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

Mayumi Ueta[1,2]*, Chie Sotozono[1], Norihiko Yokoi[1], Tsutomu Inatomi[1], Shigeru Kinoshita[1]

1 Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan, **2** Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

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.

Citation: Ueta M, Sotozono C, Yokoi N, Inatomi T, Kinoshita S (2011) Prostaglandin E Receptor Subtype EP3 Expression in Human Conjunctival Epithelium and Its Changes in Various Ocular Surface Disorders. PLoS ONE 6(9): e25209. doi:10.1371/journal.pone.0025209

Editor: Naj Sharif, Alcon Research, Ltd., United States of America

Received June 14, 2011; **Accepted** August 29, 2011; **Published** September 22, 2011

Funding: This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST from JST, a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mueta@koto.kpu-m.ac.jp

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

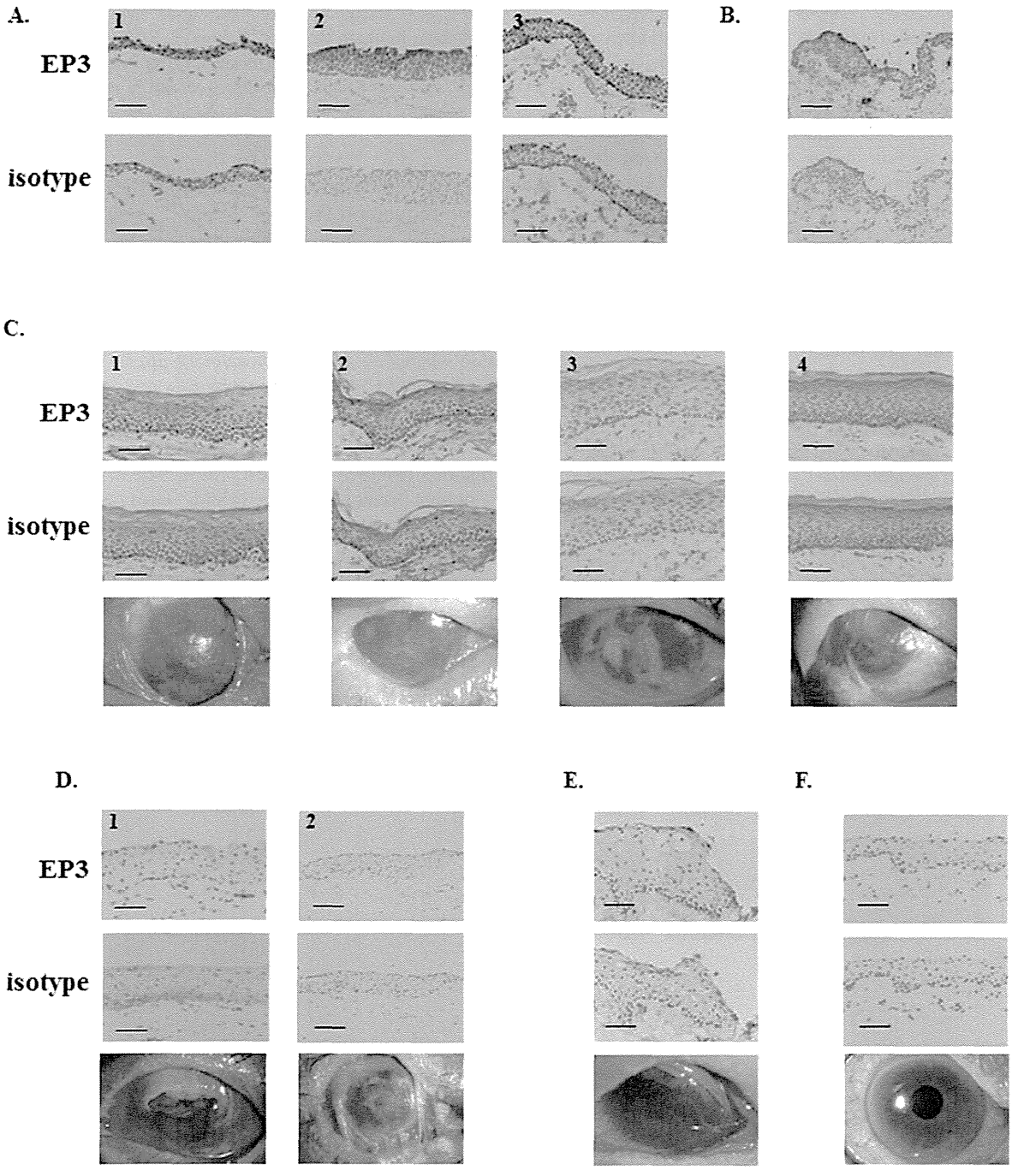

Figure 1. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of the controls and SJS/TEN patients. A. Nearly normal conjunctival tissues from patients with conjunctivochalasis. B. Normal conjunctival tissue. C. Keratinized conjunctival tissues of SJS/TEN 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.
doi:10.1371/journal.pone.0025209.g001

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

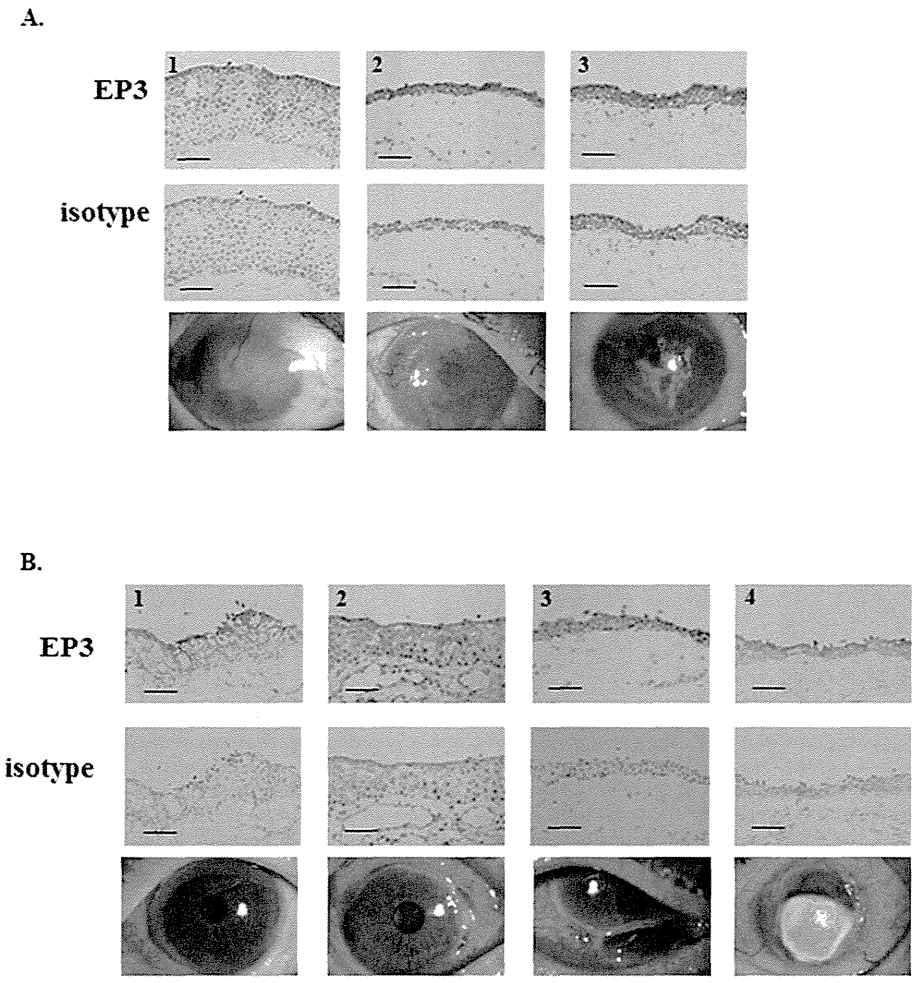

Figure 2. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with chemical eye burn and active Mooren's ulcer. A. Conjunctival tissues of patients with chemical eye burn requiring ocular surface reconstruction. B. Inflammatory conjunctival tissues 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.
doi:10.1371/journal.pone.0025209.g002

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

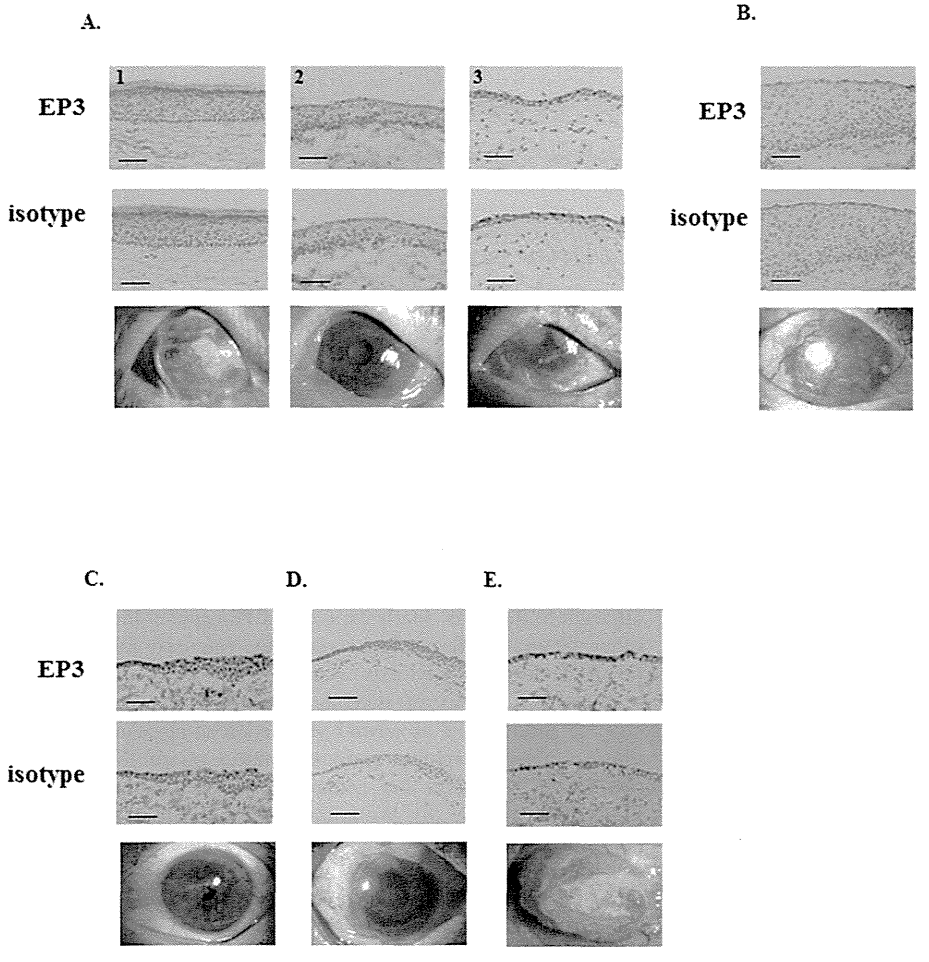

Figure 3. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with OCP (A), severe GVHD (B), pterygium (C), pseudo-OCP (D), and a giant papilla due to allergic vernal conjunctivitis (E). The 3rd lane shows the ocular surface of patients. Each 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

doi:10.1371/journal.pone.0025209.t001

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.

References

1. Matsuoka T, Narumiya S (2007) Prostaglandin receptor signaling in disease. Scientific World Journal 7: 1329–47.
2. Sotozono C, Ueta M, Koizumi N, Inatomi T, Shirakata Y, et al. (2009) Diagnosis and treatment of Stevens-Johnson syndrome and toxic epidermal necrolysis with ocular complications. Ophthalmol 116: 685–90.
3. Ueta M, Sotozono C, Nakano M, Taniguchi T, Yagi T, et al. (2010) Association between prostaglandin E receptor 3 polymorphisms and Stevens-Johnson syndrome identified by means of a genome-wide association study. J Allergy Clin Immunol 126:1218-25 e10.
4. Kunikata T, Yamane H, Segi E, Matsuoka T, Sugimoto Y, et al. (2005) Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3. Nat Immunol 6: 524–31.
5. Honda T, Matsuoka T, Ueta M, Kabashima K, Miyachi Y, et al. (2009) Prostaglandin E(2)-EP(3) signaling suppresses skin inflammation in murine contact hypersensitivity. J Allergy Clin Immunol 124: 809–18 e2.
6. Ueta M, Matsuoka T, Narumiya S, Kinoshita S (2009) Prostaglandin E receptor subtype EP3 in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis. J Allergy Clin Immunol 123: 466–71.
7. Ueta M, Matsuoka T, Yokoi N, Kinoshita S (2011) Prostaglandin E2 suppresses polyinosine-polycytidylic acid (polyI:C)-stimulated cytokine production via prostaglandin E2 receptor (EP) 2 and 3 in human conjunctival epithelial cells. Br J Ophthalmol 95: 859–63.
8. Razzaque MS, Foster CS, Ahmed AR (2003) Role of connective tissue growth factor in the pathogenesis of conjunctival scarring in ocular cicatricial pemphigoid. Invest Ophthalmol Vis Sci 44: 1998–2003.

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

Mayumi Ueta,[1,2] Toshiyuki Matsuoka,[3] Norihiko Yokoi,[1] Shigeru Kinoshita[1]

► Additional figures are published online only. To view these files please visit the journal online (http://bjo.bmj.com).

[1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan
[2]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan
[3]Department of ophthalmology, Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan

Correspondence to
Mayumi Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajiicho, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan; mueta@koto.kpu-m.ac.jp

The work described in the present paper was carried out in collaboration with Ono Pharmaceutical Co., Ltd., who supplied ONO-AE-259, ONO-AE-248 and ONO-AE-329 used in this study.

Accepted 30 January 2011
Published Online First 24 February 2011

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*

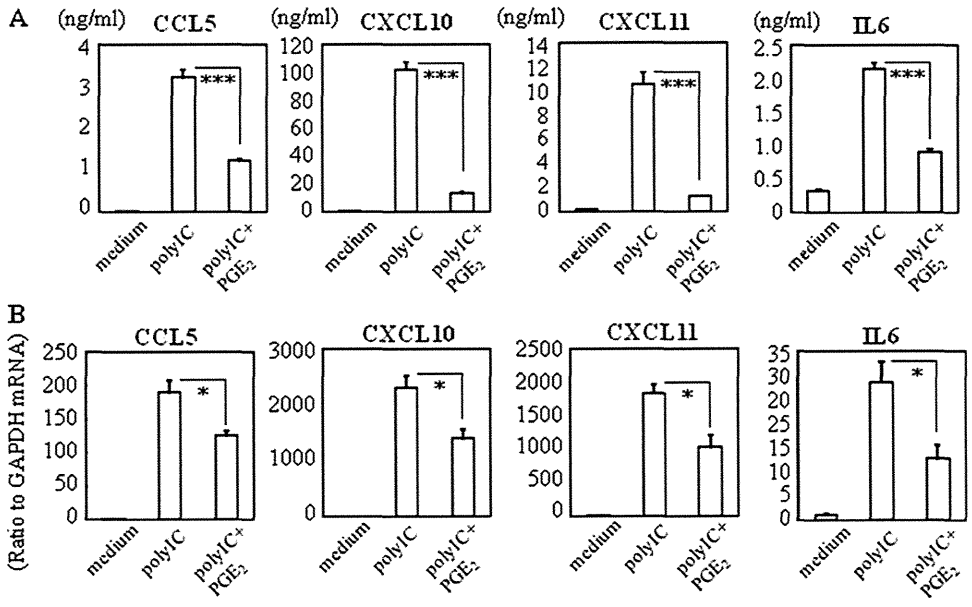

Figure 1 (A) Suppression of the production of chemokine (C-C) motif ligand (CCL) 5, chemokine (C-X-C motif) ligand (CXCL) 10, CXCL11 and interleukin (IL) 6 by prostaglandin (PG) E_2. Primary human conjunctival epithelial cells (PHCjEC) were exposed to 10 μg/ml polyinosine–polycytidylic acid (polyI:C) 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.

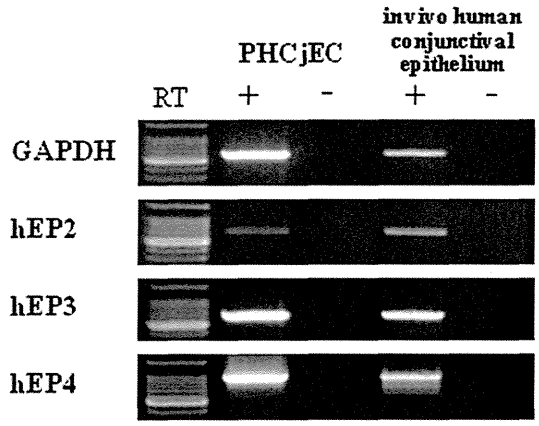

Figure 2 The mRNA expression of the prostaglandin (PG) E_2 receptors EP2, -3 and -4. Reverse-transcription (RT)-PCR analyses of the expression EP2-, ER3 and ER4-specific mRNA in in vivo human conjunctival epithelium obtained by brush cytology. RT− indicates that data were obtained without reverse transcription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

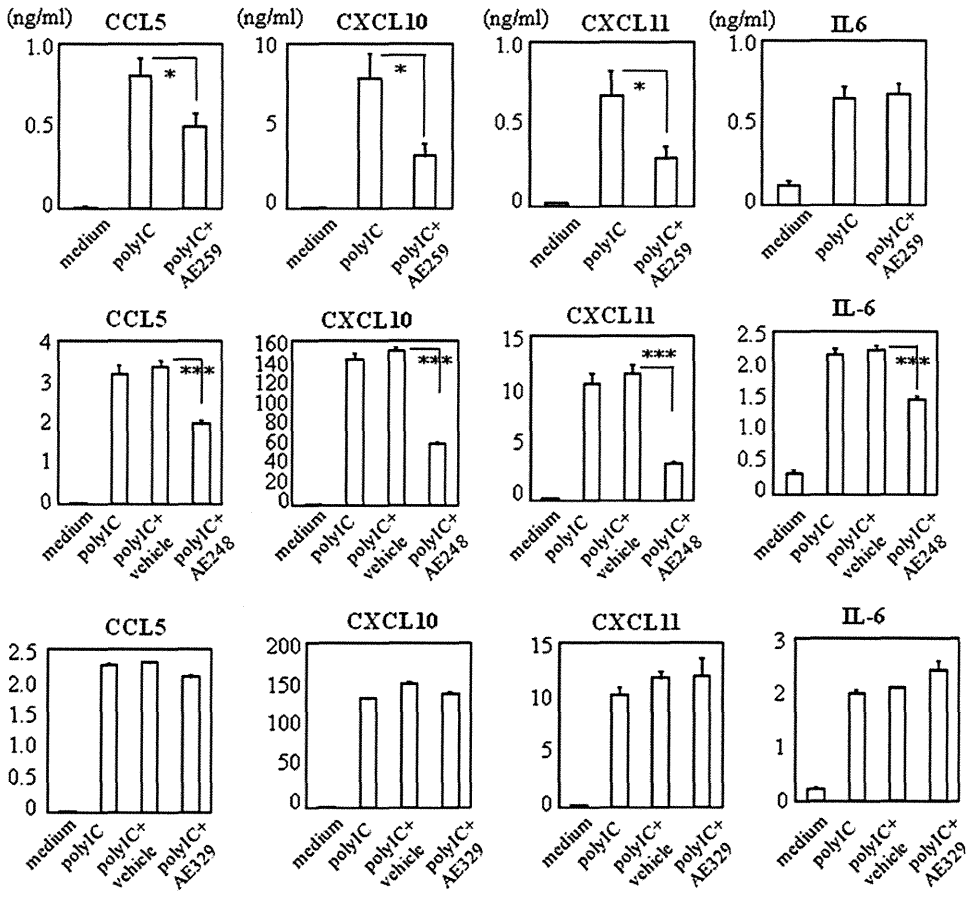

Figure 3 Effect of the prostaglandin (PG) E_2 receptors EP2, -3 and -4 on polyI:C-induced cytokine production. Primary human conjunctival epithelial cells (PHCjEC) were exposed to 10 µg/ml polyinosine–polycytidylic acid (polyI:C) and 10 µg/ml EP2 agonist (AE259), 10 µg/ml EP3 agonist (AE248) or 10 µg/ml 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.

Acknowledgements We thank Chikako Endo for technical assistance.

Funding This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare and the Japanese Ministry of Education, Culture, Sports, Science and Technology, and research grants from the Kyoto Foundation for the Promotion of Medical Science, the Intramural Research Fund of Kyoto Prefectural University of Medicine and the Shimizu Foundation.

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.

REFERENCES

1. **Matsuoka T,** Narumiya S. Prostaglandin receptor signaling in disease. *Scientific World Journal* 2007;**7**:1329–47.
2. **Takayama K,** Garcia-Cardena G, Sukhova GK, *et al*. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem* 2002;**277**:44147–54.
3. **Xu XJ,** Reichner JS, Mastrofrancesco B, *et al*. Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production. *J Immunol* 2008;**180**:2125–31.
4. **Shiraishi H,** Yoshida H, Saeki K, *et al*. Prostaglandin E2 is a major soluble factor produced by stromal cells for preventing inflammatory cytokine production from dendritic cells. *Int Immunol* 2008;**20**:1219–29.
5. **Ueta M,** Hamuro J, Kiyono H, *et al*. Triggering of TLR3 by polyI: C in human corneal epithelial cells to induce inflammatory cytokines. *Biochem Biophys Res Commun* 2005;**331**:285–94.
6. **Ueta M,** Kinoshita S. Innate immunity of the ocular surface. *Brain Res Bull* 2010;**81**:219–28.
7. **Alexopoulou L,** Holt AC, Medzhitov R, *et al*. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;**413**:732–8.
8. **Ueta M,** Kinoshita S. Ocular surface inflammation mediated by innate immunity. *Eye Contact Lens* 2010;**36**:269–81.
9. **Ueta M,** Mizushima K, Yokoi N, *et al*. Gene-expression analysis of polyI: C-stimulated primary human conjunctival epithelial cells. *Br J Ophthalmol* 2010;**94**:1528–32.
10. **Kumar A,** Zhang J, Yu FS. Toll-like receptor 3 agonist poly(I: C)-induced antiviral response in human corneal epithelial cells. *Immunology* 2006;**117**:11–21.
11. **Li J,** Shen J, Beuerman RW. Expression of toll-like receptors in human limbal and conjunctival epithelial cells. *Mol Vis* 2007;**13**:813–22.
12. **Ueta M,** Kawai T, Yokoi N, *et al*. Contribution of IPS-1 to polyI: C-induced cytokine production in conjunctival epithelial cells. *Biochem Biophys Res Commun* 2011;**404**:419–23.
13. **Ueta M,** Mizushima K, Yokoi N, *et al*. Expression of the interleukin-4 receptor alpha in human conjunctival epithelial cells. *Br J Ophthalmol* 2010;**94**:1239–43.
14. **Kojima K,** Ueta M, Hamuro J, *et al*. Human conjunctival epithelial cells express functional Toll-like receptor 5. *Br J Ophthalmol* 2008;**92**:411–16.
15. **Ueta M,** Sotozono C, Nakano M, *et al*. Association between prostaglandin E receptor 3 polymorphisms and Stevens–Johnson syndrome identified by means of a genome-wide association study. *J Allergy Clin Immunol* 2010;**126**:1218–25.e10.

16. **Suzawa T,** Miyaura C, Inada M, *et al*. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* 2000;**141**:1554–9.
17. **Ueta M,** Matsuoka T, Narumiya S, *et al*. Prostaglandin E receptor subtype EP3 in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis. *J Allergy Clin Immunol* 2009;**123**:466–71.
18. **Ueta M,** Matsuoka T, Yokoi N, *et al*. Prostaglandin E receptor subtype EP3 downregulates TSLP expression in human conjunctival epithelium. *Br J Ophthalmol*. Published Online First: 29 September 2010. doi:10.1136/bjo.2010.188748.
19. **Ueta M,** Sotozono C, Yokoi N, *et al*. Prostaglandin E receptor 4 expression in human conjunctival epithelium and its downregulation in devastating ocular surface inflammatory disorders. *Arch Ophthalmol* 2010;**128**:1369–71.

RESEARCH LETTERS

Downregulation of Monocyte Chemoattractant Protein 1 Expression by Prostaglandin E_2 in Human Ocular Surface Epithelium

Elsewhere, we reported that in the tears and serum of patients with acute-stage Stevens-Johnson syndrome or toxic epidermal necrolysis, the levels of interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) were dramatically increased.[1] We also reported that Stevens-Johnson syndrome or toxic epidermal necrolysis with severe ocular complications was associated with polymorphism of the prostaglandin E receptor 3 (EP_3) gene (*PTGER3*).[2]

Prostanoids are a group of lipid mediators that form in response to various stimuli. They include prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2. There are 4 subtypes of the PGE receptor: EP_1, EP_2, EP_3, and EP_4. We previously reported that PGE_2 suppresses polyinosine–polycytidylic acid (polyI:C)–stimulated cytokine production via EP_2 and/or EP_3 in human ocular surface epithelial cells.[3,4] PolyI:C is a ligand of Toll-like receptor 3, which is strongly expressed in ocular surface epithelium.[5] We found that PGE_2 suppresses the production of IL-6, chemokine (C-X-C motif) ligand 10, chemokine (C-X-C motif) ligand 11, and chemokine (C-C motif) ligand 5 but not IL-8 by epithelial cells on the human ocular surface[3]; it remains to be determined whether it also suppresses MCP-1 production. Monocyte chemoattractant protein 1 plays a significant role in the recruitment of monocytes and lymphocytes to the site of cellular immune reactions. In this study, we investigated whether PGE_2 downregulates polyI:C-induced MCP-1 production.

All experiments were conducted in accordance with the principles set forth in the Declaration of Helsinki. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction were performed with primary human conjunctival epithelial cells and immortalized human corneal-limbal epithelial cells using previously described methods (eAppendix, http://www.archophthalmol.com).[3]

First, we examined whether PGE_2 downregulated the production and messenger RNA (mRNA) expression of MCP-1 induced by polyI:C stimulation in human conjunctival and corneal epithelial cells. We found that it significantly attenuated the production of MCP-1 (**Figure**, A). Quantitative real-time polymerase chain reaction confirmed that the mRNA expression of MCP-1 was significantly downregulated by PGE_2 (Figure, A).

Next, we examined which PGE_2 receptor(s) contributed to the downregulation of polyI:C-induced MCP-1. We used the EP_2 agonist ONO-AE-259, the EP_3 agonist ONO-AE-248, and the EP_4 agonist ONO-AE-329. Enzyme-linked immunosorbent assay showed that the EP_2 and EP_3 agonists significantly suppressed the polyI:C-induced production of MCP-1, while the EP_4 agonist did not exert suppression (Figure, B). Quantitative real-time polymerase chain reaction confirmed that the EP_2 and EP_3 agonists significantly downregulated the mRNA expression of MCP-1 (Figure, C). Thus, our results document that PGE_2 attenuated the mRNA expression and production of MCP-1 via both EP_2 and EP_3.

In human macrophages, PGE_2 attenuated the lipopolysaccharide-induced mRNA and protein expression of chemokines including MCP-1 through EP_4.[6] On the other hand, we demonstrated that in human ocular surface epithelial cells, PGE_2 attenuated the polyI:C-induced mRNA and protein expression of MCP-1 through EP_2 and EP_3 but not EP_4. Our findings suggest that EP_2 and EP_3 play important roles in the regulation of inflammation in epithelial cells, while EP_2 and EP_4 have important roles in immune cells such as macrophages.

In the tears and serum of patients with acute-stage Stevens-Johnson syndrome or toxic epidermal necrolysis, the levels of IL-6, IL-8, and MCP-1 were dramatically increased.[1] Although IL-8 was not regulated by PGE_2, IL-6 was regulated by PGE_2 via EP_3 in human ocular surface epithelial cells.[3] Herein, we demonstrated that MCP-1 could be regulated by PGE_2 via EP_2 and EP_3. The regulation of cytokine production by PGE_2 may be associated with the pathogenesis of Stevens-Johnson syndrome or toxic epidermal necrolysis with severe ocular complications because it was associated with polymorphism of the EP_3 gene (*PTGER3*), one of the PGE receptors (EP_1, EP_2, EP_3, EP_4).[2]

In summary, our results show that MCP-1 produced by human ocular surface epithelial cells could be downregulated by PGE_2 via EP_2 and EP_3.

Mayumi Ueta, MD, PhD
Chie Sotozono, MD, PhD
Norihiko Yokoi, MD, PhD
Shigeru Kinoshita, MD, PhD

Author Affiliations: Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University (Dr Ueta) and Department of Ophthalmology, Kyoto Prefectural University of Medicine (Drs Ueta, Sotozono, Yokoi, and Kinoshita), Kyoto, Japan.
Correspondence: Dr Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Ka-

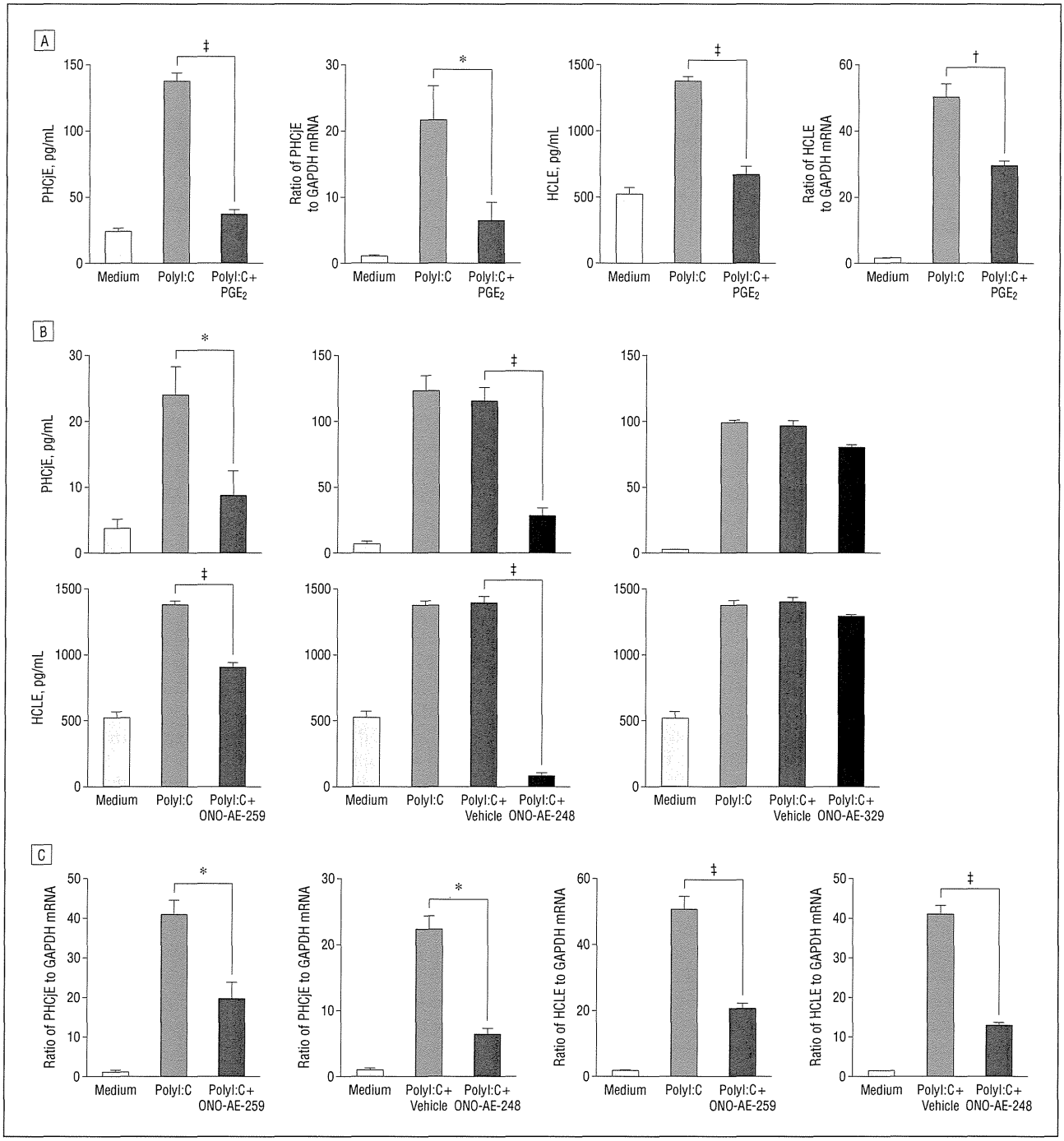

Figure. Prostaglandin E_2 (PGE_2) attenuated the messenger RNA (mRNA) expression and production of monocyte chemoattractant protein 1 via both prostaglandin E receptor 2 (EP_2) and EP_3. A, Primary human conjunctival epithelial cells (PHCjE) and human corneal-limbal epithelial cells (HCLE) were exposed to 10 μg/mL of polyinosine–polycytidylic acid (polyI:C) and 100 μg/mL of PGE_2 for 24 hours (enzyme-linked immunosorbent assay) or 6 hours (quantitative real-time polymerase chain reaction). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase. B and C, The PHCjE and HCLE were exposed to 10 μg/mL of polyI:C and 10 μg/mL of the EP_2, EP_3, or EP_4 agonist for 24 hours (enzyme-linked immunosorbent assay) (B) or 6 hours (quantitative real-time polymerase chain reaction) (C). Data are representative of 3 separate experiments and are given as the mean (SEM) from 1 experiment carried out in 6 to 8 wells (enzyme-linked immunosorbent assay) (B) or 4 to 6 wells (quantitative real-time polymerase chain reaction) (C) per group. *$P<.05$; †$P<.005$; ‡$P<.001$.

jiicho, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan (mueta@koto.kpu-m.ac.jp).

Author Contributions: Dr Ueta had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: The work described in this article was carried out in collaboration with Ono Pharmaceutical Co Ltd, who supplied ONO-AE-248 used in this study.

Funding/Support: This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour, and Welfare, the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Kyoto Foundation for the Promotion of Medical Science, the National Institute of Biomedical Innovation of Japan, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and the

Shimizu Foundation for Immunological Research Grant.
Online-Only Material: The eAppendix is available at http://www.archophthalmol.com.
Additional Contributions: Chikako Endo provided technical assistance.

1. Yagi T, Sotozono C, Tanaka M, et al. Cytokine storm arising on the ocular surface in a patient with Stevens-Johnson syndrome. *Br J Ophthalmol*. 2011; 95(7):1030-1031.
2. Ueta M, Sotozono C, Nakano M, et al. Association between prostaglandin E receptor 3 polymorphisms and Stevens-Johnson syndrome identified by means of a genome-wide association study. *J Allergy Clin Immunol*. 2010;126(6): 1218-1225, e10.
3. Ueta M, Matsuoka T, Yokoi N, Kinoshita S. Prostaglandin E2 suppresses polyinosine-polycytidylic acid (polyI:C)-stimulated cytokine production via prostaglandin E2 receptor (EP) 2 and 3 in human conjunctival epithelial cells. *Br J Ophthalmol*. 2011;95(6):859-863.
4. Ueta M, Matsuoka T, Yokoi N, Kinoshita S. Prostaglandin E receptor subtype EP3 downregulates TSLP expression in human conjunctival epithelium. *Br J Ophthalmol*. 2011;95(5):742-743.
5. Ueta M, Kinoshita S. Innate immunity of the ocular surface. *Brain Res Bull*. 2010;81(2-3):219-228.
6. Takayama K, García-Cardena G, Sukhova GK, Comander J, Gimbrone MA Jr, Libby P. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem*. 2002;277(46):44147-44154.

Depth Profile Study of Abnormal Collagen Orientation in Keratoconus Corneas

In a previous study,[1] we used femtosecond laser technology to cut ex vivo human corneas into anterior, mid, and posterior sections, after which x-ray scatter patterns were obtained at fine intervals over each specimen. Data analysis revealed the predominant orientation of collagen at each sampling site, which was assembled to show the variation in collagen orientation between central and peripheral regions of the cornea and as a function of tissue depth. We hypothesized that the predominantly orthogonal arrangement of collagen (directed toward opposing sets of rectus muscles) in the mid and posterior stroma may help to distribute strain in the cornea by allowing it to withstand the pull of the extraocular muscles. It was also suggested that the more isotropic arrangement in the anterior stroma may play a role in tissue biomechanics by resisting intraocular pressure while at the same time maintaining corneal curvature. This article, in conjunction with our findings of abnormal collagen orientation in full-thickness keratoconus corneas,[2,3] received a great deal of interest from the scientific community and prompted the following question: how does collagen orientation change as a function of tissue depth when the anterior curvature of the cornea is abnormal, as in keratoconus? Herein, we report findings from our investigation aimed at answering this question.

Methods. The Baron chamber used in our previous study[1] was adapted to enable corneal buttons to be clamped in place and inflated (by pumping physiological saline into the posterior compartment) to restore their natural curvature. A button diameter of 8 mm or larger was deemed necessary to ensure tissue stability during this process.

The next step, obtaining fresh, full-thickness, keratoconus buttons of sufficient diameter, proved to be problematic owing to the increasing popularity of deep anterior lamellar keratoplasty. Recently, however, the opportunity arose to examine an 8-mm full-thickness (300-340 μm minus epithelium) keratoconus corneal button with some central scarring and a mean power greater than 51.8 diopters (**Figure 1**). The tissue was obtained in accordance with the tenets of the Declaration of Helsinki and with full informed consent from a 31-year-old patient at the time of penetrating keratoplasty. Using techniques detailed previously,[1] the corneal button was clamped in the chamber and inflated. The central 6.3-mm region of the button was then flattened by the applanation cone and a single cut was made at a depth of 150 μm from the surface using an IntraLase 60-kHz femtosecond laser (Abbott Medical Optics Inc),[1] thus splitting the cornea into anterior and posterior sections of roughly equal thickness. Wide-angle x-ray scattering patterns were collected at 0.25-mm intervals over each cor-

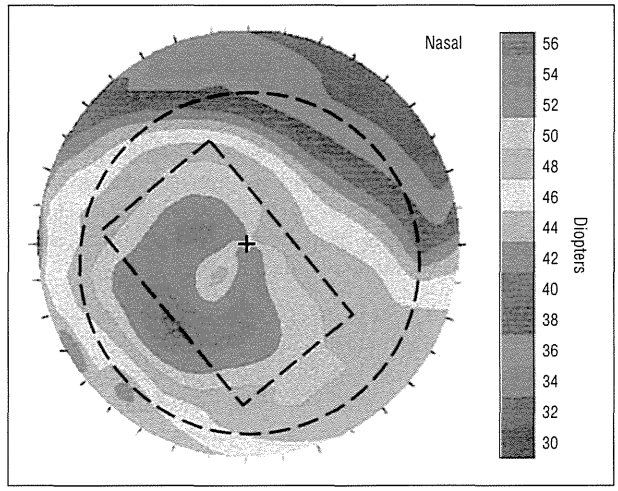

Figure 1. Corneal topography of the keratoconus cornea (recorded 12 years previously).[3] The broken lines show the 6.3-mm region of the cornea cut with the femtosecond laser (circle) and the region of greatest corneal steepening depicted in Figure 2 (rectangle).

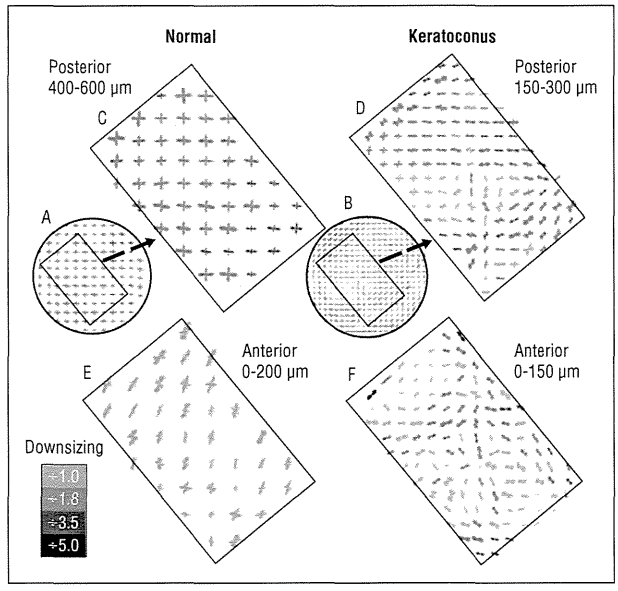

Figure 2. Collagen orientation in the normal (A) and keratoconus (B) posterior stroma (central 6.3 mm). The highlighted regions of the posterior (C and D) and anterior (E and F) stroma are expanded. Large vector plots showing high collagen alignment are downsized (key).

Prostaglandin E2 Suppresses Poly I:C-Stimulated Cytokine Production Via EP2 and EP3 in Immortalized Human Corneal Epithelial Cells

Mayumi Ueta, MD, PhD,† Toshiyuki Matsuoka, MD, PhD,‡ Chie Sotozono, MD, PhD,* and Shigeru Kinoshita, MD, PhD**

Purpose: We previously reported that prostaglandin (PG) E_2 acts as a ligand for prostaglandin E receptor 3 (EP3) in conjunctival epithelial cells, that it downregulates the progression of experimental murine allergic conjunctivitis, and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via prostaglandin E receptor 2 (EP2) and EP3, suggesting that PGE_2 might have important roles in ocular surface inflammation such as allergic conjunctivitis. Here, we investigated whether PGE_2 also downregulates polyI:C-induced cytokine production in human corneal epithelial cells.

Methods: We used enzyme-linked immunosorbent assay and quantitative reverse transcription–polymerase chain reaction to examine the effects of PGE_2 on polyI:C-induced cytokine expression by immortalized human corneal-limbal epithelial cells (HCLE). Using reverse transcription–polymerase chain reaction, we examined the messenger RNA (mRNA) expression of the PGE_2 receptor, EP1–4.

Results: PGE_2 significantly attenuated the expression of CC chemokine ligand (CCL)5 ($P < 0.0005$), CCL20 ($P < 0.0005$), C-X-C chemokine (CXCL)10 ($P < 0.0005$), CXCL11 ($P < 0.05$), and interleukin (IL)-6 ($P < 0.005$) in human corneal-limbal epithelial cells. Human corneal epithelial cells manifested the mRNA expression of EP2, EP3, and EP4, but not EP1. The EP2 agonist significantly suppressed the polyI:C-induced expression of CCL5 ($P < 0.005$), CXCL10 ($P < 0.0005$), and CXCL11 ($P < 0.05$) but not of CCL20 and IL-6. The EP3 agonist significantly suppressed the expression of CCL5 ($P < 0.05$), CCL20 ($P < 0.005$), CXCL10 ($P < 0.0005$), CXCL11 ($P < 0.0005$), and IL-6 ($P < 0.005$). The EP4 agonist failed to suppress cytokine production induced by polyI:C stimulation.

Conclusions: Our results show that in human corneal epithelial cells, PGE_2 attenuated the mRNA expression and production of CCL5, CXCL10, and CXCL11 via both EP2 and EP3, and that the mRNA expression and production of CCL20 and IL-6 was attenuated only by EP3.

Key Words: prostaglandin E_2 (PGE_2), human corneal epithelial cells, prostaglandin E receptor 3, prostaglandin E receptor 2

(*Cornea* 2012;31:1294–1298)

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 8 types of prostanoid receptors: 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]

PolyI:C, a synthetic double-stranded (ds)RNA, which mimics viral dsRNA, is the well-known ligand of Toll-like receptor 3.[2] We have reported that polyI:C stimulation induces the secretion of inflammatory cytokines such as interleukin (IL)-6, IL-8, type I interferon (IFN) such as IFN-β, IFN-inducible proteins such as C-X-C chemokine (CXCL)10 and CXCL11, and allergy-related proteins such as CC chemokine ligand (CCL)5 and thymic stromal lymphopoietin in human ocular surface epithelium, both corneal and conjunctival.[3–5] Moreover, we also reported that not only Toll-like receptor 3, but also cytoplasmic helicase proteins, RIG-I (retinoic acid–inducible protein I) and MDA5 (melanoma differentiation–associated gene 5) contribute to polyI:C-inducible responses in conjunctival epithelium.[6]

We previously reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells, that it downregulates the

Received for publication April 21, 2011; revision received October 15, 2011; accepted November 10, 2011.

From the *Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; †Department of Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan; and ‡Department of Ophthalmology, Tenri Hospital, Nara, Japan.

Supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, a research grant from the Kyoto Foundation for the Promotion of Medical Science, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and a research grant from the Shimizu Foundation.

The work described in the present article was carried out in collaboration with Ono Pharmaceutical Co, Ltd, who supplied ONO-AE-259, ONO-AE-248, and ONO-AE-329 used in this study. The authors have no other competing financial interests.

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.corneajrnl.com).

Reprints: Mayumi Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan (e-mail: mueta@koto.kpu-m.ac.jp).

progression of experimental murine allergic conjunctivitis,[7] and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via not only EP3 but also EP2,[8] suggesting that PGE_2 might have important roles in the ocular surface inflammation such as allergic conjunctivitis.

PGE_2 was reported to be produced during inflammatory responses and to suppress the production of cytokines and chemokines induced by lipopolysaccharide (LPS) stimulation in macrophages[9,10] and dendritic cells.[11] Elsewhere, we documented that human corneal and conjunctival epithelial cells produce cytokines such as IL-6, IL-8, and IFN-β in response to stimulation with polyI:C but not LPS.[3,12,13] In this study, we examined the expression of the PGE_2 receptors, EP1, EP2, EP3, and EP4, in human corneal epithelial cells and investigated whether polyI:C-induced cytokine production is downregulated by PGE_2 in these cells.

MATERIALS AND METHODS

Human Corneal Epithelial Cells

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

For reverse transcription–polymerase chain reaction (RT-PCR) assay, we obtained human corneal epithelial cells from corneal grafts of patients who had undergone corneal transplantation for bullous keratopathy. Immortalized human corneal-limbal epithelial cells (HCLE), a gift from Dr Irene K. Gipson, were cultured in low calcium–defined keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) with defined growth-promoting additives that included insulin, epidermal and fibroblast growth factors, and 1% antibiotic–antimycotic solution. The cells were used after reaching 80% confluence.[7]

Reverse Transcription–Polymerase Chain Reaction

RT-PCR assay was as previously described.[7] Briefly, total RNA was isolated from HCLE and human corneal epithelium using the Qiagen RNeasy kit (Qiagen, Valencia, CA) 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 minute, annealing for 1 minute, and 72°C for 1 minute on a commercial PCR machine (GeneAmp; PE Applied Biosystems). The primers were as previously reported.[7] RNA integrity was assessed by electrophoresis in ethidium bromide–stained 1.5% agarose gels. We performed 2 separate experiments.

Enzyme-Linked Immunosorbent Assay

Protein production was confirmed by enzyme-linked immunosorbent assay (ELISA). The amount of IL-6, CCL5, CCL20, CXCL11, and CXCL10 released into the culture supernatant was determined by ELISA using the human CCL5, CCL20, CXCL11, CXCL10 DuoSet (R&D Systems Inc, Minneapolis, MN) or the OptEIA IL-6 set (BD Pharmingen, San Diego, CA).[4,7,14]

We performed 3 separate experiments, each being carried out in 6 wells per group.

Quantitative RT-PCR

Total RNA was isolated from HCLE using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RT reaction was with the SuperScript Preamplification kit (Invitrogen). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, CA) using a previously described protocol.[4,7,14] The primers and probes were from Applied Biosystems [assay ID: CCL5 (Hs00174575), CCL20 (Hs01011368), CXCL10 (Hs00171042), CXCL11 (Hs00171138), IL-6 (Hs00174131), and human GAPDH (Hs 4326317E)]. For complementary DNA (cDNA) amplification, we performed PCR 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 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. We performed 3 separate experiments, each being carried out in 6 wells per group.

Data Analysis

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

RESULTS

PGE2 Downregulated the Production of Cytokines Induced by Poly I:C Stimulation

Using HCLE and ELISA, we examined whether PGE_2 downregulated the production of IL-6, IL-8, CCL5, CCL20, CXCL10, and CXCL11 induced by polyI:C stimulation in human corneal epithelial cells. HCLE were exposed to 10 μg/mL polyI:C and 100 μg/mL PGE_2 for 24 hours (ELISA) or 6 hours (quantitative RT-PCR). We found that PGE_2 significantly attenuated the production of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (all, $P < 0.0005$) (Fig. 1A). Quantitative RT-PCR assay confirmed that the messenger RNA (mRNA) expression of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (respectively, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$, $P < 0.05$ and $P < 0.005$) was significantly downregulated by PGE_2 (Fig. 1B).

Human Corneal 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, EP2, EP3, and EP4, in human corneal epithelial cells. PCR products of expected

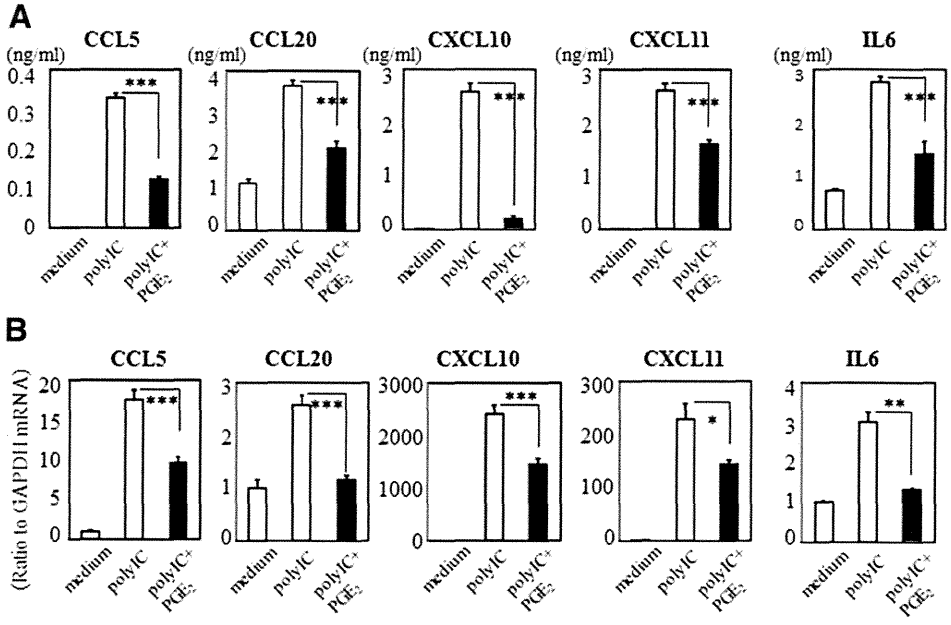

FIGURE 1. A, Suppression of the production of CCL5, CCl20, CXCL10, CXCL11, and IL-6 by PGE_2. HCLE were exposed to 10 μg/mL poly I:C and 100 μg/mL PGE_2 for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group. B, Suppression of mRNA expression of CCL5, CCL20, CXCL10, CXCL11, and IL-6 by PGE_2. HCLE were exposed to 10 μg/mL poly I:C and 100 μg/mL PGE_2 for 6 hours. 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 are given as the mean ± SEM from one experiment carried out in 6 wells per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

lengths were obtained for EP2 (683 bp), EP3 (622 bp), and EP4 (956 bp) (Fig. 2), but not for EP1 (723 bp) (data not shown), from HCLE and *in vivo* human corneal epithelial cells, suggesting that the human corneal epithelium expresses EP2, EP3, and EP4 mRNAs. To confirm the specificity for the detection of EP2-, EP3-, and EP4 mRNA, we isolated and sequenced the PCR products. The obtained sequences were identical to the human EP2-, EP3-, and EP4 cDNA sequences. Moreover, we could detect EP2, EP3 and EP4 proteins using immunoblotting (see **Figure, Supplemental Digital Content 1**, http://links.lww.com/ICO/A42).

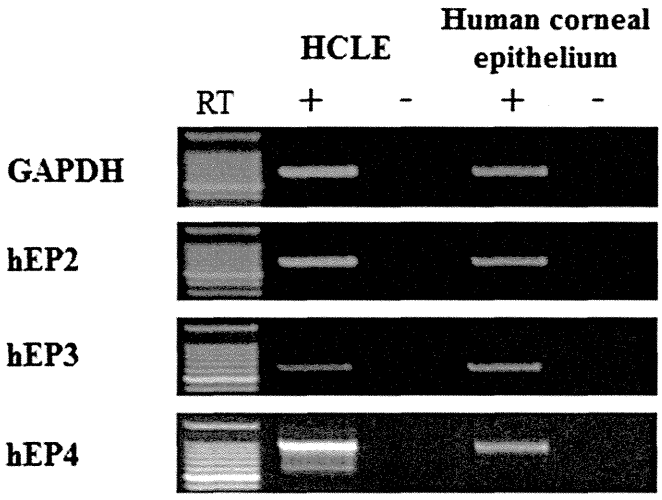

FIGURE 2. mRNA expression of the PGE_2 receptors EP2, EP3, and EP4. RT-PCR assay of the expression of PGE_2 receptor EP2, EP3, and EP4-specific mRNA in HLCE and human corneal epithelium. RT identifies data that were obtained without reverse transcription (controls).

EP2 and EP3, but not EP4 Agonists Downregulated the Production of Cytokines Induced by Poly I:C Stimulation

Using the EP2, EP3, and EP4 agonists, ONO-AE-259, ONO-AE-248, and ONO-AE-329, respectively, we also examined which PGE_2 receptor(s) contributed to their polyI:C-induced downregulation. HCLE were exposed to 10 μg/mL polyI:C and 10 μg/mL of the EP2, EP3, or EP4 agonist for 24 hours (ELISA) or 6 hours (quantitative RT-PCR). ELISA showed that the EP2 agonist significantly suppressed the polyI:C-induced production of CCL5, CXCL10, and CXCL11 (all, $P < 0.0005$) but not of CCL20 and IL-6, and that the EP3 agonist significantly suppressed the production of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (all, $P < 0.0005$). However, the EP4 agonist failed to suppress the cytokine production induced by polyI:C stimulation (Fig. 3). Quantitative RT-PCR confirmed that the EP2 agonist significantly downregulated the mRNA expression of CCL5, CXCL10, and CXCL11 (respectively, $P < 0.005$, $P < 0.0005$ and $P < 0.05$), but not of CCL20 and IL-6, and that the EP3 agonist significantly downregulated the mRNA expression of all examined cytokines (CCL5, $P < 0.05$; CCL20, $P < 0.005$; CXCL10, $P < 0.0005$; CXCL11, $P < 0.0005$; and IL-6, $P < 0,005$) (Fig. 4). 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 CCL20 and IL-6 mRNA expression and production were attenuated only by EP3 in human corneal epithelial cells.

DISCUSSION

Lipid mediators like PGE_2 regulate immune and inflammatory responses by modulating the production of cytokines and chemokines.[11] In macrophages, PGE_2 suppressed the proinflammatory gene expression induced by LPS,

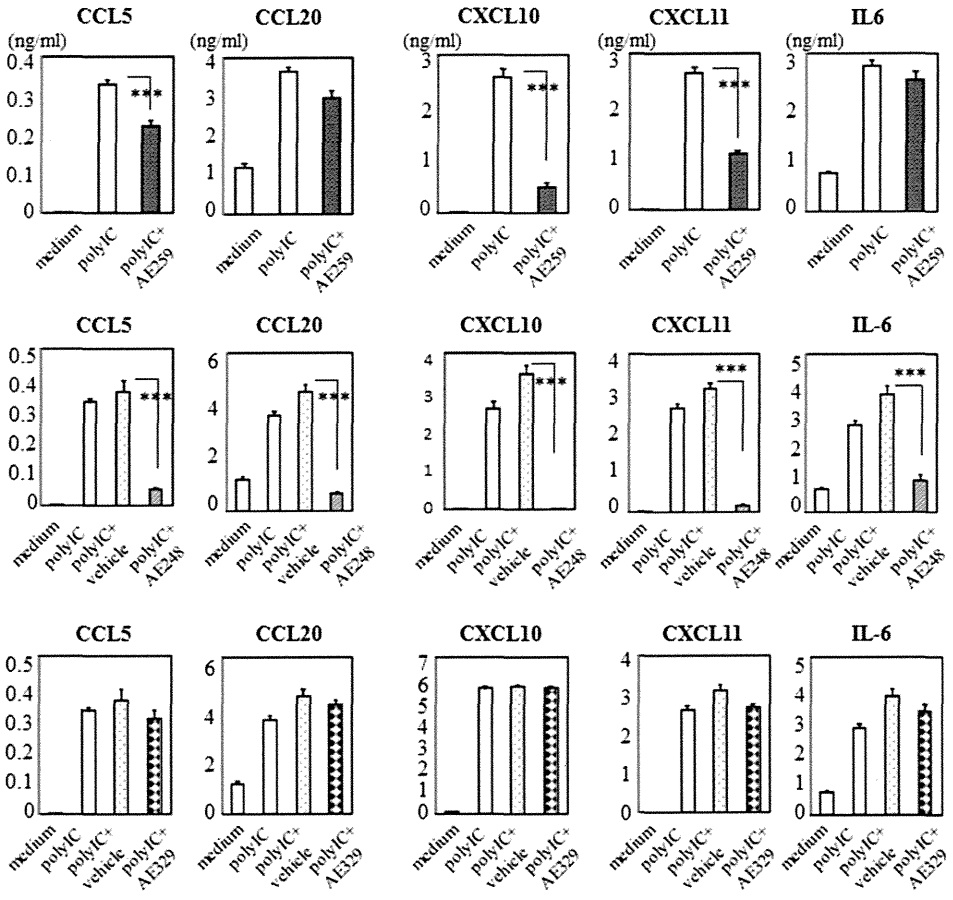

FIGURE 3. Effect of the PGE_2 receptors EP2, EP3, and EP4 on poly I:C-induced cytokine production. HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2, EP3, or EP4 agonist for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group (***$P < 0.0005$).

including macrophage inflammatory protein (MIP)-1α, MIP-1β, CCL5, CXCL10, and IL-8.[9] Here we document that PGE_2 modulates the expression and production of polyI:C-induced proinflammatory genes in not only human conjunctival epithelial cells but also corneal epithelial cells. It exerted an inhibitory effect on polyI:C-induced CCL5, CCL20, CXCL10, CXCL11, and IL-6 mRNAs (respectively, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$, $P < 0.05$ and $P < 0.005$) and on protein production in HCLE (all, $P < 0.0005$). 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,

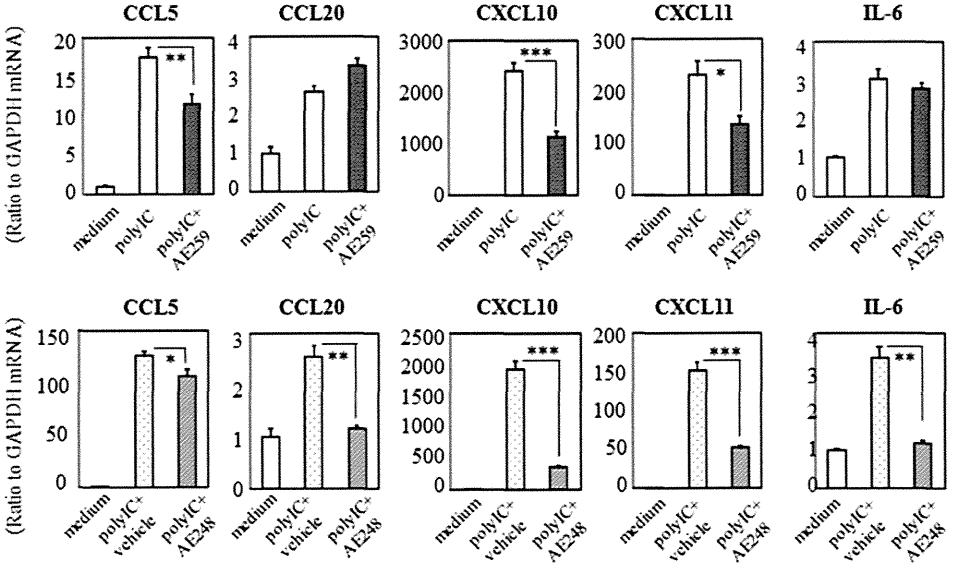

FIGURE 4. Effect of the PGE_2 receptors EP2 and EP3 on the poly I:C-induced mRNA expression of cytokines: HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2 or EP3 agonist for 6 hours. The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. The *y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

EP3, and EP4, in human corneal epithelial cells. Stimulation with either EP2- or EP3-specific agonists had a suppressive effect on polyI:C-induced CCL5, CXCL10, and CXCL11 production (both EP2- and EP3-specific agonists: all, $P < 0.0005$) , but only the EP3-specific agonist had a suppressive effect on the production of CCL20 and IL-6 (both, $P < 0.0005$).

Stimulation with PGE_2 exhibits immunosuppressive effects in various cell types including macrophages and dendritic cells via EP2 and/or EP4.[9–11] This phenomenon is explicable by the elevation of intracellular cyclic adenosine monophosphate (cAMP) via the activation of adenylcyclase.[9,10] Although PGE_2 acts on EP2 and EP4 and activates adenylcyclase, resulting in the elevation of intracellular cAMP, its action on EP3 suppresses adenylcyclase, resulting in a decrease in intracellular cAMP. In human conjunctival and corneal epithelial cells, both EP2 and EP3 contribute to the immunosuppressive effect against polyI:C stimulation; therefore, the suppressive effect cannot be explained by the elevation of intracellular cAMP. The precise molecular mechanisms underlying the immunosuppressive effects of PGE_2 in epithelial cells remain to be elucidated.

Release of PGE_2 is associated with ocular inflammation, but the exact role in inflammation has not been identified, rather PGE_2 might have been considered as inflammation-related molecules in the cornea. In this study, it is evident that PGE_2 could contribute to suppressing the production of various cytokines and chemokines in the ocular surface. Elsewhere we 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,[7] suggesting the possibility of the PGE_2 and EP3 selective agonists as antiinflammatory drugs.

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

ACKNOWLEDGEMENTS

We thank Chikako Endo for technical assistance.

REFERENCES

1. Matsuoka T, Narumiya S. Prostaglandin receptor signaling in disease. *ScientificWorldJournal*. 2007;7:1329–1347.
2. Alexopoulou L, Holt AC, Medzhitov R, et al. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 2001;413:732–738.
3. Ueta M, Kinoshita S. Ocular surface inflammation mediated by innate immunity. *Eye Contact Lens*. 2010;36:269–281.
4. Ueta M, Mizushima K, Yokoi N, et al. Gene-expression analysis of polyI:C-stimulated primary human conjunctival epithelial cells. *Br J Ophthalmol*. 2010;94:1528–1532.
5. Kumar A, Zhang J, Yu FS. Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells. *Immunology*. 2006; 117:11–21.
6. Ueta M, Kawai T, Yokoi N, et al. Contribution of IPS-1 to polyI:C-induced cytokine production in conjunctival epithelial cells. *Biochem Biophys Res Commun*. 2011;404:419–423.
7. Ueta M, Matsuoka T, Narumiya S, et al. Prostaglandin E receptor subtype EP3 in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis. *J Allergy Clin Immunol*. 2009;123:466–471.
8. Ueta M, Matsuoka T, Yokoi N, et al. Prostaglandin E2 suppresses polyinosine-polycytidylic acid (polyI:C)-stimulated cytokine production via prostaglandin E2 receptor (EP) 2 and 3 in human conjunctival epithelial cells. *Br J Ophthalmol*. 2011;95:859–863.
9. Takayama K, García-Cardena G, Sukhova GK, et al. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem*. 2002;277:44147–44154.
10. Xu XJ, Reichner JS, Mastrofrancesco B, et al. Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production. *J Immunol*. 2008;180:2125–2131.
11. Shiraishi H, Yoshida H, Saeki K, et al. Prostaglandin E2 is a major soluble factor produced by stromal cells for preventing inflammatory cytokine production from dendritic cells. *Int Immunol*. 2008;20:1219–1229.
12. Ueta M, Hamuro J, Kiyono H, et al. Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines. *Biochem Biophys Res Commun*. 2005;331:285–294.
13. Ueta M, Kinoshita S. Innate immunity of the ocular surface. *Brain Res Bull*. 2010;81:219–228.
14. Ueta M, Sotozono C, Nakano M, et al. Association between prostaglandin E receptor 3 polymorphisms and Stevens-Johnson syndrome identified by means of a genome-wide association study. *J Allergy Clin Immunol*. 2010;126:1218–1225. e10.

Atypical Continuous Keratitis in a Case of Rheumatoid Arthritis Accompanying Severe Scleritis

Masayuki Hata, MD,† Takahiro Nakamura, MD, PhD,*†‡ Chie Sotozono, MD, PhD,*†‡ Kyoko Kumagai, MD,*† Shigeru Kinoshita, MD, PhD,‡ and Yasuo Kurimoto, MD, PhD*†*

Purpose: Rheumatoid arthritis (RA) often presents with ocular complications: typically dry eye, peripheral corneal ulcer, and scleritis. We report for the first time a case of severe scleritis with RA, accompanying atypical continuous keratitis, which apparently differs from typical peripheral ulcerative keratitis (PUK).

Methods: Observational case report.

Results: A 68-year-old woman with RA presented at our hospital complaining of worsening arthritis accompanying ocular injection and discharge. On examination, nodular scleritis and peripheral corneal infiltration were noted. In addition to administering topical steroid and antibiotics, cyclosporine and an oral steroid were added because of the patient's worsening scleritis. Despite gradual improvement of the scleritis, the efficacy of the additional treatments was limited. Four months after initial treatment, the patient presented with uveitis, thought to be caused by a herpetic virus. Antivirus treatment was effective for the uveitis, but atypical continuous keratitis suddenly appeared. The keratitis was located from 4-o'clock to 10-o'clock positions continuously in the midperipheral cornea and apparently differed from herpetic keratitis or PUK as typically seen in RA cases. Immune reaction was suspected, and the keratitis improved within 2 weeks. After that, the introduction of an anti–tumor necrosis factor α drug (infliximab) completely resolved the severe scleritis and there was no recurrence of ocular inflammation.

Conclusion: As is shown in this case, RA can present with atypical continuous keratitis, thought to be a manifestation of an immunologic reaction other than PUK. In addition, although immunosuppressants are often used for the treatment of RA with scleritis, the efficacy is limited. Infliximab may be a useful treatment for treatment-resistant scleritis.

Key Words: rheumatoid arthritis, scleritis, peripheral ulcerative keratitis, uvetis, infliximab

(*Cornea* 2012;0:1–4)

Received for publication July 27, 2011; revision received August 25, 2011; accepted October 14, 2011.

From the *Department of Ophthalmology, Kobe City Medical Center General Hospital, Kobe, Japan; †Department of Ophthalmology, Institute of Biomedical Research and Innovation Hospital, Kobe, Japan; and ‡Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

The authors have no funding or conflicts of interest to disclose.

Reprints: Takahiro Nakamura, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan (e-mail: tnakamur@koto.kpu-m.ac.jp).

Rheumatoid arthritis (RA) is a chronic progressive autoimmune disease characterized by a polyarticular synovitis. RA often presents with ocular complications caused by immunocomplex deposits: typically dry eye, peripheral ulcerative keratitis (PUK), and scleritis. Moreover, RA causes a wide variety of ocular lesions, including uveitis and corneal impairment in the clinical course.[1]

Scleritis is one of the severe ocular manifestations of RA and is associated with systemic inflammation.[2,3] As compared to patients with idiopathic scleritis, RA-associated scleritis is sometimes resistant to treatments.[4] In addition, RA presents with various corneal lesions associated with the occurrence of scleritis.[5,6] PUK is a severe inflammatory disease that characteristically involves the peripheral cornea. The clinical presentation of PUK is characteristically a noninfiltrating ulcer at the periphery of the cornea that is often contiguous with adjacent scleritis.[7]

Conventional treatment of RA consists of the administration of systemic corticosteroids and immunosuppressants. Methotrexate is widely used as the first-line treatment; however, methotrexate monotherapy often fails to reduce or eradicate the inflammation. A combination with other immunosuppressants, such as cyclophosphamide, cyclosporine A, and azathioprine, is often used to lower the disease activity.[4,8] In addition, surgical intervention by keratoepithelioplasty is commonly chosen for treating the severe peripheral corneal ulcer.[9] However, very little published data exist regarding treatments for RA-related ocular inflammation resistant to conventional treatments. Tumor necrosis factor α (TNF-α) is an inflammatory cytokine, and agents blocking its action have proven to be effective in treating RA. Reports are few, but some case studies have reported the effective treatment of severe scleritis.[10,11]

In this present study, we report for the first time a case of severe scleritis with RA accompanying atypical continuous keratitis that apparently differs from typical PUK, which ultimately was effectively treated by the use of an anti-TNF drug.

CASE REPORT

A 68-year-old woman was referred to our hospital presenting with conjunctival injection and ocular discharge in her OD and worsening of arthralgia. She had been diagnosed with RA over the previous 15 years and had been treated with prednisolone (5 mg/d) and methotrexate (6 mg/wk). Her best-corrected visual acuity was 20/32 OD and 20/20 OS. In her OD, nodular scleritis at the upper and lower sclera and peripheral corneal infiltration at the 3-o'clock and 9-o'clock positions were noted (Fig. 1). There were no inflammatory cells in the anterior