivo confocal microscopy examination in all patients with Mooren's ulcer showed variable degrees of keratoconjunctival inflammation, with a greater extent of inflammatory cell infiltrates in patients with active ulcers. As we mentioned, we counted only white round cells of 5- to 15-μm diameter. At the basal cell layer level, epithelial cell nuclei are not highly reflective, and so we believe that inflammatory cells could be differentiated from epithelial cell nuclei in that location. The inflammatory cell infiltrates consist of dendritic cells and polymorphs. Since dendritic cells may be mistaken for melanocytes,[16] we chose not to include them in the total count of inflammatory cells, which represents total polymorph densities. It should also be noted that since confocal microscopy diagnosis of an inflammatory cell is based mainly on size, we found it to be logical to refrain from specifying the type of inflammatory cells and thus collectively refer to them as polymorphs. Inflammatory cells in excision specimens from patients with Mooren's ulcers have been reported to consist of neutrophils, lymphocytes, natural killer cells, and monocytes.[20] Further confocal microscopy studies comparing inflammatory cell size and density scan information with size and density of differential inflammatory cell counts in excision specimens would provide invaluable information.

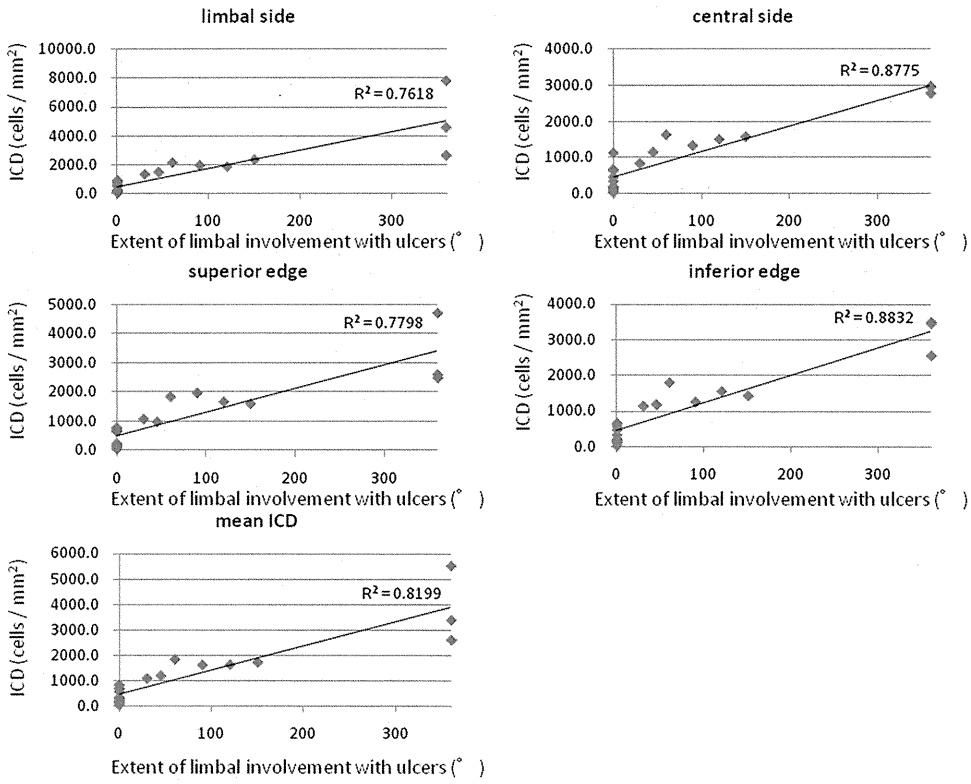

FIGURE 3. The correlation of the ICD of patients' eyes at day 0, and the extent of limbal involvement with ulcers measured by slit lamp microscopy examination. Pearson's correlation analysis revealed that the correlation was very strong.

Dark cysts harboring polymorphs were seen in six of seven patients with active ulcers. Such cysts were not observed in the patients in remission. ICD showed a time-wise decrease with treatment in all patients. The extent of decrease was less in patients 4 and 5, who eventually underwent conjunctival excision. It was noteworthy in the in vivo confocal microscopy observation performed on the day before perforation in patient 4 that the limbal conjunctiva contained two types of subconjunctival cysts: dark cysts, harboring numerous polymorphs that had presented from day 0, and fluid-filled cysts, harboring polymorphs that appeared 1 day before perforation. It is our belief that the fluid in the cysts before perforation might have been aqueous humor oozing through melting corneal tissue or serous fluid from the necrotic tissues. Observation of numerous cysts, especially the fluid-filled variety, may very well suggest imminent perforation and appears to be an important confocal microscopy finding. Since perforation in Mooren's ulcer is rare and since building up more evidence on this important finding is a challenge, observation in this perforated case, we believe, should serve as an open call to all corneal specialists who have access to in vivo confocal microscopy to pay close attention to the changes in the nature of the cysts and their relation to imminent perforation in patients with Mooren's ulcers. Ex vivo confocal microscopy examination of conjunctival histopathologic specimens showed the same architecture in the corresponding areas and revealed the intraepithelial and inflammatory cell infiltrates. ICD in confocal microscopy has been shown to correlate with the severity of ocular surface findings in Sjögren syndrome, atopic keratoconjunctivitis, and meibomian gland dysfunction.[16–18]

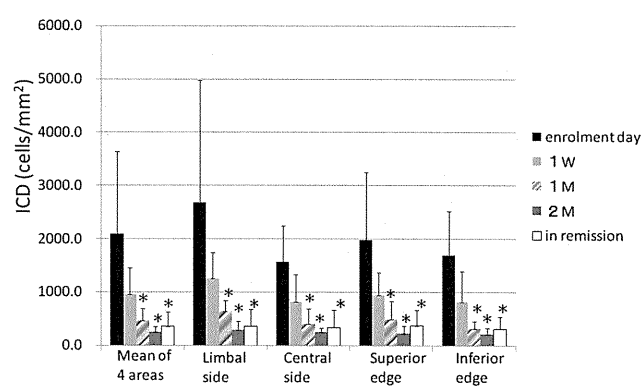

FIGURE 4. The course of ICD change in eyes with active ulcer in response to treatments, compared with the ICD in eyes in remission. Eyes 9 and 10 of patient 7 are excluded this figure, because he was lost to follow-up. The ICD of limbal side, central side, superior edge, inferior edge, and mean of these four sites, of eyes with active ulcer gradually decreased to the same level of ICD of eyes in remission with the treatment. Data are the mean ± SD of ICD values. *Statistically significant compared with the ICD at enrollment day ($P < 0.05$, Student's *t*-test).

In the present study, eyes with active Mooren's ulcer were managed with aggressive treatment including hourly topical corticosteroids, topical cyclosporine, systemic corticosteroids and cyclosporine, conjunctival excision, and/or keratoepithelioplasty. It took 4 weeks for the ICD to decrease significantly compared with day 0, and it took 8 weeks to achieve the same level of the ICD in eyes in remission.

ICD assessment adds to our armamentarium of currently existing subjective and objective diagnostic skills in judging the responsiveness of Mooren's ulcer to treatment, including evaluation of the changes in pain symptoms and slit lamp evidence of healing.

In summary, we report the first in vivo confocal scanning laser microscopy study to elucidate the keratoconjunctival alterations in Mooren's ulcer. The study provided evidence that ICD was a useful procedure in evaluating the severity of ulcers and responses to treatment. Observation of numerous limbal cysts, especially fluid-filled cysts may imply imminent perforation, which necessitates a careful follow-up of such patients.

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Prostaglandin E Receptor Subtype EP3 Expression in Human Conjunctival Epithelium and Its Changes in Various Ocular Surface Disorders

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Abstract

Background: In our earlier genome-wide association study on Stevens-Johnson Syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), we found that in Japanese patients with these severe ocular surface complications there was an association with prostaglandin E receptor 3 (EP3) gene (*PTGER3*) polymorphisms. We also reported that EP3 is dominantly expressed in the ocular surface-, especially the conjunctival epithelium, and suggested that EP3 in the conjunctival epithelium may down-regulate ocular surface inflammation. In the current study we investigated the expression of EP3 protein in the conjunctiva of patients with various ocular surface diseases such as SJS/TEN, chemical eye burns, Mooren's ulcers, and ocular cicatricial pemphigoid (OCP).

Methodology/Principal Findings: Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to SJS/TEN, chemical eye burns, and OCP, and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva. The controls were nearly normal human conjunctival tissues acquired at surgery for conjunctivochalasis. We performed immunohistological analysis of the EP3 protein and evaluated the immunohistological staining of EP3 protein in the conjunctival epithelium of patients with ocular surface diseases. EP3 was expressed in the conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer and in normal human conjunctival epithelium. However, it was markedly down-regulated in the conjunctival epithelium of SJS/TEN and OCP patients.

Conclusions: We posit an association between the down-regulation of EP3 in conjunctival epithelium and the pathogenesis and pathology of SJS/TEN and OCP, and suggest a common mechanism(s) in the pathology of these diseases. The examination of EP3 protein expression in conjunctival epithelium may aid in the differential diagnosis of various ocular surface diseases.

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Introduction

Prostanoids are comprised of prostaglandins (PGs) and thromboxanes (TXs). They are lipid mediators that form in response to various stimuli and include PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. They are released extracellularly immediately after their synthesis and they act by binding to a G-protein-coupled rhodopsin-type receptor on the surface of target cells. There are 8 types of prostanoid receptors that are conserved in mammals from mouse to human: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP) [1].

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN) are acute inflammatory vesiculobullous reactions of the skin and mucosa including the ocular surface [2]. In our earlier genome-wide association study in Japanese SJS/TEN patients with severe ocular surface complications we found associations with 6 single nucleotide polymorphisms (SNPs) in the prostaglandin E receptor 3 (EP3) gene (*PTGER3*) and we documented that compared with the controls, EP3 expression was markedly reduced in the conjunctival epithelium of SJS/TEN patients with severe ocular complications [3]. Others reported that the PGE_2-EP3 signaling pathway negatively regulates allergic reactions in a murine allergic asthma model [4] and that it inhibits keratinocyte activation and exerts anti-inflammatory actions in mouse contact hypersensitivity [5]. We also showed that EP3 is dominantly expressed in the ocular surface-, especially the conjunctival epithelium, and that PGE_2 acts as a ligand for EP3 in the conjunctival epithelium and down-regulates the progression of murine experimental allergic conjunctivitis [6]. In addition, we reported that an EP3 agonist suppressed the production of CCL5, CXCL10, CXCL11, and IL-6 in response to polyI:C stimulation

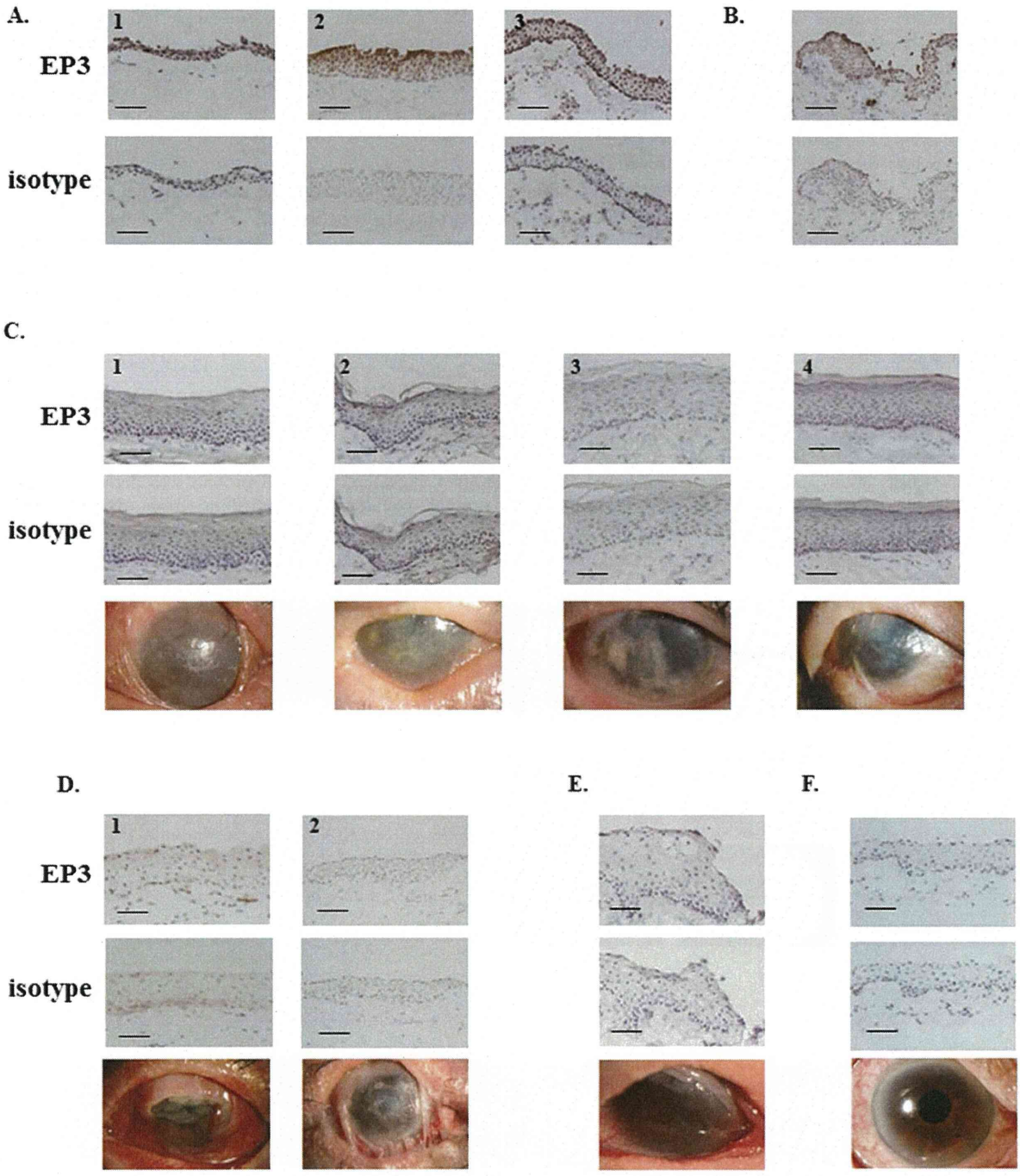

Figure 1. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of the controls and SJS/TEN patients. A. Nearly normal conjunctival tissues from patients with conjunctivochalasis. B. Normal conjunctival tissue. C. Keratinized conjunctival tissues of SJS/TEN patients in the chronic stage. D. Non-keratinized conjunctival tissues of SJS/TEN patients in the sub-acute stage. E. Non-keratinized conjunctival tissues of SJS/TEN patients in the chronic stage. F. Visibly normal conjunctival tissue of an SJS/TEN patient with minor ocular sequelae (dry eye). C-F. The 3rd lane shows the ocular surface of SJS/TEN patients. Each scale bar represents a length of 100 μm.
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of human conjunctival epithelial cells, suggesting that EP3 in the conjunctival epithelium may down-regulate ocular surface inflammation [7].

In the current study we investigated the expression of EP3 protein in the conjunctiva of patients with various ocular surface diseases such as SJS/TEN, chemical eye burns, Mooren's ulcers, and ocular cicatricial pemphigoid (OCP).

Materials and Methods

Human conjunctival tissues

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experiments were conducted in accordance with the principles set forth in the Helsinki Declaration.

Our immunohistochemistry controls were 3 nearly normal human conjunctival tissues acquired at surgery for conjunctivochalasis and one sample of normal conjunctival tissue acquired at limbal dermoid resection. Conjunctival tissues were also obtained from patients undergoing surgical reconstruction of the ocular surface due to SJS/TEN (n = 7), chemical eye burns (n = 3), OCP (n = 3), severe graft versus host disease (GVHD) (n = 1), pseudo-OCP (n = 1) and pterygium (PTG) (n = 1), from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva (n = 4), and from a patient with a giant papilla due to allergic vernal conjunctivitis. One conjunctival tissue sample was obtained from an SJS/TEN patient who did not require ocular surface reconstruction because ocular sequelae were minor (dry eye); this sample derived from additional unnecessary conjunctiva harvested just after cataract surgery.

Immunohistochemistry

For EP3 staining we used rabbit polyclonal antibody to EP3 (Cayman Chemical Co., Ann Arbor, MI) [3,6]. We previously checked and confirmed the EP3 specificity of this antibody using conjunctiva from EP3KO mice [6]. Further confirmation was by immunoblot analysis (Fig. S1). The secondary antibody (Biotin-SP-conjugated AffiniPure F(ab')$_2$ fragment donkey anti-rabbit IgG (H+L), 1:500 dilution; Jackson Immuno Research, Baltimore, MD) was applied for 30 min, then VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was added for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Evaluation of staining intensity using ImageJ software and down-regulation score

We converted the multi-color pictures into black and white pictures, and measured the gray value in the vertical line of the conjunctival epithelium. Then we recorded the average gray value on an intensity score from 5 to 16 (e.g. an average gray value of 100 was scored as 10). We also recorded the degree of down-regulation where "–" = intensity score 12–16, "+" = intensity score 8–11, and "++" = intensity score 5–7.

Results

As reported elsewhere [3], EP3 protein was detected in the nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 1A) and in the normal conjunctival

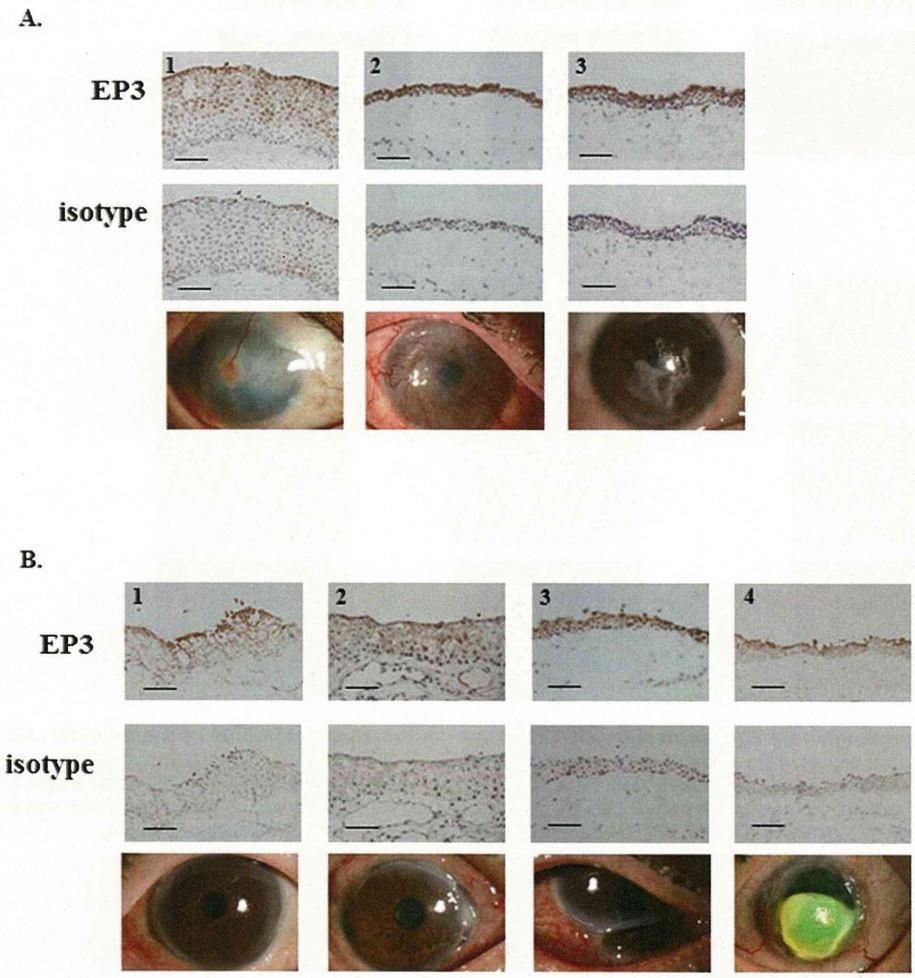

Figure 2. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with chemical eye burn and active Mooren's ulcer. A. Conjunctival tissues of patients with chemical eye burn requiring ocular surface reconstruction. B. Inflammatory conjunctival tissues of patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva. The 3rd lane shows the ocular surface of patients. Each scale bar represents a length of 100 μm.
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epithelium sample (Fig. 1B), but not in keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage (Fig. 1C). When we examined non-keratinized conjunctival epithelium from SJS/TEN patients in the sub-acute- or chronic stage (Figs. 1D, 1E) we found that EP3 was markedly down-regulated. Interestingly, even in the conjunctival epithelium from the SJS/TEN patient manifesting only dry eye, EP3 was greatly down-regulated (Fig. 1F).

Comparison with conjunctival tissues from patients with chemical eye burn showed that although ocular surface findings were similar, EP3 protein was detected in the conjunctival epithelium of 3 patients with chemical eye burn as well as in control conjunctival epithelium from conjunctivochalasis patients (Fig. 2A). We also detected EP3 protein in conjunctival epithelium from 4 patients with Mooren's ulcer, however, it appeared to be somewhat down-regulated (Fig. 2B).

Next we examined conjunctival tissues from 3 patients with OCP; their ocular surface findings were very similar to those of SJS/TEN patients. No EP3 protein was detected in conjunctival epithelium from any of these patients (Fig. 3A), nor in conjunctival epithelium from a GVHD patient with severe conjunctival invasion to the cornea (Fig. 3B). When we assessed tissues from patients with pterygium (Fig. 3C), or pseudo-OCP (Fig. 3D), we detected EP3 protein in the conjunctival epithelium of pterygium patients as we did in the control conjunctival epithelium from a patient with conjunctivochalasis. EP3 protein was also present in conjunctival epithelium from patients with pseudo-OCP although it appeared to be slightly down-regulated. We also found EP3 protein in the conjunctival epithelium of a patient with giant papillae due to chronic allergic keratoconjunctivitis (Fig. 3E). In Table 1 we show the scores obtained by our evaluation of the staining intensity and degree of down-regulation for all samples.

We document that EP3 was expressed in conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer and in normal human conjunctival epithelium. It was markedly down-regulated in the conjunctival epithelium of SJS/TEN- and OCP patients. Although we had only one patient each with severe GVHD, pterygium, pseudo-OCP, and chronic allergic keratoconjunctivitis, study of these samples suggested that EP3 is expressed in the conjunctival epithelium of patients with pterygium, pseudo-OCP, and chronic allergic keratoconjunctivitis, and that EP3 might be greatly down-regulated in the conjunctival epithelium of patients with severe GVHD.

Regarding in conjunctival epithelium, the expression of EP3 protein in the SJS/TEN and OCP patients was markedly decreased compared with normal conjunctiva. However, its expression in sub-conjunctival tissues may be up-regulated in

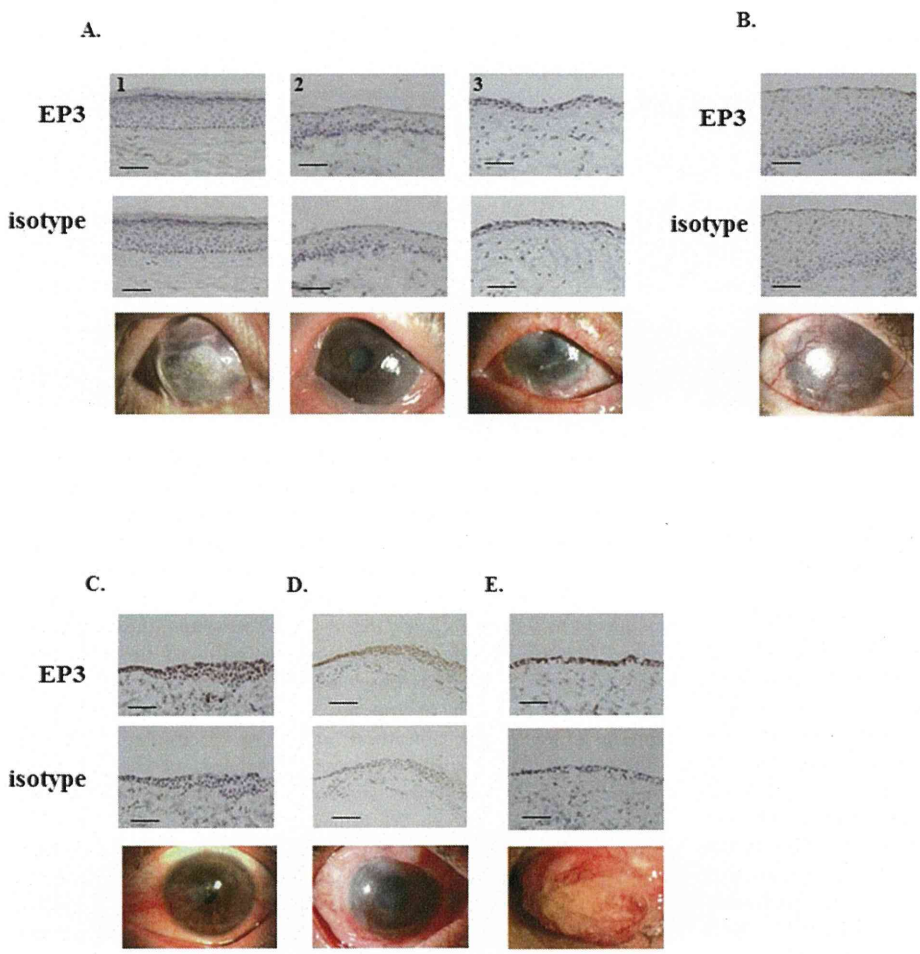

Figure 3. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with OCP (A), severe GVHD (B), pterygium (C), pseudo-OCP (D), and a giant papilla due to allergic vernal conjunctivitis (E). The 3rd lane shows the ocular surface of patients. Each scale bar represents a length of 100 μm.
doi:10.1371/journal.pone.0025209.g003

Table 1. Staining-intensity score of conjunctival epithelium.

Picture	Figure No	Intensity score	Down-regulation score	Disease
Figure 1	A1	14		Nearly normal conjunctival tissues from conjunctival chalasis
	A2	14		
	A3	14		
	B1	12		Normal conjunctival tissues
	C1	7	++	Keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage
	C2	7	++	
	C3	6	++	
	C4	7	++	
	D1	5	++	Non-keratinized conjunctival epithelium from SJS/TEN patients in the sub-acute stage
	D2	5	++	
	E	5	++	Non-keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage
	F	5	++	Conjunctival epithelium from an SJS/TEN patient manifesting only dry eye
Figure 2	A1	9	+	Chemical eye burn
	A2	16		
	A3	13		
	B1	12		Mooren's ulcer
	B2	12		
	B3	14		
	B4	10	+	
Figure 3	A1	6	++	Ocular cicatricial pemphigoid (OCP)
	A2	6	++	
	A3	6	++	
	B	6	++	GVHD with severe conjunctival invasion to the cornea
	C	13		Pterygium
	D	10	+	Pseudo-OCP
	E	16		Chronic allergic keratoconjunctivitis

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some instances because vascular endothelia expressing the EP3 protein could be increased due to the presence of inflammatory infiltrating cells in sub-conjunctival tissues (Fig. S2).

Discussion

We previously reported that in Japanese SJS/TEN patients there was a significant association between severe ocular surface complications and prostaglandin E receptor 3 gene (*PTGER3*) polymorphisms and that compared to the controls, EP3 expression was greatly reduced in their conjunctival epithelium [3]. Here we studied keratinized and non-keratinized conjunctival epithelia of SJS/TEN patients and the conjunctival epithelium of an SJS/TEN patient whose ocular sequelae were minor (dry eye). We found that EP3 was markedly down-regulated not only in keratinized- but also in non-keratinized conjunctival epithelia and even in the normal conjunctiva of a patient in the chronic stage of SJS whose only ocular sequela was dry eye. Our results suggest that the strong down-regulation of EP3 in conjunctival epithelium of SJS/TEN patients is associated with the pathogenesis and pathology of the disease because PTGER3 (EP3) polymorphisms are significantly associated with SJS/TEN.

Severe chemical eye burn results in conjunctival invasion into the cornea due to a deficiency in corneal epithelial stem cells; this leads to devastating ocular surface disorders similar to SJS/TEN. However, EP3 was not down-regulated in the conjunctival epithelium of patients with severe chemical eye burns, suggesting that the pathology of the ocular surface changes was not associated with EP3 expression.

In patients with Mooren's ulcer the peripheral stroma is destroyed first circumferentially then centrally, resulting in the characteristic overhanging inner edge. This is an inflammatory disease of the ocular surface that may require resection of the inflammatory conjunctiva adjacent to the ulcer. We found that the conjunctival epithelium of the inflammatory conjunctival tissues adjacent to the ulcer clearly expressed EP3 protein, indicating that other factors besides inflammation are required for a marked down-regulation of EP3 expression.

OCP is a subset of mucous membrane pemphigoid. It is characterized by the abnormal production of circulating autoantibodies directed against various components of the basement membrane zone and the generation of proinflammatory and fibrogenic cytokines [8]. We found that, as in SJS/TEN patients, EP3 was markedly down-regulated in the conjunctival epithelium of OCP patients with conjunctival invasion to the cornea. As in OCP patients, we failed to detect EP3 protein in the conjunctival epithelium of a patient with severe GVHD with conjunctival invasion to the cornea. This suggests that in a common mechanism(s) may underlie the pathology of SJS/TEN and OCP, especially in ocular surface epithelium such as the conjunctival epithelium. EP3 expression has been reported in skin and PGE_2 was produced abundantly during skin allergic

inflammation [5], suggesting that there is no association between decreased EP3 expression and the increased production of cornified proteins in SJS/TEN and OCP.

We found that EP3 was clearly expressed in the conjunctival epithelium of our patients with pterygium, pseudo-OCP, and a giant papilla of allergic vernal conjunctivitis. Interestingly, the expression of EP3 in conjunctival epithelium from patients with OCP and pseudo-OCP was different: EP3 was clearly present in the patient with pseudo-OCP but not the patient with OCP. The patient with pseudo-OCP had received long-term treatment with eye drops for glaucoma; this resulted in a deficiency of corneal epithelial stem cells and led to conjunctival invasion into the cornea. This suggests that different mechanisms are involved in the expression of EP3. We also detected EP3 in the conjunctival epithelium of the patient with allergic vernal conjunctivitis. Elsewhere we documented that PGE_2 acts as a ligand for EP3 in the conjunctival epithelium and down-regulates the progression of murine experimental allergic conjunctivitis [6]. Although EP3 may down-regulate allergic reactions in patients with allergic conjunctivitis, its loss may not be a causative factor.

In summary, EP3 is expressed not only in normal human conjunctival epithelium but also in the conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer. On the other hand, it is markedly down-regulated in the conjunctival epithelium of SJS/TEN- and OCP patients.

Supporting Information

Figure S1 The rabbit polyclonal antibody to EP3 we used is checked and confirmed the EP3 specificity of this antibody using immunoblot analysis.
(TIF)

Figure S2 EP3 expression in sub-conjunctival tissues in a SJS/TEN patient in the chronic stage. In some instances of SJS/TEN patients, vascular endothelia expressing the EP3 protein are found.
(TIF)

Author Contributions

Conceived and designed the experiments: MU. Performed the experiments: MU. Analyzed the data: MU. Contributed reagents/materials/analysis tools: CS NY TI SK. Wrote the paper: MU.

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Prostaglandin E_2 suppresses polyinosine–polycytidylic acid (polyI:C)-stimulated cytokine production via prostaglandin E_2 receptor (EP) 2 and 3 in human conjunctival epithelial cells

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ABSTRACT

Background Prostaglandin (PG) E_2 is produced during inflammatory responses and suppresses the production of cytokines induced by lipopolysaccharide stimulation in macrophages and dendritic cells. In this study, we examined the expression of PGE_2 receptors in human conjunctival epithelial cells and investigated whether PGE_2 downregulates polyinosine–polycytidylic acid (polyI:C)-induced cytokine production.

Methods ELISA and quantitative reverse transcription (RT)-PCR were used to examine the effects of PGE_2 on the polyI:C-induced cytokine expressions by primary human conjunctival epithelial cells (PHCjEC). Reverse transcription-PCR was performed to examine the mRNA expression of the PGE_2 receptors EP1, -2, -3 and -4.

Results PGE_2 significantly attenuated the expressions of chemokine (C-C) motif ligand (CCL) 5, chemokine (C-X-C motif) ligand (CXCL) 10, CXCL11 and interleukin (IL) 6 in PHCjECs. Human conjunctival epithelial cells exhibited expression of EP2, -3 and -4, but not of EP1. EP2 agonist significantly suppressed the polyI:C-induced the expressions of CCL5, CXCL10 and CXCL11 but not of IL-6. EP3 agonist significantly suppressed the expressions of CCL5, CXCL10, CXCL11 and IL-6. On the other hand, EP4 agonist failed to suppress the cytokine production induced by polyI:C stimulation.

Conclusion Our results show that PGE_2 attenuated the expression of CCL5, CXCL10 and CXCL11 via both EP2 and EP3, and that the expression of IL-6 was attenuated only by EP3.

INTRODUCTION

Prostanoids are a group of lipid mediators that form in response to various stimuli. They include prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane (TX) A_2. They are released extracellularly immediately after their synthesis, and they act by binding to a G-protein-coupled rhodopsin-type receptor on the surface of target cells. There are eight types of prostanoid receptors: the PGD receptor (DP), four subtypes of the PGE receptor (EP1, -2, -3 and -4), PGF receptor (FP), PGI receptor (IP) and TXA receptor (TP).[1]

It have been reported that PGE_2 is produced during inflammatory responses and suppresses the production of cytokines and chemokines induced by lipopolysaccharide (LPS) stimulation in macrophages[2 3] and dendritic cells.[4] Regarding epithelial cells, we have reported that human corneal and conjunctival epithelial cells produce cytokines such as interleukin (IL) 6, IL-8 and interferon (IFN)-β[5 6] in response to stimulation by polyinosine–polycytidylic acid (polyI:C) but not LPS.

PolyI:C, a synthetic double-stranded (ds) RNA that is mimicked with viral dsRNA, is the well-known ligand of toll-like receptor (TLR) 3.[7] It has been reported that polyI:C stimulation induces the secretion of inflammatory cytokines such as IL-6, IL-8, type I IFN such as IFN-β, interferon-inducible proteins such as chemokine (C-X-C) motif ligand (CXCL) 10 and 11, and allergy-related proteins such as chemokine (C-C motif) ligand (CCL) 5 and thymic stromal lymphopoietin (TSLP) in human ocular surface epithelium, both corneal and conjunctival.[5 6 8–11] Moreover, we previously reported that not only TLR3, but also the cytoplasmic helicase proteins retinoic-acid-inducible protein I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) contribute to polyI:C-inducible responses in conjunctival epithelium.[12]

In this study, we examined the expression of PGE_2 receptors, EP1, -2, -3 and -4, in human conjunctival epithelial cells and investigated whether PGE_2 downregulates polyI:C-induced cytokine production.

MATERIALS AND METHODS

Human conjunctival epithelial cells

For stimulation assays by polyI:C using ELISA and quantitative reverse transcription (RT)-PCR, primary human conjunctival epithelial cells (PHCjEC) were harvested from conjunctival tissue obtained at the time of conjunctivochalasis surgery, and then cultured using a previously described method.[9 13] 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 keratinocyte-serum-free medium with defined growth-promoting additives (Invitrogen, Carlsbad, California, USA) including insulin, epidermal growth factor, fibroblast growth factor and 1% antibiotic–antimycotic solution. By using this method, the cell colonies usually became visible within 3 to 4 days. After reaching 80% confluence in 7 to 10 days, the cultured PHCjECs 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 PHCjEC.[13] Each experiment was performed using PHCjEC derived from a different donor.

Table 1 Primer sets for EP1-4 and GAPDH

Gene	Primer Sense, antisense, (accession no.)	Size (bp)	Annealing
EP1 (PTGER1)	5′-GCGCGCTGGTGCTGCGTCTGTACACTGCGG-3′ 5′-AGTGGCCGCTGCAGGGAGGTAGAGCTCCAG-3′ (NM_000955)	723	60
EP2 (PTGER2)	5′-CTTCAGCCTGGCCACGATGCTCATGCTCTT-3′ 5′-CAGGAAGTTTGTGTTGCATCTTGTGTTCTT-3′ (NM_000956)	683	60
EP3 (PTGER3)	5′-CGTGTACCTGTCCAAGCAGCGTTGGGAGCA-3′ 5′-CCGTGTGTGTCTTGCAGTGCTCAACTGATG-3′ (NM_198714)	622	58
EP4 (PTGER4)	5′-TCAACCATGCCTATTTCTACAGCCACTACG-3′ 5′-AGGTCTCTGATATTCGCAAAGTCCTCAGTG-3′ (NM_000958)	956	66
GAPDH	5′-CCATCACCATCTTCCAGGAG-3′ 5′-CCTGCTTCACCACCTTCTTG-3′ (NM_002046)	575	60

For the examination of the expression in vivo human conjunctival epithelium using RT-PCR, we obtained human conjunctival epithelial cells from healthy volunteers by brush cytology. A tiny brush (Cytobrush S; Medscand AM, Malmo, Sweden) was used to scrape epithelial cells from the bulbar conjunctiva.[14]

Reverse transcription-PCR

RT-PCR assay was carried out as previously described.[9 13] Briefly, total RNA was isolated from human conjunctival epithelial cells using the Qiagen RNeasy kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. For the RT reaction we used the SuperScript Preamplification kit (Invitrogen). Amplification was with DNA polymerase (Takara, Shiga, Japan) for 38 cycles at 94°C for 1 min, annealing for 1 min, and 72°C for 1 min on a commercial PCR machine (GeneAmp; PE Applied Biosystems, Foster City, California, USA). The primers are listed in table 1. RNA integrity was assessed by electrophoresis in ethidium bromide-stained 1.5% agarose gels.

ELISA

We performed ELISA to confirm the protein productions. The amounts of IL-6, CCL5, CXCL11 and CXCL10 released into the culture supernatant fractions were determined by ELISA using the Human CCL5, CXCL11, CXCL10 DuoSet (R&D Systems, Inc., Minneapolis, Minnesota, USA) or the OptEIA IL-6 set (BD Pharmingen, San Diego, California, USA) in accordance with the manufacturer's instructions.[15] The minimum level of detection of each in the ELISA was 16 pg/ml for CCL5, 8 pg/ml for CXCL11, 31 pg/ml for CXCL10 and 5 pg/ml for IL-6.

Quantitative RT-PCR

Total RNA was isolated from PHCjECs using RNeasy Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript Preamplification kit (Invitrogen). Quantitative RT-PCR was performed using an ABI-prism 7700 (Applied Biosystems) according to a previously described protocol[9 13] and the manufacturer's instructions. The primers and probes were purchased from Applied Biosystems; assay ID: *CCL5*

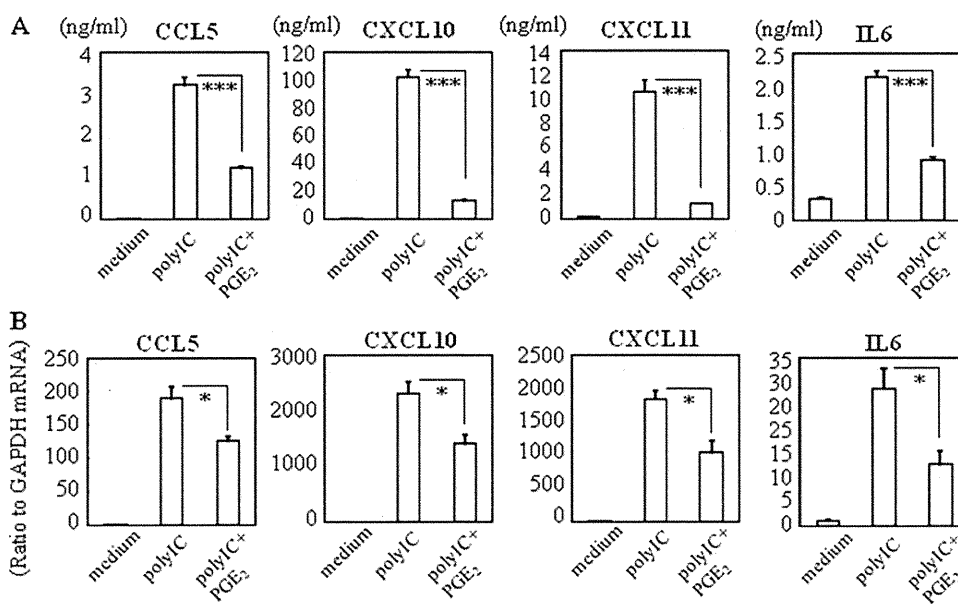

Figure 1 (A) Suppression of the production of chemokine (C-C) motif ligand (CCL) 5, chemokine (C-X-C motif) ligand (CXCL) 10, CXCL11 and interleukin (IL) 6 by prostaglandin (PG) E_2. Primary human conjunctival epithelial cells (PHCjEC) were exposed to 10 μg/ml polyinosine–polycytidylic acid (polyI:C) and 100 μg/ml PGE_2 for 24 h. The production of CCL5, CXCL10, CXCL11 and IL-6 were measured using ELISA. Data are representative of three separate experiments and are given as the mean±SEM from one experiment carried out in six wells per group. (B) Suppression of the mRNA expression of CCL5, CXCL10, CXCL11 and IL-6 by PGE_2. PHCjEC were exposed to 10 μg/ml polyI:C and 100 μg/ml PGE_2 for 6 h. The mRNA expressions of CCL5, CXCL10, CXCL11 and IL-6 were examined by quantitative reverse transcription (RT)-PCR. The quantification data were normalised to the expression of the housekeeping gene *GAPDH*. The y-axis shows the increase in specific mRNA over unstimulated samples. Data are representative of three separate experiments and are given as the mean±SEM from one experiment carried out in four wells per group. *p<0.05; ***p<0.0005.

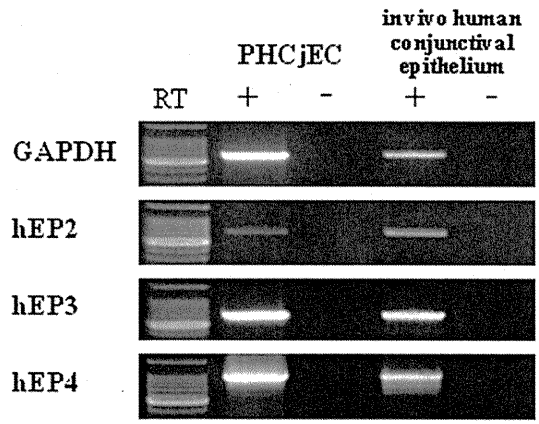

Figure 2 The mRNA expression of the prostaglandin (PG) E_2 receptors EP2, -3 and -4. Reverse-transcription (RT)-PCR analyses of the expression EP2-, ER3 and ER4-specific mRNA in in vivo human conjunctival epithelium obtained by brush cytology. RT− indicates that data were obtained without reverse transcription (controls).

(Hs00174575), *CXCL10* (Hs00171042), *CXCL11* (Hs00171138), *IL-6* (Hs00174131) and human *GAPDH* (Hs 4326317E). To amplify cDNA, PCR was performed in a 25 μl total volume that contained a 1 μl cDNA template in 2 × TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The results were analysed with sequence detection software (Applied Biosystems). The quantification data were normalised to the expression of the housekeeping gene *GAPDH*.

Selective agonists for EP2, -3 and -4

ONO-AE-259, a selective EP2 agonist, ONO-AE-248, a selective EP3 agonist, and ONO-AE-329, a selective EP4 agonist, were supplied by ONO Pharmaceutical Co., Ltd. (Osaka, Japan); the ligand-binding specificities of the compounds for each PGE receptor subtype have previously been described.[16]

Data analysis

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

RESULTS

PGE_2 downregulated the production of cytokines induced by polyI:C stimulation

First we examined whether PGE_2 downregulated the production of cytokines induced by polyI:C stimulation. Elsewhere we reported that in PHCjEC many transcripts were significantly upregulated upon polyI:C stimulation,[9] and in this study we chose to examine cytokines that can be measured using ELISA. Here we used ELISA to examine the effects of PGE_2 on the polyI:C-induced production of IL-6, IL-8, CCL5, CXCL10 and CXCL11 by PHCjEC. PHCjEC were exposed to 10 μg/ml polyI:C and 100 μg/ml PGE_2 for 24 h (ELISA) or 6 h (quantitative RT-PCR). We decided on the dose of PGE_2 according to our dose analysis (supplemental figure 1). We found that it significantly attenuated the production of CCL5, CXCL10, CXCL11 and IL-6 (figure 1A) but not of IL-8 (data not shown). Quantitative RT-PCR assay confirmed that the mRNA expression of CCL5, CXCL10, CXCL11 and IL-6 were significantly downregulated by PGE_2 (figure 1B).

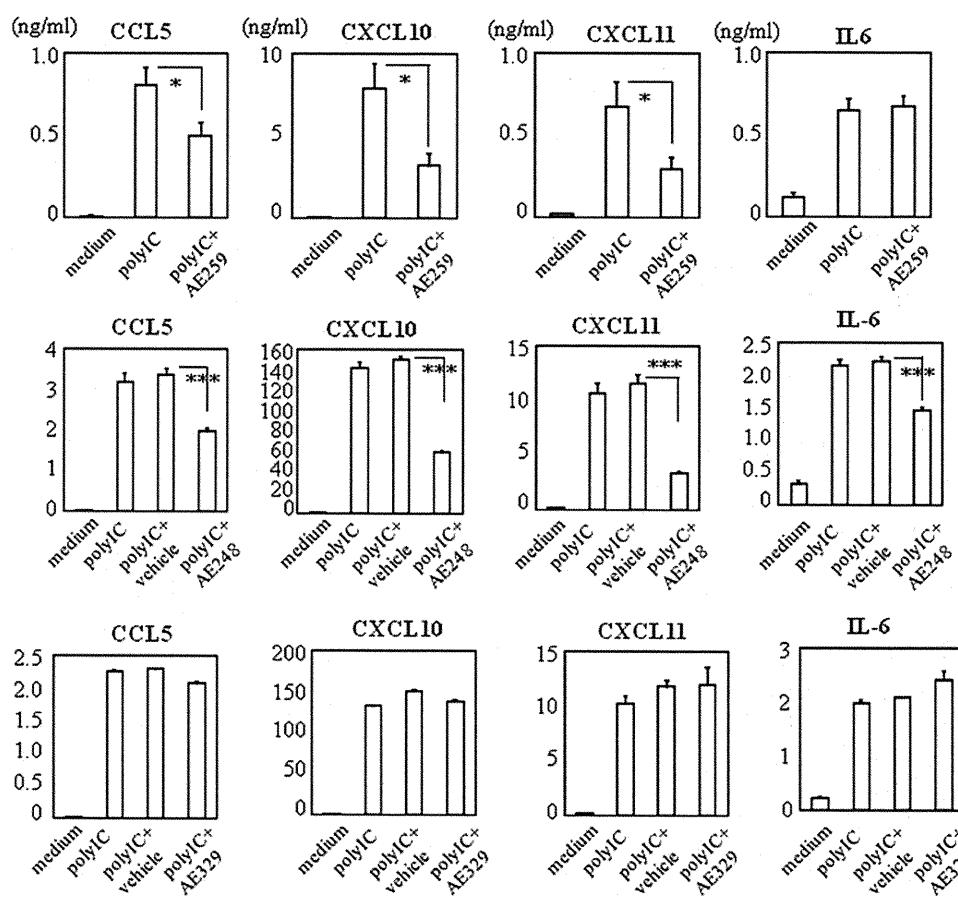

Figure 3 Effect of the prostaglandin (PG) E_2 receptors EP2, -3 and -4 on polyI:C-induced cytokine production. Primary human conjunctival epithelial cells (PHCjEC) were exposed to 10 μg/ml polyinosine–polycytidylic acid (polyI:C) and 10 μg/ml EP2 agonist (AE259), 10 μg/ml EP3 agonist (AE248) or 10 μg/ml EP4 agonist (AE329) for 24 h. The productions of chemokine (C-C) motif ligand (CCL) 5, chemokine (C-X-C motif) ligand (CXCL) 10, CXCL11 and interleukin (IL)-6 were measured using ELISA. Data are representative of three separate experiments and are given as the mean±SEM from one experiment carried out in six wells per group. *p<0.05; ***p<0.0005.

Human conjunctival epithelial cells expressed EP2-, EP3- and EP4-specific mRNA

We then performed RT-PCR to assay the mRNA expression of the PGE_2 receptors EP1, -2, -3 and -4 in human conjunctival epithelial cells. PCR products of expected lengths were obtained for EP2 (683 bp), EP3 (622 bp) and EP4 (956 bp) (figure 2), but not for EP1 (723 bp) (data not shown) from PHCjEC and in vivo human conjunctival epithelial cells, suggesting that the human conjunctival epithelium expresses EP2, -3 and -4 mRNA. To confirm the specificity for the detection of EP2, -3 and -4 mRNA we isolated and sequenced the PCR products. The obtained sequences were identical to the human EP2, -3 and -4 cDNA sequences.

The agonists of EP2 and EP3, but not EP4, downregulated the production of cytokines induced by polyI:C stimulation

Using the EP2 agonist ONO-AE259, the EP3 agonist ONO-AE248 and the EP4 agonist ONO-AE329 we also examined which PGE_2 receptor(s) contributed to their polyI:C-induced downregulation. PHCjEC were exposed to 10 μg/ml polyI:C and 10 μg/ml EP2 agonist, EP3 agonist or EP4 agonist for 24 h (ELISA) or 6 h (quantitative RT-PCR). ELISA showed that the EP2 agonist significantly suppressed the polyI:C-induced production of CCL5, CXCL10 and CXCL11 but not of IL-6, and that the EP3 agonist significantly suppressed the production of CCL5, CXCL10, CXCL11 and IL-6. On the other hand, the EP4 agonist failed to suppress the cytokine production induced by polyI:C stimulation (figure 3). Quantitative RT-PCR confirmed that the EP2 agonist significantly downregulated the mRNA expression of CCL5, CXCL10, and CXCL11 but not of IL-6, and that the EP3 agonist significantly downregulated the mRNA expression of all examined cytokines (supplemental figure 2). Thus, our results show that PGE_2 attenuated the mRNA expression and production of CCL5, CXCL10 and CXCL11 via both EP2 and EP3, and that the IL-6 mRNA expression and production of IL-6 was attenuated only by EP3.

DISCUSSION

Lipid mediators such as PGE_2 regulate immune and inflammatory responses by modulating the production of cytokines and chemokines.[4] In macrophages, PGE_2 suppresses the pro-inflammatory gene expression induced by LPS, including macrophage inflammatory protein (MIP)-1α, MIP-1β, CCL5, CXCL10 and IL8.[2] We document here that in human conjunctival epithelial cells PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes. It exhibited a inhibitory effect on polyI:C-induced CCL5-, CXCL10, CXCL11 and IL-6 mRNA and on protein production in PHCjEC. PGE_2 exerts its biological actions by binding to EP located primarily on the plasma membrane. We confirmed the presence of the PGE_2 receptor subtypes EP2, -3 and -4 in human conjunctival epithelial cells. Stimulation with either EP2- or EP3-specific agonists had a suppressive effect on polyI:C-induced CCL5, CXCL10 and CXCL11 production, but only the EP3-specific agonists exerted a suppressive effect on the production of IL-6.

Stimulation with PGE_2 exhibits immunosuppressive effects in various cell types including macrophages and dendritic cells via EP2 and/or EP4.[2–4] This phenomenon is explicable by the increased production of intracellular cAMP via the activation of adenylcyclase.[2 3] While PGE_2 acts on EP2 and EP4 and activates adenylcyclase, resulting in an increased in intracellular cAMP, its action on EP3 suppresses adenylcyclase, resulting in a decrease in intracellular cAMP. In human conjunctival epithelial cells, both EP2 and EP3 contribute to the immunosuppressive effect against polyI:C stimulation; therefore, the suppressive effect cannot be explained by the increase in intracellular cAMP, and the precise molecular mechanisms underlying the immunosuppressive effects of PGE_2 in epithelial cells remain to be elucidated.

Elsewhere we have reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells and that it downregulates the progression of murine experimental allergic conjunctivitis.[17] We also reported that PGE_2 and an EP3 agonist suppress the polyI:C-induced production of TSLP in human conjunctival epithelial cells, suggesting that a PGE_2–EP3 pathway is involved in suppressing the development of allergic conjunctivitis via the suppression of TSLP production[18] and that the levels of EP3 and EP4 are downregulated in the conjunctival epithelium in the presence of ocular surface inflammatory diseases such as Stevens–Johnson syndrome and ocular cicatricial pemphigoid.[15 19] Our current study showed that PGE_2 acts on EP2 or EP3 and suppresses the expression and production of cytokines induced by polyI:C.

In summary, our results suggest that PGE_2 and its receptors in conjunctival epithelium contribute to the regulation of ocular surface inflammation.

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Competing interests None to declare.

Patient consent Obtained.

Ethics approval This study was approved by 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.

Provenance and peer review Not commissioned; externally peer reviewed.

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アレルギー性結膜炎に対し抗アレルギー点眼薬を用いた初期療法が有用である．花粉飛散予測日の約2週間前，または症状が少しでも現れた時点で抗アレルギー点眼薬の投与を開始することで，花粉飛散ピーク時の症状が軽減される．花粉飛散開始日前から花粉の飛散は始まっていることから，少量の花粉でも反応する感受性の高い患者に対し，あるいは，花粉飛散量が多いと予測される年や地域では，積極的に取り入れたい治療法である．内服薬による眠気やステロイド点眼薬による眼圧上昇などの副作用がない，安全性の高い抗アレルギー点眼薬は初期療法に適した薬剤であると考える．

通年性アレルギー性結膜炎においても第一選択は抗アレルギー点眼薬である．抗アレルギー点眼薬だけでは効果不十分な場合，経過をみながら点眼薬の種類変更やステロイド点眼薬の併用を行う．

●春季カタル，アトピー性角結膜炎

春季カタルやアトピー性角結膜炎においても，I型アレルギー反応の抑制といった点から，抗アレルギー点眼薬が第一選択薬として用いられている．効果不十分な場合は，免疫抑制点眼薬やステロイド点眼薬を併用する．春季カタルやアトピー性角結膜炎では，I型アレルギー反応の遅発相がその病態として考えられているが，抗アレルギー薬の中には，好酸球の遊離の抑制やサブスタンスPの抑制といった，重症型の病態の改善に有効な薬理作用を有するものがある．

●コンタクトレンズ装用者

アレルギー性結膜疾患患者では，症状を有する時期には，コンタクトレンズ装用は中止し，抗アレルギー点眼薬による治療を行うことが原則である．症状が改善すれば，また，コンタクトレンズ装用を開始することはできるが，その場合，レンズは1日使い捨てレンズを選択する．原則としては，コンタクトレンズ装用前後で抗アレルギー点眼薬を点眼し，コンタクトレンズ装用時には，防腐剤無添加人工涙液をレンズ上から点眼する．コンタクトレンズ上からの抗アレルギー点眼薬の使用については，点眼薬に含有される防腐剤の吸着，コンタクトレンズの素材と抗アレルギー点眼薬のpHが問題となる．イオン性のソフトコンタクトレンズでは酸性の抗アレルギー点眼薬により含水率が低下し，コンタクトレンズのフィッティングにも影響を及ぼす可能性が考えられている．

（高村悦子）

Ⅳ-C-1

5) 副腎皮質ステロイド薬

① 作用機序

少量ステロイド投与による作用機序は近年，ほぼ明らかになってきた．ステロイド分子は脂溶性であり，細胞膜を自由に通過する．通過したステロイド分子は細胞質に存在するステロイドレセプターと2量体を形成して活性化し，細胞核内に運ばれる．そこで2量体は標的遺伝子近傍の特異塩基配列に結合し，標的遺伝子の転写活性を調節し，最終的に炎症にかかわる種々の蛋白量を増減させて抗炎症作用を示す(図Ⅳ-C-4)．しかし，ステロイド受容体は全身の細胞内に存在するため，こうした機序は免疫細胞特異的ではなく，抗炎症作用以外の糖質コルチコイド作用(これが副作用として出現してくる)も促進する．

② 投与方法の選択

a. 全身投与

眼科でのステロイド全身投与は多岐にわたる炎症性疾患に用いられる．原田病，交感性眼炎，視神経炎ではメチルプレドニゾロン1,000mg(生理的コルチゾール1日量の250倍の力価)3日間点滴後，内服薬に切り替えて数か月かけて減量するパルス療法(図Ⅳ-C-5)(I-I，p309参照)やメチルプレドニゾロンを200mg(同50倍)点滴から段階的に減量し，その後，内服に切り替える大量療法が選択される．

術後の強い炎症，角膜移植後，Behçet病の発作期，サルコイドーシスの視力低下が顕著な場合，重症の強膜炎などでは，プレドニゾロン内服を開始し数か月かけて減量していく少量投与・漸減療法が選択される．

b. 局所投与

軟膏，点眼薬の投与，そして注射用剤の局所注射(結膜下，Tenon嚢下，球後)が広範な疾患に用いられる．投与するステロイドの種類，投与頻度，他薬との組み合わせは疾患，病状によりさまざまであり，疾患別各論を参照していただきたい．

③ 投与量

すべての合成ステロイド薬は，1錠中にコルチゾール20mg(生理的コルチゾール1日量)に相当する糖質コルチコイド作用を有するように作られている．抗炎症作用は4～6錠，免疫抑制作用は

8錠以上で示すとされている．少量投与・漸減療法の開始時，ステロイド投与量はこれを基本に設定するのが好ましい．

④ 投与期間と組み合わせ

点眼治療では効果判定は多くの疾患では2週間と考えてよく，病態が改善しないときは，さらなる強力な抗炎症作用を期待して結膜下注射やTenon嚢下注射，または全身投与を行う．症状が消失したときは，直ちに，薬効の弱いステロイド(ベタメタゾン使用例なら，フルオロメトロン)に変更し離脱に入る．ぶどう膜炎の場合，リバウンド現象に特に注意が必要で，点眼治療にベタメタゾン，デキサメタゾンを使用し，離脱に際しても，点眼回数や濃度を漸次下げながら注意深く行う．

(井上幸次)

Ⅳ-C-1

6) 非ステロイド系抗炎症薬(NSAID)

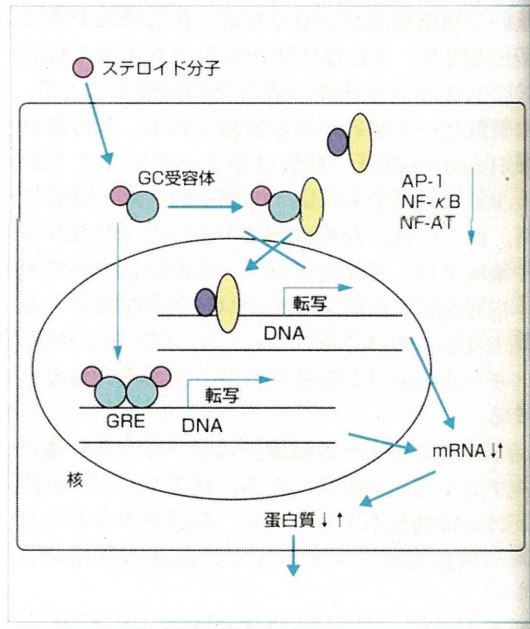
① 作用機序

局所に侵襲が加わると，細胞は細胞膜のリン脂質からホスフォリパーゼA_2を介してアラキドン酸を遊離する．このアラキドン酸にシクロオキシゲナーゼ(COX)が作用するとプロスタグランディン(PG)が産生される(図Ⅳ-C-6)．PGは細胞外でケミカルメディエーターとして非特異的炎症に加担し，眼組織では縮瞳，血管拡張，眼血液関門の障害(黄斑浮腫，前房内蛋白濃度上昇)，眼圧上昇などに関与している．

非ステロイド系抗炎症薬(NSAID)は一連の炎症反応中のCOXを阻害しPG合成を抑制する．COXにはアイソザイムが存在し，COX-1は胃粘膜，血小板などを含め多くの細胞に発現し，COX-2は炎症関連細胞を主体に発現するが，現在使われているNSAIDにはCOX-2を特異的に阻害するものはない．そのためCOX-1も同時に阻害することとなり，胃障害などの副作用を引き起こす．

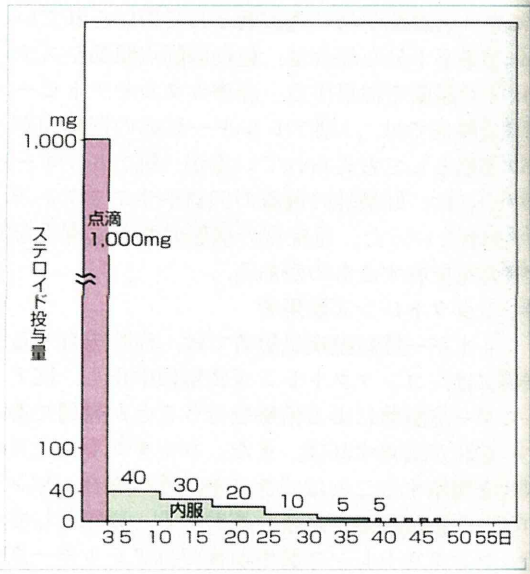
この副作用を防止すべく，drug delivery system(DDS)が進歩し，胃腸では不活性型で，肝臓で代謝されて活性型となり薬効を示すプロドラッグが開発され，使用頻度が高い．点眼薬自体も炎症局所(眼表面)の薬剤濃度を高濃度にするという意味で，一種のDDSのような効果がある．

図Ⅳ-C-4 ステロイド薬の抗炎症効果の分子機構



(小竹 聡：眼科診療プラクティス56，p80)

図Ⅳ-C-5 Vogt-小柳-原田病のステロイドパルス療法



(新藤裕実子：眼科診療プラクティス8，p183)

② 投与方法の選択

a. 全身投与

NSAIDの全身投与は眼科領域では主に手術後の疼痛や炎症の軽減に多用される．

投与方法として，経口薬，注射薬，座薬がある．座薬は，直腸から吸収された薬物が直接大静脈に入るため，肝臓で代謝される割合が少なく，した