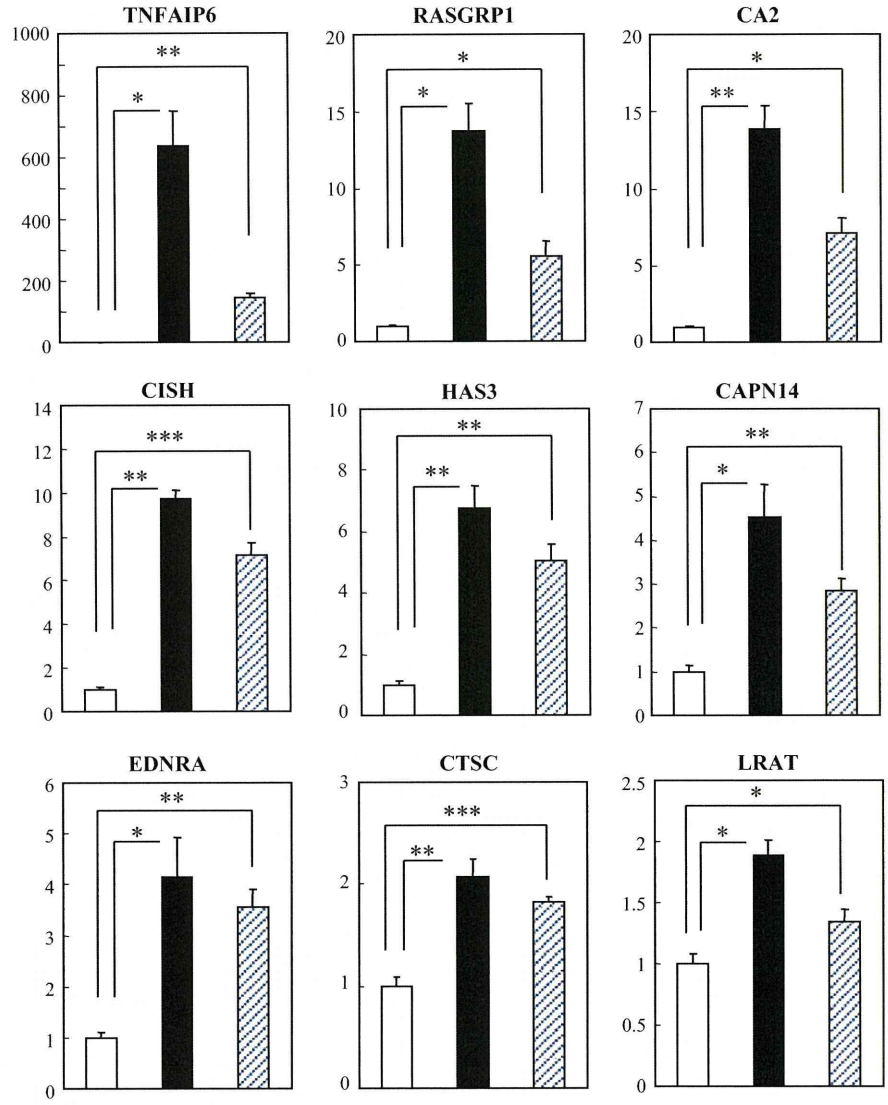

Fig. 2 Upregulation of the 9 transcripts in HCLE cells exposed to interleukin (IL)-4 or IL-13. The quantification data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The *y*-axis shows the increase ratio (arbitrary unit) of specific mRNA over nonstimulated samples [baseline unit (nonstimulated state) = 1]. Data are representative of 3 separate experiments and show the mean ± standard error of the mean (SEM) from 1 experiment carried out in 4 wells per group. *White bar* nonstimulation, *black bar* IL-4 stimulation, *shaded bar* IL-13 stimulation. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$

CTSC, HAS3, CAPN14, EDNRA, CISH, and LRAT. Thus, and as is the case with human conjunctival epithelial cells, human corneal epithelial cells express functioning IL-4Rα.

Others have reported that IL-4Rα was expressed on human corneal fibroblasts [19–21] and that IL-4 stimulation with TNF-α induced production of eotaxin and thymus activation-regulated chemokine (TARC) [22]. These reports suggest that corneal fibroblasts play a central role in the induction and amplification of ocular allergic inflammation. On the other hand, IL-4 (with or without TNF-α) stimulation of human corneal epithelial cells did not induce the production of eotaxin or TARC [22].

Our findings indicate that human corneal epithelial cells express functioning IL-4Rα and that stimulation of its ligands, IL-4 and IL-13, could induce the expression of various genes such as *TNFAIP6*, *RASGRP1*, *CA2*, *CTSC*, *HAS3*, *CAPN14*, *EDNRA*, *CISH*, and *LRAT*.

TNFAIP6 is an antiinflammatory protein present in the bronchoalveolar lavage fluid of patients with asthma; its level is increased after allergen challenge [23]. RASGRP1 is one of the diacylglycerol/phorbol ester receptors; its overexpression inhibits the expression of differentiation markers in keratinocytes [24]. CA2 is one of the carbonic anhydrases and thought to be associated with the transport of fluids and ions [25]. CISH is a member of the suppressors of the cytokine signaling family of proteins and is an important negative regulator of inflammatory cytokine signaling [26]. HAS3 is one of the hyaluronan synthases; hyaluronan has been shown to control epithelial proliferation and regeneration [27]. CAPN14 is a newly discovered member of the calpain family, which functions as calcium-dependent cysteine proteases; its function is unknown at present [28]. EDNRA is one of the receptors of endothelin-1 and known to induce epithelial-mesenchymal transition

in alveolar epithelial cells [29]. CTSC, a member of the cathepsin family, is upregulated in the bronchial biopsy tissues of asthma patients; however, its function remains unknown [30]. LRAT is one of the vitamin-A metabolism enzymes that are upregulated by cellular differentiation in human keratinocytes [31]. TNFAIP6 and CISH are anti-inflammatory molecules, while the *RASGRP1*, *HAS3*, *EDNRA*, and *LRAT* genes may play a functional role in epithelial differentiation or proliferation. Moreover, TNFAIP6, like CTSC, is reportedly upregulated in asthma patients [23]. We speculate that the upregulation of TNFAIP6 and CTSC in the bronchoalveolar lavage or biopsy tissues of asthma patients may be attributable to their derivation from epithelial cells.

Although corneal fibroblasts could produce chemokines such as eotaxin and TARC through IL4Rα and were able to augment allergic inflammation, it is possible that corneal epithelial cells suppress allergic inflammation via TNFAIP6 and CISH, which are produced through IL4Rα. Furthermore, IL4Rα in corneal epithelial cells might contribute to cellular differentiation or proliferation because corneal epithelial cells could induce cellular differentiation or proliferation-related genes such as *RASGRP1*, *HAS3*, *EDNRA*, and *LRAT* through IL4Rα. However, the exact functions of IL4Rα in corneal epithelial cells have yet to be elucidated.

In summary, we demonstrated that human corneal epithelial cells expressed functioning IL-4Rα and that the stimulation of its ligands, IL-4 and IL-13, could induce the expression of various genes, for example, antiinflammatory molecule genes such as *TNFAIP6* and *CISH*, and cellular differentiation and proliferation-related molecular genes such as *RASGRP1*, *HAS3*, *EDNRA*, and *LRAT*.

Acknowledgments The authors wish to thank Dr. Ilene K. Gipson for providing the HCLE cells and Chikako Endo for technical assistance. This work was supported in part by grants-in-aid for scientific research from the Japanese Ministries of Health, Labour and Welfare and of Education, Culture, Sports, Science and Technology; CREST from JST; a research grant from the Kyoto Foundation for the Promotion of Medical Science; the Intramural Research Fund of Kyoto Prefectural University of Medicine; a Shiseido Female Researcher Science Grant; and a research grant from the Japan Allergy Foundation.

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PLoS one

Prostaglandin E Receptor Subtype EP3 Expression in Human Conjunctival Epithelium and Its Changes in Various Ocular Surface Disorders

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Abstract

Background: In our earlier genome-wide association study on Stevens-Johnson Syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), we found that in Japanese patients with these severe ocular surface complications there was an association with prostaglandin E receptor 3 (EP3) gene (*PTGER3*) polymorphisms. We also reported that EP3 is dominantly expressed in the ocular surface-, especially the conjunctival epithelium, and suggested that EP3 in the conjunctival epithelium may down-regulate ocular surface inflammation. In the current study we investigated the expression of EP3 protein in the conjunctiva of patients with various ocular surface diseases such as SJS/TEN, chemical eye burns, Mooren's ulcers, and ocular cicatricial pemphigoid (OCP).

Methodology/Principal Findings: Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to SJS/TEN, chemical eye burns, and OCP, and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva. The controls were nearly normal human conjunctival tissues acquired at surgery for conjunctivochalasis. We performed immunohistological analysis of the EP3 protein and evaluated the immunohistological staining of EP3 protein in the conjunctival epithelium of patients with ocular surface diseases. EP3 was expressed in the conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer and in normal human conjunctival epithelium. However, it was markedly down-regulated in the conjunctival epithelium of SJS/TEN and OCP patients.

Conclusions: We posit an association between the down-regulation of EP3 in conjunctival epithelium and the pathogenesis and pathology of SJS/TEN and OCP, and suggest a common mechanism(s) in the pathology of these diseases. The examination of EP3 protein expression in conjunctival epithelium may aid in the differential diagnosis of various ocular surface diseases.

Citation: Ueta M, Sotozono C, Yokoi N, Inatomi T, Kinoshita S (2011) Prostaglandin E Receptor Subtype EP3 Expression in Human Conjunctival Epithelium and Its Changes in Various Ocular Surface Disorders. PLoS ONE 6(9): e25209. doi:10.1371/journal.pone.0025209

Editor: Naj Sharif, Alcon Research, Ltd., United States of America

Received June 14, 2011; **Accepted** August 29, 2011; **Published** September 22, 2011

Funding: This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST from JST, a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Prostanoids are comprised of prostaglandins (PGs) and thromboxanes (TXs). They are lipid mediators that form in response to various stimuli and include PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. 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. There are 8 types of prostanoid receptors that are conserved in mammals from mouse to human: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP) [1].

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN) are acute inflammatory vesiculobullous reactions of the skin and mucosa including the ocular surface [2]. In our earlier genome-wide association study in Japanese SJS/TEN patients with severe ocular surface complications we found associations with 6 single nucleotide polymorphisms (SNPs) in the prostaglandin E receptor 3 (EP3) gene (*PTGER3*) and we documented that compared with the controls, EP3 expression was markedly reduced in the conjunctival epithelium of SJS/TEN patients with severe ocular complications [3]. Others reported that the PGE_2-EP3 signaling pathway negatively regulates allergic reactions in a murine allergic asthma model [4] and that it inhibits keratinocyte activation and exerts anti-inflammatory actions in mouse contact hypersensitivity [5]. We also showed that EP3 is dominantly expressed in the ocular surface-, especially the conjunctival epithelium, and that PGE_2 acts as a ligand for EP3 in the conjunctival epithelium and down-regulates the progression of murine experimental allergic conjunctivitis [6]. In addition, we reported that an EP3 agonist suppressed the production of CCL5, CXCL10, CXCL11, and IL-6 in response to polyI:C stimulation

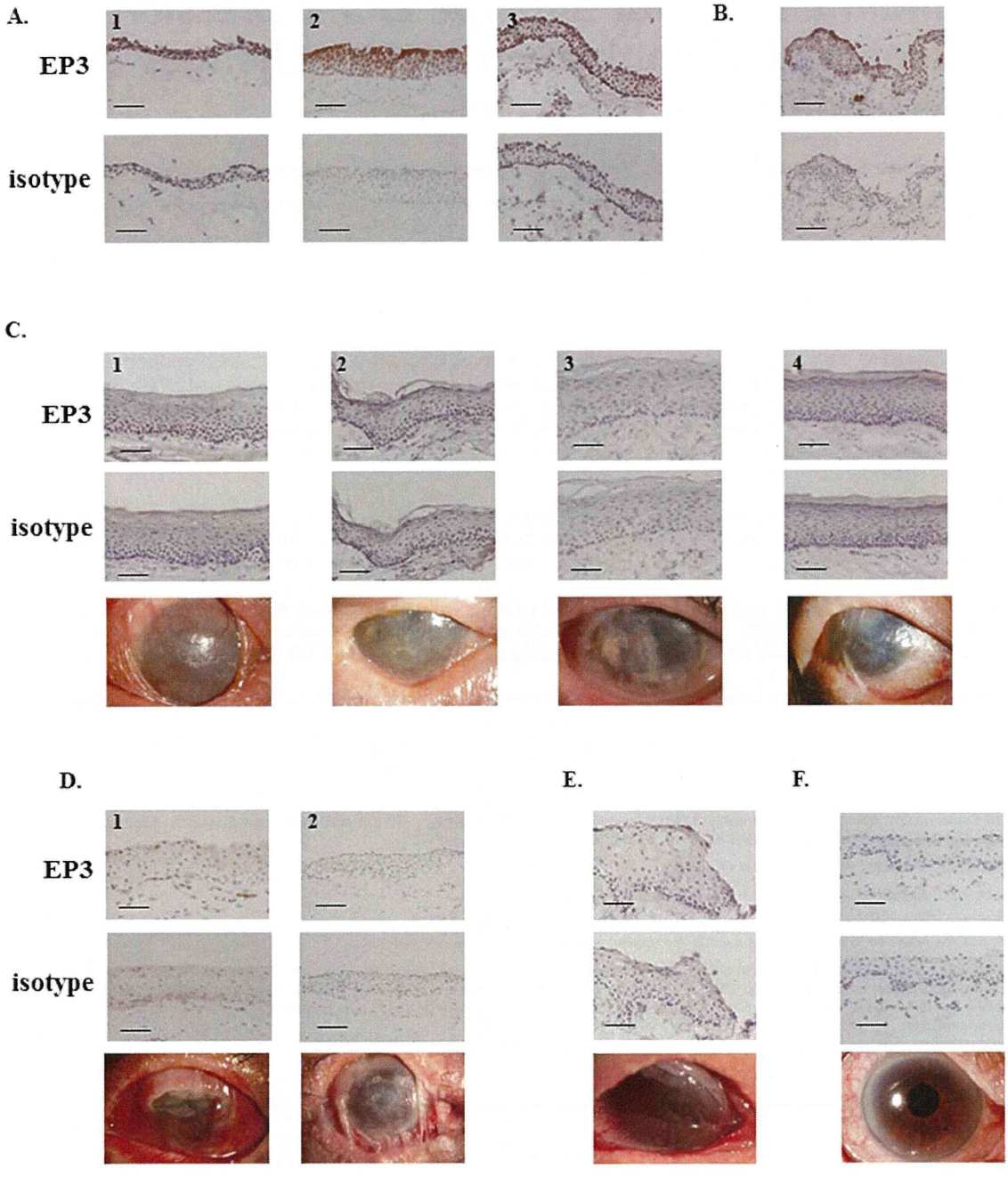

Figure 1. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of the controls and SJS/TEN patients. A. Nearly normal conjunctival tissues from patients with conjunctivochalasis. B. Normal conjunctival tissue. C. Keratinized conjunctival tissues of SJS/TEN patients in the chronic stage. D. Non-keratinized conjunctival tissues of SJS/TEN patients in the sub-acute stage. E. Non-keratinized conjunctival tissues of SJS/TEN patients in the chronic stage. F. Visibly normal conjunctival tissue of an SJS/TEN patient with minor ocular sequelae (dry eye). C-F. The 3rd lane shows the ocular surface of SJS/TEN patients. Each scale bar represents a length of 100 μm.
doi:10.1371/journal.pone.0025209.g001

of human conjunctival epithelial cells, suggesting that EP3 in the conjunctival epithelium may down-regulate ocular surface inflammation [7].

In the current study we investigated the expression of EP3 protein in the conjunctiva of patients with various ocular surface diseases such as SJS/TEN, chemical eye burns, Mooren's ulcers, and ocular cicatricial pemphigoid (OCP).

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.

Our immunohistochemistry controls were 3 nearly normal human conjunctival tissues acquired at surgery for conjunctivochalasis and one sample of normal conjunctival tissue acquired at limbal dermoid resection. Conjunctival tissues were also obtained from patients undergoing surgical reconstruction of the ocular surface due to SJS/TEN (n = 7), chemical eye burns (n = 3), OCP (n = 3), severe graft versus host disease (GVHD) (n = 1), pseudo-OCP (n = 1) and pterygium (PTG) (n = 1), from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva (n = 4), and from a patient with a giant papilla due to allergic vernal conjunctivitis. One conjunctival tissue sample was obtained from an SJS/TEN patient who did not require ocular surface reconstruction because ocular sequelae were minor (dry eye); this sample derived from additional unnecessary conjunctiva harvested just after cataract surgery.

Immunohistochemistry

For EP3 staining we used rabbit polyclonal antibody to EP3 (Cayman Chemical Co., Ann Arbor, MI) [3,6]. We previously checked and confirmed the EP3 specificity of this antibody using conjunctiva from EP3KO mice [6]. Further confirmation was by immunoblot analysis (Fig. S1). The secondary antibody (Biotin-SP-conjugated AffiniPure F(ab')$_2$ fragment donkey anti-rabbit IgG (H+L), 1:500 dilution; Jackson Immuno Research, Baltimore, MD) was applied for 30 min, then VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was added for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Evaluation of staining intensity using ImageJ software and down-regulation score

We converted the multi-color pictures into black and white pictures, and measured the gray value in the vertical line of the conjunctival epithelium. Then we recorded the average gray value on an intensity score from 5 to 16 (e.g. an average gray value of 100 was scored as 10). We also recorded the degree of down-regulation where "–" = intensity score 12–16, "+" = intensity score 8–11, and "++" = intensity score 5–7.

Results

As reported elsewhere [3], EP3 protein was detected in the nearly normal conjunctival epithelium from patients with conjunctivochalasis (Fig. 1A) and in the normal conjunctival

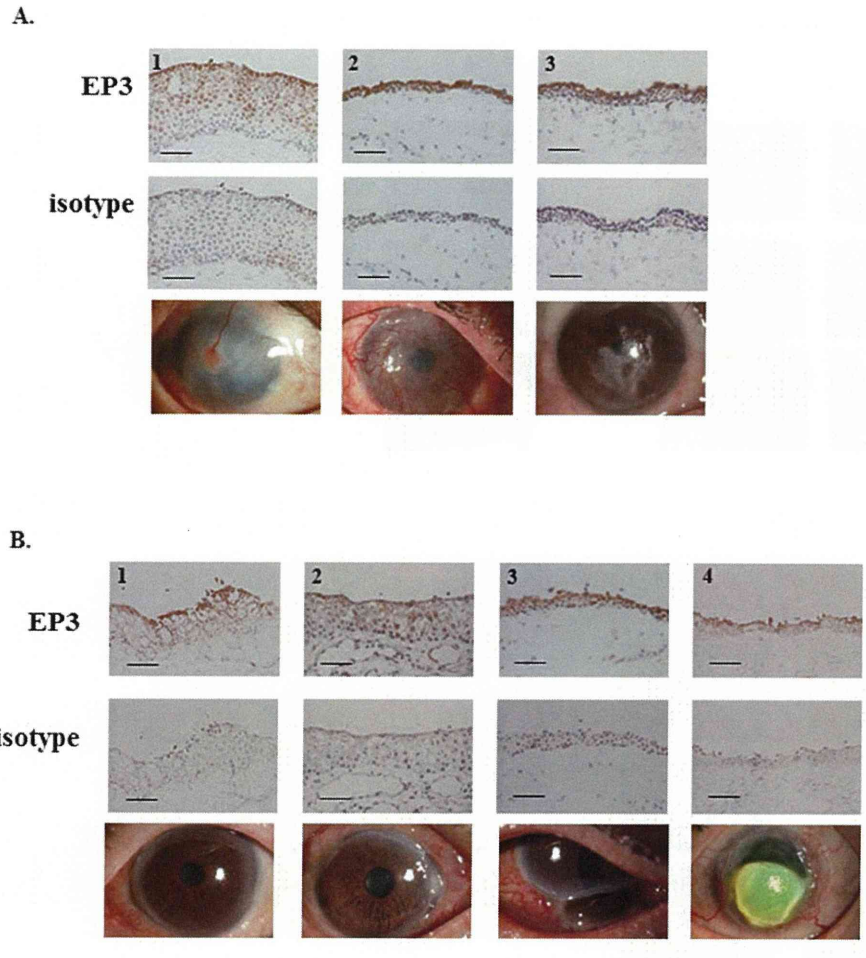

Figure 2. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with chemical eye burn and active Mooren's ulcer. A. Conjunctival tissues of patients with chemical eye burn requiring ocular surface reconstruction. B. Inflammatory conjunctival tissues of patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva. The 3rd lane shows the ocular surface of patients. Each scale bar represents a length of 100 μm.
doi:10.1371/journal.pone.0025209.g002

epithelium sample (Fig. 1B), but not in keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage (Fig. 1C). When we examined non-keratinized conjunctival epithelium from SJS/TEN patients in the sub-acute- or chronic stage (Figs. 1D, 1E) we found that EP3 was markedly down-regulated. Interestingly, even in the conjunctival epithelium from the SJS/TEN patient manifesting only dry eye, EP3 was greatly down-regulated (Fig. 1F).

Comparison with conjunctival tissues from patients with chemical eye burn showed that although ocular surface findings were similar, EP3 protein was detected in the conjunctival epithelium of 3 patients with chemical eye burn as well as in control conjunctival epithelium from conjunctivochalasis patients (Fig. 2A). We also detected EP3 protein in conjunctival epithelium from 4 patients with Mooren's ulcer, however, it appeared to be somewhat down-regulated (Fig. 2B).

Next we examined conjunctival tissues from 3 patients with OCP; their ocular surface findings were very similar to those of SJS/TEN patients. No EP3 protein was detected in conjunctival epithelium from any of these patients (Fig. 3A), nor in conjunctival epithelium from a GVHD patient with severe conjunctival invasion to the cornea (Fig. 3B). When we assessed tissues from patients with pterygium (Fig. 3C), or pseudo-OCP (Fig. 3D), we detected EP3 protein in the conjunctival epithelium of pterygium patients as we did in the control conjunctival epithelium from a patient with conjunctivochalasis. EP3 protein was also present in conjunctival epithelium from patients with pseudo-OCP although it appeared to be slightly down-regulated. We also found EP3 protein in the conjunctival epithelium of a patient with giant papillae due to chronic allergic keratoconjunctivitis (Fig. 3E). In Table 1 we show the scores obtained by our evaluation of the staining intensity and degree of down-regulation for all samples.

We document that EP3 was expressed in conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer and in normal human conjunctival epithelium. It was markedly down-regulated in the conjunctival epithelium of SJS/TEN- and OCP patients. Although we had only one patient each with severe GVHD, pterygium, pseudo-OCP, and chronic allergic keratoconjunctivitis, study of these samples suggested that EP3 is expressed in the conjunctival epithelium of patients with pterygium, pseudo-OCP, and chronic allergic keratoconjunctivitis, and that EP3 might be greatly down-regulated in the conjunctival epithelium of patients with severe GVHD.

Regarding in conjunctival epithelium, the expression of EP3 protein in the SJS/TEN and OCP patients was markedly decreased compared with normal conjunctiva. However, its expression in sub-conjunctival tissues may be up-regulated in

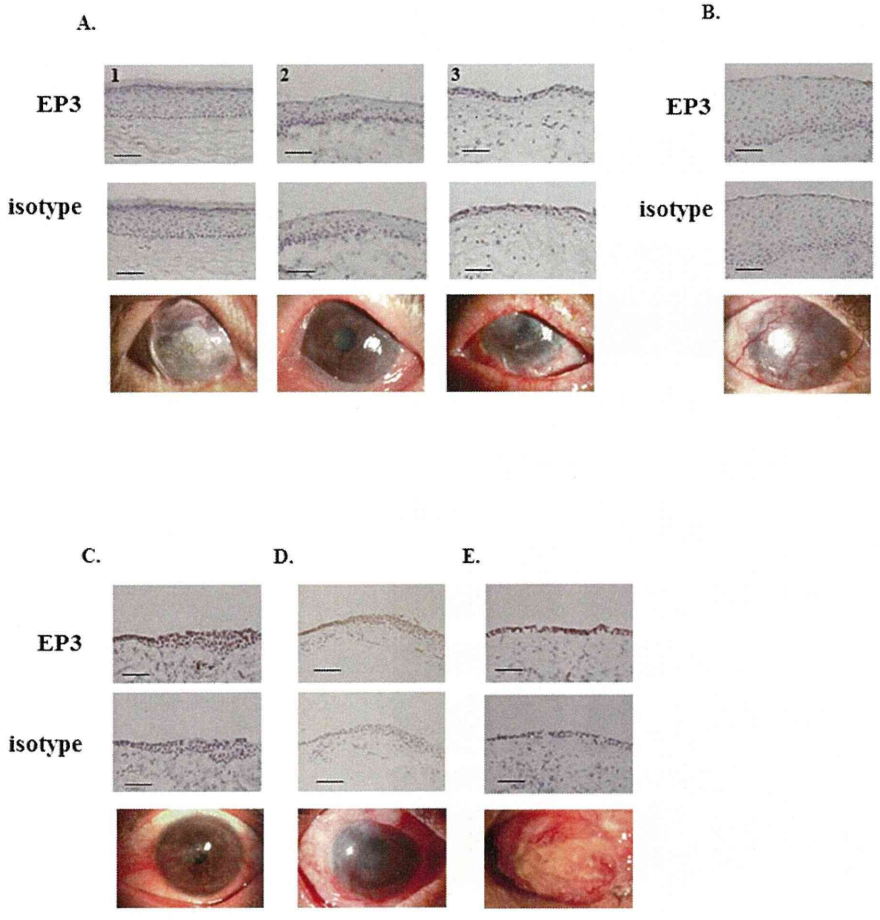

Figure 3. Immunohistological analysis of prostaglandin E receptor subtype EP3 in the conjunctival epithelium of patients with OCP (A), severe GVHD (B), pterygium (C), pseudo-OCP (D), and a giant papilla due to allergic vernal conjunctivitis (E). The 3rd lane shows the ocular surface of patients. Each scale bar represents a length of 100 μm.
doi:10.1371/journal.pone.0025209.g003

Table 1. Staining-intensity score of conjunctival epithelium.

Picture	Figure No	Intensity score	Down-regulation score	Disease
Figure 1	A1	14		Nearly normal conjunctival tissues from conjunctival chalasis
	A2	14		
	A3	14		
	B1	12		Normal conjunctival tissues
	C1	7	++	Keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage
	C2	7	++	
	C3	6	++	
	C4	7	++	
	D1	5	++	Non-keratinized conjunctival epithelium from SJS/TEN patients in the sub-acute stage
	D2	5	++	
	E	5	++	Non-keratinized conjunctival epithelium from SJS/TEN patients in the chronic stage
	F	5	++	Conjunctival epithelium from an SJS/TEN patient manifesting only dry eye
Figure 2	A1	9	+	Chemical eye burn
	A2	16		
	A3	13		
	B1	12		Mooren's ulcer
	B2	12		
	B3	14		
	B4	10	+	
Figure 3	A1	6	++	Ocular cicatricial pemphigoid (OCP)
	A2	6	++	
	A3	6	++	
	B	6	++	GVHD with severe conjunctival invasion to the cornea
	C	13		Pterygium
	D	10	+	Pseudo-OCP
	E	16		Chronic allergic keratoconjunctivitis

doi:10.1371/journal.pone.0025209.t001

some instances because vascular endothelia expressing the EP3 protein could be increased due to the presence of inflammatory infiltrating cells in sub-conjunctival tissues (Fig. S2).

Discussion

We previously reported that in Japanese SJS/TEN patients there was a significant association between severe ocular surface complications and prostaglandin E receptor 3 gene (*PTGER3*) polymorphisms and that compared to the controls, EP3 expression was greatly reduced in their conjunctival epithelium [3]. Here we studied keratinized and non-keratinized conjunctival epithelia of SJS/TEN patients and the conjunctival epithelium of an SJS/TEN patient whose ocular sequelae were minor (dry eye). We found that EP3 was markedly down-regulated not only in keratinized- but also in non-keratinized conjunctival epithelia and even in the normal conjunctiva of a patient in the chronic stage of SJS whose only ocular sequela was dry eye. Our results suggest that the strong down-regulation of EP3 in conjunctival epithelium of SJS/TEN patients is associated with the pathogenesis and pathology of the disease because PTGER3 (EP3) polymorphisms are significantly associated with SJS/TEN.

Severe chemical eye burn results in conjunctival invasion into the cornea due to a deficiency in corneal epithelial stem cells; this leads to devastating ocular surface disorders similar to SJS/TEN. However, EP3 was not down-regulated in the conjunctival epithelium of patients with severe chemical eye burns, suggesting that the pathology of the ocular surface changes was not associated with EP3 expression.

In patients with Mooren's ulcer the peripheral stroma is destroyed first circumferentially then centrally, resulting in the characteristic overhanging inner edge. This is an inflammatory disease of the ocular surface that may require resection of the inflammatory conjunctiva adjacent to the ulcer. We found that the conjunctival epithelium of the inflammatory conjunctival tissues adjacent to the ulcer clearly expressed EP3 protein, indicating that other factors besides inflammation are required for a marked down-regulation of EP3 expression.

OCP is a subset of mucous membrane pemphigoid. It is characterized by the abnormal production of circulating autoantibodies directed against various components of the basement membrane zone and the generation of proinflammatory and fibrogenic cytokines [8]. We found that, as in SJS/TEN patients, EP3 was markedly down-regulated in the conjunctival epithelium of OCP patients with conjunctival invasion to the cornea. As in OCP patients, we failed to detect EP3 protein in the conjunctival epithelium of a patient with severe GVHD with conjunctival invasion to the cornea. This suggests that in a common mechanism(s) may underlie the pathology of SJS/TEN and OCP, especially in ocular surface epithelium such as the conjunctival epithelium. EP3 expression has been reported in skin and PGE_2 was produced abundantly during skin allergic

inflammation [5], suggesting that there is no association between decreased EP3 expression and the increased production of cornified proteins in SJS/TEN and OCP.

We found that EP3 was clearly expressed in the conjunctival epithelium of our patients with pterygium, pseudo-OCP, and a giant papilla of allergic vernal conjunctivitis. Interestingly, the expression of EP3 in conjunctival epithelium from patients with OCP and pseudo-OCP was different: EP3 was clearly present in the patient with pseudo-OCP but not the patient with OCP. The patient with pseudo-OCP had received long-term treatment with eye drops for glaucoma; this resulted in a deficiency of corneal epithelial stem cells and led to conjunctival invasion into the cornea. This suggests that different mechanisms are involved in the expression of EP3. We also detected EP3 in the conjunctival epithelium of the patient with allergic vernal conjunctivitis. Elsewhere we documented that PGE_2 acts as a ligand for EP3 in the conjunctival epithelium and down-regulates the progression of murine experimental allergic conjunctivitis [6]. Although EP3 may down-regulate allergic reactions in patients with allergic conjunctivitis, its loss may not be a causative factor.

In summary, EP3 is expressed not only in normal human conjunctival epithelium but also in the conjunctival epithelium of patients with chemical eye burns and Mooren's ulcer. On the other hand, it is markedly down-regulated in the conjunctival epithelium of SJS/TEN- and OCP patients.

Supporting Information

Figure S1 The rabbit polyclonal antibody to EP3 we used is checked and confirmed the EP3 specificity of this antibody using immunoblot analysis.
(TIF)

Figure S2 EP3 expression in sub-conjunctival tissues in a SJS/TEN patient in the chronic stage. In some instances of SJS/TEN patients, vascular endothelia expressing the EP3 protein are found.
(TIF)

Author Contributions

Conceived and designed the experiments: MU. Performed the experiments: MU. Analyzed the data: MU. Contributed reagents/materials/analysis tools: CS NY TI SK. Wrote the paper: MU.

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Cytokine storm arising on the ocular surface in a patient with Stevens–Johnson syndrome

Stevens–Johnson syndrome (SJS) and its more extreme variant, toxic epidermal necrolysis (TEN), are acute, adverse systemic reactions that can affect anyone who takes medications. SJS/TEN predominantly affects the skin and mucosal membranes and predisposes patients to life-threatening complications such as sepsis, respiratory dysfunction and multi-organ failure. Even when a patient does survive this disease, serious ocular discomfort and morbidity often persists life long.[1 2]

In May 2008, a 59-year-old female inpatient had a case of red eyes, and 2 days later she presented with a sudden onset of high fever and eruption and erosion in the mucocutaneous regions including the mouth, paronychia and bilateral conjunctivitis. Slit-lamp examination revealed a large epithelial defect of the conjunctiva with severe hyperaemia in both eyes (figure 1A,B). There was no viral or bacterial infection, and skin biopsy specimens of the erythematous macules revealed necrotic keratinocytes and liquefaction, compatible with the diagnosis of SJS. Steroid pulse therapy and intensive topical betamethasone (0.1%, 10 times daily) were then initiated (figure 2).

To date, the pathogenesis of SJS/TEN is yet to be fully elucidated, although previous studies have reported that several cytokines may play a role in the accelerated apoptosis or the ocular surface inflammation.[3 4] To examine the immunological conditions existing in this patient, we measured various cytokine levels in her tear fluid and serum using a Cytometric Beads Array system. This new system makes it possible to simultaneously measure the various cytokines and chemokines using a trace amount (1 µl) of tears in a bead-based immunoassay.

Among the various cytokines measured, the levels of interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1), but not IL-1β, IL-5, eotaxin, interferon-γ or macrophage inflammatory protein-1α (MIP-1α), were dramatically increased in this patient's tear fluid and serum (figure 2). Many neutrophils were observed in the smear of the discharge from the ocular surface of this patient, compatible with the results of the tear cytokine measurement (online supplementary figure). The levels of all three cytokines in her tear fluid were extremely high compared with those in her serum at any time point.

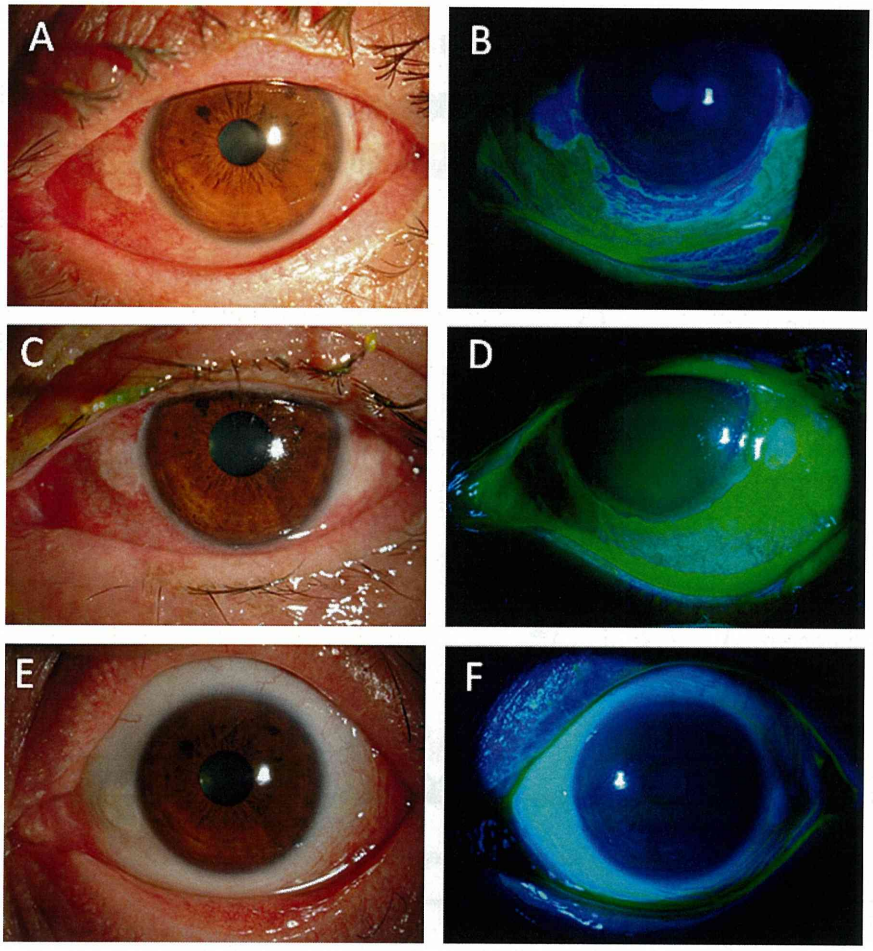

Figure 1 Appearances of the patient's left eye. Mild conjunctival hyperaemia (A) and large conjunctival epithelial defect (B) were seen on day 1 (the day that systemic eruption began). Depletion of the cilia occurred at day 3 (C) and the conjunctival epithelial defect enlarged (D). After steroid pulse therapy and intensive topical betamethasone, the conjunctival epithelium healed completely by day 23 (E, F).

While upregulated serum cytokines decreased after the corticosteroid pulse, the levels of these cytokines in her tear fluid remained high during the second week despite systemic steroid administration and intensive topical betamethasone. This study using a well-documented SJS case supports the interesting hypothesis that the ophthalmological manifestations of SJS/TEN, in association with the systemic features of the disease, are strongly correlated with a severe cytokine storm arising on the ocular surface.

Various treatments have been proposed to manage SJS/TEN during the acute phase of the disease, including steroid pulse therapy, plasmaphaeresis, intravenous immunoglobulin, cyclophosphamide therapy and amniotic membrane transplantation.[1 5] However, the choice of treatment regimens has been empirical, rather than based on the immunological mechanisms underlying the disease. Now, it is reasonable to speculate that a steroid pulse therapy is effective in controlling a cytokine storm. After steroid pulse therapy, our patient's general condition, including her skin rash, had markedly improved, and serum cytokine levels decreased coordinately. Bilateral conjunctival erosion extended to nearly the entire bulbar conjunctiva by day 3 (figure 1C,D). Thereafter, her conjunctival epithelium began to regenerate and healed completely by day 23 with decrease in tear cytokines to the baseline levels (figure 1E,F). Her visual acuity 2 years after disease onset was 20/20 in both eyes and no cicatricial changes exist. This clinical outcome correlates with our previous report describing the therapeutic importance of a corticosteroid pulse and topical betamethasone at disease onset for reducing the degree of ocular complications.[2 6] In conclusion, it is highly possible that an ocular cytokine storm and its suppression by therapeutic modalities such as those using steroids greatly influence the visual prognosis in SJS/TEN.

Acknowledgements The authors wish to thank John Bush for reviewing this manuscript.

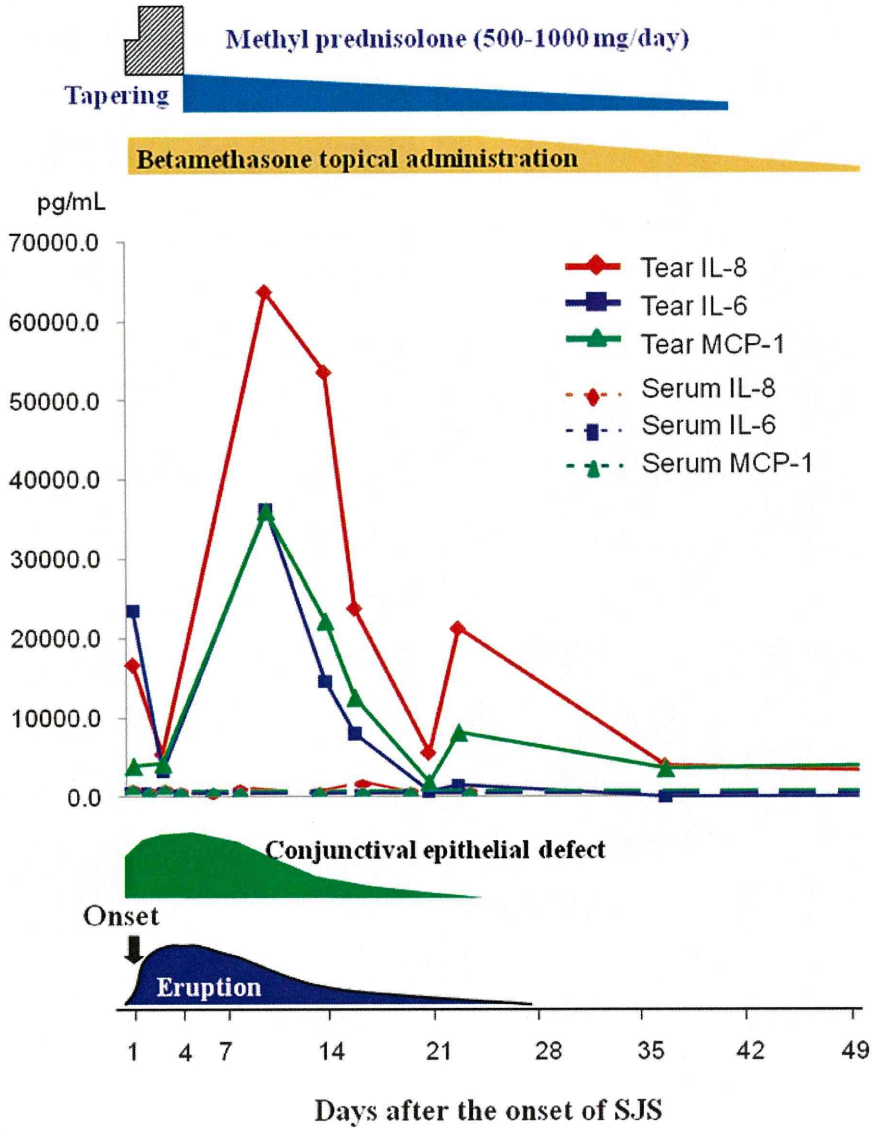

Figure 2 The interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) levels in the patient's tears and serum. SJS, Stevens–Johnson syndrome.

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Funding Health and Labor Sciences Research Grants (Research on Intractable Diseases) from the Ministry of Health, Labour and Welfare of Japan, and the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Competing interests None.

Patient consent This study was performed in accordance with the tenets set forth in the Declaration of Helsinki and written informed consent was obtained from the patient prior to involvement in the study.

Ethics approval Ethics approval was provided by the Institute Review Board (IRB) of Kyoto Prefectural University of Medicine, Kyoto, Japan.

Provenance and peer review Not commissioned; externally peer reviewed.

Accepted 20 March 2011
Published Online First 11 April 2011

Br J Ophthalmol 2011;**95**:1030–1031. doi:10.1136/bjo.2010.196295

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Authors' response

We wish to address the comments by Anderson *et al* on our paper entitled 'Strabismus-related prejudice in 5–6-year-old children'.[1] In our paper, we discussed that the absence of an effect of strabismus on participants' playmate choice in Anderson *et al*'s study[2] could be due to differential age-related cognitive and language capacities. In our study, the age range of the participants was much narrower (ie, 5–6 years) than that of the participants in Anderson *et al*'s study, which ranged from 3 to 8 years. Since Anderson *et al* did not specify the number of participants in each age group, we could not ascertain whether age was sufficiently accounted for in their data analysis. Given that children between 3 and 8 years old may be at various stages of developmental maturity, we believe that age may be a factor that needs to be controlled for when examining children's responses to images of peers with strabismus. To qualify this further, a recent study[3] demonstrated that age is an important factor that might influence perception and response to strabismus. The study clearly showed that children exhibited negative social reactions to peers with obvious exotropia. However, older children's reactions were less marked than the reactions of their younger peers. This finding suggests the need to control for participants' age when examining their perception towards strabismus. Our study design took age differences into account as opposed to Anderson *et al*'s study.

The main aim of our study was to examine young children's perception of peers with conspicuous exotropia. Since young children may not be as capable of discriminating subtle differences in facial features compared with older children, it is necessary to create images with a large magnitude of exotropia. We acknowledge and agree with Anderson *et al*'s evaluation that since we did not examine varying magnitudes of misalignment, the results of our study have limited generalisability. However, Anderson *et al* should provide a sufficient basis before assuming that children can detect a magnitude of

Cytokine storm arising on the ocular surface in a patient with Stevens –Johnson syndrome

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Br J Ophthalmol 2011 95: 1030-1031 originally published online April 11, 2011
doi: 10.1136/bjo.2010.196295

EDITOR'S CHOICE

Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders

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Accepted 21 September 2010
Published Online First 19 November 2010

ABSTRACT

Purpose To investigate the long-term outcome of autologous cultivated oral mucosal epithelial transplantation (COMET) for the treatment of the scar phase of severe ocular surface disorders.

Participants This study involved 19 eyes of 17 patients who received autologous COMET for total limbal stem-cell deficiency.

Methods Autologous cultivated oral mucosal epithelial sheets were created using amniotic membrane as a substrate. Clinical efficacy was evaluated by best-corrected visual acuity and visual acuity at the postoperative 36th month. The clinical results (clinical conjunctivalisation, corneal opacification, corneal neovascularisation and symblepharon formation) were evaluated and graded on a scale from 0 to 3 according to their severity. Clinical safety was evaluated by the presence of persistent epithelial defects, ocular hypertension and infections.

Results Autologous cultivated oral mucosal epithelial sheets were successfully generated for all 17 patients. All patients were followed up for more than 36 months; the mean follow-up period was 55 months and the longest follow-up period was 90 months. During the long-term follow-up period, postoperative conjunctivalisation and symblepharon were significantly inhibited. All eyes manifested various degrees of postoperative corneal neovascularisation, but it gradually abated and its activity was stable at 6 months after surgery. Best-corrected visual acuity was improved in 18 eyes (95%) during the follow-up periods, and visual acuity at the postoperative 36th month was improved in 10 eyes (53%).

Conclusions These long-term clinical results strongly support the conclusion that tissue-engineered cultivated oral mucosal epithelial sheets are useful in reconstructing the ocular surface of the scar phase of severe ocular surface disorders.

INTRODUCTION

Severe ocular surface disease (OSD), such as Stevens–Johnson syndrome (SJS) and ocular cicatricial pemphigoid (OCP), can be devastating and result in significant visual complications.[1–5] Since 1997, with the knowledge of tissue-specific stem cell behaviour and the development of tissue-engineering techniques, cultivated corneal limbal epithelial transplantation (CLET) has been shown to be a promising treatment modality for the management of severe OSD.[6–12] However, as most severe OSD is bilateral, surgeons were forced to use allograft donor cells, which subject the recipients to a high risk for allogeneic rejection and necessitate prolonged immunosuppression therapy. More recently, our experimental[13] and serial clinical studies[14–18] demonstrated the efficacy of autologous cultivated oral mucosal epithelial transplantation (COMET) for the treatment of severe OSD. Even though initial clinical results of COMET have been reported from several groups worldwide,[19 20] including our group, the long-term clinical assessments of COMET are entirely unknown and the feasibility of this technique still requires detailed investigation.

Here, we present the long-term clinical data on 19 eyes that received COMET, for which the mean follow-up period was 55 months; the longest follow-up period being 90 months. This study has important clinical implications and provides new information regarding the long-term visual results and survival of transplanted cultivated cells for the treatment of the scar phase of severe OSD cases.

MATERIALS AND METHODS

Subjects

All experimental procedures and clinical applications introduced in this study were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

The study included 19 eyes from 17 patients with the scar phase of severe OSD who underwent ocular surface reconstruction with COMET at our hospital from August 2002 to January 2007, and who could be followed up for more than 36 months. In this study, to precisely examine the long-term clinical results of COMET for corneal surface reconstruction, we excluded the patients who received penetrating keratoplasty after the initial COMET and patients who received COMET for conjunctival fornix reconstruction. The patients included 7 males and 10 females; their ages ranged from 20 to 80 years (mean age: 54±21 years). The patients were followed up for a mean period of 55±17 months; the longest follow-up period was 90 months. All patients were diagnosed as totally stem-cell-deficient on the basis of complete disappearance of the palisades of Vogt and 360° of conjunctivalisation. The preoperative diagnosis was SJS in 11 eyes, OCP in 4 eyes, squamous cell carcinoma in 2 eyes, thermal or chemical injury in 1 eye and graft-versus-host disease in 1 eye. Tear production was diminished but not absent in all

patients, as evidenced by the presence of a tear meniscus level with diminished tear-film break-up time.

Cultivation of oral mucosal epithelial sheets

We cultured human oral mucosal epithelial cells using a previously reported system.[14–18] Briefly, the presence of healthy oral mucosa was first confirmed by a dentist before biopsy. A small oral mucosal biopsy was performed under local anaesthesia. The oral epithelium was then incubated at 4°C for 5 h with 1.2 IU Dispase, followed by treatment with 0.05% Trypsin-EDTA solution for 10 min to separate the cells. The resultant oral epithelial cells ($1–2\times10^5$ cell/ml) were then seeded onto denuded amniotic membrane (AM) spread on the bottom of culture inserts and co-cultured with mitomycin C (MMC)-inactivated 3T3 fibroblasts. The culture medium consisted of defined keratinocyte growth medium (KGM: ArBlast Co., Ltd., Kobe, Japan) supplemented with 5% serum. The cultured cells were submerged in medium for 2 weeks and then air-lifted for 1–2 days by lowering the medium level.

Surgical procedure

The surgical procedure was as described in our previous report.[14–18] Briefly, we performed a 360° conjunctival peritomy 3 mm from the limbus and removed all perilimbal scarred or inflamed subconjunctival tissue down to bare sclera. The corneal pannus was completely removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. The cultivated oral mucosal epithelial sheet was placed over the corneal surface and secured in place with 10-0 nylon sutures at the limbus. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of the surgery, and the ocular surface was protected with a medical-use bandage contact lens (Plano B4). Postoperatively, three types of medical-use bandage contact lenses (Plano B4, Acuvue and O_2 Optics) were properly used within 1–3 months after surgery depending on the condition of the corneal surface.

Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone eye drops were instilled four times a day. The doses were tapered to a maintenance dose of—two to three times a day after 2–3 months, depending on the severity of inflammation. Oral betamethasone (1 mg/day) and cyclosporine (100 mg/day) were administered to reduce inflammation and were tapered down and then stopped 1 month postoperatively.

Evaluation of clinical efficacy

Preoperative visual acuity (VA), postoperative best-corrected visual acuity (BCVA) and VA at the postoperative 36th month were measured, and the ocular surface was inspected with a slit-lamp microscope and fluorescence staining. The clinical results (clinical conjunctivalisation (eg, invasion of conjunctival tissue), corneal opacification, corneal neovascularisation and symblepharon formation) were evaluated by two ophthalmologists and graded on a scale from 0 to 3 according to their severity in accordance with our previously reported grading system.[21]

Evaluation of clinical safety

For the assessment of postoperative complications, the patient's eyes were carefully examined for persistent epithelial defects (PEDs), ocular hypertension and infections. Epithelial defects were considered persistent if they lasted for more than 4 weeks. Ocular hypertension was considered a postoperative complication if it had not been present preoperatively. When we clearly observed the clinical bacterial focus region in the cornea, we considered it a corneal infection.

RESULTS

There were no complications during or after the excision of the oral mucosa. Autologous cultivated oral mucosal epithelial sheets were successfully generated for all 17 patients, but one case was merely fair because only 70% of the entire cultivated epithelial sheet showed mature stratification as determined by fluorescein staining under a phase-contrast microscope and an operating microscope at the end of surgery. All eyes, including the eye transplanted with the sheet whose quality we judged as only fair by use of an inverted microscope, demonstrated total re-epithelialisation of the corneal surface within 2–7 days after surgery. Successful engraftment was initially achieved in all patients with none of the grafts sloughing off. Combined surgery was performed as follows: subconjunctival MMC treatment (17 eyes, 90%), amniotic membrane transplantation (AMT) (14 eyes, 74%) and cataract surgery (5 eyes, 26%).

Preoperative BCVA in our series was light perception, hand motion (HM) or finger counting (15 eyes, 79%) and worse than 20/500 (4 eyes, 21%). Postoperative VA (BCVA, and VA at the postoperative 36th month) is summarised in figure 1 and table 1. Postoperative visual recovery ranged from HM to 20/40; during the follow-up, BCVA was improved more than 2 lines in 15 eyes (79%) and VA at the postoperative 36th month was improved in 8 eyes (42%).

The clinical grading scores pertaining to conjunctivalisation, corneal opacity, corneal neovascularisation and symblepharon formation were evaluated, and it was found that during the long-term follow-up period, postoperative clinical conjunctivalisation

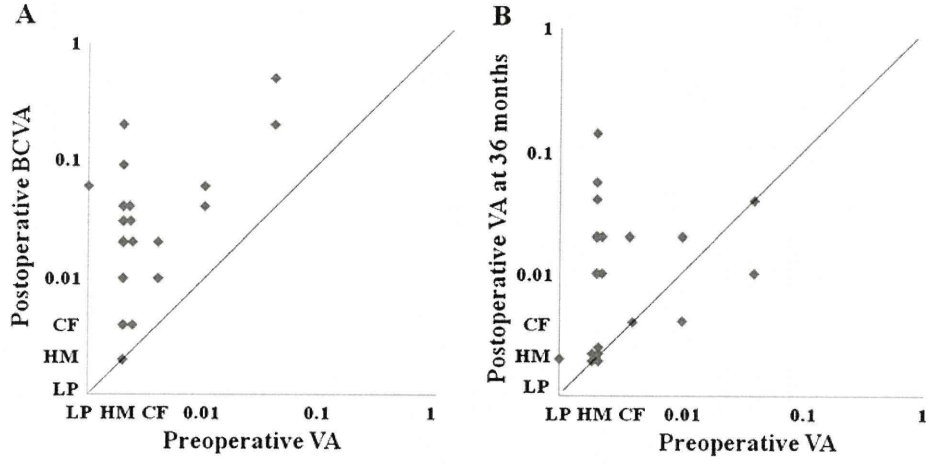

Figure 1 Postoperative best-corrected visual acuity (BCVA) (A) and visual acuity (VA) at the postoperative 36th month (B). The diagonal line indicates the values at which the preoperative and postoperative values for visual acuity were the same. CF: counting fingers; HM: hand motion; LP: light perception.

Table 1 Summary of postoperative VA

	Postoperative BCVA (%)	Postoperative VA at 36 months (%)
Improvement of VA (more than two lines)	79	42
Improvement of VA (more than one line)	95	53
No change	5	37
Decline	0	11

BCVA, best-corrected visual acuity; VA, visual acuity.

and symblepharon were significantly inhibited (figure 2). Corneal opacification tended to improve. All eyes manifested various degrees of superficial corneal vascularisation, but it gradually abated and its activity was comparatively stable from 6 months after surgery. We theorise that postoperative neovascularisation occurs because in vivo oral mucosa requires a vascular bed for maintenance.

Regarding the postoperative complications, a small but persistent epithelial defect was observed in 5–26% of the patients during the follow-up periods (table 2). Of the total 19 eyes, 7 eyes (37%) manifested PEDs once during the follow-up periods, while postoperative ocular hypertension was observed in 3 eyes (16%) (table 2). Although the intraocular pressure (IOP) of those three patients was occasionally high, they did not require glaucoma surgery. The occasional increase in IOP was mainly managed by the administration of carbonic anhydrase inhibitor (two eyes), or by the topical application of 0.05% latanoprost (one eye) or carteolol hydrochloride (one eye). Methicillin-resistant *Staphylococcus aureus* (MRSA) was the only cause of postoperative corneal infection (one eye), and that corneal infection was observed only within 6 months after transplantation (table 2).

The clinical progress of three representative patients with total limbal stem cell deficiency arising from SJS and idiopathic OSD is shown in figure 3. Before transplantation all eyes manifested severe destruction of the ocular surface with limbal stem cell deficiency (figure 3A,C,E). Postoperative appearance at 50 (figure 3B) and 71 (figure 3D) months shows a relatively smooth, epithelialised corneal surface with minimal corneal neovascularisation, scarring and inflammation. Postoperative appearance at 72 months (figure 3F) shows that due to the PED during the follow-up periods, postoperative corneal opacity was stronger than in other cases with modest neovascularisation, finally affecting the transparency of the cornea even though the ocular surface was relatively stable.

DISCUSSION

Ocular surface reconstruction for severe OSD continues to be one of the most challenging fields in ophthalmology. COMET is the most recent therapeutic method for the treatment of severe OSD, and this study provides new information regarding the long-term results of this new treatment. We found through our earlier preliminary and mid-term clinical results[14–18] that COMET is an efficacious treatment for severe OSD. The overall success rate, as measured by the improvement of BCVA and VA at the postoperative 36th month, was 95% and 53%, respectively. This success rate is similar to that of a previous report, and the patients who participated in this study were some of the most severe cases with their preoperative VA all being worse than 20/500, suggesting that our clinical results were fair and that COMET was useful for reconstructing the ocular surface of these patients with severe OSD.

In 1999, our group started the clinical application of allogeneic CLET and since 2002 we have been performing COMET for patients with severe OSD. Through these many years of clinical experience we have learned a great deal from our clinical and biological findings as follows: (1) Wearing a therapeutic soft contact lens in the early postoperative period is essential for the clinical success of COMET because it provides protection for the epithelial cells from mechanical ablation; we first used the relatively rigid-type soft contact lens (eg, the Plano B4), which is

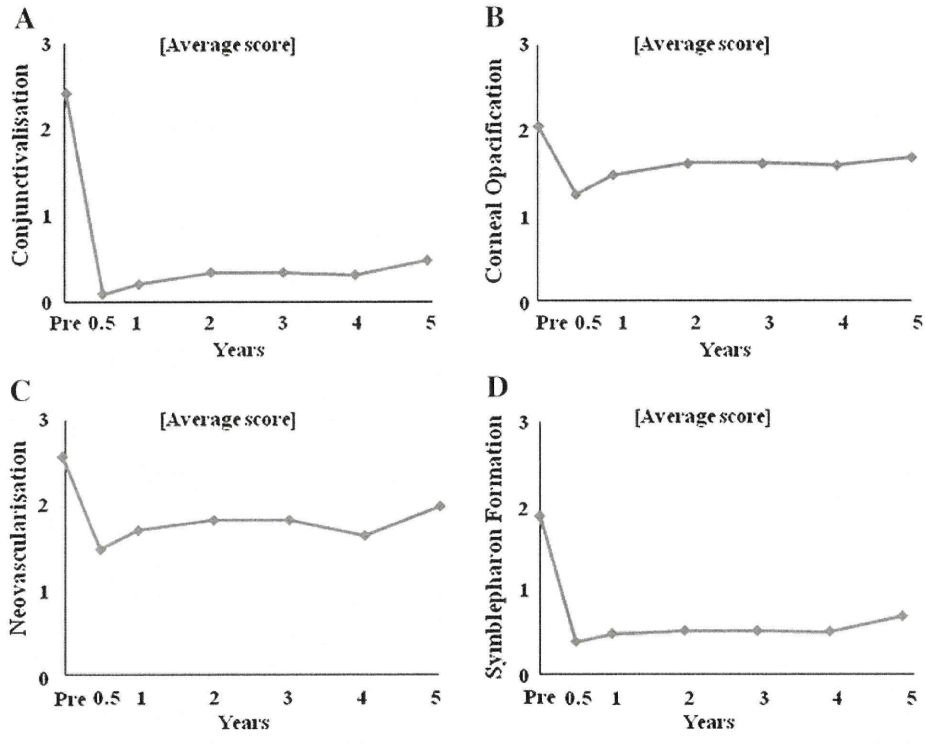

Figure 2 The results of the postoperative observation time course of clinical grading score regarding conjunctivalisation (A), corneal opacity (B), corneal neovascularisation (C), and symblepharon formation (D).

Table 2 Time course of postoperative complications

	Pre	6M	12M	24M	36M	48M	60M
Persistent epithelial defect	5% (1/19)	26% (5/19)	5% (1/19)	5% (1/19)	5% (1/19)	8% (1/12)	0% (0/8)
Ocular hypertension	5% (1/19)	10% (2/19)	15% (3/19)	5% (1/19)	5% (1/19)	8% (1/12)	12% (1/8)
Infection	0% (0/19)	5% (1/19)	0% (0/19)	0% (0/19)	0% (0/19)	0% (0/12)	0% (0/8)

M, months.

difficult to take off, and then we used the highly hydrated-type soft contact lens (Acuvue, O_2 Optics) to improve the permeability of oxygen to the corneal surface and to prevent epithelial damage due to lack of oxygen. (2) Within 1 week of COMET, almost all patients encountered dry-eye conditions, the severity of which depended upon the individual patient. Therefore, artificial eye drops should be instilled frequently (from every 15 min to 2 h).

Even though the morphological appearance of a cultivated oral mucosal epithelial sheet is quite similar to that of a cultivated corneal limbal epithelial sheet, one of the most distinguishable characteristics of a cultivated oral mucosal epithelial sheet is its distinctive fluorescein staining pattern. From our clinical experience, its staining pattern is more like that of superficial punctate keratopathy than conjunctival epithelium, but a strict discrimination between the two is somewhat difficult to observe by slit-lamp examination. Although the transplanted cultivated oral mucosal epithelial sheets in this study retained their transparency, there was a slightly high amount of light-scattering under the slit-lamp microscope examination, with or without fluorescein staining, thus affecting the postoperative corneal opacity and resulting in a BCVA of 20/40 in the 19 eyes treated by COMET. For most eyes, the BCVA was between 20/2000 and 20/200, suggesting that light-scattering on the transplanted oral mucosal epithelium on the cornea reached the level that controls VA at around 20/500. In contrast, the BCVA of some CLET patients reached 20/20, indicating that the biological character of the cells clearly affects the quality of VA.

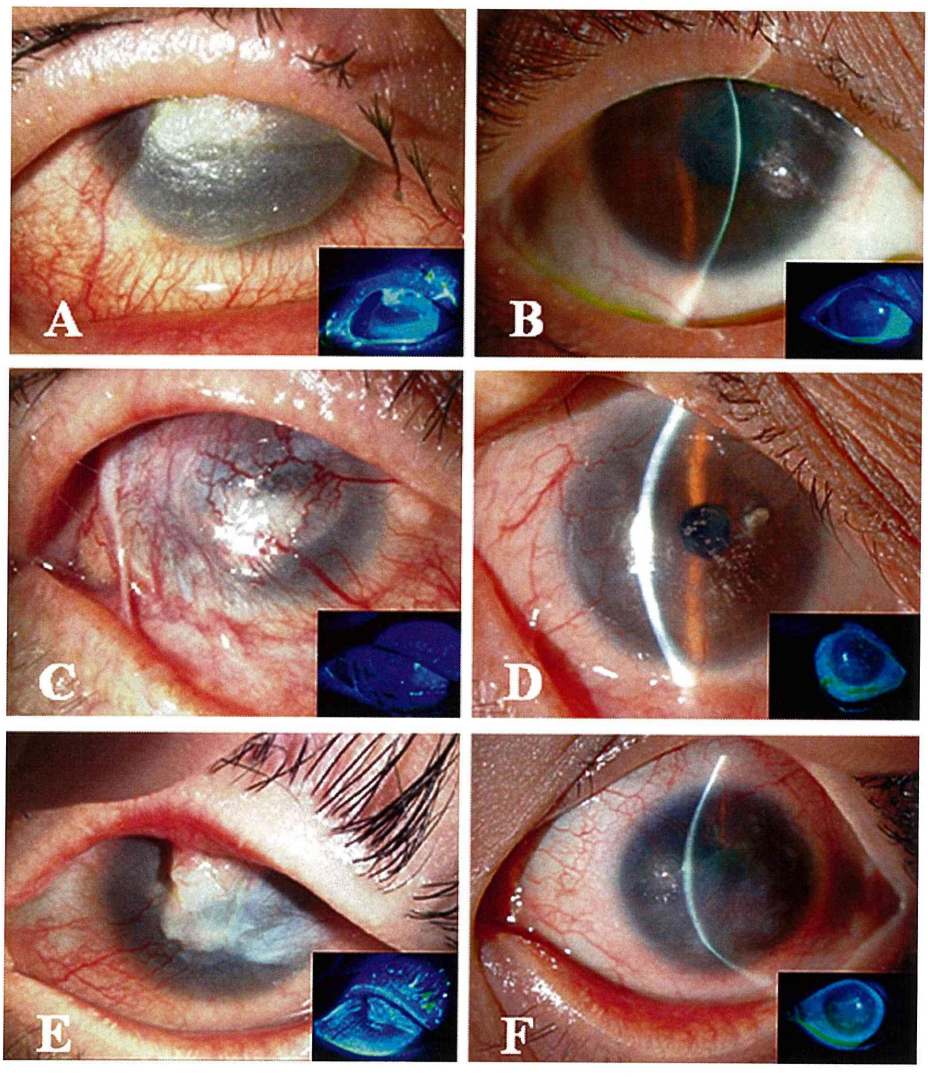

Figure 3 The clinical progress of three representative patients with severe OSD arising from SJS (A, B), idiopathic OSD (C, D) and SJS (E, F). Before transplantation all eyes manifested severe destruction of the ocular surface with limbal stem cell deficiency (A, C and E). The postoperative appearance at 50 (B) and 71 (D) months shows a relatively smooth, epithelialised corneal surface with minimal corneal neovascularisation, scarring and inflammation. The postoperative appearance at 72 months (F) shows that due to the persistent epithelial defect during the follow-up periods, postoperative corneal opacity is somehow remarkable with modest neovascularisation, finally affecting the transparency of the cornea even though the ocular surface is relatively stable. OSD, ocular surface disease; SJS, Stevens–Johnson syndrome.

The patients with OSD reported here were the most severe cases we encountered, necessitating the reconstruction of not only the corneal surface but also the surrounding ocular surface. Of the 19 eyes in this study, 90% and 74% of the cases received treatment by MMC and AMT, respectively, to prevent the postoperative proliferation activity of the subconjunctival tissue and to reconstruct the ocular surface including the conjunctival fornix. After COMET, transplanted cultivated oral epithelial cells were always observed to migrate outwards on the AM, ultimately covering the complete ocular surface. Through the simultaneous combination of these procedures, postoperative symblepharon formation was significantly inhibited during the long-term follow-up and we believe that this is the one of the most beneficial advantages of COMET. In addition, of the 19 eyes, 26% of the cases simultaneously received cataract surgery to improve the VA. Even though the intraocular visibilities in patients with OSD were often very bad, we developed the surgical technique as a step-by-step process and can now perform it safely through the use of a surgical slit-lamp microscope and indocyanine green staining of the anterior lens capsule.

We carefully assessed the clinical safety of COMET and found that postoperative PED sometimes occurred in our series. Of the 19 eyes, PED occurred in 7 eyes (37%) at least once during the long-term follow-up. Of those seven eyes, five eyes (71%; all with SJS) were systemic primary OSD. It has been reported that the health of the oral mucosal epithelium in vivo depends on the existing diseases.[22] Even though we were able to generate a cultivated oral mucosal epithelial sheet from systemic primary OSD, whose morphological features are quite similar to in vivo corneal epithelium, the biological ability of the oral epithelium cells may potentially be damaged in these patients. This issue is currently under investigation in our laboratory.

Of the 19 eyes, ocular hypertension was observed in a total of 3 eyes (16%) during the postoperative follow-up period. Since the transplanted cultivated sheets were not completely identical to in vivo epithelium, care must be taken in regard to postoperative epithelial damage caused by the use of antiseptic eye drops. Thus, a major clinical point is that in two eyes in this study, the occasional increase in IOP was found to be better managed by the administration of a carbonic anhydrase inhibitor.

In our series, postoperative corneal infections were relatively few (one eye, 5%) as compared to allogeneic CLET or limbal transplantation,[9 23] simply because COMET is an autologous transplantation and patients did not need the intensive, prolonged postoperative immunosuppressant therapy, thus resulting in the avoidance of an immunocompromised state. Interestingly, in view of the findings in this study and our recent clinical experiences, the postoperative corneal infection in our cases mainly occurred within 6 months after surgery, and all of those cases were systemic OSD patients. Furthermore, all of those cases were detected to be caused by MRSA.[24] It has been reported that MRSA is frequently detected from the ocular surface in patients with SJS as compared with normal subjects; therefore, it is important to carefully observe the corneal surface in the early postoperative periods, especially in patients with SJS.

In conclusion, this is the first study that demonstrates the long-term clinical results of COMET for ocular surface reconstruction in the treatment of the scar phase of severe OSD. We found that COMET permits sustained reconstruction of the ocular surface epithelium in many eyes with severe OSD. The management of postoperative PED and neovascularisation may further increase the efficacy of this type of transplantation.

Acknowledgements We thank Mr J Bush for editing our manuscript, and Ms Saito, Horikiri and Mano for assisting with the culture procedures.

Funding Supported in part by Grants-in-Aid for scientific research from the Japanese Ministry of Health, Labour and Welfare (H16—Saisei-007), and the Japanese Ministry of Education, Culture, Sports, Science and Technology (Kobe Translational Research Cluster), a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

Competing interests None.

Ethics approval This study was conducted with the approval of the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine.

Provenance and peer review Not commissioned; externally peer reviewed.

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Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders

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Br J Ophthalmol 2011 95: 942-946 originally published online November 19, 2010
doi: 10.1136/bjo.2010.188714

Amino Acid Profiles in Human Tear Fluids Analyzed by High-Performance Liquid Chromatography and Electrospray Ionization Tandem Mass Spectrometry

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• PURPOSE: To identify the 23 amino acid profiles in human tear fluids, and to evaluate whether the ocular disease conditions reflect the amino acid profiles.
• DESIGN: Laboratory investigation.
• METHODS: We evaluated the concentrations and relative composition of 23 amino acids in tear fluids obtained from 31 healthy volunteers using reversed-phase high-performance liquid chromatography and electrospray ionization tandem mass spectrometry, and compared them with those in plasma and aqueous humor. We also evaluated the tear-fluid amino acid profiles from 33 affected subjects.
• RESULTS: The amino acid profiles of the basal tear and reflex tear were found to be similar, and 4 distinct groups of healthy volunteers (male, female, young, and elderly) showed similar profiles. Absolute concentrations of taurine (Tau) and L-glutamine were significantly dominant in these tear fluids. The relative compositions of Tau, L-glutamic acid, L-arginine (Arg), and citrulline in the tear fluid were significantly higher than those in the plasma and aqueous humor. Analysis of the hierarchical clustering of the amino acid profiles clearly distinguished severe ocular surface diseases from non–ocular surface diseases. The relative compositions of Tau, L-methionine, and Arg decreased in severe ocular surface disease subjects compared with non–ocular surface disease subjects.
• CONCLUSIONS: Tear-fluid amino acid profiles differ from those in plasma and aqueous humor. Steady-state tear-fluid amino acid profiles might reflect ocular-surface homeostasis and the observed changes of amino acids might have a close relation with the disease conditions on the ocular surface. (Am J Ophthalmol 2011;151:799–808. © 2011 by Elsevier Inc. All rights reserved.)

Accepted for publication Nov 1, 2010.

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METABOLISM CAN BE VIEWED AS A NETWORK that can adapt to various nutritional conditions and that may become disturbed during disease and physiologic insults. Specific variations in amino acid profiles in blood have been reported in the context of liver failure,[1] renal failure,[2] cancer,[3] diabetes,[4] and so on. Conventionally, amino acid has long been considered a source of protein synthesis in the nutritional term, and the existence of a free amino acid supply to the tissues plays a pivotal role in maintaining organ and body protein homeostasis.[5] However, besides their role as substrates for protein synthesis, amino acids have multiple and critical functions, not only in maintaining baseline steady-state homeostasis, but also in the pathophysiology of diverse human disorders. It is now widely accepted that changes in amino acid availability have profound effects on many aspects of cellular functions, including the regulation of cell signaling, gene expression, and the transport of amino acids themselves.[6–8] For example, the pathophysiologic relevance of L-glutamine (Gln, GluNH2), L-arginine (Arg), and L-leucine (Leu) have been implicated in severely traumatized patients,[9] in the inflammatory response,[10] and in activating the mammalian target of rapamycin (mTOR).[11,12]

Tear fluids provide oxygen and other nutrients, as well as chemical mediators including antimicrobial and immunologic mediators. Considering the relevance of amino acids and glucose in the homeostatic metabolism of tissues, the profound understanding of the function of amino acid in tear fluids is as crucial as that of chemical mediators. Previously, a few reports have presented contradicting results on the amount of a limited number of amino acids in human tears.[13,14] The existence of a significantly higher concentration of L-valine (Val), L-isoleucine (Ile), and L-histidine (His) has been reported, and with the exception of L-aspartate (Asp), L-glutamic acid (Glu), and taurine (Tau), the quantities found in tears were at a comparable level with those found in plasma.[13,14]

Today, amino acid profiles for biological specimens are commonly analyzed by ion-exchange chromatography, a method in which amino acids and related compounds can be measured.[12,15] Recently, a new method for rapidly analyzing amino acids was developed that involves derivatization with a novel reagent, followed by reversed-phase

0002-9394/$36.00
doi:10.1016/j.ajo.2010.11.003

high-performance liquid chromatography and electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS).[16] Now, more than 100 different analytes with amino groups can be measured within 10 minutes by the combination of the precolumn derivatization and HPLC/ESI-MS/MS techniques. This method represents an alternative to traditional amino acid analysis techniques. The aim of this study was to reveal and describe the amino acid profiles in human tear fluids since this new method now makes it possible to analyze samples in trace amounts (below 0.5 μL) and with a superior reproducibility.[16] In addition, the possible physiological and pharmacologic function of amino acids will be discussed.

METHODS

• NORMAL VOLUNTEER SUBJECTS: Enrolled in this study were normal volunteer subjects with no corneal-, conjunctival-, or lacrimal-system abnormalities as assessed by slit-lamp examination. Free amino acids were evaluated in blood samples obtained from 11 healthy young male (mean age: 22.9 ± 1.4 years), 6 healthy young female (mean age: 21.0 ± 0.7 years), 6 healthy elderly male (mean age: 69.5 ± 3.8 years), and 11 healthy elderly female (mean age: 71.5 ± 4.0 years) volunteer subjects. Basal tear samples were collected from the young and elderly subjects (n = 31; young male: 10; young female: 6; elderly male: 6; elderly female: 9) and reflex tear samples were also collected from the young subjects (n = 16; male: 10; female: 6). Basal and reflex tear fluid samples (0.5−1.0 μL) were collected from the inferior tear meniscus of each subject using a microcapillary tube. Reflex-tear stimulation was initiated by inserting an applicator into the nose of each subject. Aqueous humor samples were obtained from the elderly subjects (n = 16; male: 6; female: 10) through the use of a 1-mL syringe with a 30-gauge needle prior to cataract surgery, and all of the obtained samples were free of contamination by blood fluids.

• PREPARATION OF TEAR FLUID AND AQUEOUS HUMOR SAMPLES: Each tear sample was transferred into a 0.5-mL sterile microfuge tube and then centrifuged. Supernatants were stored at −70°C until assaying for the amino acid levels. After thawing, 0.5 μL of each tear sample was diluted with 4.5 μL of sterile purified water, and then extracted by the addition of 20 μL of acetonitrile and mixing with a vortex mixer. The samples were centrifuged at 10 000 rpm for 1 minute, and the supernatants were then analyzed. A quantity of 1.0 μL of aqueous humor was used for each sample, with the same dilution as that of the tear fluid.

• PATIENTS WITH OCULAR SURFACE DISEASE AND CORNEAL OR SCLERAL DISEASE: Tear samples were collected from 33 affected subjects composed of 18 subjects with severe ocular surface diseases and 15 subjects with corneal or scleral diseases. Severe ocular surface diseases included Stevens-Johnson syndrome (SJS) (n = 10), chemical injury (n = 4), thermal burn (n = 2), and stem cell deficiency from an unknown cause (n = 2). The corneal or scleral diseases of the other 15 subjects included granular dystrophy (n = 2), band-shaped keratopathy (n = 2), keratoconus (n = 2), bullous keratopathy (n = 2), lattice dystrophy (n = 1), corneal erosion (n = 1), postcorneal infection (n = 2), necrotizing keratitis (n = 1), and scleritis (n = 2). Those 15 subjects were all classified as non–ocular surface disease.

• BIOCHEMICAL ASSAYS OF TEAR FLUID AMINO ACID AND AQUEOUS HUMOR AMINO ACID: For measurement of the amino acid concentration in the tear fluid and aqueous humor samples, we adopted precolumn derivatization with AccQ·Tag (Waters Corporation, Milford, Massachusetts, USA) to increase the ionization efficiency of the adducts before being analyzed by the multiple-reaction monitoring mode of reversed-phase HPLC HP1100 series (Agilent Technologies, Inc, Palo Alto, California, USA) and triple quadrupole tandem mass spectrometry (API4000 LC/MS/MS system; Applied Biosystems, Inc, Foster City, California, USA).[16–18] For derivatization with 20 μL of AccQ·Tag reagent, 20 μL of a deproteinized tear sample was added to 60 μL of 0.2-M borate buffer (pH 8.8) and then heated at 55°C for 10 minutes. The reaction mixture was diluted with 100 μL of 0.2% acetic acid and 5 μL of the mixture was then injected onto the HPLC column (L-Column, 50 mm × 2.0 mm, 3-μm particles; Chemicals Evaluation and Research Institute, Tokyo, Japan) prior to MS detection at a flow rate of 0.25 mL/min.

• BIOCHEMICAL ASSAYS OF PLASMA AMINO ACID: The plasma was separated and deproteinized in a final concentration of 3% sulfosalicylic acid. All samples were stored at −70°C until measurement using HPLC (SRL Inc, Tokyo, Japan). The basic amino acid and related molecules (23 compounds) were measured and used in the analysis. Those compounds are as follows: L-tyrosine (Tyr), Val, Leu, L-methionine (Met), Ile, Gln, L-serine (Ser), L-lysine (Lys), L-asparagine (Asn, AspNH2), L-threonine (Thr), L-alanine (Ala), Glu, His, L-ornithine (Orn), L-cystine (Cys2), L-proline (Pro), L-tryptophan (Trp), Arg, Tau, glycine (Gly), citrulline (Cit), Asp, and L-phenylalanine (Phe).

• STATISTICAL ANALYSIS: Statistical analysis was performed using SPSS statistical analysis software (SPSS Inc, Chicago, Illinois, USA). Hierarchical clustering analysis was performed using JMP7.0 software (SAS Institute, Inc, Cary, North Carolina, USA). The correlations among 23 amino acid concentrations as well as