

Elevated levels of prostaglandin E₂ in the tears of patients with severe allergic conjunctivitis and primary cultured conjunctival cells are suppressed by ketotifen and dexamethasone

 Ryutarō Yamanishi,¹ Naoko Okada,² Eisuke Shimizu ,¹ Hiroshi Fujishima^{1,3}

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ABSTRACT

Objective We examined the production of prostaglandin E₂ (PGE₂), which is the key prostaglandin involved in inflammatory disorders of the ocular surface. Tears and conjunctival fibroblasts were evaluated in order to assess allergic inflammation and the effect of specific drugs. **Methods and analysis** PGE₂ was measured in tears from both patients and normal volunteers. Primary cultures of human conjunctival fibroblasts were incubated with interleukin (IL)-4 and tumour necrosis factor (TNF)-α with or without ketotifen fumarate or dexamethasone. The culture supernatants were removed 24 hours after exposure and the concentrations of PGE₂ were quantified by ELISA.

Results Significantly higher levels of PGE₂ were observed in the tears of patients with severe allergic conjunctivitis than in those with post-surgical inflammation (p=0.02), and this production was reduced by eye drops. Stimulation with IL-4 and TNF-α induced the generation of PGE₂ in supernatants of conjunctival fibroblasts, and this production was significantly downregulated by ketotifen fumarate or steroids.

Conclusion PGE₂ may participate in the pathogenesis of severe ocular allergic disease, and both ketotifen fumarate and steroid reduce the production of PGE₂.

INTRODUCTION

Evidence suggests that ocular surface tissues such as the cornea and conjunctiva play active roles in ocular allergy; histamine receptors are expressed on conjunctival epithelial cells, and proinflammatory cytokines such as interleukin (IL)-4 and tumour necrosis factor (TNF)-α are released in tears in ocular allergic inflammation.^{1 2} Arachidonic acid (AA), released from cell membrane phospholipids of activated cells, is metabolised to prostaglandins (PGs) by the sequential activity of cyclooxygenase (COX).³ It is known that corneal and conjunctival cells also release AA in inflammatory conditions.⁴ We have previously reported that PGE₂ is produced by human conjunctival and corneal cells in response to IL-4 and TNF-α stimulation.⁵ In this

Key messages

What is already known about this subject?

- ▶ Prostaglandin E₂ (PGE₂) is produced in the tears of patients with inflammatory disorders in the ocular surface as severe allergic conjunctivitis.

What are the new findings?

- ▶ Significantly higher levels of PGE₂ were observed in the tears of patients with severe allergic conjunctivitis. Moreover, ketotifen fumarate affected PGE₂ production and reduced allergic inflammation.

How might these results change the focus of research or clinical practice?

- ▶ Prostaglandins have potential importance in the management of ocular allergic inflammation and could be the pathogenesis of severe ocular allergic disease.

report, we analysed PGE₂ production, which has been thought to contribute to the development of inflammation, in allergic inflammation within two representative types of ocular tissues: epithelial cells and fibroblasts.⁵ PGs have been reported to mediate various inflammatory responses in ophthalmological diseases.⁶ COX-2 is an inducible enzyme and highly associated with ocular surface injections.^{7 8} TNF-α is a potent inducible inflammatory cytokine and is capable of inducing PG production by cells.^{9–12} Corneal keratocytes produce chemokines such as regulated on activation normal T cell expressed and secreted (RANTES),¹³ monocyte chemoattractant protein (MCP)-1,^{14 15} and eotaxin¹⁶ following stimulation by IL-4 and TNF-α. These chemokines recruit eosinophils into local tissues. In our previous study, stimulation with IL-4 and TNF-α also affected the expression of many other genes in keratocytes, including those of various chemokines, such as IP-10,¹⁷ the fourth most induced transcript I-TAC,¹⁷ the sixth most induced transcript RANTES¹⁸ and the ninth most induced



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¹Department of Ophthalmology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

²Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, Kita adachi-gun, Saitama, Japan

³Ophthalmology, Tsurumi University Dental Hospital, Yokohama, Kanagawa, Japan

Correspondence to

Dr Eisuke Shimizu;
ophthalmolog1st.acek39@keio.jp

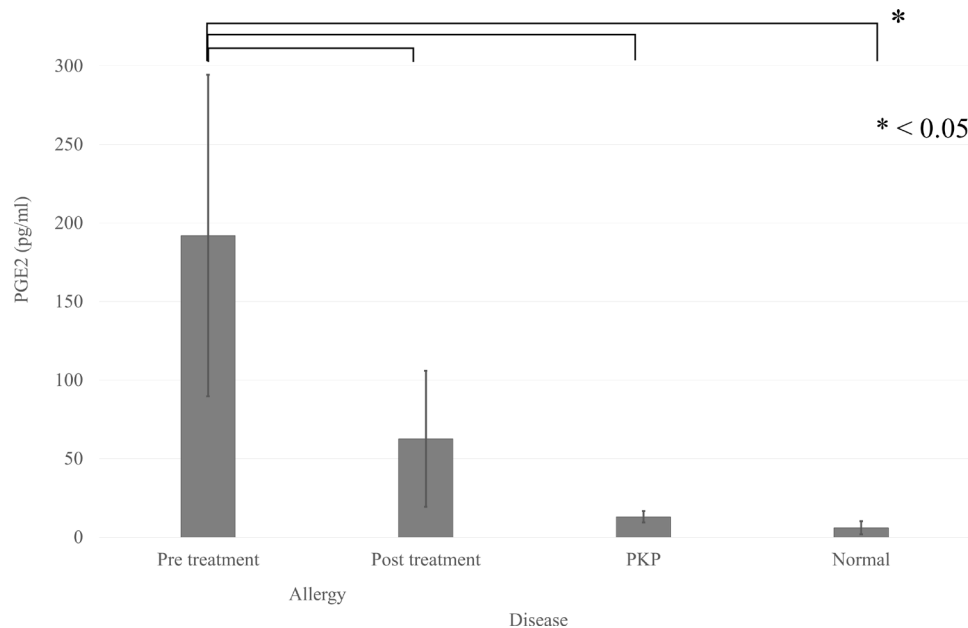


Figure 1 PGE₂ production in the tears of patients, and its downregulation by ketotifen fumarate and dexamethasone. The mean production of PGE₂ in the tears of patients with severe allergic inflammation (n=5) was significantly higher than that in patients following 4 weeks of eye drop treatment, in patients post-surgical inflammation (n=5) and normal controls (n=5). Results are presented as means±SD. Student's t-test was performed. Allergy indicates patients with severe allergic inflammation. PKP indicates patients with post-surgical inflammation. PGE₂, prostaglandin E₂; PKP, penetrating keratoplasty.

transcript MCP-2. Activated conjunctival epithelial cells release biologically active compounds which play essential roles in the pathogenesis of ocular inflammation, including lipid mediators, eicosanoids, and a variety of cytokines and chemokines.

Ketotifen fumarate is non-competitive antagonist of the histamine H1 receptor and a mast cell stabiliser, which inhibits the release of inflammatory mediators from mast cells. In addition, its ophthalmic solution was safe, well tolerated, and effective in preventing the signs and symptoms of allergic conjunctivitis.¹⁹

Although previous ocular allergy studies have provided useful insights, the specific mechanisms of the regulated drug processes in severe ocular allergic diseases are not fully understood. In this study, we examined PGE₂ production in the tears of patients with severe ocular allergic inflammation, followed by primary cultures of the human conjunctival fibroblasts.²⁰ This study aimed to investigate the effects of ketotifen fumarate and steroids on the release of PGE₂ by primary cultures of human conjunctival fibroblasts.

MATERIALS AND METHODS

Written informed consent was obtained from all subjects before participation. Patient data were anonymised before access and analysis.

Clinical samples

Five patients who underwent simple penetrating keratoplasty (PKP) surgery (mean age: 27.7±9.1 years) were recruited to obtain samples 1 hour post-surgical inflammation. Four patients had been performed PKP due to keratoconus, and the other had been due to bullous keratopathy. We chose

PKP case as disease control because under the epithelial defect, continuous stimulation of stromal cell may increase the production of PGE₂.⁵ In addition, five patients (mean age: 15.1±3.4 years) with atopic keratoconjunctivitis or vernal keratoconjunctivitis (VKC) with corneal erosion were recruited to obtain samples exhibiting severe allergic inflammation.²¹ Their treatment included Zaditen (0.05% ketotifen fumarate, Santen, Osaka, Japan) and Santeson ophthalmic solution (0.1% dexamethasone, Santen, Osaka, Japan), both administered four times per day for 4 weeks. We included an additional five participants as normal controls (mean age: 31.6±4.3 years). The diagnosis of atopic dermatitis was made by a dermatology specialist based on the criteria of Hanifin *et al.*²² No treatment was supplied prior to tear sample collections. In addition, samples of normal cases were collected from normal volunteers. Samples were collected before and 4 weeks after topical treatment in the control group, and a day after the operation in patients with PKP. Using a micropipette, 20 µL of tears were collected at the lateral canthus of the eyelid without anaesthetic; patients were in the supine position with their heads tilted to the side. Tear samples were centrifuged immediately at 4°C to remove cells and transferred to new tubes. Tear samples were stored at -80°C until further examination.

Measurements

PGE₂ concentration was measured by ELISA (R&D, Minneapolis, Minnesota, USA). The cytokines, RANTES and IL-8 were purchased from R&D. Eotaxin was received as a gift from Dr Hiroshi Kawasaki of the Department of Clinical Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan.

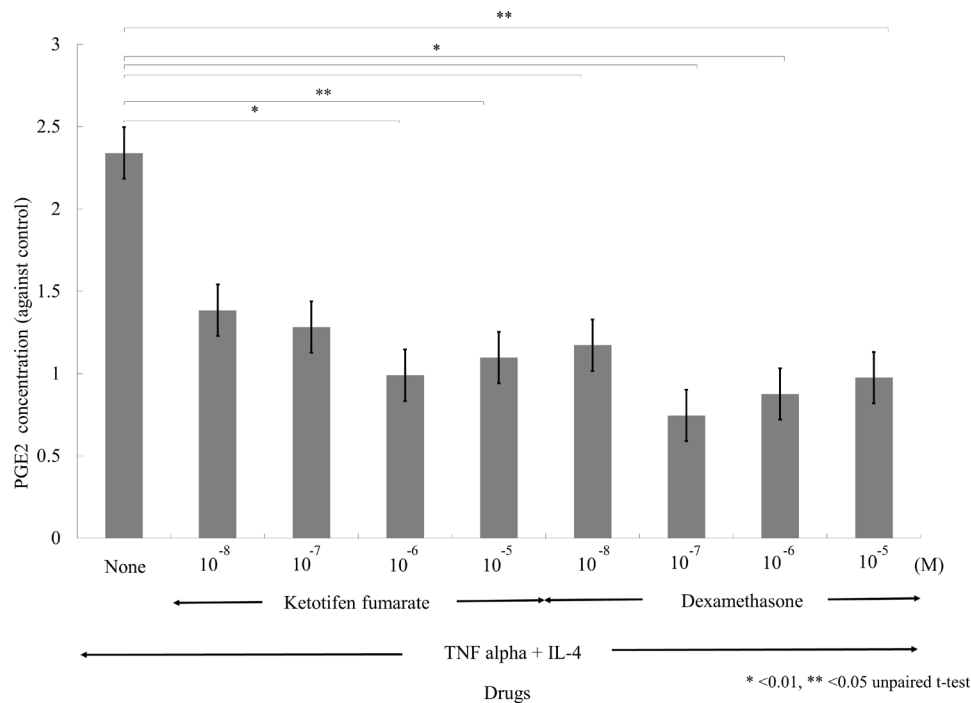


Figure 2 Effects of ketotifen fumarate and dexamethasone on PGE₂ production by conjunctival fibroblasts. Human conjunctival fibroblasts were maintained in 96 well plates at a concentration of 1×10^5 cells/mL. Cells were incubated with IL-4 (10 ng/mL or 100 ng/mL) and/or TNF- α (10 or 100 ng/mL) and harvested 24 hours later. Ketotifen fumarate and dexamethasone were added at concentrations from 10^{-8} nM to 10^{-5} nM. The 'None' bar indicates medium alone. The generation of PGE₂ was determined in cell-free supernatants by ELISA. Results are presented as means \pm SD of seven independent experiments. Significant differences at the levels of $p < 0.05$ and $p < 0.01$ vs time 0 are indicated by * and **, respectively (Student's t-test). IL-4, interleukin 4; PGE₂, prostaglandin E₂; TNF, tumour necrosis factor.

Primary culture

Human conjunctival samples were collected using scissors from normal volunteers from whom informed consent had been obtained. Samples were incubated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to grow fibroblasts. Specimens were cultured in complete medium at 37°C in a humidified atmosphere supplemented with 5% CO₂ in air for a few weeks. The cells were grown with incubation in a flask. Fibroblasts were subcultured in 96 well plates at a concentration of 1×10^5 cells/mL. One day later, cells were incubated with TNF- α (30 ng/mL) and IL-4 (30 ng/mL) in combination with ketotifen fumarate and dexamethasone: 10^{-6} – 10^{-8} M (Sigma-Aldrich, St. Louis, Missouri, USA). Twenty-four hours later, culture supernatants were collected and PGE₂ levels were determined.

Statistical analysis

The data were analysed using Prism software (V.6.04 for Mac; GraphPad Software, San Diego, California, USA). The D'Agostino-Pearson omnibus normality test was used to assess whether the data showed a normal distribution. Student's t-test was performed to assess the differences in tear and PGE₂ production associated with IL-4 and TNF- α . The same test was also used to assess differences in drug inhibition associated with ketotifen fumarate and dexamethasone. Values are expressed as mean \pm SD. P

values were not adjusted for multiple comparison testing. Values below 0.05 were considered statistically significant.

RESULTS

Mean tear PGE₂ levels are shown in figure 1; the level was 13.0 ± 3.6 pg/mL in post-PKP inflammation, 6.0 ± 4.2 pg/mL in normal controls and 190 ± 100 pg/mL in the severe allergic inflammatory condition, which decreased significantly to 62.6 ± 43.3 pg/mL after 4 weeks of treatment with ketotifen fumarate and dexamethasone ($p = 0.04$).

Patients with severe allergic inflammation produced significantly higher levels of PGE₂ than those found after 4 weeks of topical treatment (0.05% ketotifen fumarate and 0.1% dexamethasone), and in those post-PKP inflammation, and in normal controls ($p < 0.05$; figure 1).

Stimulation with 30 ng of IL-4 and TNF- α for 24 hours induced the production of PGE₂ by fibroblasts, and this production was significantly downregulated by ketotifen fumarate at concentrations of 10^{-6} M ($p < 0.01$) and 10^{-5} M ($p < 0.05$). Dexamethasone reduced the PGE₂ by fibroblasts at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M (all $p < 0.01$), as well as 10^{-5} M ($p < 0.05$; figure 2). Chemokines such as IL-8, RANTES and eotaxin did not induce the production of PGE₂ by fibroblasts (figure 3).

DISCUSSION

This study identified the production of PGE₂ in tears of patients with severe allergic conjunctivitis, and that this

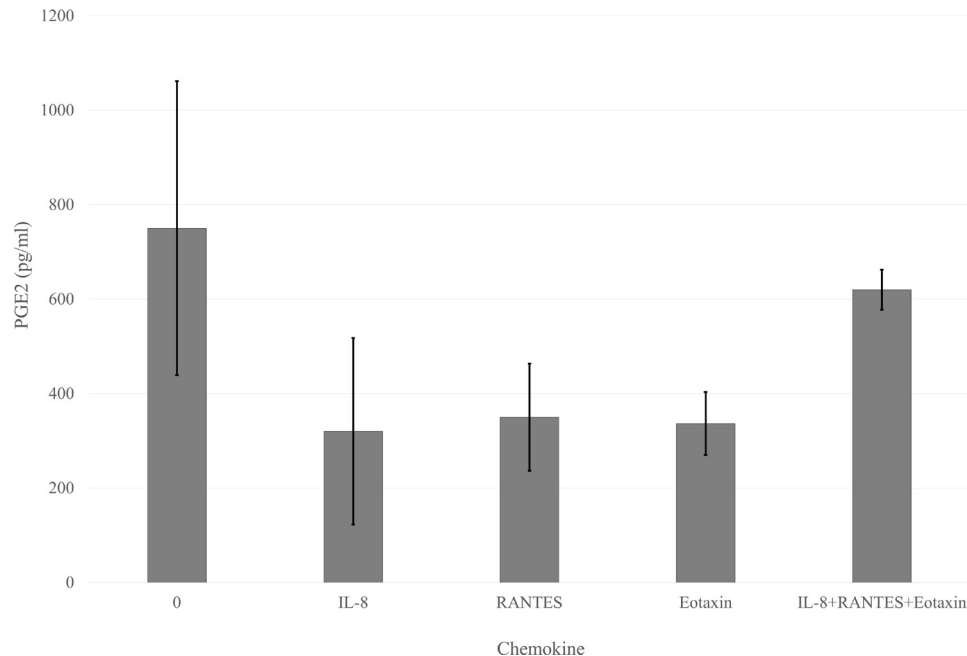


Figure 3 PGE₂ production by conjunctival fibroblasts stimulated with chemokines. Human conjunctival fibroblasts were maintained in 96 well plates at a concentration of 1×10^5 cells/mL. Cells were incubated with IL-8 (30 ng/mL), RANTES (30 ng/mL) and eotaxin (30 ng/mL), and harvested 24 hours later. The left bar '0' indicates medium alone. The generation of PGE₂ was determined in cell-free supernatants by ELISA. Results are presented as means \pm SD of at least three independent experiments. No significant differences were observed between the chemokine combinations (Student's t-test). IL-8, interleukin 8; PGE₂, prostaglandin E₂; RANTES, regulated on activation normal T cell expressed and secreted.

production was decreased by the administration of 0.05% ketotifen fumarate and 0.1% dexamethasone. PGE₂ was also produced by primary cultured conjunctival fibroblasts from normal volunteers following stimulation with IL-4 and TNF- α , and this production was also decreased by ketotifen fumarate or dexamethasone. PGs induce vasodilation, which is an important feature of local inflammation.²³ AA, which is released from the cell membrane phospholipids of activated cells, is metabolised to PGs by the sequential activity of COX-1 and COX-2.²⁴

It has been reported previously that a conjunctival allergic reaction can be induced with antigen in sensitised guinea pigs, and that in the acute phase of allergic conjunctivitis, the PGE₂ levels can be determined in lavage fluid; PGE₂ was produced simultaneously in the conjunctiva and exhibited identical profiles of synthesis in response to antigen provocation.²⁵ In this study, PGE₂ was identified in the tears of patients with severe allergic conjunctivitis, who were thought to be in the late phase of this condition. Levels of PGE₂ were higher in the tears of patients with severe allergy than in tears 4 weeks after eye drop treatment, and in post-surgical patients with PKP. The PKP tear samples, used as inflammatory controls, likely involve quite different inflammatory mechanisms and are not chronic. It is therefore not surprising that PGE₂ levels in patients with PKP were close to baseline. While our PKP surgical procedure usually concludes with a subconjunctival steroid injection and coverage with a medical use contact lens, PGE₂ production was significantly higher in the tears from patients with a severe

allergy. This may be due to the corneal ulcers in patients with severe allergic disease.⁵ PGE₂ was produced from the conjunctival fibroblasts by the action of IL-4 or TNF- α in tears,⁵ as discussed below.

In vitro concentrations of procollagens and inflammatory cytokines in fibroblasts from patients with VKC, including TNF- α and other cytokines, have been shown to be increased.²⁶ TNF- α levels have been shown to be increased in the tears of patients with atopy after conjunctival allergen challenge.¹¹ We have also reported increased levels of IL-4 in tears from patients with VKC.²⁷ We have stimulated ocular surface cells by IL-4 and TNF- α through allergic inflammation in vitro. Cooperation between proinflammatory cytokines is well documented in the literature. Production of PGE₂ was induced in primary cultured corneal keratocytes, conjunctival epithelial cells, and fibroblasts by IL-4 and TNF- α . Kinetic studies have demonstrated that PGE₂ is significantly increased in a time-dependent manner.⁵ In this paper, corneal epithelial cells produced almost no PGE₂. The reason for this is still unknown, but we hypothesise that corneal epithelial cells did not release PGE₂ and may be the target cell of this inflammatory response. Thus, we are now investigating how PGE₂ affects corneal epithelial cells. Repeated induction of PGE₂ production by conjunctival cells may cause corneal epithelial injury, and once the epithelial barrier is broken, keratocytes could produce more PGE₂ and epithelial erosions or ulcers can occur in severe allergic conjunctivitis. The effects of PGE₂ on corneal epithelial cells are

still unclear. We are studying these effects because we believe the corneal epithelium is a target of PGE₂. Any event which induces the production of PGE₂ may play an important role in allergic inflammation and result in severe clinical manifestations.

The effect of steroids on COX enzyme inhibition is now widely accepted. The anti-inflammatory effects of ketotifen fumarate are therefore likely to be due to the inhibition of COX. The identification of selective inhibitors of COX-2 will therefore lead to advances in therapy.²⁴ In this study, the production of PGE₂ by conjunctival fibroblasts was decreased by ketotifen fumarate, which is an antihistamine drug with an incompletely understood efficacy. Ketotifen fumarate has a strong inhibitory effect of platelet-activating factor (PAF), which enhances AA metabolism in addition to its role in both the acute and delayed phases of allergy. There is one speculation that under the circumstances an environment without mast cells, PGE₂ production was suppressed by its PAF inhibitory effect. Although this agent has distinct antihistamine molecular mechanisms of action and exhibits different immunoregulatory profiles, the direct effects on antigen presentation processes remain unknown. This is why we have studied the effects of this drug on primary cultured ocular surface cells. The pathogenesis of topical therapeutic antihistamine drugs in the inhibition of allergic inflammation may be due to other reasons.

CC chemokines, such as eotaxin and RANTES, are important in recruiting eosinophils into tissues affected by allergy.²⁸ RANTES has been found in the tears of patients with allergic conjunctivitis,²⁹ and is produced by conjunctival and corneal cells. We have previously reported that eotaxin is present in the tears of patients with allergy and severe corneal injury, collating with the number of eosinophils in the tears.³⁰ We have also found that IL-4 induces eotaxin production by human corneal keratocytes.¹⁶ In this study, we investigated the induction of PGE₂ production by fibroblasts associated with IL-8, RANTES and eotaxin. No effects on fibroblasts were observed (figure 3). Chemokines have no direct effects on keratocytes during the induction of PGE₂ production. This result implies that PGE₂ production by fibroblasts is not promoted by chemokines via autocrine or paracrine stimulations, but instead by the direct stimulation of IL-4 and TNF-α.

In summary, we found that PGE₂ was produced in the tears of patients with severe allergic conjunctivitis, and produced by cultured conjunctival fibroblast cells in a sequential manner following stimulation with IL-4 and TNF-α. Ketotifen fumarate affected this PGE₂ production and reduced allergic inflammation with unknown antihistamine efficacy. PGs have an important role in the study of allergic inflammation and have potential importance in managing ocular allergic inflammation.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was performed in accordance with the Declaration of Helsinki and was approved by the institutional ethics review board of Tsurumi University School of Dental Medicine (Kanagawa, Japan; IRB number: 1111).

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Data availability statement Data may be obtained from a third party and are not publicly available.

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ORCID iD

Eisuke Shimizu <http://orcid.org/0000-0002-3422-8578>

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