chamber and vitreous cavity. A fundus examination showed normal macula, peripheral retina, disk, and vessels in the OD.

The patient was initially treated with topical betamethasone and levofloxacin but was found to be resistant to those treatments. One month after the initial treatment, oral steroids and cyclosporine (50 mg) were added to the treatment. As a result, the scleritis and peripheral corneal infiltration showed gradual improvement, yet the efficacy was limited. Four months after the initial treatment, she presented with anterior uveitis with mutton fat keratic precipitates with pigment and ocular hypertension (Fig. 2). From these findings, an accompanying herpetic uveitis was highly suspected. Thus, antiherpetic drugs were administered and the anterior uveitis responded well and improved immediately. After that, and most interestingly, an atypical continuous keratitis appeared, which apparently differs from typical PUK. The lesion was located from the 4-o'clock to 10-o'clock positions continuously in the midperipheral cornea, with diffuse superficial punctate keratitis (SPK) (Fig. 3), and we continued to administer antivirus drugs and topical steroids. Two weeks later, the atypical keratitis gradually disappeared, yet conjunctival invasion and hurricane keratopathy were temporarily noted. Subsequently, administration of an anti–TNF-α drug (infliximab) was initiated because of the persistence of ocular and systemic inflammation. As a result, both scleritis and infiltrative keratitis were completely improved, and there was no recurrence of ocular diseases (Fig. 4).

DISCUSSION

The ophthalmologic manifestations of RA are dry eye, PUK, scleritis, and other ocular complications.[1] RA also presents with various corneal impairments. It is reported that the scleritis is often accompanied by corneal lesions and that the activity of infiltrative keratitis correlates with that of the scleritis.[5,6] In addition to severe scleritis, this case presented for the first time a unique corneal complication, one that is clearly different from typical PUK.

In the present case, the continuous keratitis suddenly appeared in the midperipheral cornea in the course of treatment for uveitis, thought to be caused by a herpetic virus. Considering the shape and the location of the epithelial lesions, an immunologic mechanism was highly suspected. Although the accompanying diffuse SPK, the subsequent hurricane keratopathy, and the effect of the reduced antivirus drug may support the indication of a drug-induced mechanism, the shape and location of the epithelial lesions were atypical for drug-induced corneal damage. Herpetic keratitis was also suspected, but the SPK surrounding the ulcer and the effect of the reduced antivirus drug were incompatible with that hypothesis.

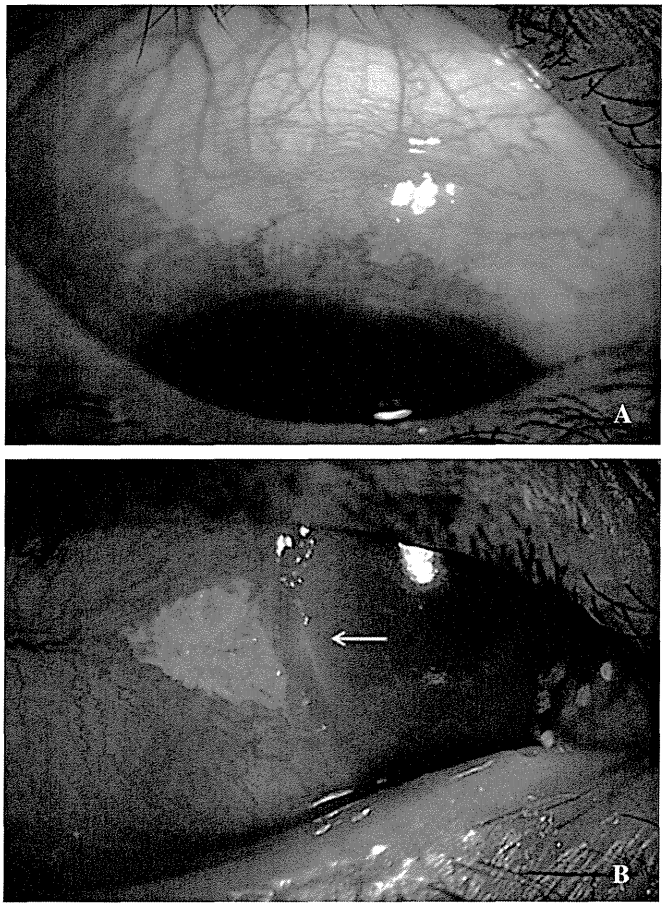

FIGURE 1. Slit-lamp photograph of the cornea and sclera in diffuse illumination showing nodular scleritis at the upper sclera (A) and peripheral corneal infiltration at the 3-o'clock and 9-o'clock positions (white arrow) (B).

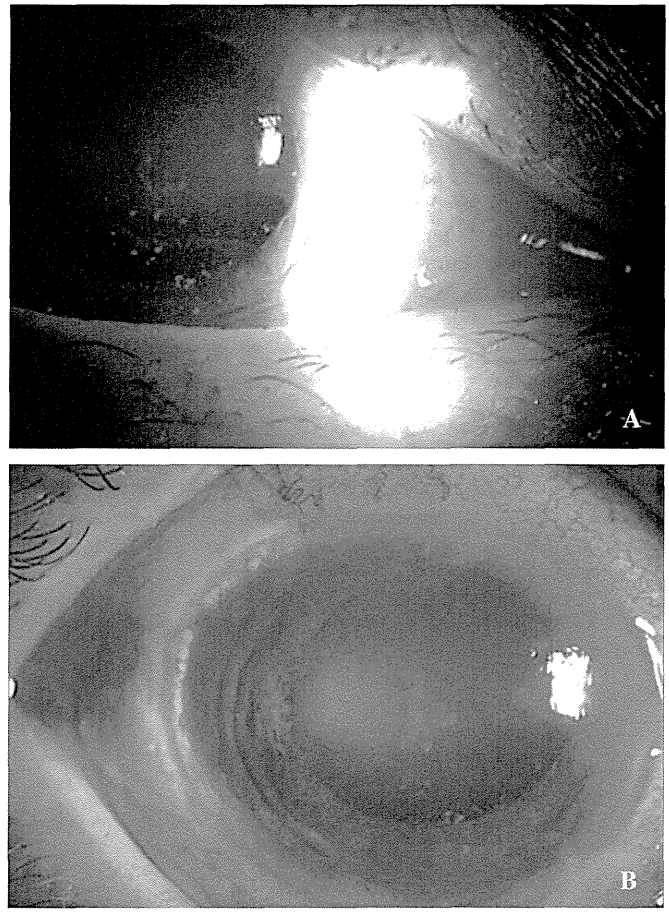

FIGURE 2. A, Slit-lamp photograph of the anterior chamber with scleral scattering showing anterior uveitis with mutton fat keratic precipitates with pigment. B, Slit-lamp photograph of the cornea showing the residual nodular scleritis and peripheral corneal infiltration.

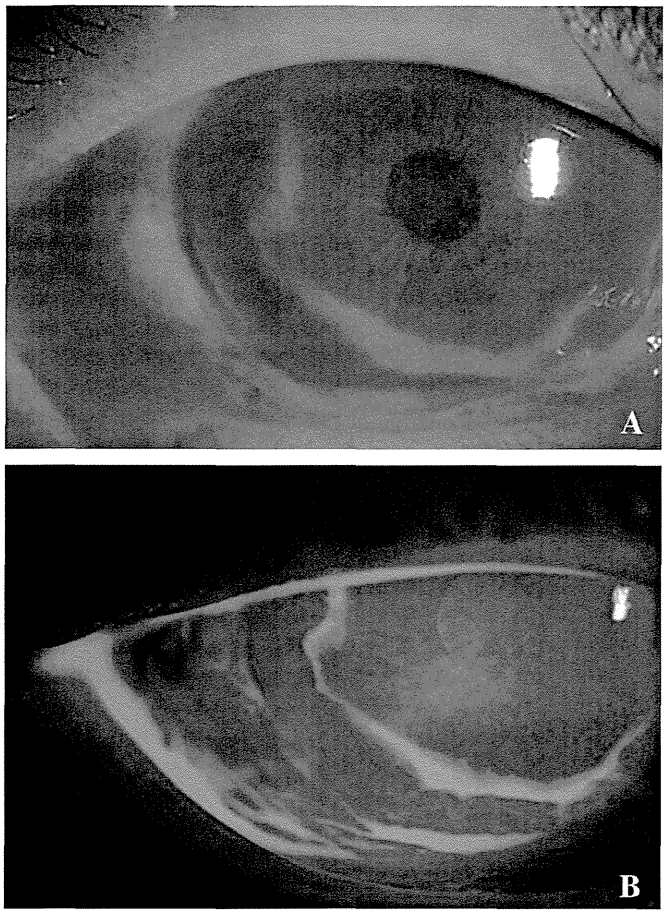

FIGURE 3. A, Slit-lamp photograph of the cornea showing continuous corneal epithelial defect, located from the 4-o'clock to 10-o'clock positions in the midperipheral cornea. B, Slit-lamp photograph of the cornea with fluorescein stain showing diffuse SPK and the corneal epithelial defect.

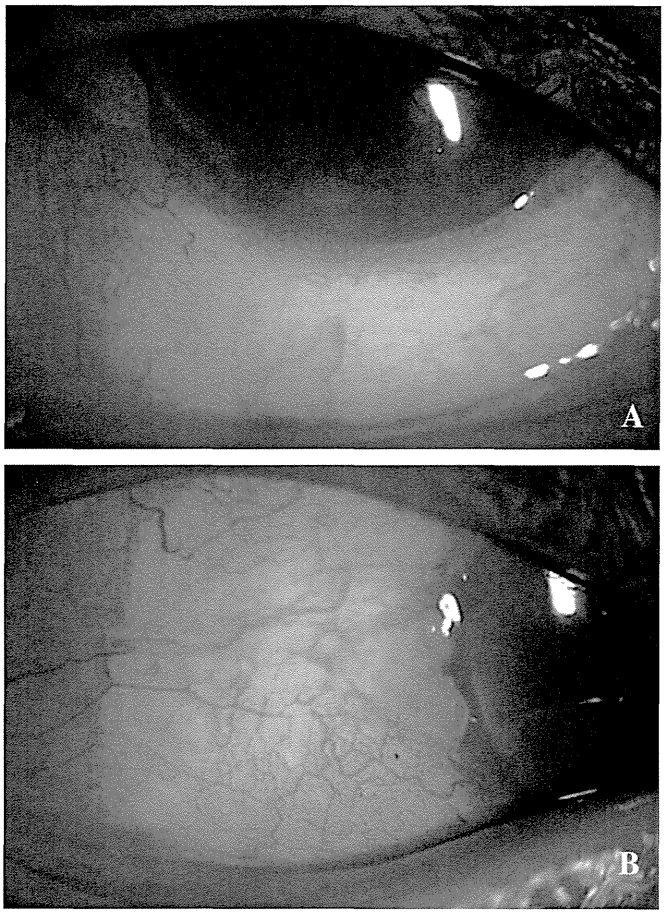

FIGURE 4. A, B, Slit-lamp photograph of the cornea and sclera in diffuse illumination showing the complete improvement of the scleritis and peripheral corneal infiltration after the administration of infliximab.

The standard treatment for RA-associated scleritis is the administration of topical betamethasone, but RA is often unresponsive to topical treatments. Previous reports have shown that cyclosporine is effective for treating severe scleritis; however, side effects that limit the effect of that treatment frequently occurred in elderly patients.[12–14] In the case presented in this study, we added cyclosporine and an oral steroid because of the disease's resistance to topical treatments. The activity of scleritis and peripheral corneal infiltration improved gradually, but the efficacy was limited.

The anti–TNF-α monoclonal antibody infliximab is widely used for the treatment of RA.[15] Previous reports suggested that infliximab is effective for the treatment of ocular inflammation associated with RA, especially refractory scleritis.[10,16] In this present case, infliximab was initiated after the administration of cyclosporine because ocular activity and systemic inflammation remained. Considering the fact that both the scleritis and the peripheral corneal infiltration were completely resolved without any side effects, infliximab may prove to be the optimal treatment option in refractory cases of RA-associated scleritis and corneal ulcer, especially in elderly patients.

In conclusion, RA can present with atypical continuous keratitis that is thought to be caused by an immunologic mechanism, as is shown in this case. The pathophysiology is complicated because of the modification of the disease by its long clinical course and the various drugs that are administered for treatment. In addition, although immunosuppressants are often used for the treatment of RA with severe scleritis, the efficacy of those drugs is limited and side effects can frequently occur. Infliximab could be considered a treatment choice in patients who are found to be resistant to topical or conventional treatments.

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

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ABSTRACT

Objectives: To confirm the downregulation of PTGER4 mRNA in the conjunctiva of Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and ocular cicatricial pemphigoid (OCP) patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study.

Setting and participants: We performed quantitative reverse transcription-PCR (RT-PCR) analysis of *PTGER4* mRNA in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the downregulation of *PTGER4* mRNA expression. We also analysed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, subacute SJS/TEN or chronic SJS/TEN, chronic OCP, severe graft versus host disease (GVHD) and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of *PTGER4* mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

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

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

ARTICLE SUMMARY

Article focus

- We previously reported that EP4 protein was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP). Article focus of this study are to confirm the downregulation of *PTGER4* mRNA, which protein is EP4, in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders in addition chronic SJS/TEN and OCP.

Key messages

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

Strengths and limitations of this study

- The function of EP4 in conjunctival epithelial cells is not elucidated.

INTRODUCTION

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are lipid mediators that form in response to various stimuli. They are released extracellularly immediately after their synthesis and they act by binding to a G protein-coupled rhodopsin-type receptor on the surface of target cells.[1] PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide-stimulated macrophages[2 3] and dendritic cells.[4] Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced proinflammatory genes in human conjunctival epithelial cells.[5]

There are four PGE receptor subtypes, EP1, EP2, EP3 and EP4. The intestinal epithelium has been reported to express EP4 mRNA,[6] and intestinal homeostasis was

maintained and the immune response downregulated by EP4.[7] The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused on the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.

We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP).[8] Here we examined the mRNA expression of *PTGER4*, which is the gene of EP4 protein, in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that *PTGER4* mRNA EP4 is down-regulated in their conjunctiva. We also examined the expression of *PTGER4* mRNA protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD) and of patients in the subacute stage of SJS/TEN.

MATERIALS AND METHODS

Human conjunctival tissues

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

For quantitative reverse transcription-PCR (RT-PCR) the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in four patients in the chronic stage of SJS/TEN and four patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in three patients with chemical (alkali) eye burn (two in the chronic stage and one in the subacute stage), two patients with subacute SJS/TEN, one patient with severe GVHD and from four patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute stage but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections using the RNeasy mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The RT reaction was with the SuperScript preamplification kit (Invitrogen, Carlsbad, California, USA). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, California, USA). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For cDNA amplification we performed PCR in a 25 µl total volume that contained a 1 µl cDNA template in 2×TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The results were analysed with sequence detection software (Applied Biosystems). The quantification data were normalised to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan, USA). The secondary antibody (Biotin-SP-conjugated AffiniPure $F(ab')_2$ fragment donkey antirabbit IgG (H+L), 1 : 500 dilution; Jackson Immuno Research, Baltimore, Maryland, USA) was applied for 30 min. The VECTASTAIN ABC reagent (Vector Laboratories, Inc, Burlingame, California, USA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Data analysis

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

RESULTS

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

Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders. EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (figure 2A) and in conjunctival tissues from three patients with chemical eye burn (figure 2B). Its expression varied in conjunctival epithelium from four patients with Mooren's ulcer (figure 2C): in one patient is was similar to the control,

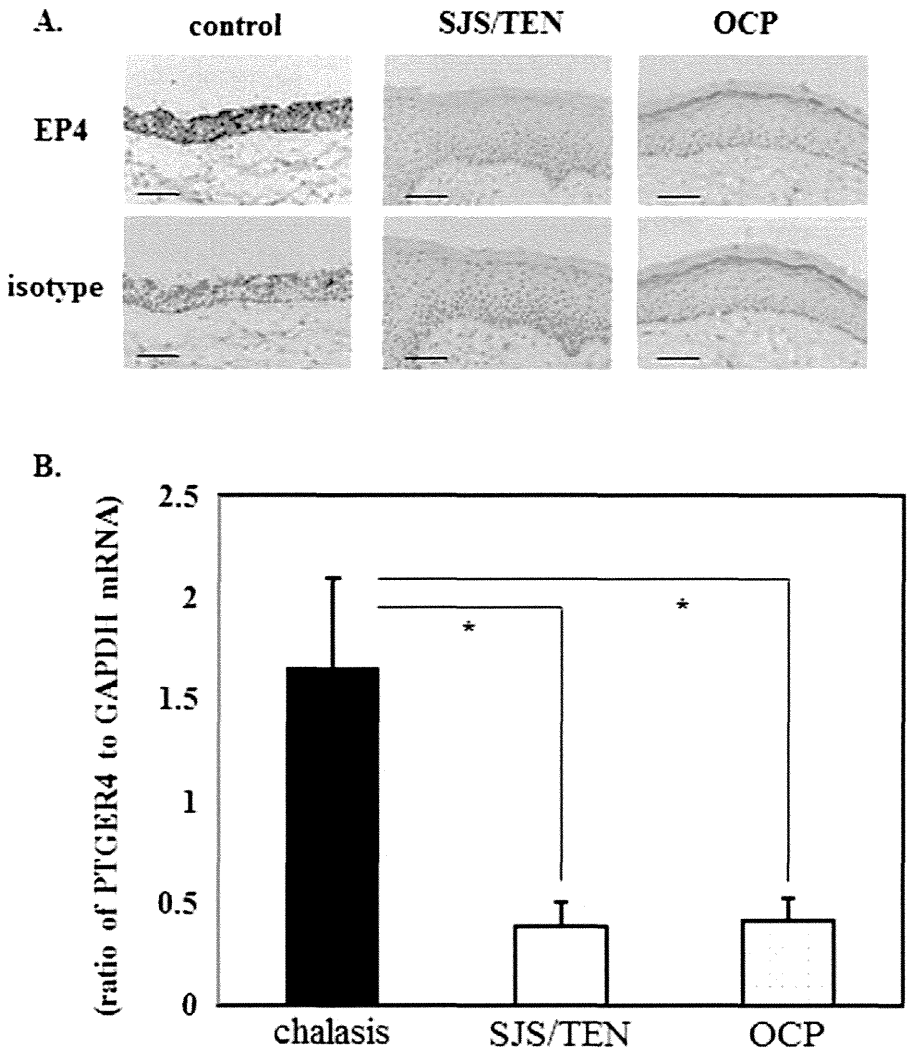

Figure 1 The expression of *PTGER4* mRNA in conjunctival tissues from patients with Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN), ocular cicatricial pemphigoid (OCP) and the controls. (A) Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP). (B) Expression of *PTGER4* mRNA in human conjunctival tissues (*p<0.05).

in two it was slightly lower than in the control and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from two patients with subacute SJS/TEN (figure 2D), a patient with severe GVHD (figure 2E) as same as patients with chronic SJS/TEN or OCP.[8]

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

DISCUSSION

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

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

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

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

Kabashima *et al*[7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced

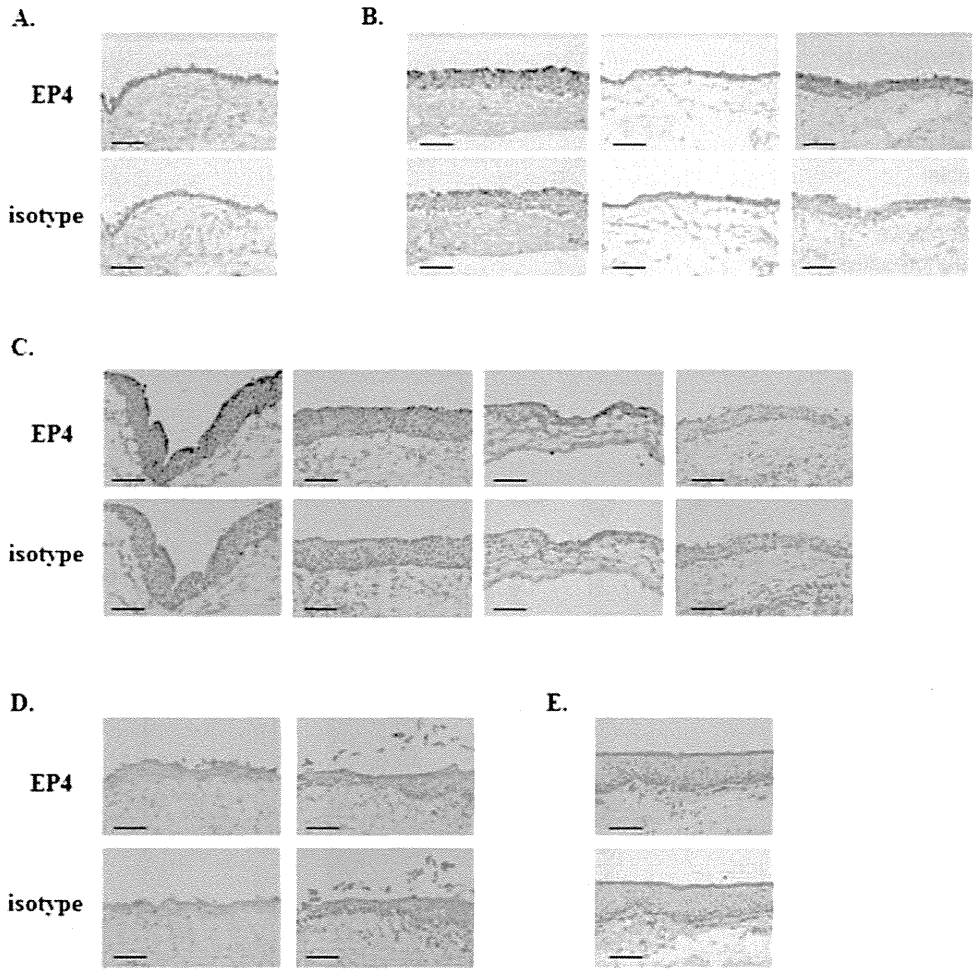

Figure 2 Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases. (A) Nearly normal conjunctival tissues from patients with conjunctivochalasis. (B) Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction. (C) Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva. (D) Conjunctival tissues from Stevens-Johnson syndrome/toxic epidermal necrolysis patients in the subacute stage. (E) Conjunctival tissues from a patient with severe graft versus host disease. Each scale bar represents 100 μm.

the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao *et al*[9] found that PGE_2 acting on its receptor EP4 on T cells and dendritic cells not only facilitated T helper 1 (T_H1) cell differentiation but also amplified interleukin-23-mediated T_H17-cell expansion in vitro. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signalling promotes immune inflammation.

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

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

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

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Contributors All the authors substantially contributed to the conception and design, acquisition of data, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be published.

Competing interests None.

Ethics approval Ethics—Human Subjects.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement There are no additional data available.

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

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Immunohistochemical analysis of inflammatory limbal conjunctiva adjacent to Mooren's ulcer

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ABSTRACT

Background/aims To examine the characteristics of infiltrating cells in conjunctival tissues adjacent to the peripheral corneal ulcers of Mooren's ulcer.

Methods This study involved four eyes of four patients with Mooren's ulcer and who were considered to be in need of surgical treatment. The patients' resected conjunctival tissues were embedded and frozen. The tissue sections were then subjected to H&E and immunohistochemical staining. The stained sections were observed and the characteristics of the infiltrating cells in the conjunctival tissues were pathologically examined.

Results In all patients, infiltration of inflammatory cells was observed in the submucosal connective tissue of the conjunctiva. Immunohistochemical analysis revealed inflammatory cell infiltration into the submucosal layer of the conjunctiva that was mainly composed of CD3-positive and CD45RO-positive cells. Some of these cells also showed positive reactivity with CD4, yet very few cells showed positive reactivity with CD8. In addition, infiltration of the cells indicating CD68 positivity was frequent in a few cases.

Conclusions In the four Mooren's ulcer cases, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were found to be mainly composed of helper T lymphocytes and macrophages. Our results show that helper T cells and macrophages contribute to the pathogenesis of Mooren's ulcer.

INTRODUCTION

Mooren's ulcer (rodent corneal ulcer) is a rare disorder first described by Mooren in 1867[1] involving chronic and painful ulceration of the cornea.[2] It occurs in the absence of any systemic disorder such as collagen diseases. The ulcerative lesion with overhanging edges typically starts on the periphery of the cornea and tends to spread progressively to the entire circumference or towards the centre of the cornea.[2–5] In such cases, severe inflammation sometimes occurs on the ocular surface, progresses rapidly and may cause corneal perforation. Since Mooren's ulcer is a rare disorder, the aetiology or mechanisms of pathogenesis remain uncertain. Topical administration of corticosteroids[6] and systemic[7 8] or topical[6 9] administration of immunosuppressive agents such as cyclosporin A are commonly used as a conservative treatment for the disorder. However, for cases that are resistant to such medical treatments, various surgical treatments such as peritectomy or keratoepithelioplasty[10] are indicated.

A few studies have reported that autoimmunity[11 12] is involved in Mooren's ulcer. Histopathologically, inflammatory cell infiltration is observed in the cornea and conjunctiva adjacent to Mooren's ulcer.[2 13] Moreover, steroid and/or immunosuppressive therapies have been shown to be effective. Therefore, it is considered that the primary pathogenesis of Mooren's ulcer is an immunological reaction. However, few reports have focused on examining the pathological findings of this disease in detail.

To elucidate the pathology of Mooren's ulcer, in this study we examined the characteristics of the infiltrating cells in the conjunctival tissues adjacent to the peripheral corneal ulcers using an immunohistochemical technique.

MATERIALS AND METHODS

Patients

This study involved four eyes of four patients (two men and two women; age range: 56–82 years) who were diagnosed with Mooren's ulcer at the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan. These patients were considered to be in need of surgical treatment because they were resistant to the systemic administration of betamethasone and cyclosporin A, and the topical administration of betamethasone. Macroscopic images of the ocular surface in these four patients are shown in figure 1.

Limbal conjunctival tissues adjacent to the ulcerative lesions resected during each patient's surgery were used for this study. As a control, the conjunctival tissues resected at the time of surgery for one woman (79 years of age) with conjunctivochalasis (CCh) were also used.[14]

This research was approved by the Committee for Ethical Issues on Human Research, Kyoto Prefectural University of Medicine and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients after they had received a detailed explanation of the procedures.

Histological and immunohistochemical analysis

Preparation of the sections and H&E staining

The resected conjunctival tissues were embedded in Tissue Tek OCT compound (Sakura Finetek Japan Co., Tokyo, Japan) and snap frozen in liquid nitrogen. Next, serial sections approximately 5 μm in thickness were cut using a cryostat (CM3050S; Leica Biosystems Nussloch GmbH., Nussloch, Germany). The sections were then placed on aminosilane-coated glass slides (MAS slide glass; Matsunami Glass Ind., Osaka, Japan), air dried, fixed with Zamboni's fixative (phosphate buffer containing 2% paraformaldehyde and 0.19% picric acid) for 1 min, and subjected to standard H&E staining.

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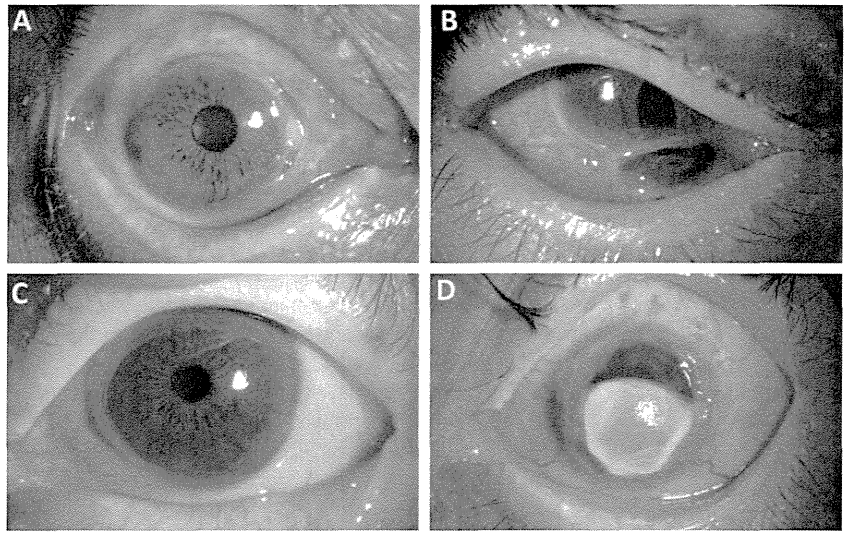

Figure 1 Macroscopic images of the patients' ocular surface. (A) Case 1, the right eye of a 60-year-old man. (B) Case 2, the right eye of a 56-year-old man. (C) Case 3, the left eye of an 82-year-old woman. (D) Case 4, the left eye of a 64-year-old woman.

Immunohistochemical staining

The prepared tissue sections were subjected to an indirect immunohistochemical staining. In brief, the sections were fixed with Zamboni's fixative for 10 min at 4°C. They were then washed in 0.01 mol/litre phosphate buffered saline (PBS), preincubated with PBS containing 2% bovine serum albumin (Nacalai Tesque Inc., Kyoto, Japan) at room temperature (RT) to eliminate any non-specific reaction, and continuously diluted primary antibody solutions (table 1) were applied to the sections for approximately 16 h at 4°C. To confirm specificity of the immunohistochemical staining, diluted solutions of normal mouse IgG_1 or mouse IgG_{2a} (Dako Japan, Tokyo, Japan) were applied instead of the primary antibody solutions. The sections were then washed with PBS and immersed in methanol containing 0.3% H_2O_2 for 30 min at RT to eliminate endogenous peroxidase activity. The sections were washed again with PBS and peroxidase conjugated secondary antibody (Histofine Simple Stain MAX-PO MULTI; Nichirei BioSciences Inc., Tokyo, Japan) was applied for 45 min at RT. The sections were then washed with PBS and purified water, and incubated with 3, 3′-diaminobenzidin solution (Peroxidase Substrate Kit DAB; Vector Laboratories, Inc., Burlingame, California, USA) for 1–2 min to visualise the immunoreaction. After counterstaining was performed with haematoxylin, the sections were dehydrated and mounted.

Analysis

The H&E and immunohistochemical stained sections were observed by light microscopy (AX-70; Olympus Corporation, Tokyo, Japan) and pathologically examined. Images of the sections were obtained using a CCD camera (DP50; Olympus).

RESULTS

H&E staining

In all four cases of Mooren's ulcer in this study, infiltration of small to slightly large-sized round-shaped cells was observed in the submucosal connective tissue of the conjunctiva (figure 2A–D). In case 1 (figure 2A) and case 2 (figure 2B), round-shaped cells and a number of slightly large-sized cells with many vacuoles were found to have been infiltrated. In case 3 (figure 2C), severe fibrosis and necrotic change was observed in the submucosa of the lesion site of the conjunctiva. In the tissue obtained from the female patient with CCh (figure 2E), no remarkable changes were observed in the mucosa or submucosa of the conjunctiva.

Immunohistchemical staining

The results of the immunohistochemical staining are shown in table 2. In all four cases, inflammatory cell infiltration mainly composed of CD3-positive cells (figure 3A,E,I,M) and

Table 1 List of primary antibodies

Antibody	Maker	Immunised animal/clonality	Subtype of immunoglobulin	Clone name	Cat. No.	Specificity
CD3	Dako Japan	Mouse Monoclonal	IgG1-k	T3-4B5	M0756	Pan T cell (mature)
CD4	Dako Japan	Mouse Monoclonal	IgG1-k	MT310	M0716	Helper/inducer T cell, monocyte
CD8	Dako Japan	Mouse Monoclonal	IgG1-k	C8/144B	M7103	Suppressor/cytotoxic T cell
CD20cy	Dako Japan	Mouse Monoclonal	IgG2a-k	L26	M0755	Pan B cell (except plasma cell)
CD45RO	Dako Japan	Mouse Monoclonal	IgG2a-k	UCHL1	M0742	Pan T cell
Mast cell tryptase	Dako Japan	Mouse Monoclonal	IgG1-k	AA1	M7052	Mast cell
Neutrophil elastase	Dako Japan	Mouse Monoclonal	IgG1-k	NP57	M0752	Neutrophil, monocyte
CD68	Dako Japan	Mouse Monoclonal	IgG1-k	KP1	M0814	Macrophage, histiocyte

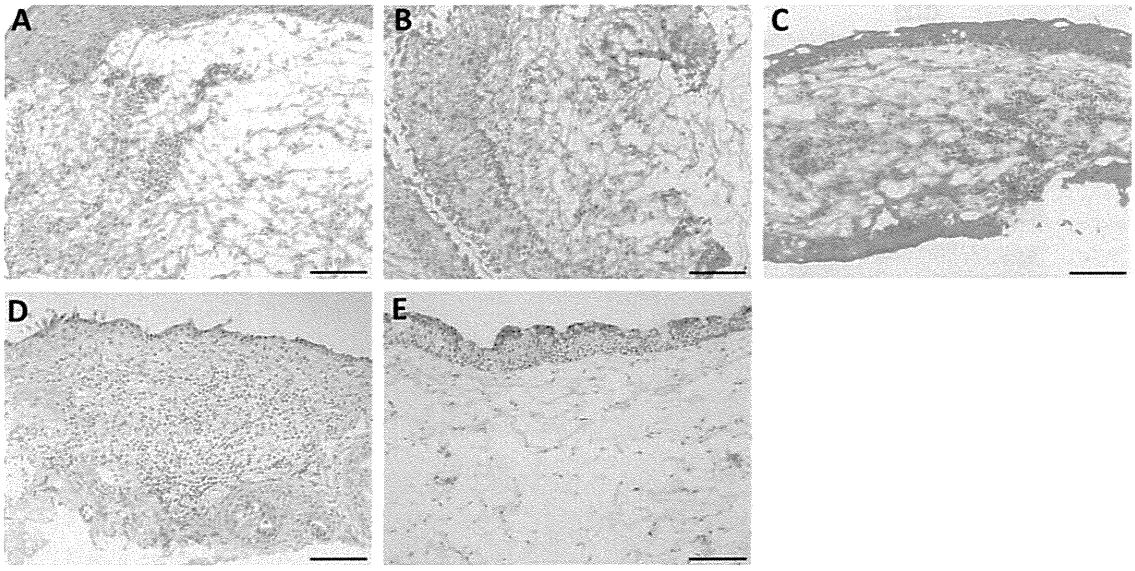

Figure 2 H&E staining images. (A) Case 1, (B) case 2, (C) case 3, (D) case 4 and (E) control with conjunctivochalasis (CCh). Some case-specific differences of grade can be seen, and small to slight large-sized round-shaped cells infiltrate into the submucosal connective tissue of the conjunctiva in all cases. (A) In case 1, infiltration of slight large-sized cells which have many vacuoles can be observed. (E) In CCh, no remarkable changes can be seen. Magnification ×200. Scale bar 100 μm.

CD45RO-positive cells (figures not shown) was observed in the submucosal layer of the conjunctiva. These cells were characterised as T lymphocytes due to the pattern of their immunoreactivity. Some of these cells also showed positive reactivity with CD4 (figure 3B,F,J,N), although very few cells showed positive reactivity with CD8 (figure 3C,G,K,O). In addition, T lymphocytes and the infiltration of cells indicating CD3 negativity and CD4 positivity were frequent in case 1 (figure 3A,B). These cells were thought to be macrophages because, as with the large cells, they showed vacuolisation of their cytoplasm on H&E staining and positive reactivity with CD68. In addition, during immunohistochemical analysis, a large number of macrophages showing positive reactivity with CD68 were observed in case 1 (figure 3D) and also in case 2 (figure 3H), case 3 (figure 3L) and case 4 (figure 3P). In contrast, a small number of CD20cy-positive, mast cell tryptase-positive or neutrophil elastase-positive cells were observed in all four cases, and there were no specific localisation patterns (figures not shown).

In the CCh specimen, a small number of cells showed positive reactivity to antibodies (figure 3Q–T). In the negative control, sections stained using normal mouse IgG_1 or IgG_{2a} showed no positive reactivity (figure 3U,V).

In summary, in the four cases of Mooren's ulcer in this study, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were mainly composed of CD3-positive, CD45RO-positive and CD4-positive helper T lymphocytes and CD68-positive macrophages, whereas the infiltration of B lymphocytes, neutrophils and mast cells was minimal.

Table 2 Results of immunohistochemistry

Antibody/cases	1	2	3	4	CCh
CD3	+*	+	++	++	+/–
CD4	++	+	+/–	+	+/–
CD8	+/–	+/–	+/–	+/–	+/–
CD20cy	+/–	+/–	+/–	+/–	+/–
CD45RO	++	++	+	++	+/–
Mast cell tryptase	+/–	+/–	–	+/–	+/–
Neutrophil elastase	+/–	+	+/–	+/–	+/–
CD68	++	+	+	+	+/–

*Infiltration of inflammatory cells was scored as follows: –, no positive cells are observed; +/–, a small number of positive cells are observed; +, a large number of positive cells are observed; ++, any of numerous positive cells are observed, and/or aggregations of numerous positive cells are observed.
CCh, conjunctivochalasis.

DISCUSSION

In the four patients with Mooren's ulcer in this study, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were found to be mainly composed of CD3-positive, CD45RO-positive and CD4-positive helper T lymphocytes and CD68-positive macrophages. In addition, the CD4-positive ratio of infiltrating T lymphocytes was clearly higher than the CD8-positive ratio. However, a small number of CD20cy-positive, mast cell tryptase-positive and neutrophil elastase-positive cells were observed in the submucosa of the conjunctival tissues but there were no characteristic patterns.

Wang *et al*[15] previously reported that in the adjacent bulbar conjunctiva of Mooren's ulcer the CD4/CD8 ratio is significantly higher than in normal controls, which is consistent with our results.

It is known that immunosuppressive reagents such as cyclosporin A are effective for the treatment of Mooren's ulcer.[6–9] The primary effective treatment mechanism of cyclosporin A is the inhibition of the activation of helper T cells by suppressing the production of inflammatory cytokines such as interleukin-2 by binding to calcineurin.[16 17] In the inflammatory lesion of Mooren's ulcer, it is thought that cyclosporin A inhibits the function of helper T lymphocytes and stimulates suppressor/cytotoxic T lymphocytes.[7–9] Therefore, the helper T lymphocytes are more likely to participate in Mooren's ulcer.

In this study, infiltration of macrophages was also observed in the conjunctival submucosa. Since infiltration of T lymphocytes

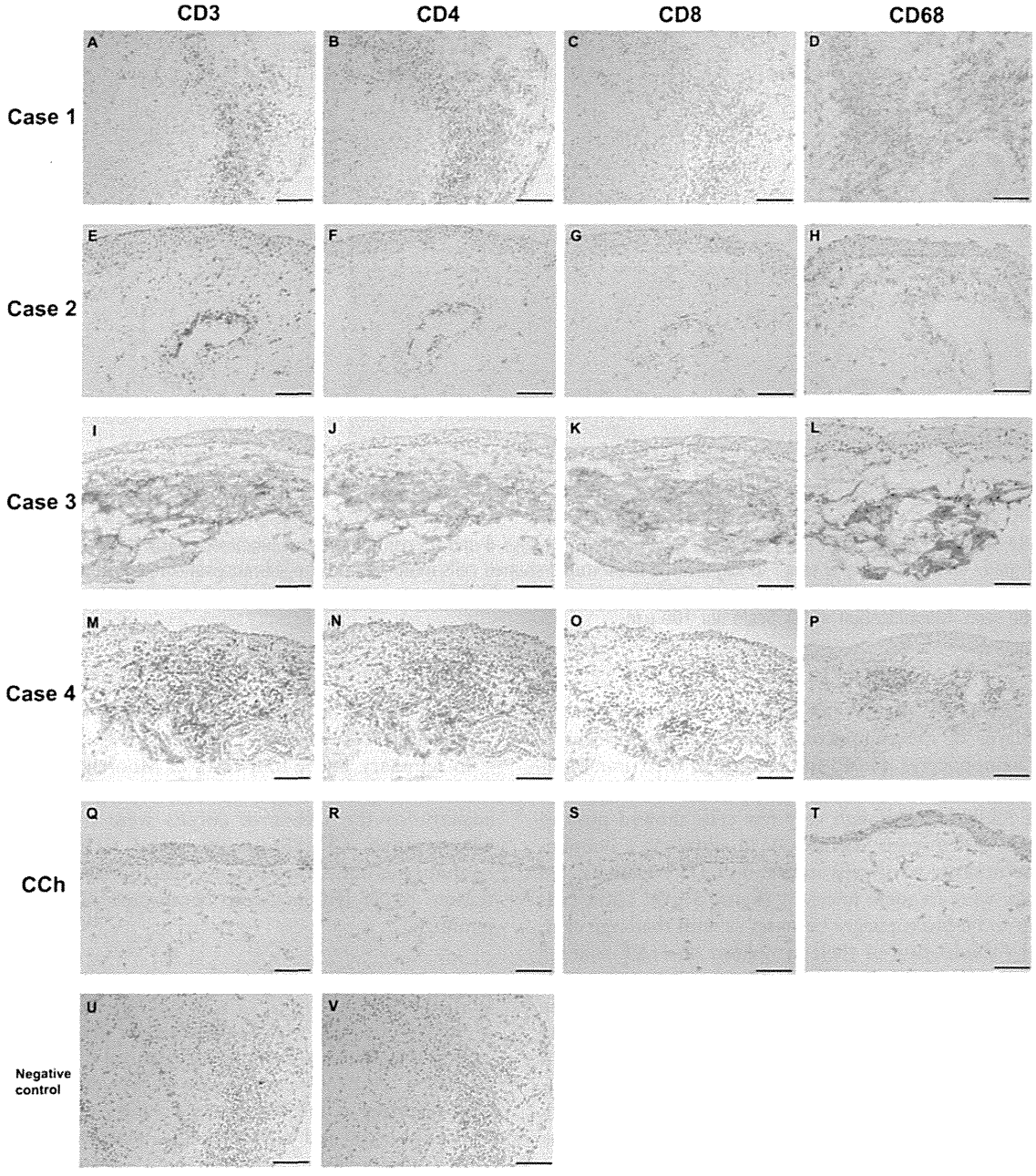

Figure 3 Immunohistochemical staining images (CD3, CD4, CD8 and CD68). (A–D and U, V) Case 1, (E–H) case 2, (I–L) case 3, (M–P) case 4, (Q–T) control with conjunctivochalasis (CCh). (A, E, I, M) Submucosal infiltrating cells show positive reactivity with CD3 in all cases. (B, F, J, N) Some CD3-positive cells also show positive reactivity with CD4. (C, G, K, O) A small number of cells show positive reactivity with CD8. (D, H, L, P) Many submucosal infiltrating cells show positive reactivity with CD68, indicating that they are macrophages. (D) In case 1, a large number of CD68-positive cells form a granulomatous lesion in the submucosa. (Q, R, S, T) In the control with CCh, only a small number of cells show positive reactivity with CD3, CD4, CD8 and CD68. (U,V) Negative controls, no positive reactivity is observed. Magnification ×200. Scale bar 100 μm.

and macrophages was observed in the Mooren's ulcer lesion site, it seems that some abnormalities of the immune system are involved in the pathogenesis of the disorder. Previous reports have shown that an autoantibody against cornea-associated antigen was significantly increased in the serum of patients with Mooren's ulcer.[12]

As for the four cases involved in this study, systemic or topical treatments were applied consecutively. The histological or immunohistochemical findings may undergo various modifications with treatment. At a minimum, it can be posited that the infiltration of helper T lymphocytes and macrophages might be related to the pathogenesis of Mooren's ulcer.

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Contributors MU designed this study. KS and MU organised the whole study and wrote the manuscript. KS organised pathological examinations. CS, TI and NY collected the specimens of patients. SK supervised this study. Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: MU, KS, CS, TI, NY, NK, SK. Drafting the article or revising it critically for important intellectual content: MU, KS, CS, TI, NY, NK, SK. Final approval of the version to be published: MU, KS, CS, TI, NY, NK, SK.

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Competing interests None.

Patient consent Obtained.

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Immunohistochemical analysis of inflammatory limbal conjunctiva adjacent to Mooren's ulcer

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特集 第66回日本臨床眼科学会講演集（5）原著（展示 1-269） 臨眼 67(7)：000-000，2013

タクロリムスが奏効した難治性 Mooren 潰瘍の 1 例

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要約 **目的**：タクロリムスが奏効した難治性 Mooren 潰瘍の症例報告。**症例**：65 歳男性。左眼痛で近医を受診し，周辺部角膜潰瘍と診断され，4 か月後に紹介受診した。**所見**：結膜充血，輪部浮腫，周辺部下掘れ潰瘍を認めた。結膜切除術と角膜上皮形成術を施行し，術後，ベタメタゾン点眼，シクロスポリン点眼，プレドニゾロン内服を行うも病勢は抑えきれず，自家作製タクロリムス眼軟膏を追加し，増悪傾向が抑えられた。タクロリムス眼軟膏のコンプライアンスが悪く，角膜菲薄化，浸潤が増強したため，初回手術から 2 年後，結膜切除術と角膜上皮形成術を施行した。以後，タクロリムス点眼の併用で鎮静化している。**結論**：難治性の Mooren 潰瘍には，手術と薬剤治療の併用が必要で，タクロリムスはその選択肢の 1 つとなる。

Tacrolimus ointment was effective in a protracted case of Mooren's ulcer

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Abstract. **Purpose**：To report a protracted case of Mooren's ulcer treated by topical tacrolimus. **Case**：A 65-year-old male was referred to us for corneal ulcer since 4 months before. He had suffered from alkhali burn in the right eye 8 years before. **Finding**：Corrected visual acuity was no light perception right and 1.2 left. The left eye showed conjunctival injection, perilimbal edema, and peripheral corneal ulcer with undermining. The left eye was treated by conjunctival resection and keratoepithelioplasty followed by instillation of betamethasone and cyclosporine. He started receiving tacrolimus ointment 2 months later. Pain in the left eye persisted with corneal thinning and progression of corneal ulcer involving three quadrants. The left eye was treated again by conjunctival resection and keratoepithelioplasty with instillation of tacrolimus ophthalmic solution 2 years later. The corneal lesion subsided another 18 months later. **Conclusion**：Repeated surgery with instillation of tacrolimus was effective for protracted Mooren's ulcer in the present case.

Rinsho Ganka (Jpn J Clin Ophthalmol) 67(7)：000-000, 2013

緒言

Mooren 潰瘍の発症には，角膜上皮・実質に対する自己抗体が関与していると考えられている[1,2]。上皮基底膜に免疫グロブリンの沈着がみられるとの報告もあり，抗原抗体反応による角膜組織障害の病態と考えられている。治療はステロイドの投与が主体であり，シクロスポリンを併用する場合もある。しかし，内科的治療に抵抗することも多く，角膜潰瘍近傍の結膜を切除する Brown 手術や角膜上皮形成術を行うなど，治療は複合的に行われる[3]。今回筆者らは，ステロイド点眼・内服，シクロスポリン点眼，手術では病勢を抑えられず，タクロリムス局所投与が奏効した症例を経験したので報告する。

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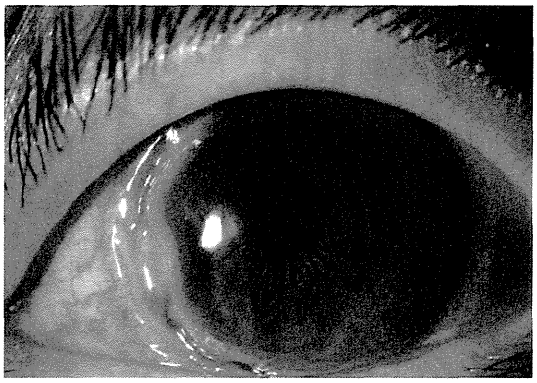

図 1　初診時の左眼前眼部写真
輪部浮腫，毛様充血，周辺部下掘れ潰瘍・浸潤を認める。

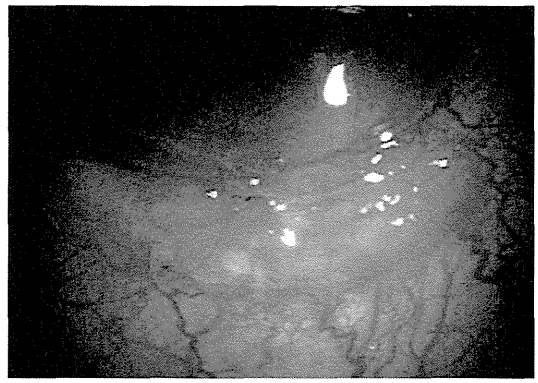

図 2　初回手術 1 か月後の左眼前眼部写真
移植した lenticule が融解し，角膜輪部の菲薄化を認める。

症 例

患者：65 歳，男性

既往歴：高血圧症

現病歴：右眼は 2000 年ごろ，アルカリ外傷で失明したとのことだが詳細不明。その 7 年後に周辺部角膜潰瘍と診断され，近医にて結膜被覆術を施行した。2008 年 4 月上旬，左眼眼痛にて近医受診し，鼻側輪部に角膜潰瘍を認め，ステロイド結膜下注射などでいったん軽快したが，その後再発し，近医受診の 4 か月後に鳥取大学眼科へ紹介受診となった。

初診時所見：視力は右眼光覚なし，左眼は 1.0 (矯正 1.2) で，眼圧は右眼 8 mmHg，左眼 10 mmHg であった。スリット所見で，左眼は輪部浮腫，毛様充血と，5 時から 11 時にかけて周辺部下掘れ潰瘍・浸潤を認め，特に 9 時の位置で強い菲薄化を認めた（**図 1**）。Mooren 潰瘍と診断し，初期治療はレボフロキサシン点眼（左眼 1 日 4 回），ベタメタゾン点眼（左眼 1 日 4 回）で開始した。しかし潰瘍はかなり進行しており，右眼は失明していることから手術を考慮する必要があると判断した。2008 年 8 月下旬，結膜切除術と角膜上皮形成術を施行した。術後もベタメタゾン点眼を使用していたが，角膜菲薄化の進行が抑えられず，術後 2 週間目にシクロスポリン点眼（左眼 1 日 4 回）を追加した。

術後 1 か月目より，移植した lenticule が融解し，角膜輪部の菲薄化が認められた（**図 2**）ため，プレドニゾロン内服（20 mg/日）を追加した。しかし，その後も菲薄化と lenticule の融解が進行し，術後 2 か月目に自家作製タクロリムス眼軟膏（左眼 1 日 3 回）を追加した。その後，増悪傾向が抑えられ，ステロイド内服の漸減終了が可能となった。この時点で左眼視力は 1.5 に維持されていた（**図 3**）。

タクロリムス眼軟膏使用後に眼灼熱感と霧視の訴えがあり，また軟膏であるため使用感が悪く，2009 年以降コンプライアンス不良となり，タクロリムス眼軟膏の継続が難しい状況となった。そのため加療を弱めると浸潤が悪化する状況が続き，左眼視力は 1.2 を維持しているものの，潰瘍は 4 分の 3 周を越える状態となった。

2010 年以降もコンプライアンスは不良で，タクロリムス眼軟膏を不定期に使用する状況が続き，浸潤の出現を繰り返すうちに菲薄化した部分は拡大していった（**図 4**）。手術後 2 年が経過したころ，左眼鼻下側の菲薄化が悪化し，侵入した結膜が角膜を蚕食する状態となり，左眼視力は 0.5 に低下した。

2010 年 12 月，再度結膜切除術と角膜上皮形成術を施行した。術後はステロイド点眼・内服，シクロスポリン点眼を行い，強く消炎を図った。術後は lenticule の浮腫・混濁などはなかったが，小浸潤の出没を認め，血管の勢いを抑制できないため，さらに強く消炎しなければステロイドの減量は難しいと考え（**図 5**），タクロリムス点眼（左眼 1 日 2 回）を追加した。タクロリムス点眼も点眼後の灼熱感の訴えがあったが，タクロリムス眼軟膏よりも使用感が改善したことでコンプライア

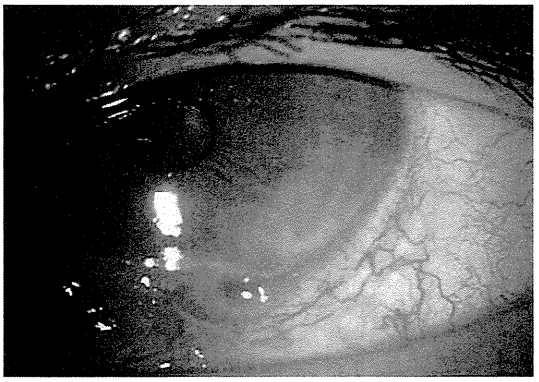

図 3　初回手術 2 か月後の左眼前眼部写真
自家作製タクロリムス眼軟膏を追加し，所見の増悪が抑えられた。

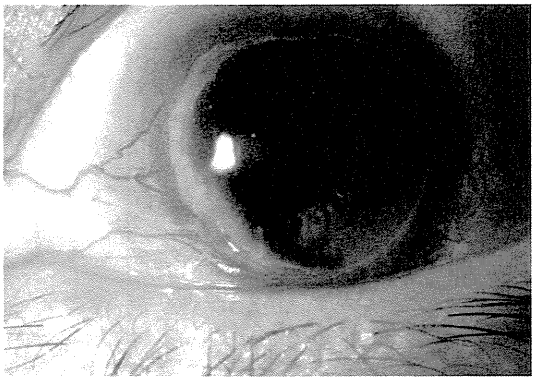

図 4　初回手術 2 年後の左眼前眼部写真
浸潤の出現を繰り返すうちに菲薄化した部分が拡大している。

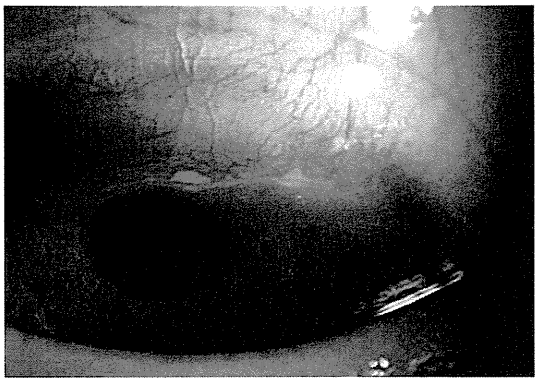

図 5　2 回目手術の 3 週間後の左眼前眼部写真
小さな浸潤を認め，血管の勢いは抑制できていない。

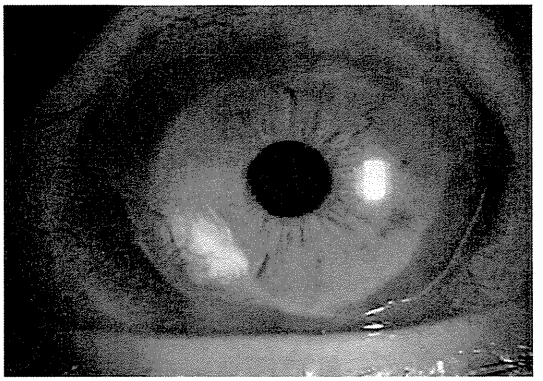

図 6　2 回目手術の 1 年 6 か月後の左眼前眼部写真
タクロリムス点眼が追加され，鼻下側に lipid keratopathy を認めるが，浸潤は認めない。

ンスも改善した。これでようやく鎮静化し，プレドニゾロン内服・シクロスポリン点眼の漸減終了が可能となった。

2 回目の手術後 1 年 6 か月が経過した時点で，鼻下側に lipid keratopathy を認めるものの（図 6），炎症所見・角膜浸潤はなく落ち着いており，タクロリムス点眼を中止した。この時点での加療はレボフロキサシン点眼（左眼 1 日 2 回），ベタメタゾン点眼（左眼 1 日 2 回）で，その後も炎症所見の増悪はなく経過している。

考按

タクロリムスは免疫抑制薬の一種で，臓器移植または骨髄移植を行った患者の拒絶反応を抑制する薬剤として認可された[4]。そして近年，タクロリムス軟膏がアトピー性皮膚炎の治療薬として用いられており[5]，本症例で用いたタクロリムス眼軟膏は，アトピー性皮膚炎用外用剤として 1999 年に発売されたプロトピック® 軟膏をタリビット® 眼軟膏で 5 倍に希釈して自家作製したものであり，鳥取大学倫理委員会の承認を得て使用した。

タクロリムスの作用機序は，T 細胞抗原受容体からの刺激伝達経路中のカルシニューリンの脱リン酸化を阻害することで，サイトカイン産生の抑制，細胞障害性 T 細胞の誘導の阻害，肥満細胞の脱顆粒の阻害などをもたらす[6]。免疫抑制薬のシクロスポリンとタクロリムスを比較すると，2 つの作用機序は同じで，免疫抑制効果は，タクロリムスがシクロスポリンの 10〜100 倍あるとされている[7,8]。

タクロリムス眼軟膏による眼表面疾患治療に関

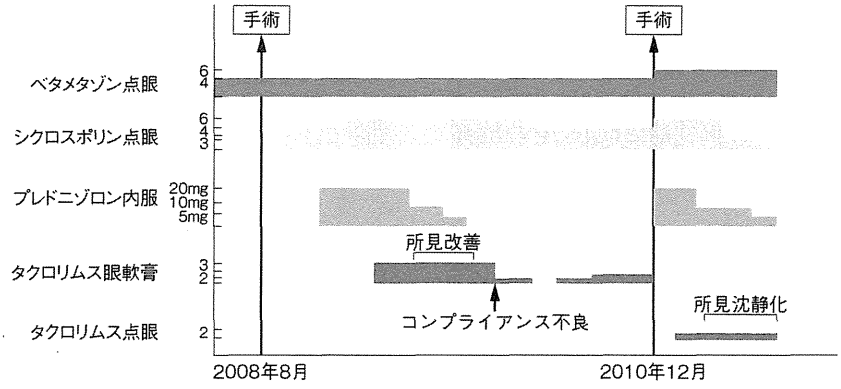

図 7　使用した薬剤の経過

して，共著者の Miyazaki ら[9]が，ステロイドレスポンダーあるいは副腎皮質ステロイド薬治療に対して抵抗性の難治性眼表面疾患症例 10 例を対象に，タクロリムス眼軟膏の治療効果を検討した報告がある。タクロリムス眼軟膏使用の全症例において臨床所見の改善を認め，ステロイドレスポンダーにおいてはステロイド減量が可能であり，眼圧の下降が得られた。このようにタクロリムス眼軟膏は，高用量のステロイド加療あるいは外科的治療が考慮される症例における代替療法となりうると考えられる。

今回使用した薬剤の経過を**図 7** にまとめた。1 回目の手術後，ベタメタゾン点眼とシクロスポリン点眼に加え，ステロイド内服を追加しても病状は悪化したが，タクロリムス眼軟膏の使用により所見の改善が得られ，ステロイドの内服を漸減終了することができた。しかしその後，タクロリムス眼軟膏の使用感の悪さからコンプライアンスが不良となり，病状悪化のため 2 回目の手術を行った。

術直後よりステロイド内服も併用して強く消炎し病状は落ち着いたが，タクロリムス眼軟膏よりも使用感のよいタクロリムス点眼が使用可能となり，これを追加し，コンプライアンスの改善により継続的に使用できるようになったことからより強い消炎ができたため，ステロイド内服の漸減終了が可能となった。

タクロリムス軟膏の副作用として，投与開始直後の灼熱感が挙げられる[10]。本症例でも眼灼熱感の訴えがみられた。しかしこの症状は一時的であることが多く[9]，連用し炎症が改善するにつれ，灼熱感は減少する傾向にある[11]。春季カタルの治療にタクロリムス軟膏を眼軟膏として使用した例でも，1〜2 週間以内にこの症状は消失したとの報告もある[12]。本症例でもこの副作用の訴えはあったが，それに加え，本患者はもともと点眼指示を守ることが困難な性格であり，眼軟膏の使用感の悪さが影響してコンプライアンスの改善が得られなかった。

タクロリムス点眼が春季カタルの治療薬として開発され，副作用に目の灼熱感や異物感が認められるが，多くはこれも一過性である。タクロリムス軟膏を眼軟膏として使用するよりも，使用方法が簡便になり，何よりも眼軟膏使用後の霧視が改善されることにより，本患者のコンプライアンスがある程度改善され，所見の改善が得られた。

角膜上皮形成術で Mooren 潰瘍は治癒させることができると報告されているが[13]，本症例ではその後も病勢を止めることができず，lenticule の融解を招いた。そのような重症例でもタクロリムスは効果があった。春季カタルの治療薬としてタクロリムス点眼が開発され，タクロリムス眼軟膏よりも使用感が改善したことでコンプライアンスが改善し，Mooren 潰瘍の鎮静化を得ることができた。今後は，Mooren 潰瘍などの難症例に対して，タクロリムス点眼の保険適用が広がり，ステロイドとの併用治療が可能となることが期待される。

利益相反：該当なし

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