

Prostaglandin E receptor subtype EP3 in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis

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Background: We previously demonstrated that the prostaglandin E₂ (PGE₂)–EP3 pathway negatively regulates allergic reactions in a murine allergic asthma model.

Objectives: We investigated whether the PGE₂–EP3 pathway also regulates the development of murine experimental allergic conjunctivitis (EAC).

Methods: The expression of EP3 was examined by means of RT-PCR and immunohistochemistry in wild-type mice, as well as by means of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining in mice deficient in EP3 (*Ptger3*^{−/−} mice) carrying the β-galactosidase gene at the EP3 gene locus. EAC was induced by immunization of mice with short ragweed pollen (RW), followed by challenge with eye drops of RW, and eosinophil infiltration and *eotaxin-1* mRNA expression in the conjunctiva were examined. Mice were also treated with a topical application of an EP3-selective agonist during the elicitation phase.

Quantitative RT-PCR was used to detect expression of *COXs* and *prostaglandin E synthases*, and **ELISA** was used to measure PGE₂ production in the eyelid.

Results: EP3 was constitutively expressed in conjunctival epithelium on the ocular surface. *Ptger3*^{−/−} mice demonstrated significantly increased eosinophil infiltration in conjunctiva after RW challenge compared with wild-type mice. Consistently, significantly higher expression of *eotaxin-1* mRNA was observed

in *Ptger3*^{−/−} mice. Conversely, treatment of wild-type mice with an EP3-selective agonist resulted in a significant decrease in eosinophil infiltration, which was blunted in *Ptger3*^{−/−} mice. Expression of *COX-2* and *prostaglandin E synthases* was upregulated and PGE₂ content was increased in the eyelids after RW challenge.

Conclusion: These data suggest that PGE₂ acts on EP3 in conjunctival epithelium and downregulates the progression of EAC. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Conjunctival epithelium, prostaglandin E receptor subtype EP3, allergic conjunctivitis, eosinophilic conjunctival inflammation, EP3 agonist

Allergy, especially type I allergy, such as asthma, allergic rhinitis, allergic conjunctivitis, allergic dermatitis, food allergy, and severe anaphylactic response, has increased enormously in prevalence during the past 2 decades¹ and has become one of the major health problems in our society. The course of allergic diseases can be divided into 2 phases: the early-phase reaction and the late-phase reaction. The early-phase reaction occurs within 1 hour after allergen exposure and is driven by the cross-linking of allergen-specific IgE bound to the high-affinity IgE receptor FcεRI on the surface of resident mast cells, the key effector cell in the early-phase reaction. The late-phase reaction occurs 12 to 24 hours after allergen challenge and is characterized by eosinophil-dominant inflammatory cell infiltration in which chemokines such as *eotaxin* play a key role.

Allergic conjunctivitis is ocular-surface inflammation associated with type I hypersensitivity reactions accompanied by the characteristic symptoms (itching, tearing, conjunctival edema, redness, and photophobia) during the early phase, and eosinophil infiltration occurs in conjunctivas during the late phase. The signs and symptoms of allergic conjunctivitis have a significant effect on patients' comfort, health, and quality of life. Patients with allergic conjunctivitis have a poor quality of life associated with their symptoms, and current treatments for allergic conjunctivitis are inadequate to cure them completely and sometimes lead to side effects, such as an increased risk for the development of cataracts and glaucoma caused by corticosteroids.² Therefore safer and more effective treatments are being sought.

One candidate for such treatment is the manipulation of prostanoids and their receptor-signaling pathways because they were reported to regulate allergic reactions in a mouse allergic asthma model. Although prostaglandin D₂ acts on its receptor, DP, and functions as a mediator of allergic asthma,³ prostaglandin E₂ (PGE₂) acts on one of its 4 receptor subtypes, the prostaglandin E receptor subtype EP3, and negatively regulates allergic

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Abbreviations used

alum:	Aluminum hydroxide
cPGES:	Cytosolic prostaglandin E synthase
EAC:	Experimental allergic conjunctivitis
mPGES:	Membrane-bound prostaglandin E synthase
PGE ₂ :	Prostaglandin E ₂
PGES:	Prostaglandin E synthase
RT:	Room temperature
RW:	Short ragweed pollen
TARC:	Thymus and activation-regulated chemokine
X-gal:	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

reactions.⁴ In the latest analysis significantly more pronounced allergic inflammation developed in EP3-deficient (*Ptger3*^{-/-}) mice than in wild-type mice, and the EP3-selective agonist suppressed the inflammation. Intriguingly, EP3 is expressed in airway epithelial cells but not infiltrating cells.⁴ On the other hand, nonsteroidal anti-inflammatory drugs, which reduce the increase in both prostaglandin D₂ and PGE₂ levels, are used not only for prevention of posterior capsular opacification⁵ but also at times for the treatment of allergic conjunctivitis.⁶

In this study we tested a hypothesis that ocular-surface epithelial cells express EP3 and regulate the inflammation of allergic conjunctivitis through the PGE₂-EP3 pathway, and we addressed this issue by examining ocular-surface EP3 expression and analyzing its role in murine experimental allergic conjunctivitis (EAC) by using *Ptger3*^{-/-} mice and a selective EP3 agonist, ONO-AE-248.

METHODS**Mice, compounds, and reagents**

BALB/c mice were purchased from CLEA (Tokyo, Japan) and used at 6 to 12 weeks of age for sensitization. *Ptger3*^{-/-} mice were generated as previously described,⁷ back-crossed more than 10 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age with age-matched, wild-type BALB/c mice as control animals. Mice were maintained on a 12-hour/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology "Statement for the use of animals in ophthalmic and vision research."

ONO-AE-248, a selective EP3 agonist, was supplied by ONO Pharmaceutical Co, Ltd (Osaka, Japan); the ligand-binding specificities of the compounds for each prostaglandin E receptor subtype have previously been described.⁸ Short ragweed pollen (RW), RW extract, and aluminum hydroxide (alum) were purchased from Polysciences, Inc (Warrington, Pa); LSL Co, Ltd (Tokyo, Japan); and Sigma (St Louis, Mo), respectively.

RT-PCR analysis

By using TRIzol (Invitrogen, Carlsbad, Calif), total RNA was isolated from mouse kidney, corneal, and conjunctival tissues, according to the manufacturer's instructions. For the RT-PCR, we used the SuperScript preamplification system (Invitrogen). Amplification was performed with DNA polymerase (Takara, Shiga, Japan) for 40 cycles at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute for mouse *EP3* (GeneAmp; PE Applied Biosystems, Foster City, Calif). The primers for mouse *EP3* were as follows: forward 5'-ATCCTCGTGACCTGTACAGCGACGCTGG-3' and reverse 5'-TGCTCAACCTACATCTGATTGAAGATCATT-3'. For mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT), they were as follows: forward 5'-GTTGGATACAGGCCAGACTTTGTT-3' and reverse 5'-GAGGGTAGGCTGCCTATAGGCT-3'. A kidney, conjunctiva, or cornea was isolated from wild-type mice.

Histochemistry and histologic analysis

For staining for β-galactosidase activity, freshly isolated eye tissue with the eyelids and conjunctiva was embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and then flash-frozen in liquid nitrogen and cut into 8-μm-thick sections with a cryostat. The sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS; washed with PBS with 1 mmol/L MgCl₂; incubated for 24 hours at 37°C with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 1 mg/mL), 5 mmol/L K₃Fe(CN)₆, 5 mmol/L K₄Fe(CN)₆, and 1 mmol/L MgCl₂; and then counterstained with hematoxylin and eosin.

For staining of eosinophils, the whole eyeball, together with the eyelids and conjunctiva, were dissected and fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Vertical 6-μm-thick sections were affixed to microscope slides and deparaffinized. The slides were then stained with the Luna method (stain in a working 1% Biebrich scarlet solution, dip in lithium carbonate solution, wash in running water, counterstain in a working hematoxylin solution, wash in running water, and dehydrate), and eosinophils were examined with a light microscope. The number of infiltrating eosinophils in the lamina propria mucosae of the tarsal of the conjunctivas in the entire section was counted. The sections used were those from the central portion of the eye, which included the pupil and optic nerve head. Because the cell numbers vary depending on the counting area, the cell-count data are expressed as infiltrating eosinophil numbers divided by the area (in square millimeters), as measured with Scion Image (Scion Corp, Frederick, Md). The data are presented as means ± SEMs of all the mice examined.

Immunohistochemistry

The whole eyeball, together with the eyelids and conjunctiva, was embedded in OCT compound (Sakura Finetek) and then flash-frozen in liquid nitrogen. Sections 6 μm in thickness were cut and fixed with 100% acetone at 4°C for 10 minutes and blocked for 30 minutes with 10% normal donkey serum in PBS. Rabbit polyclonal antibody was to EP3 or to membrane-bound prostaglandin E synthase (mPGES) 1 (Cayman Chemical Co, Ann Arbor, Mich). Nonspecific rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was used as the negative control. For EP3 staining, *Ptger3*^{-/-} mice also were used as the negative control. 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 minutes. VECTASTAIN ABC Reagents (Vector Laboratories, Inc, Burlingame, Calif) were used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories, Inc) as a chromogenic substrate.

Sensitization and challenge

Mice were immunized by means of subcutaneous injection into their left hind footpads of RW adsorbed on alum (200 μg of RW and 2.6 mg of alum in a total volume of 200 μL) on day 0, followed by intraperitoneal injection of RW adsorbed on alum on day 7. On day 18, the eyes of the immunized mice were challenged with RW in PBS (500 μg in 5 μL per eye) or with PBS alone (5 μL per eye). For histologic analysis, the eyes were collected 24 hours after challenge.⁹ For RT-PCR analysis of *eotaxin-1*-specific mRNA, the upper and lower eyelids were isolated 6 hours after challenge. For measurement of *COX* values, *prostaglandin E synthase* (*PGES*)-specific mRNA, and PGE₂ content, the upper and lower eyelids were harvested with a time course of 0, 1, 3, 6, and 12 hours after challenge.

Quantitative RT-PCR of *eotaxin-1*, *COXs*, and *PGES*-specific mRNA in eyelids

Quantitative RT-PCRs of *eotaxin-1*, *COXs*, and *PGES*-specific mRNA in the eyelids were performed as previously reported.⁹ Briefly, the upper and lower lids were collected with the previously specified time course after RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTra Ace (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *eotaxin-1*, *COX-1*, *COX-2*, *mPGES-1*, *mPGES-2*, *cytosolic PGES* (*cPGES*),

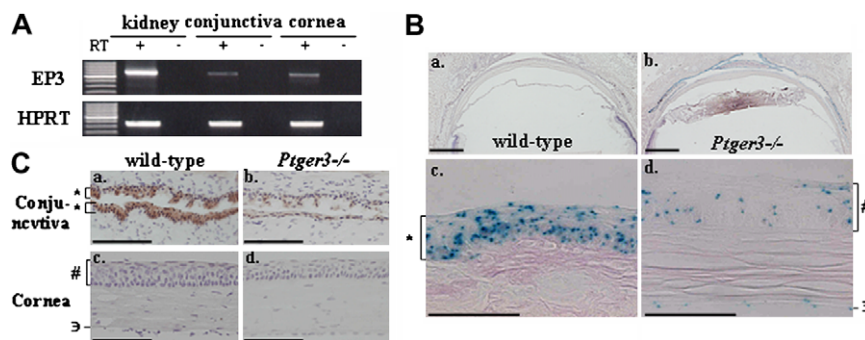


FIG 1. Expression and localization of EP3 in the conjunctiva and cornea. **A**, RT-PCR analyses of the expression of *EP3*-specific mRNA in the cornea and conjunctiva of wild-type mice. **B**, Histochemical staining for EP3 (X-gal): wild-type mice (a) and *Ptger3*^{-/-} mice (b-d). Data are representative of 3 experiments. **C**, Immunohistologic analysis for EP3. Each bar represents a length of 500 (B, a, b), 50 (B, c, d), or 100 (c) μ m. Data are representative of 3 experiments. *Conjunctival epithelium, #corneal epithelium, Δ Corneal endothelium.

and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) were from Applied Biosystems. The results were analyzed with sequence-detection software (Applied Biosystems); the expression level of *eotaxin-1*, *COXs*, and *PGES*-specific mRNA was normalized to the expression of the mouse housekeeping gene *GADPH*. The time after RW challenge, 6 hours, was optimal for maximum induction of *eotaxin-1* mRNA expression.

Measurement of PGE₂

PGE₂ measurement in eyelids were assessed by means of ELISA with the Prostaglandin E2 Biotrak Enzyme-immunoassay System (Amersham Biosciences, Buckinghamshire, United Kingdom), according to the manufacturer's recommendation.

Data analysis

Data were expressed as the mean $\pm$ SEM, and statistical analyses were performed by means of ANOVA and the Student *t* test.

RESULTS

Expression and localization of EP3 in the ocular surface

To examine the expression of *EP3* in the ocular surface, we performed RT-PCR analyses. As a positive control, we used mRNA isolated from the kidney. The expected length of PCR products (608 bp) was obtained from kidney, corneal, and conjunctival samples, suggesting that the ocular-surface tissues, both cornea and conjunctiva, express *EP3* mRNA (Fig 1, A). PCR products were isolated and sequenced to confirm the specificity of *EP3* mRNA detection. The sequences obtained from these PCR products were identical to the mouse *EP3* cDNA sequence. We next examined *EP3* localization using *Ptger3*^{-/-} mice in which the β -galactosidase gene was "knocked in" at the *EP3* gene locus and with immunohistochemistry of wild-type mice. In *Ptger3*^{-/-} mice X-gal staining of ocular-surface tissue revealed dense positive signals in conjunctival epithelia and scattered positive signals in corneal epithelia (Fig 1, B, b-d), suggesting a strong *EP3* expression in conjunctival epithelial cells and a slight expression in corneal epithelial cells. Interestingly, a few positive signals were seen in corneal endothelia (Fig 1, B, d). In other eye compartments, such as the lens, retina, uvea, and sclera, of the *Ptger3*^{-/-} mice, there were no positive signals (Fig 1, B, b). X-gal staining of ocular-surface tissue did not reveal positive signals in wild-type mice (Fig 1, B, a). Immunohistochemistry of conjunctival and corneal tissue

revealed that conjunctival epithelial cells expressed EP3 protein (Fig 1, C, a), although we could not find the positive signal in corneal epithelial and endothelial cells, presumably because of decreased expression of EP3 protein (Fig 1, C, c).

Antibody responses to RW sensitization

We next examined whether immunization of RW induced immune responses specific to RW equally in wild-type and *Ptger3*^{-/-} mice (See the Methods section of the Online Repository at www.jacionline.org). RW sensitization significantly increased the serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 in both wild-type and *Ptger3*^{-/-} mice compared with levels seen in the unsensitized control mice. There were no significant differences in serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 between the sensitized wild-type and *Ptger3*^{-/-} mice (see Fig E1 in this article's Online Repository at www.jacionline.org). These results suggest that wild-type and *Ptger3*^{-/-} mice had been equally sensitized to RW.

Eosinophil accumulation in the lamina propria mucosae of the conjunctivas

To investigate whether EP3 plays a role in the late-phase reaction of EAC, we examined conjunctival tissue from wild-type and *Ptger3*^{-/-} mice. Histologic analyses of conjunctivas revealed that the RW challenge on RW-sensitized mice led to inflammatory cell infiltrations in the lamina propria mucosae of the conjunctivas at 24 hours after challenge in our EAC model (Fig 2). The infiltrated cells consisted predominantly of eosinophils; only a few lymphocytes and neutrophils were detected. Both wild-type and *Ptger3*^{-/-} mice had eosinophil-dominant inflammatory cell infiltrations in the lamina propria mucosae at 24 hours after challenge; however, the numbers of eosinophils in *Ptger3*^{-/-} mice were significantly greater than in wild-type mice (Table I). Either RW sensitization or RW challenge alone did not significantly increase the numbers of eosinophils in wild-type and *Ptger3*^{-/-} mice (data not shown). These results suggest that the PGE₂-EP3 pathway negatively regulates EAC development in the late-phase reaction, which cause the pronounced allergic inflammation in *Ptger3*^{-/-} mice.

Expression of *eotaxin-1*-specific mRNA in eyelids

Given the critical roles of *eotaxin-1* in eosinophil recruitment,¹⁰⁻¹² we examined the *eotaxin-1*-specific mRNA expression

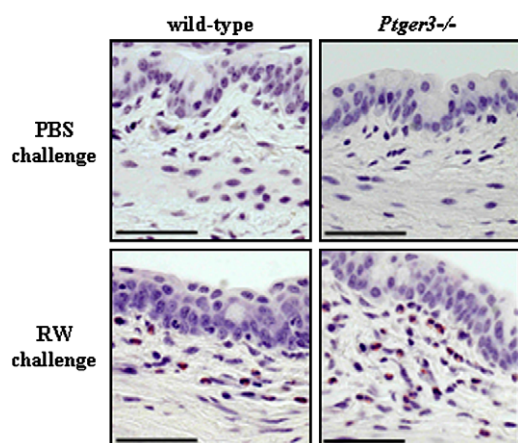


FIG 2. Histologic analyses of eosinophil inflammation in conjunctiva. Infiltration of eosinophils into the conjunctiva of wild-type and *Ptger3*^{-/-} mice were detected by using the Luna method, which stained eosinophil granules with a distinctive red. Scale bars = 50 μ m.

TABLE I. Number of eosinophils in the lamina propria mucosae of the tarsal conjunctiva

	Wild-type mice		<i>Ptger3</i> ^{-/-} mice	
Sensitization	+		+	
Challenge	-	+	-	+
No. of mice	19	19	19	19
No. of eosinophils, mean $\pm$ SEM/0.1 mm ²	4.6 $\pm$ 1.1	42.4 $\pm$ 3.1	4.2 $\pm$ 0.8	86.5 $\pm$ 5.4*

* $P < .0005$ (42.4 $\pm$ 3.1 vs 86.5 $\pm$ 5.4).

in the eyelids from RW-sensitized wild-type and *Ptger3*^{-/-} mice at 6 hours after RW challenge.

Quantitative real-time RT-PCR analyses demonstrated that the RW challenge significantly increased the *eotaxin-1* mRNA expression in both genotypes compared with that in vehicle-treated control animals. The *eotaxin-1* mRNA expression increase in RW-challenged *Ptger3*^{-/-} mice is significantly larger than that seen in wild-type mice (Fig 3), which is consistent with pronounced eosinophil inflammation in *Ptger3*^{-/-} mice.

Upregulation of COX-2, PGES-specific mRNA expression, and PGE₂ contents in eyelids during EAC

Because COX and PGES action is necessary for PGE₂ synthesis,¹³ we examined the expression of mRNA for COX isoforms and PGES isoforms in eyelids during EAC. After RW challenge, the relative expression levels of COX-2 mRNA increased and peaked at 1 hour (about 9 times higher than the basal level at 0 hour) and then gradually decreased (Fig 4, A), whereas the expression of COX-1 mRNA stayed at an almost basal level (data not shown). After RW challenge, the relative expression levels of *mPGES-1* mRNA also increased and peaked at 3 hours and then gradually decreased (Fig 4, A), and similar gene expression patterns of *mPGES-2* and *cPGES* were also observed (data not shown). We also examined the PGE₂ contents in eyelids during EAC. After RW challenge, the PGE₂ contents in eyelids increased time dependently until 12 hours (Fig 4, B). These results suggest that RW challenge increased PGE₂ synthesis in eyelids through

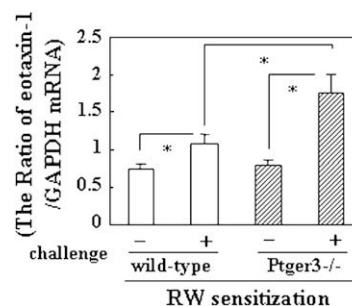


FIG 3. Real-time PCR analyses of the expression of *eotaxin-1* mRNA in the eyelids 6 hours after RW challenge. Relative mRNA expression levels for *eotaxin-1* normalized by *GAPDH* are shown. Data are shown as the mean $\pm$ SEM of 7 samples per group. * $P < .05$.

upregulation of inducible enzymes, such as COX-2 and mPGES-1, during the elicitation phase of EAC. Furthermore, we examined the localization of PGE₂ synthesis by using immunohistochemistry of mPGES-1. Immunohistologic analysis of ocular-surface tissue, which was obtained from RW-sensitized wild-type mice at 6 hours after RW challenge, revealed that conjunctival epithelial cells expressed mPGES-1 protein (Fig 4, C), suggesting that PGE₂ synthesis through mPGES-1 might occur in conjunctival epithelium during the elicitation phase of EAC.

Effect of an EP3-selective agonist

To investigate whether allergic inflammation can be suppressed by stimulating the PGE₂–EP3 pathway in EAC, we next examined the effects of an EP3-selective agonist, ONO-AE-248. We topically administered ONO-AE-248 to the eyes of RW-sensitized mice 3 times at 1 hour before and at 3 and 7 hours after RW challenge. First, to determine the optimal dose, ONO-AE-248 was administered at various doses (0.01, 0.1, 1, 10, and 100 ng) in 5 μ L of PBS per eye to wild-type mice. ONO-AE-248 suppresses eosinophil infiltration dose dependently until 1 ng, and then at the higher doses of 10 ng and 100 ng, its suppression becomes weak (see Fig E2 in this article's Online Repository at www.jacionline.org). We therefore determined that the optimal dose of AE248 was 1 ng in 5 μ L of PBS (0.02%). In wild-type mice topical administration of 1 ng of ONO-AE-248 significantly inhibited the antigen-induced infiltration of eosinophils compared with that seen in the vehicle-treated groups (Fig 5 and see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, this inhibitory effect of ONO-AE-248 was absent in *Ptger3*^{-/-} mice, confirming that it was mediated by EP3 (Fig 5 and see Fig E3). Although vehicle treatment mildly decreased eosinophil infiltration, it is very likely that a 3-time vehicle administration washed out the challenged RW antigen and resulted in reduced allergic responses. These results suggest that allergic inflammation can be suppressed by stimulating EP3 with a selective agonist.

DISCUSSION

In this study we investigated the roles of the PGE₂–EP3 pathway in allergic conjunctivitis by using RW-induced EAC as a model in mice. First, we examined EP3 expression in ocular tissues and found that EP3 is expressed in the conjunctival epithelium on the ocular surface in mice. Because *Ptger3*^{-/-} mice have allergic inflammation that was much more pronounced

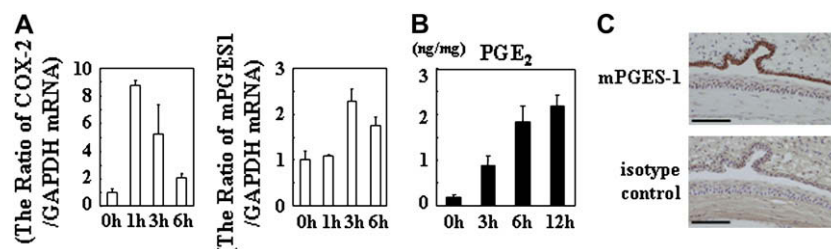


FIG 4. RW challenge increased PGE₂ synthesis in eyelids. **A**, Time course of *COX-2* and *mPGES-1* mRNA expression in eyelids during EAC. The *y*-axis shows the increase of specific mRNA over that seen in 0-hour samples. The *x*-axis shows the time after the RW challenge. Data are shown as the mean $\pm$ SEM of 3 samples. **B**, Time course of PGE₂ content in eyelids during EAC. The *x*-axis shows the time after the RW challenge. Data are shown as the mean $\pm$ SEM of 4 samples. **C**, Localization of mPGES-1 on the ocular surface. Each bar represents a length of 100 μ m.

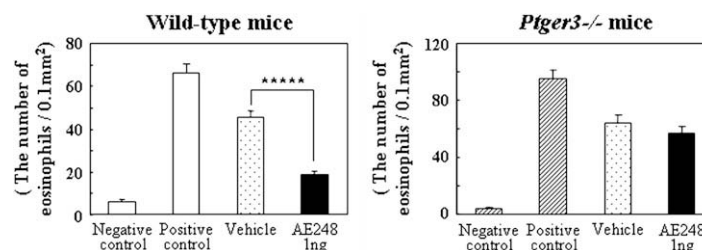


FIG 5. The effects of an EP3-selective agonist on eosinophil infiltration in conjunctiva. Data are shown as the mean $\pm$ SEM of samples from all the mice examined. Negative control (RW sensitization without challenge), positive control (RW sensitization with challenge) only vehicle, and AE248 are shown. (Wild-type mice: negative control, *n* = 20; positive control, *n* = 20; vehicle only, *n* = 25; AE248, *n* = 30; *Ptger3*^{-/-} mice: negative control, *n* = 11; positive control, *n* = 11; vehicle only, *n* = 14; AE248, *n* = 16). ******P* < .0005.

than that seen in wild-type mice, negative regulation of the development of EAC through the PGE₂–EP3 pathway was suggested. Consistently, significantly greater *eotaxin-1* mRNA expression was observed in *Ptger3*^{-/-} mice. Conversely, treatment with an EP3-selective agonist resulted in significantly decreased eosinophil infiltration in wild-type mice, which was blunted in *Ptger3*^{-/-} mice. We examined the expression of mRNA of *COX* isoforms, *PGES* isoforms, and PGE₂ content in eyelids during EAC and found that *COX-2*, *PGES* mRNA, and PGE₂ content in eyelids increased after RW challenge. Interestingly, the expression of mPGES-1 was localized in conjunctival epithelium, suggesting that PGE₂ synthesis through mPGES-1 might occur in conjunctival epithelium. These results suggest not only the critical roles of the PGE₂–EP3 pathway in allergic conjunctivitis but also a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE₂–EP3 pathway.

The allergic response in conjunctivitis is typically elicited by ocular exposure to an allergen, such as grass or tree pollen, that causes cross-linkage of membrane-bound IgE, which triggers mast cell degranulation, releasing a cascade of allergic and inflammatory mediators. Thus mast cells play an important role in the early-phase reactions. Given the predominant role of mast cells in the early-phase reaction, most patients presenting with allergic conjunctivitis are treated with eye drops containing mast cell stabilizers or antihistamines. It is also suggested that mediators released by mast cells during the early-phase reactions contribute to the development of the late-phase reaction.¹⁴ However, we previously demonstrated that mast cells do not play an essential role in the development of a late-phase reaction of eosinophilic conjunctival inflammation by showing that mast

cell-deficient mice similarly had eosinophilic conjunctival inflammation compared with congenic littermates with EAC.⁹ In this study we showed that conjunctival epithelial cells might be also implicated in the eosinophilic conjunctival inflammation seen in allergic conjunctivitis.

Late-phase reaction is characterized by eosinophil-dominant infiltrations in the local tissue.^{9,12} The increase of eosinophil infiltration correlates well with both the severity and condition of this disease.¹⁰⁻¹² For eosinophil recruitment, *eotaxin-1* is now accepted as the central mediator, and it was reported that its deficiency ablates eosinophilic responses in allergic conjunctivitis.¹² Hence we examined the *eotaxin-1*-specific mRNA expression in the eyelids from RW-sensitized wild-type and *Ptger3*^{-/-} mice at 6 hours after RW challenge. Because we observed a large number of eosinophils in our EAC at 24 hours after RW challenge, this condition is thought to mimic the late-phase reaction in human allergic conjunctivitis. Recently, T cells¹⁵ and fibroblasts¹⁶ were also reported to contribute to the development of the late-phase reaction. Although various cell types can contribute to orchestrate the allergic response and various mechanisms are thought to develop the late-phase reaction, our findings in this study strongly suggest that conjunctival epithelium, which predominantly expresses EP3 among ocular tissues, substantially contributes to the late-phase reaction in our EAC.

In an allergic asthma model PGE₂ was reported to act at EP3 on airway epithelial cells and regulate the extent of the late-phase reaction by attenuating the expression of chemokine genes, such as *eotaxin-1* and *thymus and activation-regulated chemokine (TARC)*.⁴ Given that RW challenge-induced *eotaxin-1* mRNA increase in eyelids was much more pronounced in *Ptger3*^{-/-} mice, it was suggested that the PGE₂–EP3 pathway negatively regulates

allergic reactions by suppressing chemokine gene expression in EAC. With regard to the clinical relevance of eotaxin-1 in allergic conjunctivitis, it was reported that eotaxin-1 was expressed in the conjunctival epithelium of patients with vernal keratoconjunctivitis,¹⁷ although we failed to show the eotaxin-1 or TARC production by cultured conjunctival epithelial cells stimulated with combinations of TNF- α and IL-4 (data not shown). The difference between *in vivo* and *in vitro* conditions might contribute to the different cell responses. On the other hand, cultured conjunctival fibroblasts could release eotaxin-1 or TARC in response to stimulation with combinations of TNF- α and IL-4 (data not shown). The mechanisms of eosinophilic conjunctival inflammation at the late-phase reaction are still elusive. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis.

It is well known that prostaglandins are produced in substantial amounts during allergen exposure and disease development^{18,19} and that COX enzymes are involved in the synthesis of prostaglandins.²⁰ COX-1 is an ubiquitous housekeeping enzyme and thought to produce a basal level of prostaglandins, whereas COX-2 is an inducible enzyme and thought to be involved in pathologic conditions. Consistently, we detected unchanged *COX-1* and markedly increased *COX-2* mRNA expression in conjunctiva after RW challenge. Furthermore, we also detected increased *PGES* mRNA values and PGE₂ contents in eyelids and mPGES-1 expression in conjunctival epithelium. Of 3 PGESs, cPGES and mPGES-2 are constitutive enzymes, whereas mPGES-1 is an induced enzyme and upregulated by proinflammatory stimuli.²¹ Because the functional coupling of COX-2 to mPGES-1 for PGE₂ biosynthesis has been suggested,²² the coordinate increased expression of COX-2 and mPGES-1 might contribute to an increase in PGE₂ production in the conjunctival epithelium during the elicitation phase of our EAC. Thus we propose the endogenous negative-feedback regulation of allergic inflammation; the PGE₂–EP3 pathway is activated on challenge and functions to suppress development of EAC. Notably, this endogenous feedback system can be augmented by stimulating EP3 with a selective agonist.

It is possible that the PGE₂–EP3 pathway might function to suppress the development of human allergic conjunctivitis because we have confirmed the expression of EP3 in human conjunctival epithelium at both the mRNA and protein levels (see Fig E4 in this article's Online Repository at www.jacionline.org).

In summary, we demonstrated that EP3 is expressed in the ocular surface and that the PGE₂–EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis. Although species differences between human subjects and mice should be considered cautiously and extrapolation from the mouse models to human pathologies must be performed with some reservation, stimulating the PGE₂–EP3 pathway with a selective agonist might be useful for treating allergic conjunctivitis in human subjects. Furthermore, we propose a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE₂–EP3 pathway.

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Clinical implications: EP3 in conjunctival epithelium can be a target for drug development to treat allergic conjunctivitis.

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METHODS

Measurement of immunoglobulins in serum

Twenty-four hours after RW challenge, blood was collected, and serum was prepared. Serum total IgE levels were assessed by means of ELISA with the OptEIA mouse IgE ELISA set (BD Biosciences PharMingen, San Diego, Calif), according to the manufacturer's recommendation. RW-specific immunoglobulin levels in the serum were assessed as previously reported (Kweon, 2000). Briefly, EIA plates (Costar, Corning, NY) were coated with RW extract (5 μ g/mL) at 4°C overnight. After blocking with 1% BSA in PBS, serum samples were added and incubated for 4 hours at room temperature. The plates were washed with PBS plus 0.05% Tween 20 (Wako, Osaka, Japan) and incubated for 2 hours at room temperature with goat anti-mouse IgG1–horseradish peroxidase–conjugated antibody (Southern Biotechnology Associates, Inc, Birmingham, Ala) for RW-specific IgG1. In the case of IgE, biotin-conjugated rat anti-mouse IgE mAb (BD Bioscience PharMingen) was added to each well for 1 hour at room temperature, and then after washing, avidin–horseradish peroxidase conjugate (BD Bioscience PharMingen) was added to each well for 30 minutes at room temperature. After washing, the color reaction was developed with 3, 3', 5, 5'-tetramethyl-benzidine (Moss, Inc, Pasadena, Calif).

End point titers of RW-specific immunoglobulins were expressed as the reciprocal log2 of the last dilution that showed a level of OD units 2-fold higher than the background.

Human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocol were explained to all patients, and their informed consent was obtained.

For RT-PCR, we obtained human conjunctival epithelial cells from healthy volunteers by means of impression cytology. For immunohistochemistry, serial sections of human conjunctivas were prepared from samples obtained at conjunctivochalasis surgery.

For the RT-PCR, the primers for human *EP3* and human *GADPH* were as follows: forward 5'- GCG CGC TGG TGC TGC GTC TGT ACA CTG CGG - 3' and reverse 5'- AGT GGC CGC TGC AGG GAG GTA GAG CTC CAG -3' and forward 5'- CCA TCA CCA TCT TCC AGG AG-3' and reverse 5'- CCT GCT TCA CCA CCT TCT TG-3', respectively.

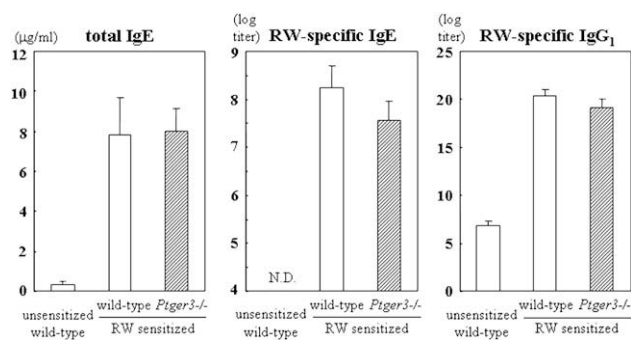


FIG E1. Antibody responses to RW sensitization and challenge. The serum concentration of total IgE (*left*) and the log titer of RW-specific IgE (*middle*) and RW-specific IgG1 (*right*) assessed by means of ELISA are shown. Sera were prepared from sensitized wild-type and *Ptger3*^{-/-} mice at 24 hours after RW challenge. Sera from unsensitized wild-type mice were used as controls. Data are shown as the means ± SEMs of 8 samples.

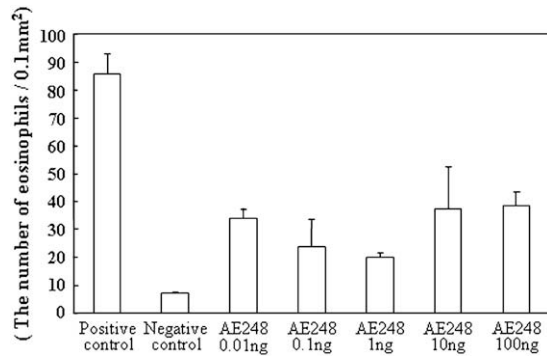


FIG E2. The optimal dose of ONO-AE-248. ONO-AE-248 was administered at various doses (0.01 ng, 0.1, 1, 10, 100 ng) in 5 μ L of PBS per eye to wild-type mice at 1, 3, and 7 hours after RW challenge. The quantified eosinophil numbers in conjunctivas of wild-type mice were shown. The data from the mice without both RW challenge and topical treatment and the mice without topical treatment were used as negative and positive controls, respectively. Data are shown as the means $\pm$ SEMs of samples from all mice examined. Positive control (RW sensitization with challenge), $n = 19$; negative control (RW sensitization without challenge), $n = 19$. AE248: 0.01 ng, $n = 18$; 0.1 ng, $n = 6$; 1 ng, $n = 27$; 10 ng, $n = 6$; 100 ng, $n = 17$.

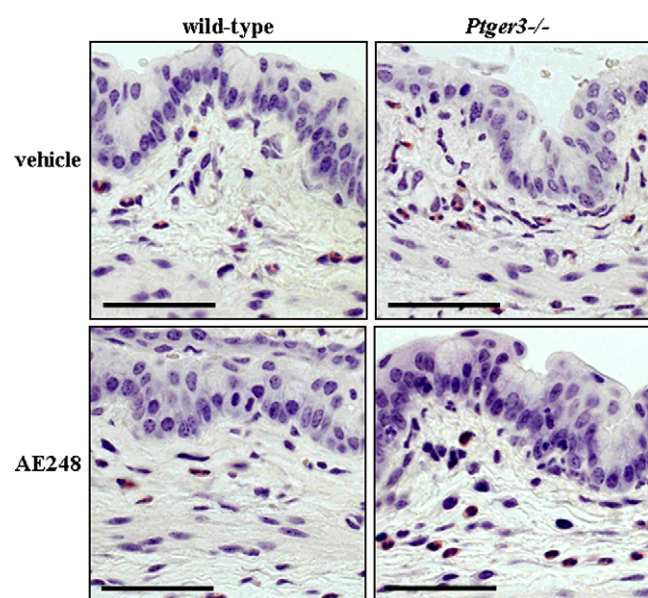


FIG E3. Infiltration of eosinophils into the conjunctiva treated with an EP3-selective agonist. Infiltrations of eosinophils were detected by using the Luna method, which stained eosinophil granules with a distinctive red. Scale bars = 50 μ m.

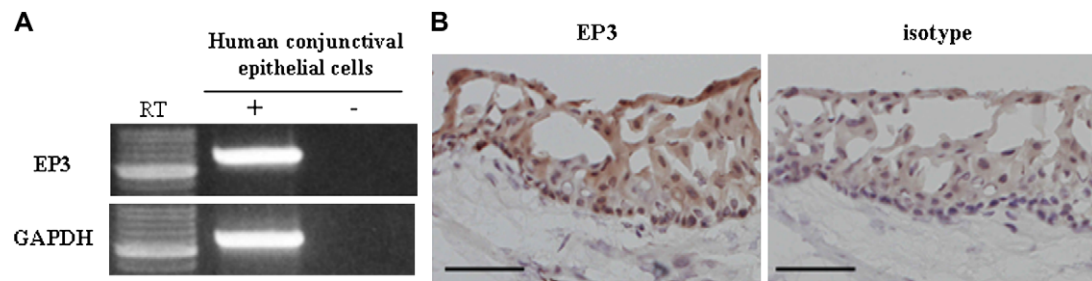


FIG E4. The expression of EP3 in human conjunctival epithelium. **A**, RT-PCR analyses of the expression of *EP3*-specific mRNA. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **B**, Immunohistologic analysis for EP3. Each *bar* represents a length of 50 μ m.