BMJ open

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001330
Article Type:	Research
Date Submitted by the Author:	30-Apr-2012
Complete List of Authors:	Ueta, Mayumi; Kyoto Prefectural University of Medicine, Department of Ophthalmology Sotozono, Chie; Kyoto Prefectural Univ of Med, Ophthalmology Yamada, Keiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Yokoi, Norihiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Inatomi, Tsutomu; Kyoto Prefectural University of Medicine, Department of Ophthalmology Kinoshita, Shigeru; Kyoto Prefectural University of Medicine, Department of Ophthalmology
Primary Subject Heading:	Ophthalmology
Secondary Subject Heading:	Ophthalmology
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, Histology < BASIC SCIENCES

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: case-control study

Mayumi Ueta, MD, PhD,[a,b] Chie Sotozono, MD, PhD,[a] Keiko Yamada, MD,[a]

Norihiko Yokoi, MD, PhD,[a] Tsutomu Inatomi, MD, PhD,[a] Shigeru Kinoshita, MD, PhD[a]

[a]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[b]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Corresponding author:

Mayumi Ueta, MD, PhD

Department of Ophthalmology

Kyoto Prefectural University of Medicine

465 Kajiicho, Hirokoji

Kawaramachi, Kamigyoku

Kyoto 602-0841, Japan

Telephone: 81-75-251-5578

Fax: 81-75-251-5663

e-mail: mueta@koto.kpu-m.ac.jp

Key words

Prostaglandin E receptor subtype EP4, human conjunctival epithelium, chemical eye burn, Mooren's ulcer

Abbreviations

EP4: Prostaglandin E receptor subtype EP4

SJS: Stevens-Johnson syndrome

TEN: Toxic epidermal necrolysis

GVHD: graft versus host disease

OCP: ocular cicatricial pemphigoid

RT-PCR: Reverse transcription polymerase chain reaction

PG: Prostaglandin

1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

2) drafting the article or revising it critically for important intellectual content; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

3) final approval of the version to be published; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

There is no additional data available.

Competing interest statement: none

Abstract

Objectives: To examine the expression of EP4 in conjunctival epithelium of patients with various ocular surface disorders.

Design: case-control study

Setting & Participants: Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute and chronic stage SJS/TEN, chronic stage ocular cicatricial pemphigoid (OCP) or severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva. We performed immunohistological analysis of EP4 and quantitative RT-PCR analysis of conjunctival tissue sections to confirm the down-regulation of EP4 mRNA expression in patients with SJS/TEN and OCP.

Primary and secondary outcome measures: Expression of EP4 protein by immunohistological methods and expression of EP4 mRNA.

Results: EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. The expression of EP4 protein varied in conjunctival epithelium from patients with Mooren's ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN, or OCP.

Conclusions: The strong down-regulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

Introduction

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are lipid mediators that form in response to various stimuli. 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. [1] PGE_2 was produced during inflammatory responses and it suppressed the production of cytokines and chemokines induced by lipopolysaccharide (LPS)-stimulated macrophages [2, 3] and dendritic cells. [4] Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes in human conjunctival epithelial cells. [5]

There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4. Intestinal epithelium has been reported to express EP4 mRNA, [6] and intestinal homeostasis was maintained- and the immune response was down-regulated by EP4. [7] We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/Toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP). [8] Here we examine the expression of EP4 in conjunctival epithelium of patients with ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the subacute stage of SJS/TEN.

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.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical eye burn, 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva.

For quantitative RT-PCR the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 4 patients in the chronic stage of SJS/TEN and 4 OCP patients in the chronic stage.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co., Ann Arbor, MI). 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. The VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.

Results

EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 1A) and in conjunctival tissues from 3 patients with chemical eye burn (Fig. 1B). Its expression varied in conjunctival epithelium from 4 patients with Mooren's ulcer (Fig. 1C): in one patient is was similar to the control, in 2 patients it was slightly lower than in the control, and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from 2 patients with subacute SJS/TEN (Fig. 1D), nor from patients with severe GVHD (Fig. 1E), as same as chronic SJS/TEN, or OCP. [8]

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, it was strongly down-regulated in conjunctival epithelium from patients with SJS/TEN, OCP, or severe GVHD.

To confirm the down-regulation of EP4 in the ocular surface of SJS and OCP patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 2A. Although EP4 protein was detected in tissues from patients with conjunctivochalasis (controls), conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples (Fig. 2B).

Discussion

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP. [8] Here we document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder. We also confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated.

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the sub-acute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Kabashima et al. [7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao et al. [9] found that PGE_2 acting on its receptor EP4 on T- and dendritic cells

not only facilitated T_H1 cell differentiation but also amplified interleukin-23-mediated T_H17 cell expansion *in vitro*. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signaling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer because as in the acute or sub-acute stage, patients in the chronic phase of OCP and SJS/TEN manifested mucosal inflammation on the ocular surface.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11, and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells. [5] Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic- and subacute SJS/TEN.

Figure Legends

Figure 1

Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.

A. Nearly normal conjunctival tissues from patients with conjunctivochalasis.

B. Conjunctival tissues of chemical eye burn patients in need of ocular surface reconstruction.

C. Inflammatory conjunctival tissues of patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.

D. Conjunctival tissues of SJS/TEN patients in the sub-acute stage.

E. Conjunctival tissues of a patient with severe GVHD.

Each scale bar represents 100 μm.

Figure 2

The expression of PTGER4 mRNA in conjunctival tissues from patients with SJS/TEN, OCP and the controls.

A. Representative findings of EP4 immunoreactivity of each group (control, SJS/TEN, OCP).

B. Expression of PTGER4 mRNA in human conjunctival tissues (*$p < 0.05$).

References

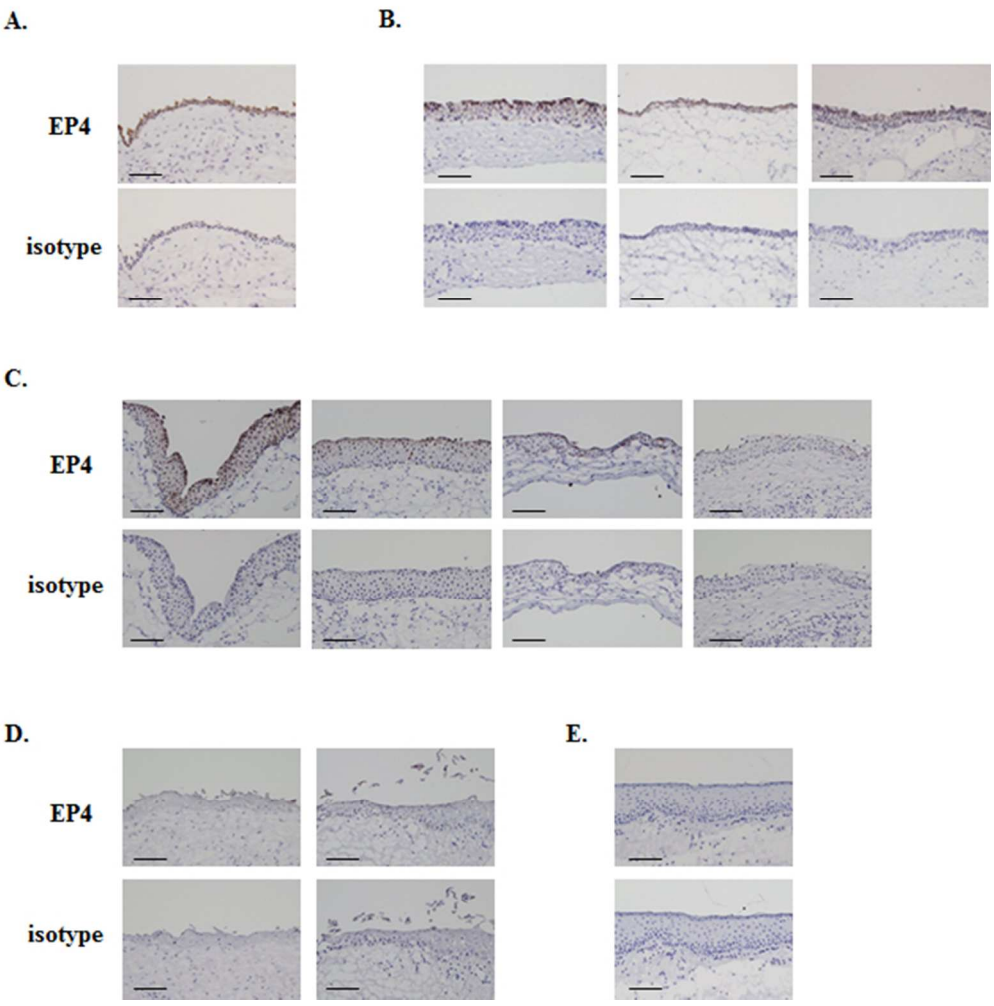
1. Matsuoka T, Narumiya S: **Prostaglandin receptor signaling in disease**. *ScientificWorldJournal* 2007, **7**:1329-1347.
2. Takayama K, Garcia-Cardena G, Sukhova GK, *et al.*: **Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor**. *J Biol Chem* 2002, **277**:44147-44154.
3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al.*: **Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production**. *J Immunol* 2008, **180**:2125-2131.
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**:1219-1229.
5. 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.
6. Morimoto K, Sugimoto Y, Katsuyama M, *et al*: **Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract**. *Am J Physiol* 1997, **272**:G681-687.
7. Kabashima K, Saji T, Murata T, *et al*: **The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut**. *J Clin Invest* 2002, **109**:883-893.
8. 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**(10):1369-1371.

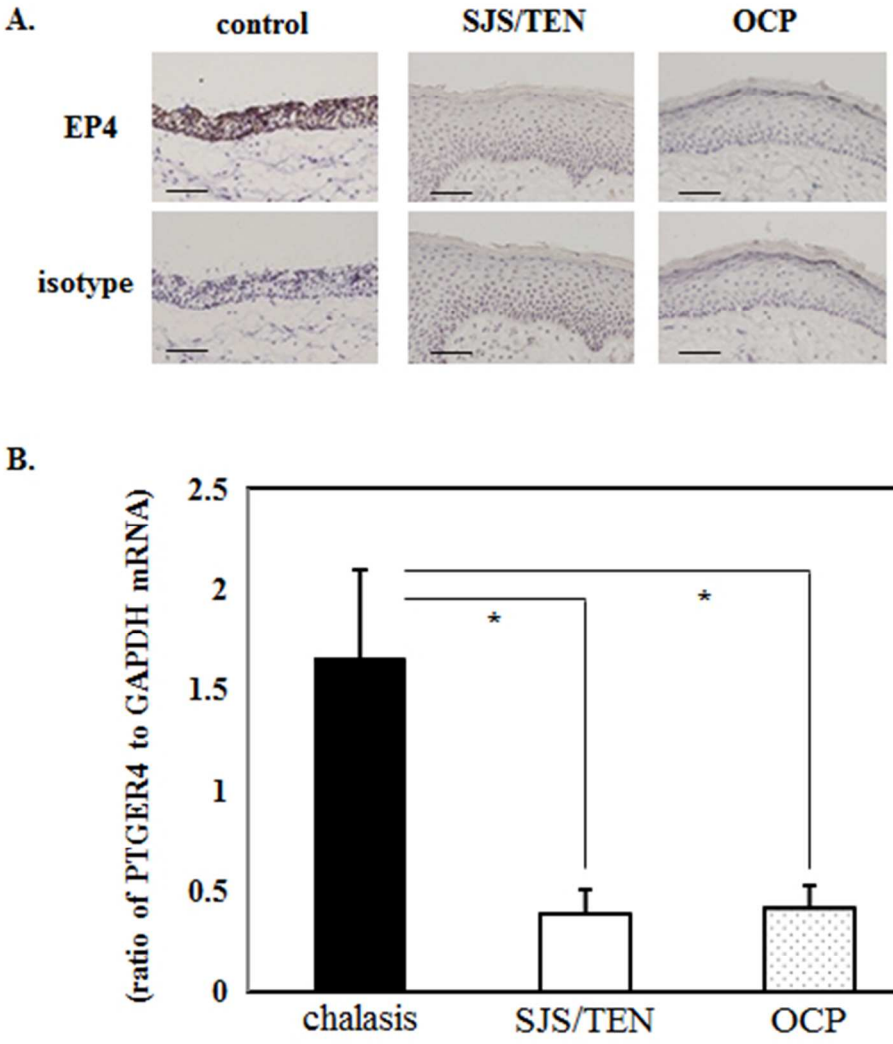
9. Yao C, Sakata D, Esaki Y, *et al*: **Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion**. *Nat Med* 2009, **15**:633-640.

ACKNOWLEDGMENTS

We thank Chikako Endo for technical assistance. 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, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and an Immunological Research Grant from the Shimizu Foundation.



Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.
53x53mm (300 x 300 DPI)



40x45mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	*(*a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how matching of cases and controls was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	7
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	8
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1 3

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ open

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001330.R1
Article Type:	Research
Date Submitted by the Author:	28-Jun-2012
Complete List of Authors:	Ueta, Mayumi; Kyoto Prefectural University of Medicine, Department of Ophthalmology Sotozono, Chie; Kyoto Prefectural Univ of Med, Ophthalmology Yamada, Keiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Yokoi, Norihiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Inatomi, Tsutomu; Kyoto Prefectural University of Medicine, Department of Ophthalmology Kinoshita, Shigeru; Kyoto Prefectural University of Medicine, Department of Ophthalmology
Primary Subject Heading:	Ophthalmology
Secondary Subject Heading:	Ophthalmology
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, Histology < BASIC SCIENCES

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how matching of cases and controls was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	7
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	8
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1 3

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: Case-control study

Mayumi Ueta, MD, PhD,[a,b] Chie Sotozono, MD, PhD,[a] Keiko Yamada, MD,[a]

Norihiko Yokoi, MD, PhD,[a] Tsutomu Inatomi, MD, PhD,[a] Shigeru Kinoshita, MD, PhD[a]

[a]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[b]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Corresponding author:

Mayumi Ueta, MD, PhD

Department of Ophthalmology

Kyoto Prefectural University of Medicine

465 Kajiicho, Hirokoji

Kawaramachi, Kamigyoku

Kyoto 602-0841, Japan

Telephone: 81-75-251-5578

Fax: 81-75-251-5663

e-mail: mueta@koto.kpu-m.ac.jp

Key words

Prostaglandin E receptor subtype EP4, human conjunctival epithelium, chemical eye burn, Mooren's ulcer

Abbreviations

EP4: Prostaglandin E receptor subtype EP4

SJS: Stevens-Johnson syndrome

TEN: Toxic epidermal necrolysis

GVHD: Graft versus host disease

OCP: Ocular cicatricial pemphigoid

RT-PCR: Reverse transcription polymerase chain reaction

PG: Prostaglandin

1) Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

2) Drafting the article or revising it critically for important intellectual content: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

3) Final approval of the version to be published; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

No additional data are available.

Competing interest statement: None

Abstract

Objectives: To confirm the down-regulation of PTGER4 mRNA in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study

Setting & Participants: We performed quantitative RT-PCR analysis of EP4 in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the down-regulation of EP4 mRNA expression. We also analyzed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute- or chronic SJS/TEN, chronic ocular cicatricial pemphigoid (OCP), severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of EP4 mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

Results: PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren’s ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN, or OCP.

Conclusions: The strong down-regulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

Introduction

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are lipid mediators that form in response to various stimuli. 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 [1]. PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide (LPS)-stimulated macrophages [2, 3] and dendritic cells [4]. Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes in human conjunctival epithelial cells [5].

There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4. The intestinal epithelium has been reported to express EP4 mRNA [6] and intestinal homeostasis was maintained and the immune response down-regulated by EP4 [7]. We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP) [8]. Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is down-regulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN.

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.

For quantitative RT-PCR the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 4 patients in the chronic stage of SJS/TEN and 4 patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co., Ann Arbor, MI). 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. The VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Results

To confirm the down-regulation of EP4 in the ocular surface of SJS and OCP patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples (Fig. 1B).

EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 2A) and in conjunctival tissues from 3 patients with chemical eye burn (Fig. 2B). Its expression varied in conjunctival epithelium from 4 patients with Mooren's ulcer (Fig. 2C): in one patient is was similar to the control, in 2 it was slightly lower than in the control, and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from 2 patients with subacute SJS/TEN (Fig. 2D), a patient with severe GVHD (Fig. 2E), and patients with chronic SJS/TEN or OCP [8].

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, EP4 immunoreactivity was not detected in conjunctival epithelium from patients with SJS/TEN, OCP, or severe GVHD. We did not detect EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined.

Discussion

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP [8]. Here we confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated, and we also document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder.

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the sub-acute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Kabashima et al. [7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao et al. [9] found that PGE_2 acting on its receptor EP4 on T- and dendritic cells

not only facilitated T_H1 cell differentiation but also amplified interleukin-23-mediated T_H17 cell expansion *in vitro*. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signaling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11, and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells [5]. Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic- and subacute SJS/TEN.

Figure Legends

Figure 1

The expression of PTGER4 mRNA in conjunctival tissues from patients with SJS/TEN, OCP and the controls.

A. Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP).

B. Expression of PTGER4 mRNA in human conjunctival tissues (*$p < 0.05$).

Figure 2

Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.

A. Nearly normal conjunctival tissues from patients with conjunctivochalasis.

B. Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction.

C. Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.

D. Conjunctival tissues from SJS/TEN patients in the sub-acute stage.

E. Conjunctival tissues from a patient with severe GVHD.

Each scale bar represents 100 μm.

References

1. Matsuoka T, Narumiya S: **Prostaglandin receptor signaling in disease**. *ScientificWorldJournal* 2007, **7**:1329-1347.
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**:44147-44154.
3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al.*: **Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production**. *J Immunol* 2008, **180**:2125-2131.
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**:1219-1229.
5. 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.
6. Morimoto K, Sugimoto Y, Katsuyama M, *et al*: **Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract**. *Am J Physiol* 1997, **272**:G681-687.
7. Kabashima K, Saji T, Murata T, *et al*: **The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut**. *J Clin Invest* 2002, **109**:883-893.
8. 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**(10):1369-1371.

9. Yao C, Sakata D, Esaki Y, *et al*: **Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion**. *Nat Med* 2009, **15**:633-640.

ACKNOWLEDGMENTS

We thank Chikako Endo for technical assistance. 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, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and an Immunological Research Grant from the Shimizu Foundation.

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: Case-control study

Mayumi Ueta, MD, PhD,[a,b] Chie Sotozono, MD, PhD,[a] Keiko Yamada, MD,[a]

Norihiko Yokoi, MD, PhD,[a] Tsutomu Inatomi, MD, PhD,[a] Shigeru Kinoshita, MD, PhD[a]

[a]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[b]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Corresponding author:

Mayumi Ueta, MD, PhD

Department of Ophthalmology

Kyoto Prefectural University of Medicine

465 Kajiicho, Hirokoji

Kawaramachi, Kamigyoku

Kyoto 602-0841, Japan

Telephone: 81-75-251-5578

Fax: 81-75-251-5663

e-mail: mueta@koto.kpu-m.ac.jp

Key words

Prostaglandin E receptor subtype EP4, human conjunctival epithelium, chemical eye burn, Mooren's ulcer

Abbreviations

EP4: Prostaglandin E receptor subtype EP4

SJS: Stevens-Johnson syndrome

TEN: Toxic epidermal necrolysis

GVHD: Graft versus host disease

OCP: Ocular cicatricial pemphigoid

RT-PCR: Reverse transcription polymerase chain reaction

PG: Prostaglandin

1) Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

2) Drafting the article or revising it critically for important intellectual content: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

3) Final approval of the version to be published; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

No additional data are available.

Competing interest statement: None

Abstract

Objectives: To confirm the down-regulation of PTGER4 mRNA in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study

Setting & Participants: ~~Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute and chronic stage SJS/TEN, chronic stage ocular cicatricial pemphigoid (OCP) or severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.~~ We performed quantitative RT-PCR analysis ~~immunohistological analysis~~ of EP4 ~~and quantitative RT-PCR analysis of~~ in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the down-regulation of EP4 mRNA expression. We also analyzed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute- or chronic SJS/TEN, chronic ocular cicatricial pemphigoid (OCP), severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of EP4 mRNA and ~~expression of~~ EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods. ~~and expression of EP4 mRNA~~.

Results: PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control

conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren's ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN, or OCP.

Conclusions: The strong down-regulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

Introduction

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are lipid mediators that form in response to various stimuli. 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 [1]. PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide (LPS)-stimulated macrophages [2, 3] and dendritic cells [4]. Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes in human conjunctival epithelial cells [5].

There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4. The intestinal epithelium has been reported to express EP4 mRNA [6] and intestinal homeostasis was maintained and the immune response down-regulated by EP4 [7]. We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP) [8]. Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is down-regulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN.

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.

For quantitative RT-PCR the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 4 patients in the chronic stage of SJS/TEN and 4 patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co., Ann Arbor, MI). 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. The VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

~~*Quantitative RT-PCR*~~

~~Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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~~

~~min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.~~

Results

To confirm the down-regulation of EP4 in the ocular surface of SJS and OCP patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples (Fig. 1B).

EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 2A) and in conjunctival tissues from 3 patients with chemical eye burn (Fig. 2B). Its expression varied in conjunctival epithelium from 4 patients with Mooren's ulcer (Fig. 2C): in one patient is was similar to the control, in 2 it was slightly lower than in the control, and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from 2 patients with subacute SJS/TEN (Fig. 2D), a patient with severe GVHD (Fig. 2E), and patients with chronic SJS/TEN or OCP [8].

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, EP4 immunoreactivity was not detected ~~it was strongly down-regulated~~ in conjunctival epithelium from patients with SJS/TEN, OCP, or severe GVHD. We did not detect EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined.

~~To confirm the down-regulation of EP4 in the ocular surface of SJS and OCP~~

patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 2A. Although EP4 protein was detected in tissues from patients with conjunctivochalasis (controls), conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples (Fig. 2B).

Discussion

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP [8]. Here we confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated, and we also document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder. ~~We also confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated.~~

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the sub-acute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Kabashima et al. [7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other

hand, Yao et al. [9] found that PGE_2 acting on its receptor EP4 on T- and dendritic cells not only facilitated T_H1 cell differentiation but also amplified interleukin-23-mediated T_H17 cell expansion *in vitro*. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signaling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer~~ because as in the acute or sub-acute stage, patients in the chronic phase of OCP and SJS/TEN manifested mucosal inflammation on the ocular surface~~.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11, and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells [5]. Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic- and subacute SJS/TEN.

Figure Legends

~~**Figure 1**~~

~~Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.~~

~~A. Nearly normal conjunctival tissues from patients with conjunctivochalasis.~~

~~B. Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction.~~

~~C. Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.~~

~~D. Conjunctival tissues from SJS/TEN patients in the sub-acute stage.~~

~~E. Conjunctival tissues from a patient with severe GVHD.~~

~~Each scale bar represents 100 μm.~~

~~**Figure 2**~~

Figure 1

The expression of PTGER4 mRNA in conjunctival tissues from patients with SJS/TEN, OCP and the controls.

A. Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP).

B. Expression of PTGER4 mRNA in human conjunctival tissues (*$p < 0.05$).

~~**Figure 1**~~

Figure 2

Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.

F. Nearly normal conjunctival tissues from patients with conjunctivochalasis.

G. Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction.

H. Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.

I. Conjunctival tissues from SJS/TEN patients in the sub-acute stage.

J. Conjunctival tissues from a patient with severe GVHD.

Each scale bar represents 100 μm.

References

1. Matsuoka T, Narumiya S: **Prostaglandin receptor signaling in disease**. *ScientificWorldJournal* 2007, **7**:1329-1347.
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**:44147-44154.
3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al*.: **Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production**. *J Immunol* 2008, **180**:2125-2131.
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**:1219-1229.
5. 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.
6. Morimoto K, Sugimoto Y, Katsuyama M, *et al*: **Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract**. *Am J Physiol* 1997, **272**:G681-687.
7. Kabashima K, Saji T, Murata T, *et al*: **The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut**. *J Clin Invest* 2002, **109**:883-893.
8. Ueta M, Sotozono C, Yokoi N, *et al*: **Prostaglandin E receptor 4 expression in human conjunctival epithelium and its downregulation in devastating**

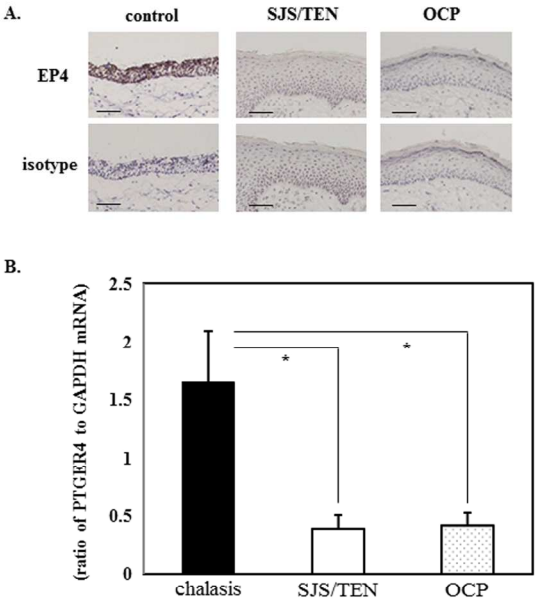
ocular surface inflammatory disorders. *Arch Ophthalmol* 2010, **128**(10):1369-1371.

9. Yao C, Sakata D, Esaki Y, *et al*: **Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion**. *Nat Med* 2009, **15**:633-640.

ACKNOWLEDGMENTS

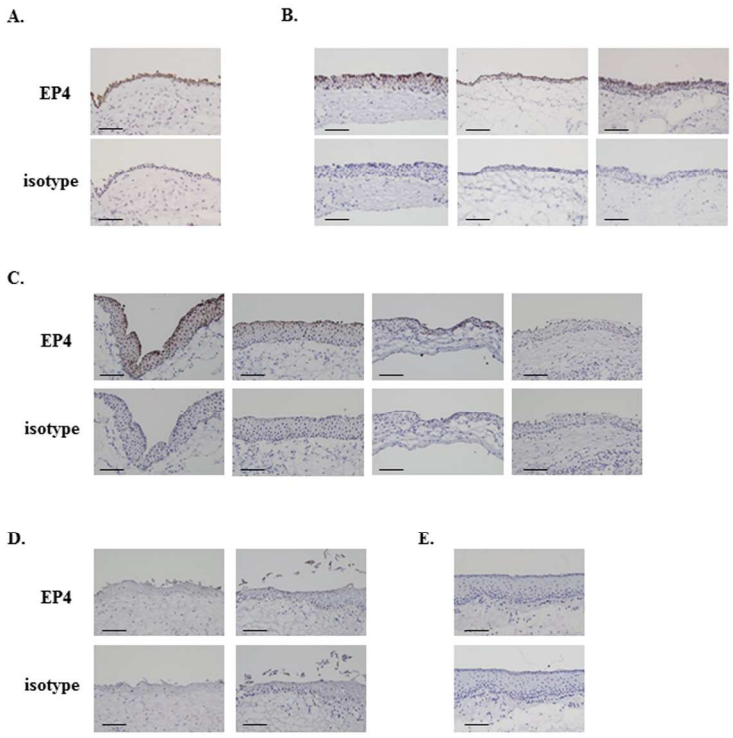
We thank Chikako Endo for technical assistance. This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST from JST, a research grant from the Kyoto Foundation for the Promotion of Medical Science, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and an Immunological Research Grant from the Shimizu Foundation.

Figure 1



190x275mm (300 x 300 DPI)

Figure 2



190x275mm (300 x 300 DPI)

BMJ open

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001330.R2
Article Type:	Research
Date Submitted by the Author:	28-Jul-2012
Complete List of Authors:	Ueta, Mayumi; Kyoto Prefectural University of Medicine, Department of Ophthalmology Sotozono, Chie; Kyoto Prefectural Univ of Med, Ophthalmology Yamada, Keiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Yokoi, Norihiko; Kyoto Prefectural University of Medicine, Department of Ophthalmology Inatomi, Tsutomu; Kyoto Prefectural University of Medicine, Department of Ophthalmology Kinoshita, Shigeru; Kyoto Prefectural University of Medicine, Department of Ophthalmology
Primary Subject Heading:	Ophthalmology
Secondary Subject Heading:	Genetics and genomics
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, Histology < BASIC SCIENCES

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	*(*a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how matching of cases and controls was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	7
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	8
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1 3

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: Case-control study

Mayumi Ueta, MD, PhD,[a,b] Chie Sotozono, MD, PhD,[a] Keiko Yamada, MD,[a]

Norihiko Yokoi, MD, PhD,[a] Tsutomu Inatomi, MD, PhD,[a] Shigeru Kinoshita, MD, PhD[a]

[a]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[b]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Corresponding author:

Mayumi Ueta, MD, PhD

Department of Ophthalmology

Kyoto Prefectural University of Medicine

465 Kajiicho, Hirokoji

Kawaramachi, Kamigyoku

Kyoto 602-0841, Japan

Telephone: 81-75-251-5578

Fax: 81-75-251-5663

e-mail: mueta@koto.kpu-m.ac.jp

Key words

Prostaglandin E receptor subtype EP4, human conjunctival epithelium, chemical eye burn, Mooren's ulcer

Abbreviations

EP4: Prostaglandin E receptor subtype EP4

SJS: Stevens-Johnson syndrome

TEN: Toxic epidermal necrolysis

GVHD: Graft versus host disease

OCP: Ocular cicatricial pemphigoid

RT-PCR: Reverse transcription polymerase chain reaction

PG: Prostaglandin

1) Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

2) Drafting the article or revising it critically for important intellectual content: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

3) Final approval of the version to be published; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

No additional data are available.

Competing interest statement: None

Abstract

Objectives: To confirm the down-regulation of PTGER4 mRNA in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study

Setting & Participants: We performed quantitative RT-PCR analysis of EP4 in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the down-regulation of EP4 mRNA expression. We also analyzed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute- or chronic SJS/TEN, chronic ocular cicatricial pemphigoid (OCP), severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of EP4 mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

Results: PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren's ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN, or OCP.

Conclusions: The strong down-regulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

Introduction

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are lipid mediators that form in response to various stimuli. 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 [1]. PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide (LPS)-stimulated macrophages [2, 3] and dendritic cells [4]. Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes in human conjunctival epithelial cells [5].

There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4. The intestinal epithelium has been reported to express EP4 mRNA [6] and intestinal homeostasis was maintained and the immune response down-regulated by EP4 [7]. The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.

We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP) [8]. Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is down-regulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN.

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.

For quantitative RT-PCR the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 4 patients in the chronic stage of SJS/TEN and 4 patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co., Ann Arbor, MI). 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. The VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Data analysis

Data were expressed as the mean ± SEM and evaluated by the Student's t-test using the Microsoft Excel software program.

Results

We previously documented that EP4 protein expression was down-regulated in conjunctival epithelium of devastating ocular surface inflammatory disorders such as chronic SJS/TEN and chronic OCP [8]. In this study, to confirm the down-regulation of EP4 in the ocular surface of SJS/TEN and OCP patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS/TEN and OCP patients than in the control conjunctivochalasis samples (Fig. 1B).

Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders. EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 2A) and in conjunctival tissues from 3 patients with chemical eye burn (Fig. 2B). Its expression varied in conjunctival epithelium from 4 patients with Mooren's ulcer (Fig. 2C): in one patient is was similar to the control, in 2 it was slightly lower than in the control, and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from 2 patients with subacute SJS/TEN (Fig. 2D), a patient with severe GVHD (Fig. 2E) as same as patients with chronic SJS/TEN or OCP [8].

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, EP4

immunoreactivity was not detected in conjunctival epithelium from patients with SJS/TEN, OCP, or severe GVHD. We did not detect EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined.

Discussion

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP [8]. Here we confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated, and we also document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder.

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the sub-acute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Our results suggest that it is possible that EP4 in conjunctival epithelium might contribute the ocular surface homeostasis, while the EP4 may not necessarily be down-regulated in all devastating ocular surface disorders.

Kabashima et al. [7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis

was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao et al. [9] found that PGE_2 acting on its receptor EP4 on T- and dendritic cells not only facilitated T_H1 cell differentiation but also amplified interleukin-23-mediated T_H17 cell expansion *in vitro*. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signaling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11, and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells [5]. Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic- and subacute SJS/TEN.

Figure Legends

Figure 1

The expression of PTGER4 mRNA in conjunctival tissues from patients with SJS/TEN, OCP and the controls.

A. Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP).

B. Expression of PTGER4 mRNA in human conjunctival tissues (*$p < 0.05$).

Figure 2

Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.

A. Nearly normal conjunctival tissues from patients with conjunctivochalasis.

B. Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction.

C. Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.

D. Conjunctival tissues from SJS/TEN patients in the sub-acute stage.

E. Conjunctival tissues from a patient with severe GVHD.

Each scale bar represents 100 μm.

References

1. Matsuoka T, Narumiya S: **Prostaglandin receptor signaling in disease**. *ScientificWorldJournal* 2007, **7**:1329-1347.

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**:44147-44154.

3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al.*: **Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production**. *J Immunol* 2008, **180**:2125-2131.

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**:1219-1229.

5. 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.

6. Morimoto K, Sugimoto Y, Katsuyama M, *et al*: **Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract**. *Am J Physiol* 1997, **272**:G681-687.

7. Kabashima K, Saji T, Murata T, *et al*: **The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut**. *J Clin Invest* 2002, **109**:883-893.

8. 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**(10):1369-1371.

9. Yao C, Sakata D, Esaki Y, *et al*: **Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion**. *Nat Med* 2009, **15**:633-640.

ACKNOWLEDGMENTS

We thank Chikako Endo for technical assistance. 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, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and an Immunological Research Grant from the Shimizu Foundation.

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: Case-control study

Mayumi Ueta, MD, PhD,[a,b] Chie Sotozono, MD, PhD,[a] Keiko Yamada, MD,[a]

Norihiko Yokoi, MD, PhD,[a] Tsutomu Inatomi, MD, PhD,[a] Shigeru Kinoshita, MD, PhD[a]

[a]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[b]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Corresponding author:

Mayumi Ueta, MD, PhD

Department of Ophthalmology

Kyoto Prefectural University of Medicine

465 Kajiicho, Hirokoji

Kawaramachi, Kamigyoku

Kyoto 602-0841, Japan

Telephone: 81-75-251-5578

Fax: 81-75-251-5663

e-mail: mueta@koto.kpu-m.ac.jp

Key words

Prostaglandin E receptor subtype EP4, human conjunctival epithelium, chemical eye burn, Mooren's ulcer

Abbreviations

EP4: Prostaglandin E receptor subtype EP4

SJS: Stevens-Johnson syndrome

TEN: Toxic epidermal necrolysis

GVHD: Graft versus host disease

OCP: Ocular cicatricial pemphigoid

RT-PCR: Reverse transcription polymerase chain reaction

PG: Prostaglandin

1) Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

2) Drafting the article or revising it critically for important intellectual content: Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

3) Final approval of the version to be published; Mayumi Ueta, Chie Sotozono, Keiko Yamada, Norihiko Yokoi, Tsutomu Inatomi, Shigeru Kinoshita

No additional data are available.

Competing interest statement: None

Abstract

Objectives: To confirm the down-regulation of PTGER4 mRNA in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study

Setting & Participants: We performed quantitative RT-PCR analysis of EP4 in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the down-regulation of EP4 mRNA expression. We also analyzed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, sub-acute- or chronic SJS/TEN, chronic ocular cicatricial pemphigoid (OCP), severe graft versus host disease (GVHD), and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of EP4 mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

Results: PTGER4 mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren’s ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN, or OCP.

Conclusions: The strong down-regulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

Introduction

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are lipid mediators that form in response to various stimuli. 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 [1]. PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide (LPS)-stimulated macrophages [2, 3] and dendritic cells [4]. Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced pro-inflammatory genes in human conjunctival epithelial cells [5].

There are four PGE receptor subtypes, EP1, EP2, EP3, and EP4. The intestinal epithelium has been reported to express EP4 mRNA [6] and intestinal homeostasis was maintained and the immune response down-regulated by EP4 [7]. The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.

We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP) [8]. Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is down-regulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN.

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.

For quantitative RT-PCR the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 4 patients in the chronic stage of SJS/TEN and 4 patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections 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). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For 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 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co., Ann Arbor, MI). 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. The VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Data analysis

Data were expressed as the mean ± SEM and evaluated by the Student's t-test using the Microsoft Excel software program.

Results

We previously documented that EP4 protein expression was down-regulated in conjunctival epithelium of devastating ocular surface inflammatory disorders such as chronic SJS/TEN and chronic OCP [8]. In this study, to confirm the down-regulation of EP4 in the ocular surface of SJS/TEN and OCP patients we examined the expression of PTGER4 mRNA in control conjunctival tissues from 6 conjunctival chalasis patients and in conjunctival tissues from 4 SJS/TEN- and 4 OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in Fig. 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS- and OCP patients did not manifest EP4 immunoreactivity. PTGER4 mRNA was significantly lower in conjunctival tissues from SJS/TEN and OCP patients than in the control conjunctivochalasis samples (Fig. 1B).

Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders. EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 2A) and in conjunctival tissues from 3 patients with chemical eye burn (Fig. 2B). Its expression varied in conjunctival epithelium from 4 patients with Mooren's ulcer (Fig. 2C): in one patient is was similar to the control, in 2 it was slightly lower than in the control, and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from 2 patients with subacute SJS/TEN (Fig. 2D), a patient with severe GVHD (Fig. 2E) as same as patients with chronic SJS/TEN or OCP [8].

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, EP4

immunoreactivity was not detected in conjunctival epithelium from patients with SJS/TEN, OCP, or severe GVHD. We did not detect EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined.

Discussion

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP [8]. Here we confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated, and we also document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder.

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the sub-acute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Our results suggest that it is possible that EP4 in conjunctival epithelium might contribute the ocular surface homeostasis, while the EP4 could not necessarily be down-regulated in all devastating ocular surface disorders.

Kabashima et al. [7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis

was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao et al. [9] found that PGE_2 acting on its receptor EP4 on T- and dendritic cells not only facilitated T_H1 cell differentiation but also amplified interleukin-23-mediated T_H17 cell expansion *in vitro*. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signaling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11, and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells [5]. Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic- and subacute SJS/TEN.

Figure Legends

Figure 1

The expression of PTGER4 mRNA in conjunctival tissues from patients with SJS/TEN, OCP and the controls.

A. Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP).

B. Expression of PTGER4 mRNA in human conjunctival tissues (*$p < 0.05$).

Figure 2

Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases.

A. Nearly normal conjunctival tissues from patients with conjunctivochalasis.

B. Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction.

C. Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva.

D. Conjunctival tissues from SJS/TEN patients in the sub-acute stage.

E. Conjunctival tissues from a patient with severe GVHD.

Each scale bar represents 100 μm.

References

1. Matsuoka T, Narumiya S: **Prostaglandin receptor signaling in disease**. *ScientificWorldJournal* 2007, **7**:1329-1347.

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**:44147-44154.

3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al.*: **Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production**. *J Immunol* 2008, **180**:2125-2131.

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**:1219-1229.

5. 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.

6. Morimoto K, Sugimoto Y, Katsuyama M, *et al*: **Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract**. *Am J Physiol* 1997, **272**:G681-687.

7. Kabashima K, Saji T, Murata T, *et al*: **The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut**. *J Clin Invest* 2002, **109**:883-893.

8. Ueta M, Sotozono C, Yokoi N, *et al*: **Prostaglandin E receptor 4 expression in human conjunctival epithelium and its downregulation in devastating**

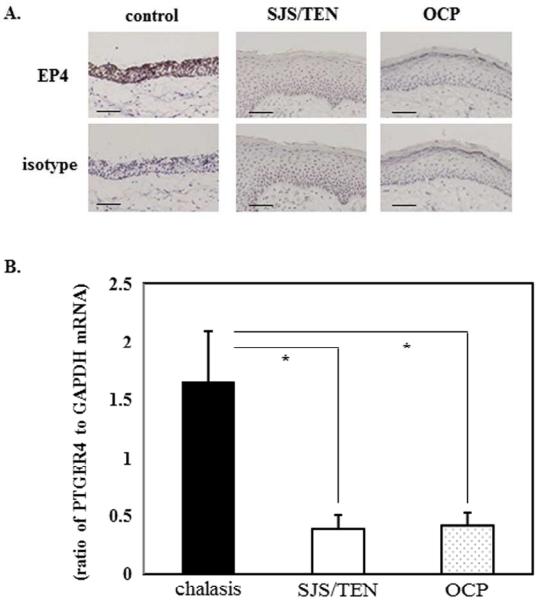
ocular surface inflammatory disorders. *Arch Ophthalmol* 2010, **128**(10):1369-1371.

9. Yao C, Sakata D, Esaki Y, *et al*: **Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion**. *Nat Med* 2009, **15**:633-640.

ACKNOWLEDGMENTS

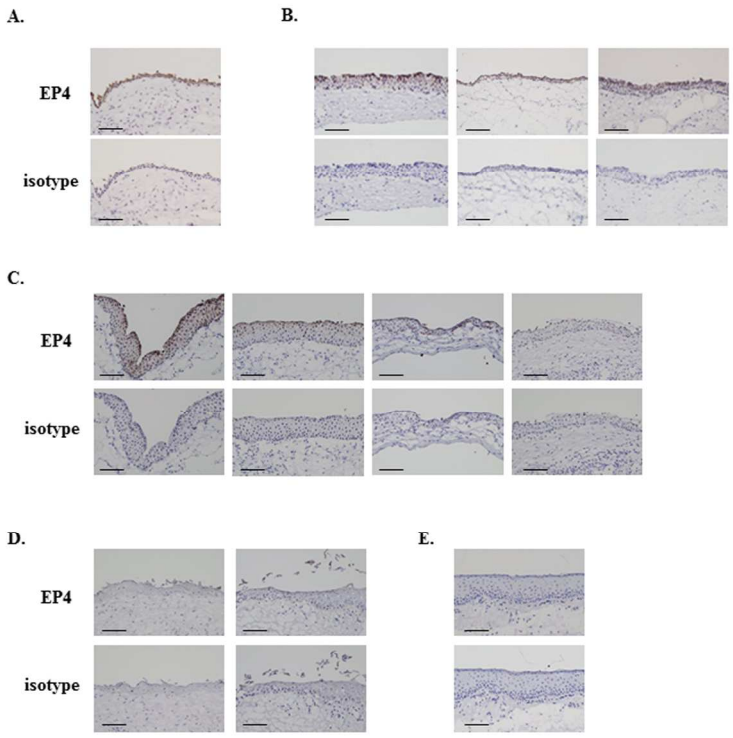
We thank Chikako Endo for technical assistance. This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST from JST, a research grant from the Kyoto Foundation for the Promotion of Medical Science, the Intramural Research Fund of Kyoto Prefectural University of Medicine, and an Immunological Research Grant from the Shimizu Foundation.

Figure 1



190x275mm (300 x 300 DPI)

Figure 2



190x275mm (300 x 300 DPI)