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Cyclosporin A Eye Drops Inhibit Fibrosis and Inflammatory Cell Infiltration in Murine Type I Allergic Conjunctivitis without Affecting the Early-Phase Reaction

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ABSTRACT

Purpose: To understand the mechanisms of action of cyclosporin A eye drops in severe allergic diseases such as vernal keratoconjunctivitis, the inhibitory effects of cyclosporin A eye drops on fibrosis and inflammatory cell infiltration in murine allergic conjunctivitis were evaluated. **Methods:** BALB/c mice that had been actively sensitized with ovalbumin were challenged with ovalbumin on days 10–14 after initial sensitization. Cyclosporin A (0.1%) or betamethasone (0.1%) eye drops were instilled 1, 4, and 7 hours after each challenge. Ocular tissue was harvested for histological evaluation 24 hours after the last challenge, and conjunctival tissue was collected for the measurement of collagen content and quantitative PCR 8 hours after the last challenge. **Results:** Scores for fibrosis and inflammatory cell infiltration and collagen content in the conjunctiva were higher after 5 days of antigen challenge than in normal non-challenged conjunctiva. Instillation of cyclosporin A or betamethasone reduced the antigen-induced increases in scores for fibrosis and inflammatory cell infiltration in the conjunctiva, and cyclosporin A significantly reduced the antigen-induced increase in conjunctival collagen content. Betamethasone also showed a tendency to reduce the increase in collagen content. Cyclosporin A and betamethasone decreased the numbers of CD3⁺ and CD4⁺ T-cells and eosinophils in the conjunctiva, but did not affect the number of mast cells. Neither type of eye drop suppressed the increase in vascular permeability that occurred for 30 minutes after the last antigen challenge. In quantitative PCR, cyclosporin A suppressed the expression of IL-4 and IL-5 mRNA but did not suppress the expression of transforming growth factor (TGF)- β 1, whereas betamethasone suppressed the expression of IL-4, IL-5, and TGF- β 1. **Conclusion:** The results suggest that cyclosporin A eye drops inhibited fibrosis and inflammatory cell infiltration by the suppression of Th2 cytokine production in repeatedly antigen-challenged conjunctiva without affecting the early-phase reaction.

Keywords: allergic conjunctivitis; cyclosporin A; fibrosis; inflammatory cell; mice; Th2 cytokine production

INTRODUCTION

Vernal keratoconjunctivitis (VKC) is one of the most severe refractory allergic conjunctival diseases, with frequent remissions and exacerbations. VKC involves severe allergic inflammation sufficient to cause the formation of proliferative conjunctival giant papillae,

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intense itching, strong pain, sensation of a foreign body, mucous discharge, and corneal damage. Proliferative changes in the conjunctiva, such as giant papilla formation, are the most preventable symptom of VKC. An increase in extracellular matrix material, including collagen, has been reported in giant papillae.¹ Therefore, fibrosis is thought to be involved in the formation of giant papillae.

The therapeutic effect of cyclosporin A eye drops in the treatment of giant papillae in VKC patients has been described in several articles.^{2–4} Cyclosporin A is a cyclic polypeptide calcineurin inhibitor that was first purified by Novartis Pharma (formerly Sandoz Pharma, Switzerland) from cultures of the fungus *Tolypocladium inflatum* Gams in the 1970s. It has long been a trusted agent for the suppression not only of graft rejection after transplantation but also of the immune reaction disorder often seen in autoimmune diseases. Cyclosporin A inhibits cytokine production from T-cells by binding to calcineurin,⁵ resulting in the suppression of T-cell activation/proliferation, IgE production, and the induction of apoptosis in eosinophils. These actions of cyclosporin A are considered to be effective in the treatment of VKC when used in topical preparations, but the mechanism by which cyclosporin A eye drops inhibit the formation of giant papillae is not well understood.

Eosinophil infiltration is essential for the late-phase reaction in type I allergy, and an increase in the number of eosinophils in the conjunctiva of VKC patients has been demonstrated.⁶ In addition, transforming growth factor (TGF)- β produced by eosinophils is reported to be associated with the induction of fibrosis in allergic disease.^{7,8} Therefore, the late-phase reaction induced by eosinophil infiltration may play a critical role in the aetiology of VKC. Thus, eosinophil infiltration followed by the early-phase reaction is thought to be involved in the development of fibrosis in type I allergic conjunctivitis.

In this study, we demonstrate that repeated antigen challenge in an animal model of type I allergic conjunctivitis induces fibrosis and infiltration of eosinophils and T-cells into the conjunctiva. To understand the mechanism of the therapeutic effect of cyclosporin A in VKC, we used this model to investigate the effects of instillation of cyclosporin A eye drops on fibrosis, eosinophil, and T-cell infiltration and the expression of IL-4, IL-5, and TGF- β mRNA in the conjunctiva.

MATERIALS AND METHODS

Animals

Male BALB/c mice (Charles River Japan, Kanagawa, Japan), age 4–6 weeks, were used for this study. They

were housed under a 12-hr light/dark cycle (07.00 to 19.00 hr), with the room temperature maintained at $23 \pm 3^\circ\text{C}$ and humidity at $50 \pm 20\%$. Food and water were given *ad libitum*. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Santen Institutional Animal Care and Use Committee.

Chemicals

Cyclosporin A was obtained from Novartis Ringaskiddy Ltd. (Co. Cork, Ireland). Cyclosporin A eye drops (Papilock Mini ophthalmic solution 0.1%) and vehicle were prepared at Santen Pharmaceutical Co. Ltd. (Osaka, Japan). Betamethasone sodium phosphate eye drops (Rinderon solution 0.1%) was purchased from Shionogi Co., Ltd. (Osaka, Japan). Ovalbumin (Grade V; Sigma, St Louis, MO, USA), Evans blue dye (Sigma), rat anti-mouse CD3 monoclonal antibody (clone KT3; Chemicon, Temecula, CA, USA), rat anti-mouse CD4 monoclonal antibody (clone GK1.5; Santa Cruz Biotechnology, Santa Cruz, CA, USA), normal rat IgG (Santa Cruz Biotechnology), and a SircolTM soluble collagen assay kit (Biocolor Ltd., Newtownabbey, UK) were used in the experiments.

Induction of Experimental Allergic Conjunctivitis

Actively sensitized allergic conjunctivitis is widely used as a model of allergic conjunctivitis with the type I allergic reaction.^{9,10} The experimental schedule is shown in Figure 1. Mice were actively sensitized by intraperitoneal injection of 0.5 ml ovalbumin adsorbed on aluminium hydroxide gel (containing 10 μg ovalbumin and 1 mg aluminium hydroxide gel) on days 0 and 5, and then challenged by topical instillation of 2.5 μl of 15% ovalbumin solution in both eyes on each of days 10–12 or 10–14 in the histological studies. Drug solutions (2.5 μl) were instilled in both eyes three times—1, 4, and 7 hr after each challenge—on each of days 10–14. Normal groups were only subjected to sensitization.

Histological Analysis

Conjunctival tissue was removed 24 hr after the last challenge on day 12 or 14, fixed with 10% neutral buffered formalin solution, and embedded in paraffin, following which 2 μm thick vertical sections were cut. Sections were cut from the central portion of the eye, which included the pupil and optic nerve head. The sections were stained with haematoxylin-eosin,

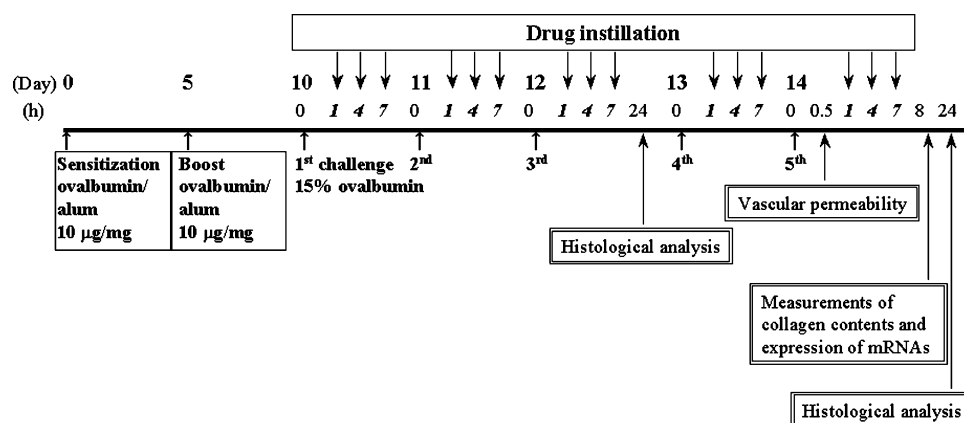


Figure 1. Schedule of the experiments. Allergic conjunctivitis was induced in BALB/c mice by active sensitization with ovalbumin. This was followed by repeated antigen challenge with ovalbumin eye drops daily for 5 days. Drug solution was instilled in each eye 1, 4, and 7 hr after each challenge.

Congo red, or toluidine blue. Fibrosis and infiltration of inflammatory cells, detected by HE staining, were evaluated numerically using a graded scale. Fibrosis in lamina propria of the tarsal conjunctiva was scored as follows: 0, no changes; 1, mild conjunctival fibroblast proliferation; 2, conjunctival fibroblast proliferation and thickening; 3, strong conjunctival fibroblast proliferation and conjunctival papillary hyperplasia; and 4, marked conjunctival papillary hyperplasia. Inflammatory cell infiltration was scored as follows: 0, no change; 1, dispersed inflammatory cells in the subepithelial area; 2, widespread inflammatory cells in the subepithelial area; 3, features of 2+ aggregations of several inflammatory cells; 4, features of 2+ aggregations of numerous inflammatory cells. Congo red-stained infiltrating eosinophils and toluidine blue-stained mast cells were counted. Cells were counted in the lamina propria of the tarsal and bulbar conjunctiva in each section according to the method of Fukushima et al.¹¹

Tissues for detection of CD3⁺ or CD4⁺ T cells were fixed with periodate-lysine-paraformaldehyde, embedded in OCT compound, and snap-frozen in dry ice/isopentane. Frozen vertical 4 µm thick sections were cut from the central portion of the eye, which included the pupil and optic nerve head. Next, rat anti-mouse CD3 or CD4 monoclonal antibody was applied overnight at 4°C, and the sections were then immersed in methanol containing 0.3% H₂O₂ for 15 min at room temperature to block endogenous peroxidase activity. The sections were then overlaid with Histofine Simple Stain Mouse MAX PO (rat) (peroxidase-labeled anti-rat secondary antibody; Nichirei, Tokyo, Japan). All samples were visualized using a DAB kit (Dako Japan, Kyoto, Japan). CD3⁺ and CD4⁺ T-cells were counted in the lamina propria of the tarsal and bulbar conjunctiva in each section according to the method of Fukushima et al.¹¹

Measurement of Collagen Content

Eyes were collected 8 hr after the last challenge on day 14. To extract soluble collagen, conjunctival tissue was carefully separated from the eyelid, minced in 0.5 M acetic acid, and incubated overnight at 4°C. Total soluble collagen was quantified using a Sircol™ Soluble Collagen Assay kit according to the manufacturer's instructions. Briefly, after centrifugation, 100 µl of each supernatant was assayed. One milliliter of Sircol dye reagent, which binds to collagen, was added to each sample, and the solutions were mixed for 30 min. After centrifugation, the pellet was suspended in 1 ml of the alkali reagent included in the kit and read at 540 nm with a spectrophotometer. Results were normalized for conjunctiva weight.

Measurement of mRNA Expression

Conjunctival tissue was removed 8 hr after the last challenge on day 14 and placed in RNeasy (Qiagen, Valencia, CA, USA). Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. The total RNA content was measured spectrophotometrically at 260 and 280 nm. cDNA from total RNA (0.04–1 µg) was synthesized using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) with M-MLV Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with SYBR Green Master Mix (Qiagen) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR primers for IL-4, IL-5, and cyclophilin A (CYPA) were designed using Primer 3 or OligoPerfect™ Designer software, and primers for TGF-β1 were purchased from Qiagen (catalogue number QT00145250). Primer sequences were as follows: IL-4,

5'-GGAGATGGATGTGCCAAACGTC-3' and 5'-GAAGCACCTTGGAAGCCCTACA-3'; IL-5, 5'-GACAAGCAATGAGACGATGAGG-3' and 5'-CC-CACGGACAGTTTGATTCTTC-3'; CYPA, 5'-GACCAAACACAAACGGTTCCCA-3' and 5'-CACCTTCCCAAAGACCACATGC-3'. A comparative threshold cycle (C_T) was used to determine gene expression relative to vehicle treatment (calibrator). Hence, steady-state mRNA levels were expressed as n -fold differences relative to the calibrator. For each sample, the C_T value was normalized using the formula $\Delta C_T = C_{T,\text{sample}} - C_{T,\text{CYPA}}$. Relative expression levels were determined using the formula $\Delta\Delta C_T = \Delta C_{T,\text{sample}} - \Delta C_{T,\text{calibrator}}$, and the value used to plot the relative expression level of each mRNA was calculated using the expression $2^{-\Delta\Delta C_T}$.

Vascular Permeability in the Conjunctiva

A solution of 0.1% Evans blue dye (15 ml/kg) was administered intravenously following the last challenge on day 14, and the tissues exhibiting dye leakage, including the conjunctiva, were removed 30 min after the challenge. Evans blue dye was extracted for more than 48 hr after the tissues were removed, and the absorbance of the extracts was determined with a spectrophotometer at 620 nm.

Statistical Analysis

Data from two groups were evaluated statistically using the student's t -test or the Aspin-Welch t -test based on the variance of data examined with the F test and examined using the non-parametric Wilcoxon test. Significance was set at $p < 0.05$.

RESULTS

Effect of Cyclosporin A Eye Drops on Fibrosis and Infiltration of Inflammatory Cells into the Conjunctiva

We examined histopathological changes of conjunctiva tissues 24 hr after 5 days of challenge with ovalbumin. Representative patterns found in the conjunctiva after repeated antigen challenge are shown in Figure 2. Hypertrophy of the conjunctival lamina propria was observed, and excessive fibroblast proliferation and massive infiltration with inflammatory cells, such as neutrophils, eosinophils, and lymphocytes, were evident in the lamina propria of the tarsal and bulbar conjunctival tissue after repeated antigen challenge (Fig. 2B) compared with normal conjunctiva (Fig. 2A). Panels C and D in Figure 2 show the effects of 0.1% cy-

Table 1. Number of mice in the fibrosis score (0–4), respectively was indicated

The pathological score of the fibrosis	3rd challenge		5th challenge	
	Normal (n = 5)	Vehicle (n = 10)	Normal (n = 6)	Vehicle (n = 14)
0	5	0	6	0
1	0	5	0	1
2	0	5	0	13
3	0	0	0	0
4	0	0	0	0

Allergic conjunctivitis was induced as described in the legend to Figure 1. Conjunctiva tissues were removed 24 h after the last challenge on day 12 or 14, and stained with haematoxylin-eosin.

closporin A and 0.1% betamethasone eye drops, respectively, on the histopathological changes in conjunctiva tissues 24 hr after 5 days of antigen challenge. Instillation of 0.1% cyclosporin A or 0.1% betamethasone decreased hypertrophy of the conjunctival lamina propria, and suppressed fibroblast proliferation and infiltration of inflammatory cells compared with the conjunctiva from vehicle-treated eyes.

To confirm the inhibitory effect of cyclosporin A, we examined the changes in scores for fibrosis and inflammatory cell infiltration. As shown in Table 1, 5 of 10 mice showed a score of 2 after the third daily antigen challenge, but after the fifth daily antigen challenge, this number increased, i.e., 13 of 14 mice showed a score of 2. This clearly shows that the fifth topical application of ovalbumin induced fibrosis to a greater extent than the third antigen challenge. Marked fibrosis and inflammatory cell infiltration were detected in the conjunctiva after 5 days of antigen challenge compared with normal conjunctiva; instillation of 0.1% cyclosporin A significantly decreased scores for fibrosis and inflammatory cell infiltration to the same degree as 0.1% betamethasone eye drops (Fig. 3A and 3B).

Effect of Cyclosporin A Eye Drops on Collagen Content and Infiltration of Eosinophils, CD3⁺ T-cells, and CD4⁺ T-cells in the Conjunctiva

Next, we measured the total soluble collagen content, which reflects fibrosis, in the conjunctiva after challenge with ovalbumin for 5 days (Fig. 4). Antigen challenge for 5 days induced an increase in collagen content compared with normal conjunctiva. Instillation of 0.1% cyclosporin A significantly inhibited the

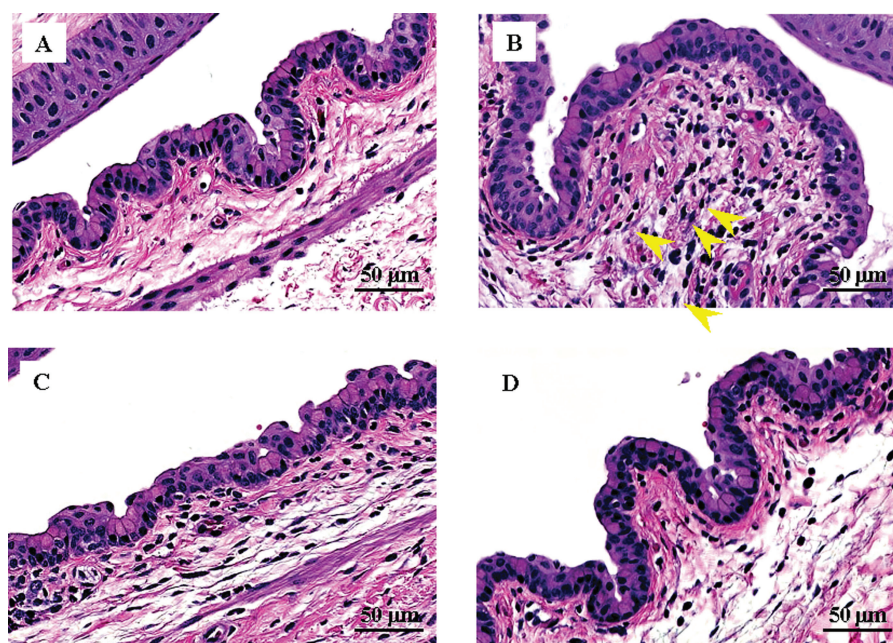


Figure 2. Histological section of the conjunctiva after repeated antigen challenge for 5 days and the effects of cyclosporin A and betamethasone eye drops. Haematoxylin-eosin staining. Allergic conjunctivitis was induced as shown in Figure 1. Conjunctival tissue was removed 24 hr after the last challenge on day 14. (A) No antigen. (B) Antigen-challenged mice, vehicle. (C) Antigen-challenged mice, 0.1% cyclosporin A. (D) Antigen-challenged mice, 0.1% betamethasone. Fibrosis, proliferation of spindle-shaped fibroblasts (arrowhead), and infiltration of inflammatory cells, such as lymphocytes, neutrophils, and eosinophils, was observed in the vehicle group (B) but not in the normal group (A). In the groups receiving (C) cyclosporin A and (D) betamethasone eye drops, there was less fibrosis, proliferation of fibroblasts, and infiltration of inflammatory cells compared with (B) the vehicle group.

increase in collagen content, and instillation of 0.1% betamethasone showed a tendency to decrease the collagen content compared with the vehicle group.

The number of eosinophils infiltrating the conjunctiva after antigen challenge for 5 days, evaluated by Congo red staining (Fig. 5), was markedly higher compared with normal conjunctiva. Instillation of 0.1% cyclosporin A or 0.1% betamethasone significantly inhibited the increase in the number of eosinophils infiltrating the conjunctiva compared with the vehicle-treated conjunctiva.

To investigate the effect of cyclosporin A eye drops on T-cells, sections were prepared for immunohistochemistry to detect infiltrating CD3⁺ and CD4⁺ T-cells (Fig. 6). Compared with normal conjunctiva (Fig. 6A), more CD3⁺ and CD4⁺ T-cells were detected in the lamina propria of the tarsal and bulbar conjunctival tissue after antigen challenge for 5 days (Fig. 6B). Instillation of 0.1% cyclosporin A (Fig. 6C) or 0.1% betamethasone (Fig. 6D) decreased infiltration of CD3⁺ and CD4⁺ T-cells into the conjunctiva. Counts of CD3⁺ and CD4⁺ T-cells that infiltrated into the conjunctiva are shown in Figure 6E. Antigen challenge for 5 days significantly increased the numbers of CD3⁺ and CD4⁺ T-cells in-

filtrating the conjunctiva compared with conjunctiva from normal mice; CD4⁺ and CD3⁺ T-cell counts were almost the same. This result suggests that helper T-cells were dominant among the T-cells that infiltrated the conjunctiva after 5 days of antigen challenge. Instillation of 0.1% cyclosporin A or 0.1% betamethasone significantly inhibited the infiltration of both CD3⁺ and CD4⁺ T-cells into the conjunctiva compared with vehicle-treated conjunctiva.

Effect of Cyclosporin A Eye Drops on Expression of IL-4, IL-5, and TGF- β 1 mRNA in the Conjunctiva

To elucidate the effect of cyclosporin A eye drops on the production of Th2-type cytokines (IL-4, IL-5) and TGF- β 1, the levels of mRNA expression of IL-4, IL-5, and TGF- β 1 in the conjunctiva were measured by real-time PCR (Fig. 7). Levels of mRNA expression of IL-4 and IL-5 were very low in normal conjunctiva, but after antigen challenge for 5 days, increases in expression of IL-4 and IL-5 mRNA were observed. Instillation of 0.1% cyclosporin A or 0.1% betamethasone significantly suppressed the expression of IL-4 and IL-5 mRNA compared with vehicle-treated

Effect of Cyclosporin A in Allergic Conjunctivitis

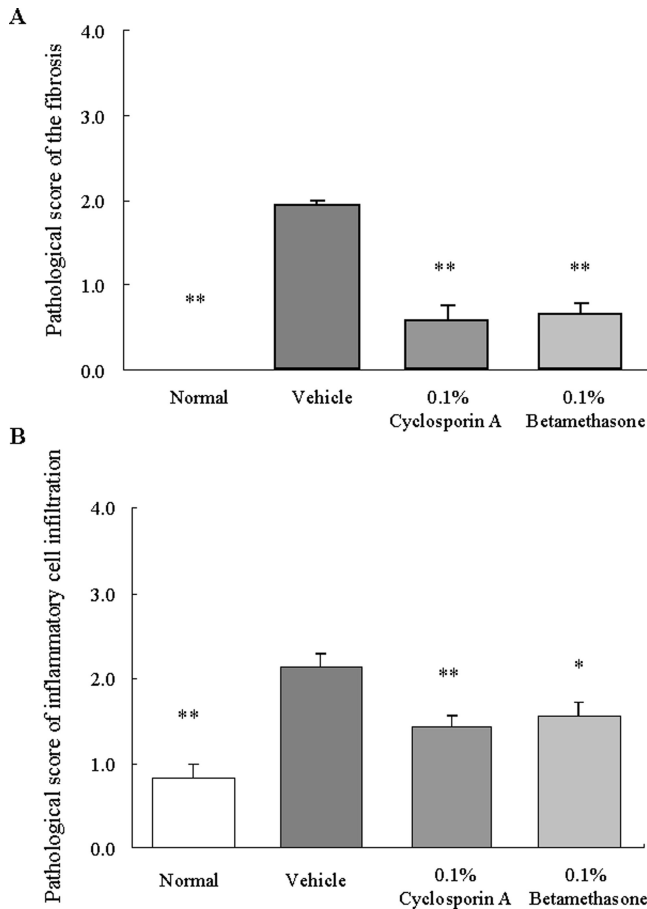


Figure 3. Effects of cyclosporin A and betamethasone eye drops on histopathological scores for (A) fibrosis and (B) infiltration of inflammatory cells. Allergic conjunctivitis was induced as described in Figure 1. Conjunctival tissue was removed 24 hr after the last challenge on day 14. Pathological scores for fibrosis and inflammatory cell infiltration were significantly reduced by treatment with cyclosporin A or betamethasone compared with the vehicle group. Each column shows the mean and SEM for 6 normal (non-challenged) or 14 antigen-challenged mice. * $p < 0.05$, ** $p < 0.01$ compared with the vehicle group (Wilcoxon test).

conjunctiva. Antigen challenge for 5 days tended to increase the expression of TGF- β 1 mRNA compared with normal conjunctiva. Expression of TGF- β 1 mRNA was not decreased by instillation of 0.1% cyclosporin A but was significantly decreased by instillation of 0.1% betamethasone compared with vehicle-treated conjunctiva.

Effect of Cyclosporin A Eye Drops on the Number of Mast Cells and Vascular Permeability in the Conjunctiva

Repeated challenge with ovalbumin for 5 days did not induce an increase in the number of mast cells

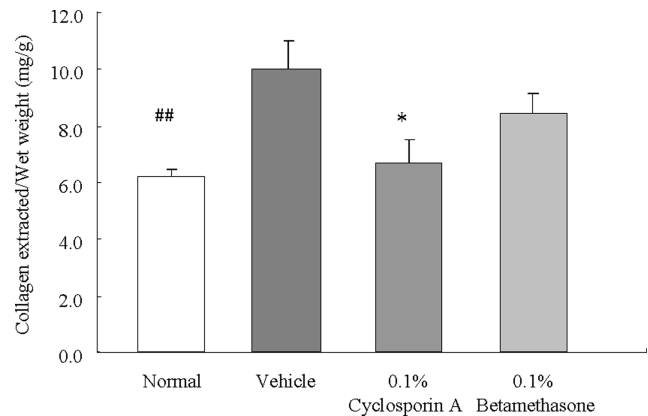


Figure 4. Effects of cyclosporin A and betamethasone eye drops on soluble collagen content in the conjunctiva. Allergic conjunctivitis was induced as shown in Figure 1. Conjunctival tissue was removed 8 hr after the last challenge on day 14, and the content of soluble collagen was quantified as described in the text. Cyclosporin A significantly inhibited the increase in collagen content, and betamethasone tended to decrease the collagen content compared with the vehicle group. Each column shows the mean and SEM for 4 normal (non-challenged) or 7 antigen-challenged mice. * $p < 0.05$ compared with the vehicle group (student's t -test); ## $p < 0.01$ compared with the vehicle group (Aspin-Welch t -test).

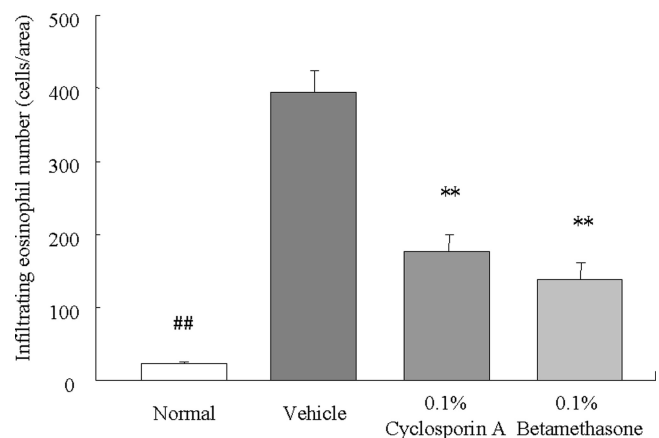


Figure 5. Effects of cyclosporin A and betamethasone eye drops on infiltration of eosinophils into the conjunctiva. Allergic conjunctivitis was induced as shown in Figure 1. Conjunctival tissue was removed 24 hr after the last challenge on day 14, samples were stained with Congo red, and the number of eosinophils infiltrating the lamina propria of the tarsal and bulbar conjunctiva was counted. Cyclosporin A and betamethasone significantly inhibited the increase in the number of eosinophils infiltrating the conjunctiva compared with the vehicle group. Each column shows the mean and SEM of 6 normal (non-challenged) or 14 antigen-challenged mice. ** $p < 0.01$ compared with the vehicle group (student's t -test); ## $p < 0.01$ compared with the vehicle group (Aspin-Welch t -test).

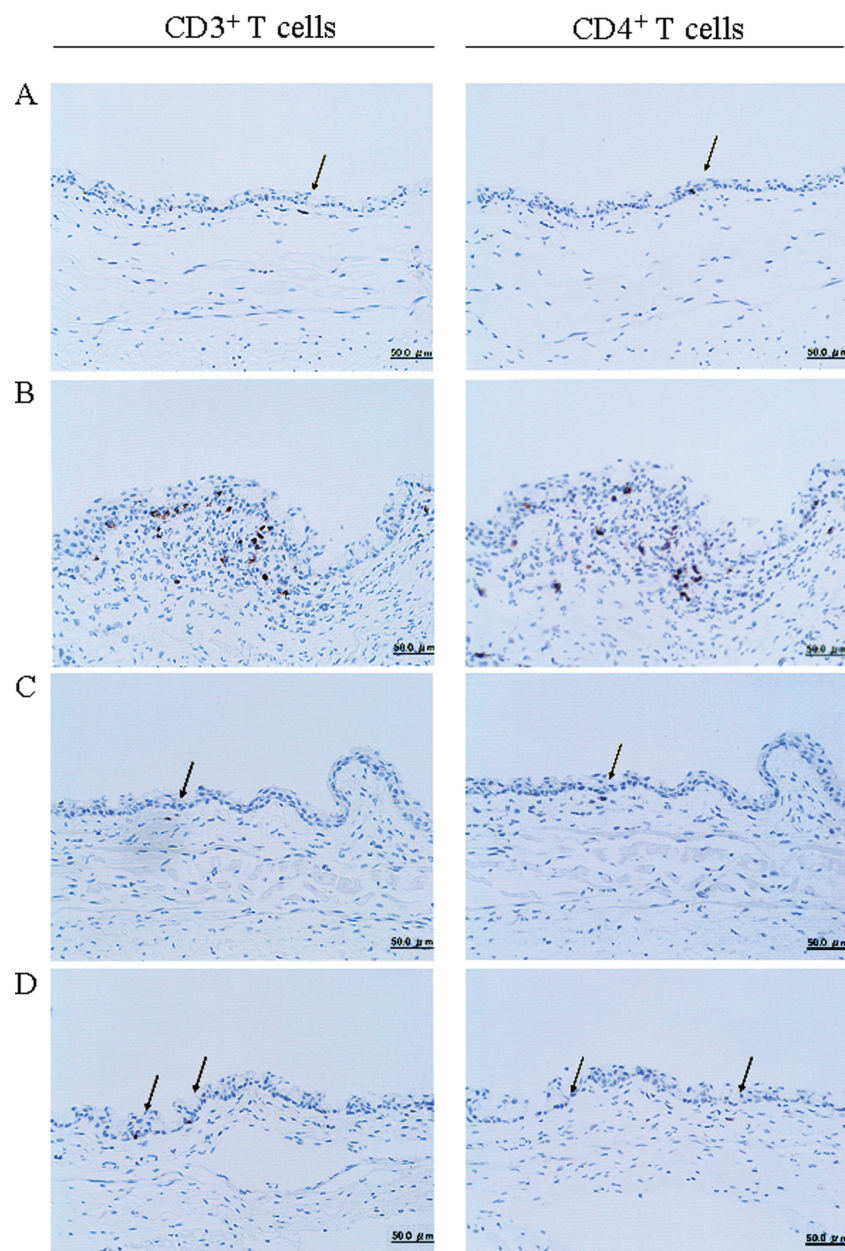


Figure 6. Effects of cyclosporin A and betamethasone eye drops on infiltration of CD3⁺ and CD4⁺ T cells into the conjunctiva. (A) Normal mice. (B) Antigen-challenged mice, vehicle. (C) Antigen-challenged mice, 0.1% cyclosporin A. (D) Antigen-challenged mice, 0.1% betamethasone. Allergic conjunctivitis was induced as shown in Figure 1. Conjunctival tissue was removed 24 hr after the last challenge on day 14, and samples were immunostained for CD3⁺ and CD4⁺ T-cells. Increase of infiltrating CD3⁺ and CD4⁺ T-cells (arrows) was observed in the (B) vehicle-treated group compared with the (A) normal group. In the groups treated with (C) cyclosporin A or (D) betamethasone eye drops, there were fewer infiltrating CD3⁺ and CD4⁺ T-cells than in the (B) vehicle-treated group. Numbers of infiltrating CD3⁺ and CD4⁺ T-cells in the lamina propria of the tarsal and bulbar conjunctiva were counted (E). Cyclosporin A and betamethasone eye drops significantly inhibited the infiltration of both CD3⁺ and CD4⁺ T-cells into the conjunctiva compared with the vehicle group. Each column shows the mean and SEM of 6 normal (non-challenged) or 14 antigen-challenged mice. ***p* < 0.01 compared with the vehicle group (student's *t*-test); ##*p* < 0.01 compared with the vehicle group (Aspin-Welch *t*-test). (Continued)

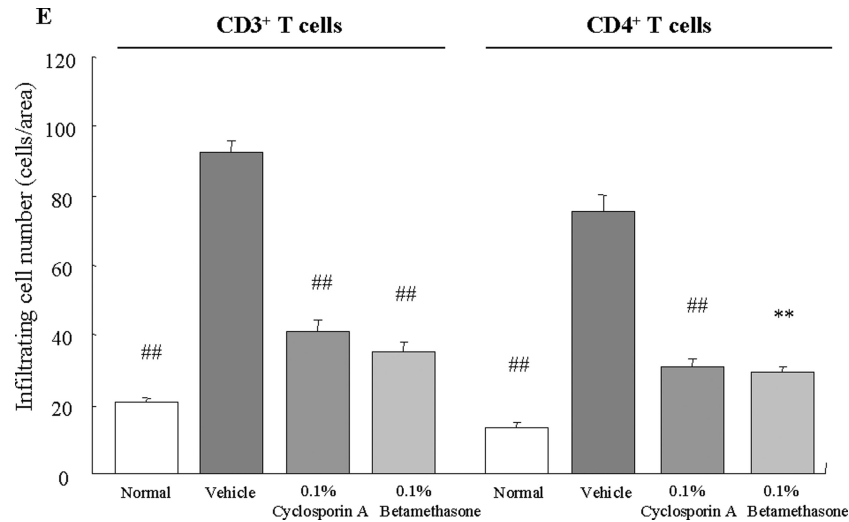


Figure 6. (Continued)

compared with normal conjunctiva (Fig. 8A). Instillation of 0.1% cyclosporin A or 0.1% betamethasone did not affect the number of mast cells. To evaluate the effect of cyclosporin A eye drops on the early-phase reaction, induced by degranulation of mast cells, vascular permeability determined (from Evans blue leakage) in the conjunctiva was measured 30 min after last antigen challenge (Fig. 8B). After the last antigen challenge, an increase in permeability was detected compared with normal conjunctiva, suggesting that mast cells normally present in the conjunctiva had degranulated in this experiment. Instillation of 0.1% cyclosporin A or 0.1% betamethasone did not significantly reduce vascular permeability compared with vehicle-treated conjunctiva.

DISCUSSION

It is well known that type I allergy induces an increase in vascular permeability in the early-phase reaction and infiltration of eosinophils in the late-phase reaction, but the relationship between type I allergy and fibrosis in the conjunctiva is not well understood. In this study, we investigated the effect of type I allergy induced by repeated antigen challenge on fibrosis in the conjunctiva. Leonardi et al.¹ reported highly positive immunostaining for collagen and infiltrated inflammatory cells in the giant papillae of VKC conjunctiva. Collagen is a major component of the extracellular matrix produced by fibroblasts, and a relatively large amount of collagen is a key marker of fibrosis.¹² We therefore examined the influence of antigen challenge for 5 days on the conjunctival collagen content in addition to the histological evaluation of fibrogenesis. We found hypertrophy of the conjunctival lamina propria, fibroblast prolifera-

tion, and increased collagen content in the conjunctiva after antigen challenge for 5 days compared with normal conjunctiva. This suggested that induction of type I allergy by repeated antigen challenge induced fibrosis in the conjunctiva. This is the first report, to our knowledge, to show that repeated antigen challenge induces fibrosis in the conjunctiva in a model of type I allergy. Our results suggest that type I allergy in the conjunctiva may be involved in the induction of fibrosis, including formation of papilla in VKC and AKC. However, we only detected the proliferation of fibroblasts and the increase of collagen contents, which is not exactly the same as fibrosis-like papilla in VKC and AKC, and the typical type I allergy-mediated conjunctivitis-like seasonal allergic conjunctivitis does not usually evolve into clinical fibrosis. Therefore, other factors might be needed to induce formation of papilla in such severe allergic conjunctivitis.

Many reports show that cyclosporin A inhibits the activation of mast cells *in vitro*.^{13,14} Histamine, a typical chemical mediator in the early-phase reaction, has been shown to increase collagen production by fibroblasts in VKC patients.¹⁵ Therefore, the instillation of cyclosporin A before antigen challenge may be able to inhibit the early-phase reaction that leads to fibrosis in type I allergy. The early-phase reaction reaches a peak within 1 hr after antigen challenge; hence, to prevent the inhibitory effect of cyclosporin A on the early-phase reaction, it was instilled 1 hr after antigen challenge. In our experiments, instillation of cyclosporin A 1, 4, and 7 hr after antigen challenge significantly inhibited the increases in both the fibrosis score and the collagen content in the conjunctiva induced by antigen challenge. Therefore, we tried to examine the effects of cyclosporine A eye drops instilled 1, 4, and

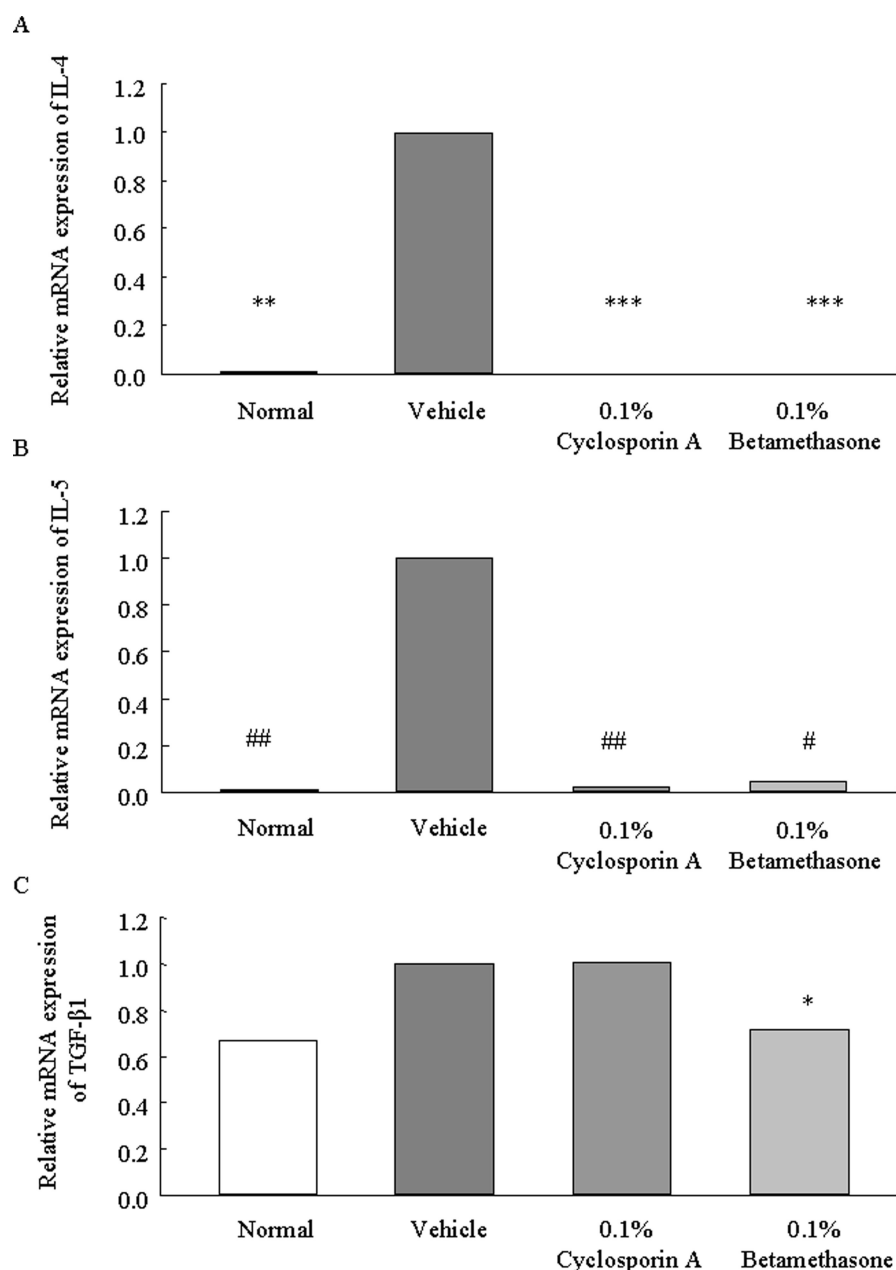


Figure 7. Effects of cyclosporin A and betamethasone eye drops on IL-4, IL-5, and TGF- β 1 mRNA expression in the conjunctiva. Allergic conjunctivitis was induced as shown in Figure 1. Conjunctival tissue was removed 8 hr after the last challenge on day 14, and expression of IL-4 (A), IL-5 (B), and TGF- β 1 (C) was determined by quantitative real-time PCR. The level of gene expression normalized to CYPB was given by $2^{-\Delta\Delta CT}$. Cyclosporin A significantly suppressed the expression of IL-4 and IL-5 mRNA but not that of TGF- β 1 mRNA. Betamethasone significantly decreased IL-4, IL-5, and TGF- β 1 mRNA compared with the vehicle group. Each column shows the mean of 10 (IL-4, IL-5) or 9 (TGF- β 1) samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the vehicle group (student's t -test); # $p < 0.05$, ## $p < 0.01$ compared with the vehicle group (Aspin-Welch t -test).

7 hr after antigen challenge on the vascular permeability and the number of mast cells to confirm that cyclosporine A eye drops in this dosing schedule did not inhibit early-phase reaction. In this study, instillation of cyclosporin A 1, 4, and 7 hr after antigen challenge did not affect the increase of vascular permeability and the number of mast cells in the conjunctiva. Therefore,

the inhibition of the early-phase reaction may not be necessary for the therapeutic effect of cyclosporin A on fibrosis in this model. These results suggest that the inhibitory action of cyclosporin A eye drops in the late-phase reaction or the activation of T-cells may be involved in the therapeutic effect of cyclosporin A on fibrosis.

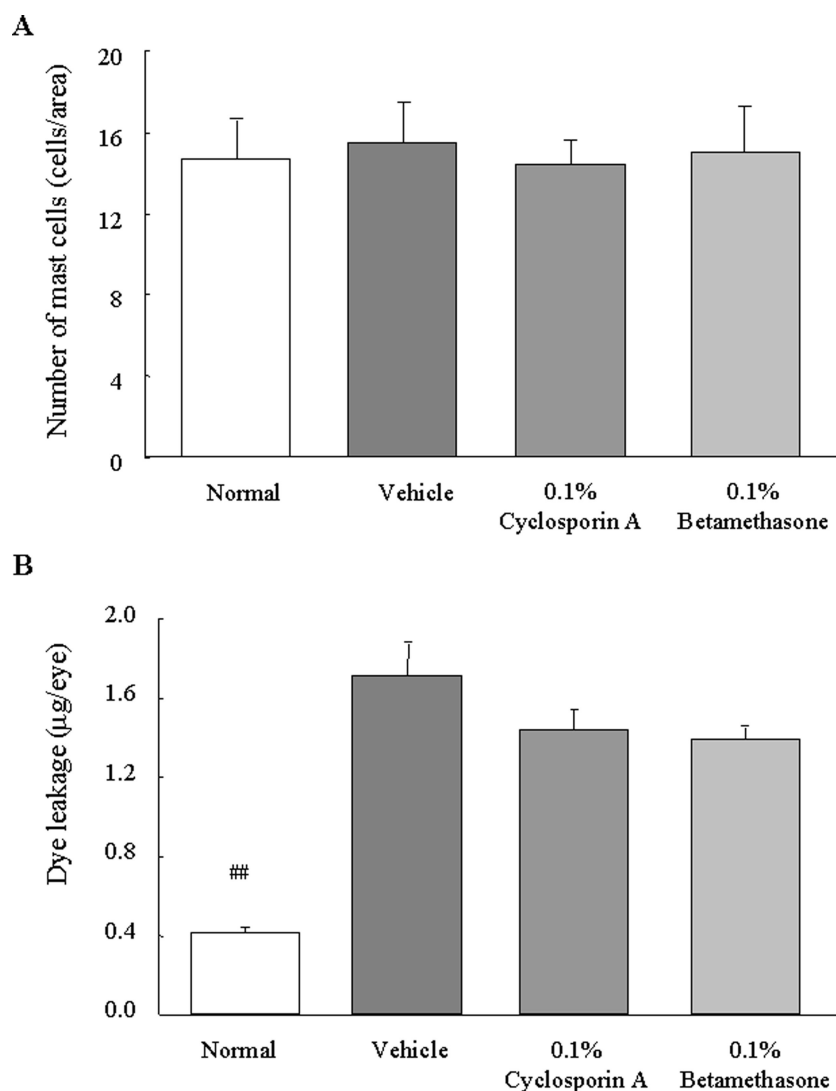


Figure 8. Effects of cyclosporin A and betamethasone eye drops on the number of mast cells and increase in conjunctival vascular permeability. Allergic conjunctivitis was induced as shown in Figure 1. (A) Conjunctival tissue was removed 24 hr after the last challenge on day 14 and stained with toluidine blue. Mast cells were counted in the lamina propria of the tarsal and bulbar conjunctiva. Cyclosporin A and betamethasone had no effect on the number of mast cells. Each column shows the mean and SEM of 6 normal (non-challenged) or 14 antigen-challenged mice. (B) Cyclosporin A and betamethasone did not significantly decrease conjunctival vascular permeability, assessed by measuring leakage of Evans blue, compared with the vehicle group. Each column shows the mean and SEM of 10 mice. ## $p < 0.01$ compared with the vehicle group (Aspin-Welch t -test).

Except for mast cells, infiltration of inflammatory cells such as eosinophils and T-cells ($CD3^+$ and $CD4^+$ T-cells) into the conjunctiva was observed after repeated antigen challenge compared with normal conjunctiva, and instillation of cyclosporin A after the early-phase reaction inhibited the infiltration of these cells. In the late-phase reaction in the type I allergic conjunctivitis model, eosinophils are dominant among the infiltrating inflammatory cells. Whitcup et al.¹⁶ reported that the instillation of cyclosporin A inhibited the infiltration of inflammatory cells into the conjunctiva induced by activation of mast cells after administration

of compound 48/40 in the conjunctiva, and it was suggested that cyclosporin A inhibited the late-phase reaction by suppressing the early-phase reaction in type I allergy. The inhibitory effect of cyclosporin A eye drops on eosinophil infiltration in allergic conjunctivitis was also demonstrated in a report by Fukushima et al.¹¹ These authors observed that cyclosporin A inhibited eosinophil infiltration during passive immunization with antigen-primed splenocytes, but failed to inhibit infiltration during passive immunization by IgE transfer. They concluded that cyclosporin A eye drops predominantly inhibit eosinophil

infiltration via the suppression of T-cells in type IV allergic conjunctivitis. However, the effect of cyclosporin A on the early-phase reaction was not evaluated in either of these two studies. Chemical mediators released from mast cells, such as histamine, prostaglandin D₂, and platelet-activating factor, have been shown to induce eosinophil infiltration,^{17,18} and it has also been reported that the late-phase reaction is significantly diminished in histamine H1 receptor knockout mice.¹⁹ Thus, chemical mediators in the early-phase reaction may induce the late-phase reaction, including infiltration by eosinophils, but our experiments demonstrated that the instillation of cyclosporin A without inhibiting the early-phase reaction inhibited the late-phase reaction, including eosinophil infiltration. In addition to the increase in eosinophils, we demonstrated that repeated antigen challenge clearly increased the infiltration of helper T-cells and expression of IL-4 and IL-5 mRNA compared with normal conjunctiva, and that cyclosporin A eye drops inhibited the infiltration of helper T-cells and mRNA expression of IL-4 and IL-5, which are important cytokines in the infiltration and activation of eosinophils in this model.^{20,21} These results suggest that the inhibitory effect of cyclosporin A on the late-phase reaction may not be mediated by the early-phase reaction, but that T-cells might mediate the late-phase reaction in the type I allergy model, as reported by Fukushima et al.¹¹

TGF- β has been reported to induce the production of collagen from fibroblasts,⁷ and eosinophils are thought to induce fibrosis mediated by the production of TGF- β in allergic disease²²; hence, TGF- β is thought to be one of the main factors inducing fibrosis in allergic disease. We therefore measured mRNA expression of TGF- β in the conjunctiva. In our study, the expression of TGF- β 1 did not significantly increase after repeated antigen challenge, and the instillation of cyclosporin A did not show any inhibitory effects. Those results suggest that TGF- β 1 is not associated with the therapeutic effect of cyclosporin A eye drops on fibrosis in type I allergic conjunctivitis. Typical Th2 cytokines, such as IL-4, were also demonstrated to induce the production of collagen by fibroblasts.^{23,24} As described above, repeated antigen challenge increased the expression of IL-4, and the instillation of cyclosporin A significantly inhibited mRNA expression of IL-4. Therefore, Th2 cytokines such as IL-4 may play a predominant role in the inhibitory effect of cyclosporin A on fibrosis in this model. In addition, the number of T-cells is reported to be increased in the papillae of VKC patients,²⁵ and elevations in Th2 cytokine levels have been detected in tears of VKC patients.^{26,27} Thus, Th2 cells are thought to be involved in VKC via the production of Th2 cytokines. Therefore, the inhibition of Th2 cytokines such as IL-4, produced by T-cells, might be important in the

therapeutic mechanisms of cyclosporin A in VKC. It had been reported that cyclosporin A directly inhibited the proliferation and procollagen production of human conjunctival fibroblasts.²⁸ Further investigation is needed to understand the contribution of direct inhibition to the therapeutic effects of cyclosporin A eye drops in VKC.

Corticosteroids are used for the treatment of VKC and are reported to have potent anti-inflammatory and immunosuppressant effects.^{29,30} In addition to cyclosporin A, betamethasone, a strong steroid, showed inhibitory effects on the fibrosis score as well as infiltration by eosinophils and T-cells in the present study. In the instillation schedule described here, betamethasone did not show any effects on the number of mast cells or the early-phase reaction. However, its effect on cytokine expression was different from that of cyclosporin A. Betamethasone significantly inhibited the expression of IL-4, IL-5, and TGF- β 1 mRNA in the conjunctiva. TGF- β is produced by inflammatory cells such as eosinophils.³¹ Corticosteroids have been reported to have a more potent anti-inflammatory effect on eosinophils than cyclosporin A,³² but the potency of cyclosporin A with regard to fibrosis was similar to that of betamethasone in the present experiment. Therefore, inhibition of the expression of Th2 cytokines may be more important in the therapeutic effects of steroid eye drops rather than inhibition of TGF- β 1 in this model.

In our experiments, antigen challenge for 5 days significantly increased the fibrosis score, collagen content, inflammatory cell infiltration score, infiltration by inflammatory cells (CD3⁺ and CD4⁺ T-cells, eosinophils), expression of IL-4 and IL-5 mRNA, and vascular permeability, but did not increase the number of mast cells or expression of TGF- β 1 in the conjunctiva compared with normal conjunctiva. Furthermore, instillation of cyclosporin A after the early-phase reaction showed inhibitory effects on all these increases except the increase in vascular permeability. These results suggest that (1) cyclosporin A eye drops may inhibit fibrosis, including collagen synthesis, and (2) infiltration of eosinophils and T-cells into the conjunctiva without causing pathological changes in the early-phase reaction (e.g., mast cell degranulation) and inhibition of Th2 cytokine production might be important in the therapeutic mechanisms of cyclosporin A with respect to the induction of fibrosis in type I allergy.

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