TABLE 1. Concentration (Mean ± SEM, μM) of Each Amino Acid in the Basal Tears, Reflex Tears, Aqueous Humor, and Plasma of Normal Subjects

	Basal Tear (n = 31)	Reflex Tear (n = 16)	Aqueous Humor (n = 16)	Plasma (n = 34)
Gly	10.7 ± 2.8	14.4 ± 7.1	0.4 ± 0.1	231.3 ± 11.1
Ala	23.8 ± 4.8	19.5 ± 8.5	14.4 ± 2.7	385.5 ± 14.5
Val	3.5 ± 0.9	3.8 ± 1.4	6.7 ± 1.1	244.7 ± 8.5
Leu	10.1 ± 1.9	6.4 ± 1.9	3.3 ± 0.6	125.4 ± 6.1
Ile	1.1 ± 0.3	1.1 ± 0.5	1.5 ± 0.3	68.7 ± 3.6
Ser	14.4 ± 4.2	26.4 ± 13.0	3.9 ± 0.8	113.6 ± 3.3
Thr	5.4 ± 1.2	8.4 ± 3.6	3.5 ± 0.7	127.6 ± 3.8
Met	2.4 ± 0.1	2.2 ± 0.1	0.9 ± 0.2	28.1 ± 1.3
AspNH2 (Asn)	1.4 ± 0.3	1.5 ± 0.7	1.0 ± 0.2	49.6 ± 1.5
GluNH2 (Gln)	42.1 ± 6.8	25.4 ± 6.3	28.7 ± 5.2	589.5 ± 11.0
Pro	8.4 ± 1.4	6.7 ± 1.5	0.5 ± 0.1	180.2 ± 8.5
Phe	10.7 ± 1.9	5.7 ± 1.5	2.8 ± 0.5	63.7 ± 2.0
Tyr	1.3 ± 0.5	1.1 ± 0.5	2.8 ± 0.6	73.5 ± 3.8
Trp	7.1 ± 0.7	6.6 ± 1.0	0.9 ± 0.1	52.9 ± 1.8
Asp	13.2 ± 4.5	18.2 ± 11.6	0.0 ± 0.0	1.8 ± 0.2
Glu	30.2 ± 4.8	20.9 ± 4.8	0.1 ± 0.0	42.3 ± 2.8
Lys	5.7 ± 1.2	3.5 ± 1.2	3.1 ± 0.6	195.7 ± 7.2
Arg	18.7 ± 1.6	14.4 ± 2.5	2.5 ± 0.5	82.7 ± 3.0
His	1.9 ± 0.7	3.2 ± 1.9	1.5 ± 0.3	83.7 ± 2.0
Cit	10.1 ± 0.8	8.2 ± 1.5	1.2 ± 0.2	35.1 ± 1.7
Orn	3.3 ± 1.1	7.1 ± 3.0	0.4 ± 0.1	79.3 ± 4.9
Cys2	1.0 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	49.0 ± 2.4
Tau	195.1 ± 26.9	100.1 ± 18.7	1.2 ± 0.3	57.7 ± 2.0

Ala = L-alanine; Arg = L-arginine; Asp = L-aspartate; AspNH2 (Asn) = L-asparagine; Cit = citrulline; Cys2 = L-cystine; Glu = L-glutamic acid; GluNH2 (Gln) = L-glutamine; Gly = glycine; His = L-histidine; Ile = L-isoleucine; Leu = L-leucine; Lys = L-lysine; Met = L-methionine; Orn = L-ornithine; Phe = L-phenylalanine; Pro = L-proline; Ser = L-serine; Tau = taurine; Thr = L-threonine; Trp = L-tryptophan; Tyr = L-tyrosine; Val = L-valine.

among plasma and/or ophthalmic fluid (basal tear, reflex tear, and aqueous humor) samples were clustered using the Ward method. For the red>gray>blue color system, the depth in the red color or blue color reflected the different degree of values above or below the mean (gray color), respectively. Graphic presentations of box plots were generated using R (R Foundation for Statistical Computing, Vienna, Austria). In each box plot, the top and bottom of the boxes were the first and third quartiles, respectively, and the length of the box represented the inter-quartile range within 50% of the values that were included. The horizontal line within each box represented the median, the vertical lines showed the largest/smallest observation that fell within a distance of 1.5 times the box size from the nearest quartile in the box, and the additional points were considered "extreme" values and are shown separately.

These presentations and analyses were performed based on each of the absolute amino acid concentrations (μM) or relative amino acid composition (percentage), calculated by each amino acid concentration/total 23 amino acid concentrations.

RESULTS

• **COMPARISON OF AMINO ACID PROFILES AMONG TEAR FLUID, AQUEOUS HUMOR, AND PLASMA SAMPLES OF NORMAL SUBJECTS:** Amino acid concentrations in a trace amount of tear fluids of 0.5 to 1.0 μL were able to be measured reproducibly by HPLC/ESI-MS/MS. The reproducibility of the measurements was confirmed in separate experiments using normal tear fluids. The Table shows the concentrations of each amino acid in the basal tear, the reflex tear, the aqueous humor, and the plasma of normal subjects. A significant difference of amino acid profiles between the tear fluid, the aqueous humor, and the plasma samples was confirmed. The concentration of total amino acids in the tear fluid samples (means ± SEM: 382.1 ± 42.6 μM) was lower than that in the plasma samples (2961.6 ± 63.1 μM) but higher than that in the aqueous humor samples (81.6 ± 15.2 μM). Ala, Val, and Gln were dominant components in the plasma samples (more than 240 μM). Gly, Pro, and Lys were also major components in the plasma samples.

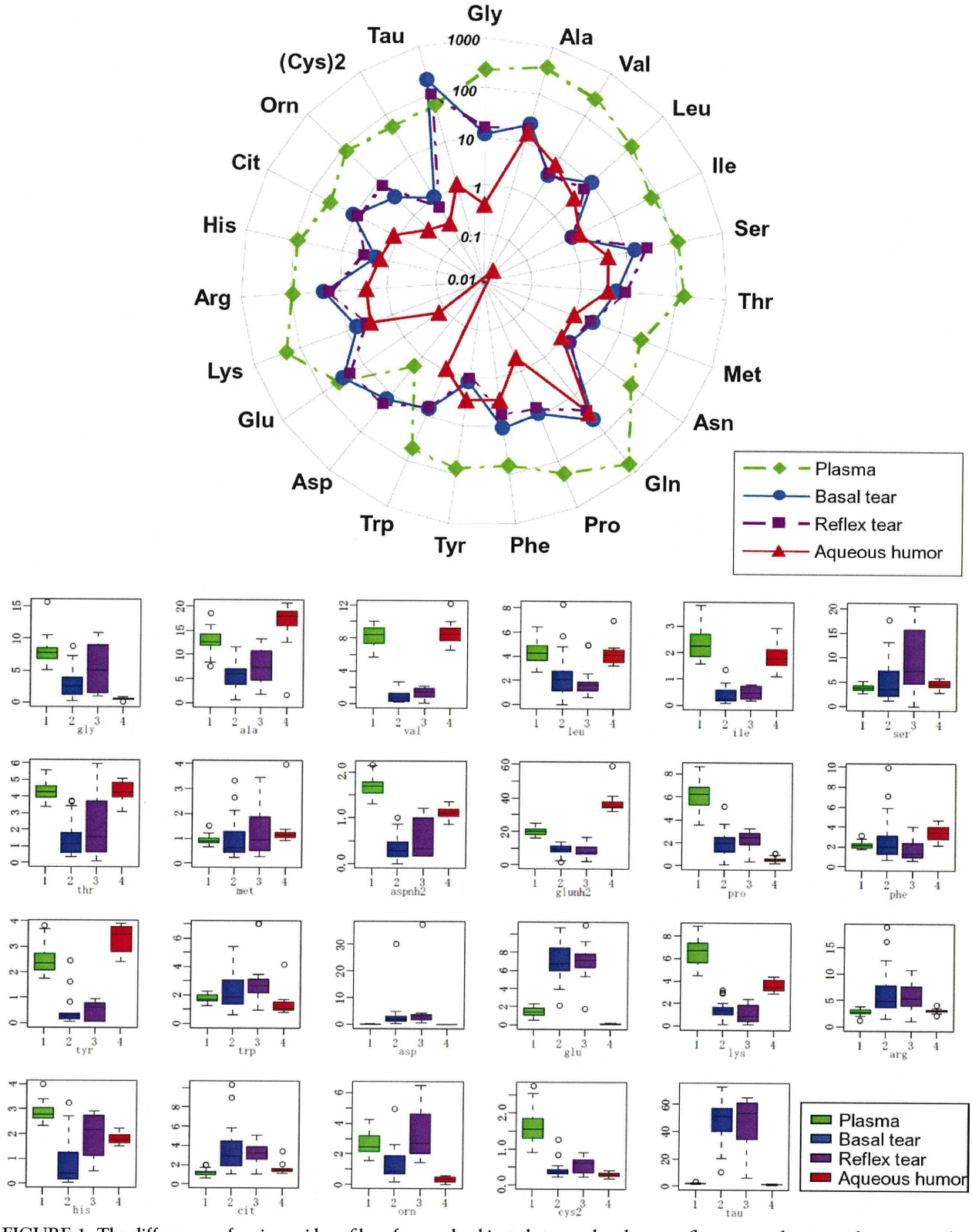

FIGURE 1. The differences of amino acid profiles of normal subjects between basal tear, reflex tear, and aqueous humor samples, compared with that of plasma samples. (Top) Mean values of 23 amino acid concentrations (μM) of basal tear (n = 31) (blue), reflex tear (n = 16) (purple), aqueous humor (n = 16) (red), and plasma (n = 34) (green) samples from normal subjects are shown in

In the tear fluids, Tau, Glu, and Gln were dominant components (more than 25 μM). Arg and Cit were of a higher concentration than other amino acid concentrations, whereas Val, Ile, Met, Asn, Tyr, His, and Orn were of a lower concentration (below 5 μM) (Table, and Figure 1, Top). Notably, the concentrations of Tau and Glu were significantly higher than (3.4 times higher) or comparable with (0.7 times higher) those in plasma samples. Asp also was of a higher value than that in plasma samples. The amino acid concentrations and profiles of the basal tear fluid and reflex tear fluid were found to be similar.

Since it has been shown that the metabolomic profiling of amino acid can be helpful in revealing specific physiological conditions or states, and that the ratios between some specific amino acids can be useful in diagnosing them,[19] we performed some analyses based on not only amino acid concentrations but also relative amino acid composition (Figure 1, Bottom). The composition of amino acid in aqueous humors was rather analogous to that in plasmas, although the levels of Gly, Pro, and Orn were low. In the tear fluid samples, Tau and Glu were of a higher relative amino acid composition (25.0 times higher and 4.9 times higher, respectively; $P < .001$) (Tukey test) than those in the plasma samples, yet the aqueous humor samples were not (Tau: 0.73 times higher, Glu: 0.09 times higher). The relative compositions of Arg and Cit in the tear fluid samples were smaller but statistically significantly higher than those in the plasma and aqueous humor samples. There was no significant difference between the basal tear fluid and reflex tear fluid in 23 relative amino acid compositions (Tukey test).

In an aim to build up a simple visual presentation, the correlations among 23 amino acid concentrations and the relative amino acid composition of 31 basal tear fluid, 16 reflex tear fluid, 16 aqueous humor, and 34 plasma samples were clustered (Figure 2). Plasma samples were clearly clustered at the highest position, followed by tear fluid samples and then aqueous humor samples.

• COMPARISON OF TEAR-FLUID AMINO ACID PROFILES AMONG YOUNG AND ELDERLY VOLUNTEERS: The change of amino acid concentration attributable to aging is known to exist in other body fluids.[20] In this study, a preliminary analysis was carried out on the basal tear fluid and plasma samples of adults that differed by both age and sex. The mean values of 23 amino acids in relation to concentration and composition that were obtained from young male (n = 10), young female (n = 6), elderly male (n = 6), and elderly female (n = 9) normal subjects were examined. The Tukey test showed that the differences were not statistically significant. Hierarchical clustering charts of both the concentration and composition did not exhibit any distinct clustering trends among these 4 groups (data not shown).

• DISTINCTIVE CHANGE OF AMINO ACID PROFILES IN TEAR FLUIDS FROM DISEASED EYES: The difference of amino acid concentrations and profiles among tear fluids from individuals of severe ocular surface disease and non–ocular surface disease was investigated. Means of the 23 amino acid concentrations (μM) from severe ocular surface disease (n = 18) and non–ocular surface disease (n = 15) were shown in a radar chart (Figure 3, Top). The concentrations of amino acid were highly elevated in tear fluids from severe ocular surface disease subjects compared with those from non–ocular surface disease subjects. Four amino acids (Val, Ile, Tyr, and Glu) showed a significant P value of less than .01, and 15 amino acids showed a P value of less than .05 (Student t test). Of important note is the fact that the changes were not restricted to the concentration, but also extended to the amino acid profile, namely composition.

Next, the distribution pattern of the amino acid composition in tear fluids from the severe ocular surface disease subjects was compared with those from the non–ocular surface disease subjects. Amino acid composition (%) of tear fluids from 15 non–ocular surface disease subjects (column 1, left) and 18 severe ocular surface disease subjects (column 2, right) are shown in the box plots (Figure 3, Bottom). The significant changes were the decrease in Arg, Met, and Tau and the increase in Orn, Lys, and Thr in the severe ocular surface disease subjects.

Analysis of the hierarchical clustering of the amino acid profiles of tear fluids from severe ocular surface disease subjects and non–ocular surface disease subjects indicated clear clustering of 12 of the 18 specimens from individuals with severe ocular surface disease when classified on the basis of amino acid composition (Figure 4). Interestingly, the composition-based clustering exhibited 5 distinctive clusters of amino acid, namely Tau/Met/Arg, Asn/Tyr/Val/Ile/Lys, Glu/Gln, Cit/Leu/Phe/Trp/Pro, and Asp/His/Thr/Orn/Ser/Gly/Ala. Decreased amino acid, Tau, Met, and Arg were included in 1 cluster, which suggests a correlation of the metabolism and/or transport of these amino acids in inflammatory eyes.

DISCUSSION

IN THIS STUDY, WE PERFORMED THE SYSTEMATIC QUANTIFICATION of free amino acids in human tear fluids by HPLC/ESI-MS/

the radar chart. (Bottom) Amino acid composition data (percentages) from plasma (n = 34) (green), basal tear (n = 31) (blue), reflex tear (n = 16) (purple), and aqueous humor (n = 16) (red) samples are shown in the box plots. The box represents the first and third quartiles, and the horizontal line within each box represents the median. The vertical lines show the largest/smallest observation, and the additional points are "extreme" values.

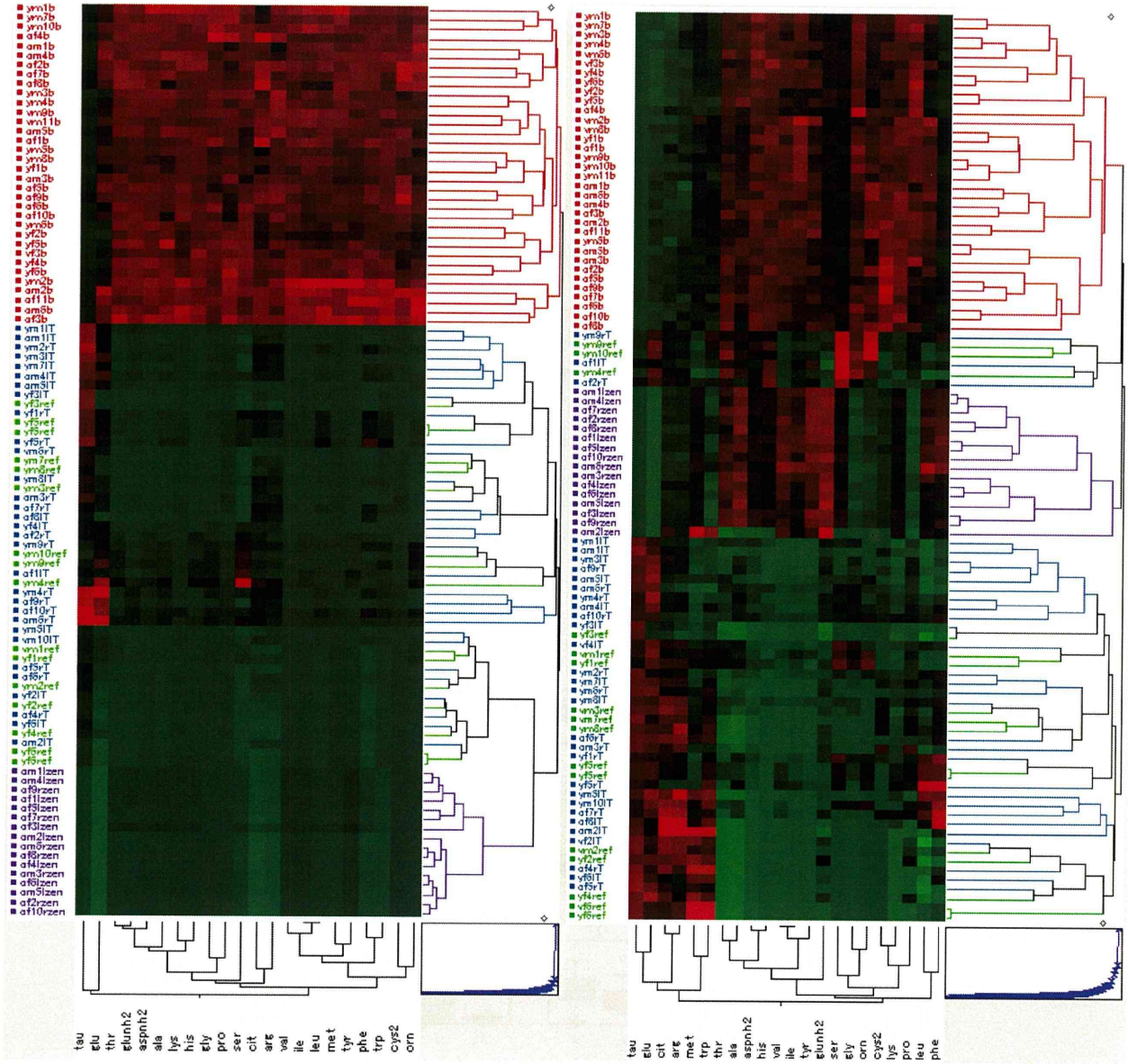

FIGURE 2. Hierarchical clustering of the amino acid profiles of basal tear, reflex tear, aqueous humor, and plasma samples of normal subjects. The correlations among 23 amino acid concentrations (Left) and relative amino acid compositions (Right) of basal tear (n = 31) (blue), reflex tear (n = 16) (green), aqueous humor (n = 16) (purple), and plasma (n = 34) (red) samples were clustered using the Ward method.

MS. The amino acid profiles showed the following characteristics: 1) the amino acid profiles in tear fluids differ from those in aqueous humors and plasmas, 2) the absolute concentrations of Tau, Glu, and Asp in tear fluids are significantly higher than, or comparable with, those in plasma samples, 3) the amino acid profile in the aqueous humor samples was rather analogous to that in the plasma samples, 4) the amino acid profiles of the tear fluid of distinct groups (male vs female, young vs elderly) are similar, and 5) amino acid profiles of tear fluids from severe ocular surface disease subjects differed from those of non–ocular surface disease subjects.

The combination of the precolumn derivatization and HPLC/ESI-MS/MS techniques enabled the analysis of 120 body fluid samples in a single day and may possibly provide an alternative to traditional techniques used for amino acid analysis. From this viewpoint, we theorize that our high-speed, reliable procedure for amino acid analysis will prove to be a useful technique for the diagnosis and management of inherited disorders of amino acid metabolism in the clinical setting. In this study, we discovered that almost all amino acids exist in tear fluid. The plasma concentration of amino acid is the sum of its rates of appearance in and disappearance from plasma, and amino acid appearance

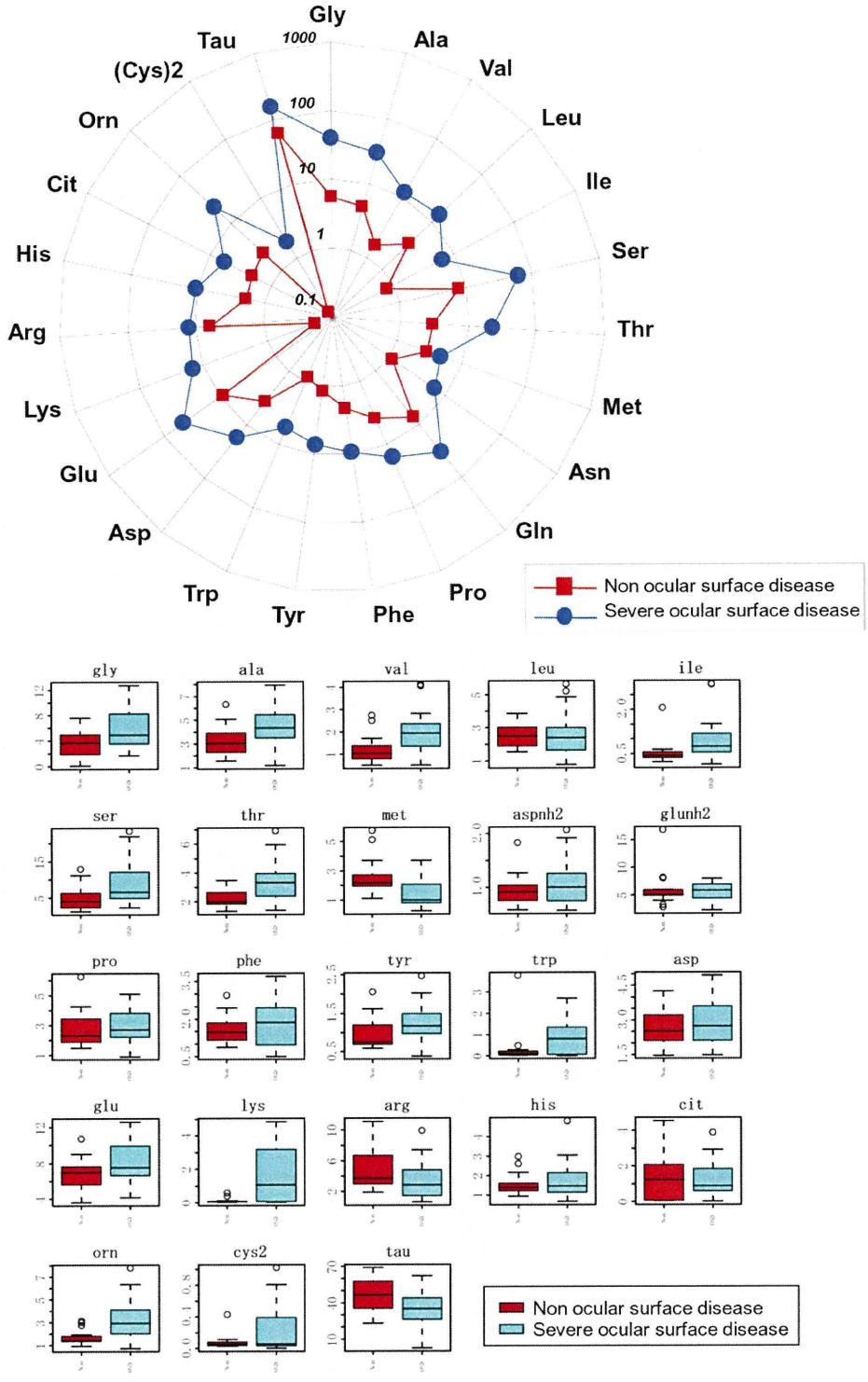

FIGURE 3. The differences of amino acid concentrations between tear fluids from severe ocular surface disease subjects and non–ocular surface disease subjects. (Top) Means values of 23 amino acid concentrations (μM) of tears from severe ocular surface disease subjects (n = 18) (blue) and non–ocular surface disease subjects (n = 15) (red) are shown in the radar chart. (Bottom) Amino acid composition data (percentages) of tears from non–ocular surface disease (n = 15) (red; left) and severe ocular surface disease (n = 18) (blue; right) subjects are shown in the box plots.

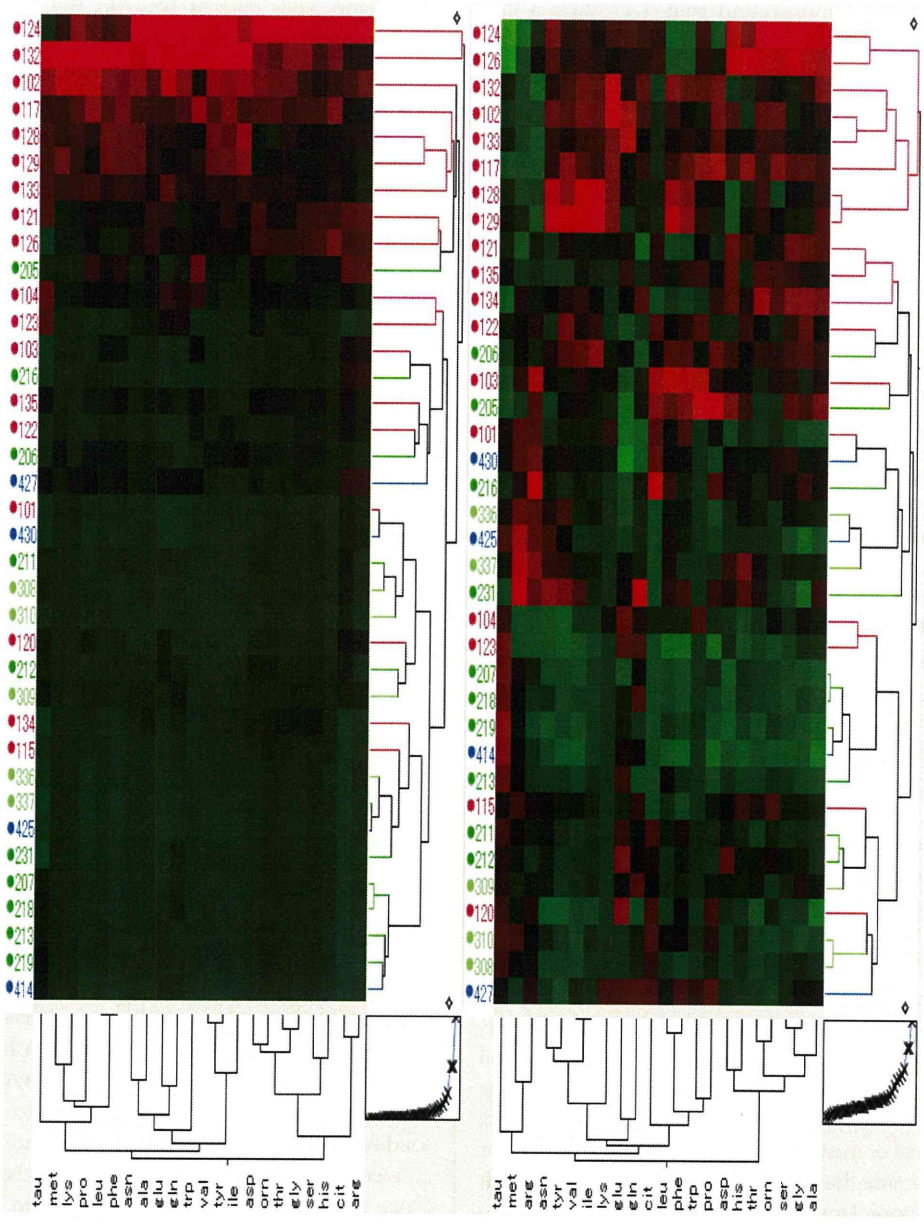

FIGURE 4. Hierarchical clustering of the amino acid profiles of tear fluids from severe ocular surface disease subjects and non–ocular surface disease subjects. The correlations among 23 amino acid concentrations (Left) and relative amino acid compositions (Right) of tear samples from severe ocular surface disease subjects (n = 18) (red) and non–ocular surface disease subjects (n = 15) (green) were clustered using the Ward method. The concentrations and relative composition of 23 amino acids in tear fluids differ from those in plasma and aqueous humor. Analysis of the hierarchical clustering of the amino acid profiles distinguished severe ocular surface diseases from non-ocular surface diseases. 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.

and disappearance are tightly regulated. In some circumstances, tissue uptake of amino acid and further metabolism depend on plasma amino acid concentrations. Mere determinations of plasma amino acid concentrations at the basal state (ie, postabsorptive) provide a rather limited amount of information.[21]

Previously, a few reports have presented contradicting results on the amount of limited kinds of free amino acids in human tears.[13,14] In those studies, the quantities found in tear fluids were at a comparable level with those found in plasma, except for the levels of Asp, Glu, and Tau. The high level for Tau in tear fluids was reported previously,[14] and Tau has been identified as a major player in numerous biological functions[22,23] and is involved in the regulation of the proinflammatory responses.[22] Very

recently, it has been demonstrated that Tau plays a key role in regulating epithelial barrier function.[24] In this study, the concentration of Tau was confirmed to be very high in the tear fluid samples compared with that in the plasma samples, consistent with the previous reports. These findings signal the necessity to discriminate the subtle function of Tau under physiological and pathologic environments. Plasma Tau levels are usually high, although decreases are observed in response to surgical injury and numerous pathologic conditions, including cancer and sepsis.[25] The observed higher concentration of Glu (and probably that of Asp as well) may reflect the energy demand at the luminal side of corneal/conjunctival epithelial cells. Mucosal tissue exhibits a high rate of aerobic glycolysis, and in intestinal metabolism, luminal Gln, Glu, and Asp, but not glucose, has been found to contribute critically to the respiratory CO_2 produced in this tissue.[26] Although considered nonessential, Gln becomes conditionally essential during severe catabolic stress in which intracellular and plasma Gln levels decrease rapidly.[27–29]

It is very difficult to discuss with clarity the underlying mechanism for the regulation of amino acid profiles in tear fluids, because to date, almost no information has been presented on the dynamic roles of ocular surface epithelial cells in regard to amino acid transportation. The presence of the Na^+-dependent neutral amino acid transporter has been reported in a rabbit primary corneal epithelial cell culture and rabbit corneas.[30] Whether all or part of the amino acid component of tear fluids is attributable to secretion, filtration, local synthesis, or local degradation of proteins is unknown. However, it is speculated that the amino acids are transported from tear fluids into the corneal tissues by this transport system. It is important to discuss the characteristic variance of amino acid profiles in tear fluid between the individuals with and without ocular surface disease. Diseases that develop because of the loss of corneal epithelial stem cells are known as limbal stem cell deficiency. Among limbal stem cell deficiency, SJS, ocular cicatricial pemphigoid, and severe chemical or thermal burns are known as severe ocular surface disease, because these diseases are intractable, even with corneal epithelial transplantation, and visual prognosis is poor. In cases of severe ocular surface disease, corneal neovascularization, ingrowth of fibrous tissue, and stromal scarring occur and often progress with chronic inflammation. However, the mechanisms of chronic inflammation and pathophysiology are still unclear. In this study, both the concentrations and relative composition of the amino acids differed between the severe ocular surface disease subjects and the non–ocular surface disease subjects. Hierarchical clustering on the amino acid profiles of tears clearly distinguished severe ocular surface disease subjects from non–ocular surface disease subjects. Surprisingly, hierarchical clustering also distinguished the eyes with SJS at the chronic stage as severe ocular surface disease, in which at least clinically, no inflammation and no scarring were detected. Amino acid profiles might be a sensitive marker of ocular surface inflammation, or they may be a new method to reflect ocular surface pathologic dynamics.

It is well known that supplemental Arg promotes wound healing following trauma shock. The major catabolic products of Arg are Orn and Cit. Because of the competition between nitric oxidase synthase and arginase for the same substrate Arg, their activities are regulated reciprocally. As for conditionally essential amino acids, Arg exhibited a significant decrease with a moderate decrease in Cit in the eyes with severe ocular surface disease, while Orn exhibited a prominent increase, indicating the possible polarization of tissue inflammation to the axis of arginase (oxidative tissue regeneration).[31–34] In this context, the reason why the increased composition of Orn over Cit, reflecting the high arginase activity, exists only in the tear fluids of individuals with chronic ocular surface inflammation is a subject that requires further investigation.

We can now harness information buried in amino acid profiles for the generation of diagnostic and surrogate markers. The methods described in this study might be applicable to the clinical setting and prove useful in diagnosing various physiologic and disease states at the ocular surface. Our results using cluster analysis of amino acid profiles in tear fluids suggest that the analysis can help us understand the complex interrelations that make up the metabolism of the ocular surface. In the future, a far more stringent study with controlled background factors will need to be performed to solidify the present observations. Amino acid represents a convenient set of metabolites that can be easily measured. Once the association of abnormalities in individual amino acid concentrations and/or composition with specific ocular surface diseases or physiologic conditions has been addressed, amino acid profiles for the generation of diagnostic and surrogate markers may prove to help advance biomedical and nutritional science in the field of ocular physiology.

PUBLICATION OF THIS ARTICLE WAS SUPPORTED IN PART BY HEALTH AND LABOR SCIENCES RESEARCH GRANTS (RESEARCH ON Intractable Diseases) from the Ministry of Health, Labour and Welfare of Japan, Tokyo, Japan, and the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan; a research grant from the Kyoto Foundation for the Promotion of Medical Science, Kyoto, Japan; and the Intramural Research Fund of Kyoto Prefectural University of Medicine, Kyoto, Japan. The authors indicate no financial conflict of interest. Involved in conception and design (C.S., J.H., S.K.); analysis and interpretation (M.N., C.S., K.S., N.O., H.M., J.H.); writing the article (M.N., C.S., A.O., J.H.); critical revision of the article (C.S., J.H., S.K.); final approval of the manuscript (M.N., C.S., K.S., N.O., H.M., A.O., J.H., S.K.); data collection (M.N., C.S.); provision of materials, patients, or resources (M.N., C.S.); statistical expertise (N.O., A.O.); obtaining funding (C.S., S.K.); and literature search (M.N., C.S., J.H.). All experimental procedures were approved by the Institutional Review Board for Human Studies at Kyoto Prefectural University of Medicine (KPUM). Prior informed consent was obtained from all patients, and this study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

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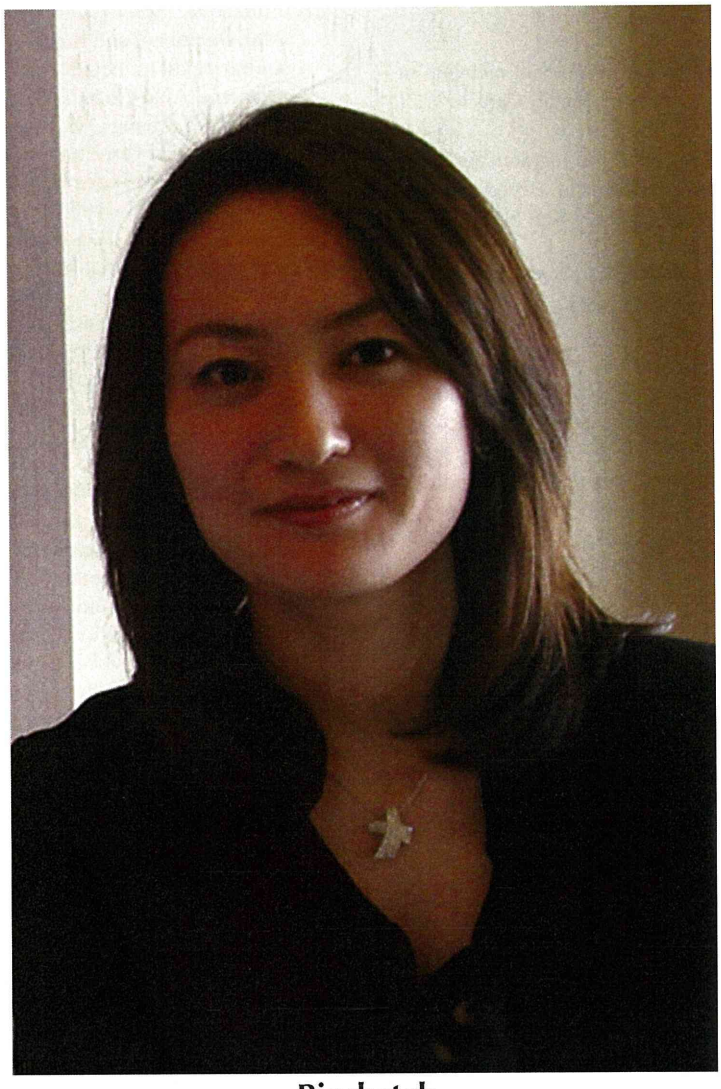

Biosketch

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Ocular Surface Reconstruction Using the Combination of Autologous Cultivated Oral Mucosal Epithelial Transplantation and Eyelid Surgery for Severe Ocular Surface Disease

KAZUNORI TAKEDA, TAKAHIRO NAKAMURA, TSUTOMU INATOMI, CHIE SOTOZONO, AKIHIDE WATANABE, AND SHIGERU KINOSHITA

• PURPOSE: To assess the surgical combination of autologous cultivated oral mucosal epithelial transplantation and eyelid surgery used to treat patients with severe ocular surface disease and entropion.

• DESIGN: Observational case series.

• METHODS: Three patients with severe thermal and chemical injury were treated by the surgical combination of autologous cultivated oral mucosal epithelial transplantation and everting sutures to correct entropion. Their clinical outcomes and the efficacy of this surgical procedure were assessed.

• RESULTS: The ocular surfaces were successfully reconstructed with autologous cultivated oral mucosal epithelial sheets and everting sutures without any complications during the operations. In the course of a mean follow-up period of 30 months their clinical outcomes were assessed. Postoperative follow-up showed that the simultaneous everting sutures caused no problems with the cultivated oral mucosal epithelial sheet, and there were no severe complications such as infection or inflammation. During the follow-up period, in 2 of the 3 eyes the ocular surface and eyelid remained stable with no recurrence of entropion.

• CONCLUSION: This case series presents a surgical approach to treat severely scarred ocular surfaces using the combination of autologous cultivated oral mucosal epithelial transplantation and everting sutures. Clinical outcomes suggest that this combined surgical procedure is a safe and useful method for the treatment of patients with severe ocular surface disease and entropion. (Am J Ophthalmol 2011;152:195–201. © 2011 by Elsevier Inc. All rights reserved.)

Accepted for publication Jan 15, 2011.

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CORNEAL EPITHELIAL STEM CELL DEFICIENCY RESULTING from severe ocular surface disease (OSD) such as thermal and chemical injury, Stevens-Johnson syndrome, and ocular cicatricial pemphigoid leads to visual loss attributable to conjunctivalization, vascularization, opacification, and symblepharon.[1–3] Specifically, various degrees of pathologic symblepharon and entropion frequently occur in patients with severe OSD and disturb the stability of the ocular surface. Moreover, eyelid abnormalities can often make a severe corneal disease worse because eyelid margin rotation or structural abnormalities impair tear spreading and corneal wetting. The eyelid pathologies include keratinization and tarsal scarring that causes and aggravates limbal stem cell deficiency (LSCD), as first reported by Di Pascuale and associates.[4] These abnormalities were later graded by Sotozono and associates.[5] Malfunction of the fornix by symblepharon can cause ocular surface diseases such as dry eye, blindness-related microtrauma, and an inflamed ocular surface resulting from cicatricial entropion, mal-aligned lashes, and restriction of ocular motility. Therefore, it was recently reported that the selection of the proper surgical approach depends on the severity of pathogenic symblepharon.[6,7] Moreover, it was also recommended that the eyelid and fornix abnormalities be corrected prior to performing ocular surface reconstruction.[7–9] In these types of patients, it is necessary to reconstruct not only the ocular surface epithelium but also the formation of the eyelid.

To reconstruct the ocular surface in patients with severe OSD, a variety of surgical approaches such as keratoepithelioplasty, limbal transplantation, and amniotic membrane (AM) transplantation have been used.[10–17] Due to the recent progress that has been made with regenerative medicine techniques, there have now been studies reporting the use of cultivated epithelial transplantation.[18–20] Since 2002, we have performed 61 cases of autologous cultivated oral mucosal epithelial transplantation for patients with severe OSD.[21,22] Among those cases, we have experienced 3 cases of severe entropion in thermal and chemical injury. The treatment of those 3 cases required the surgical combination of autologous cultivated oral

0002-9394/$36.00
doi:10.1016/j.ajo.2011.01.046

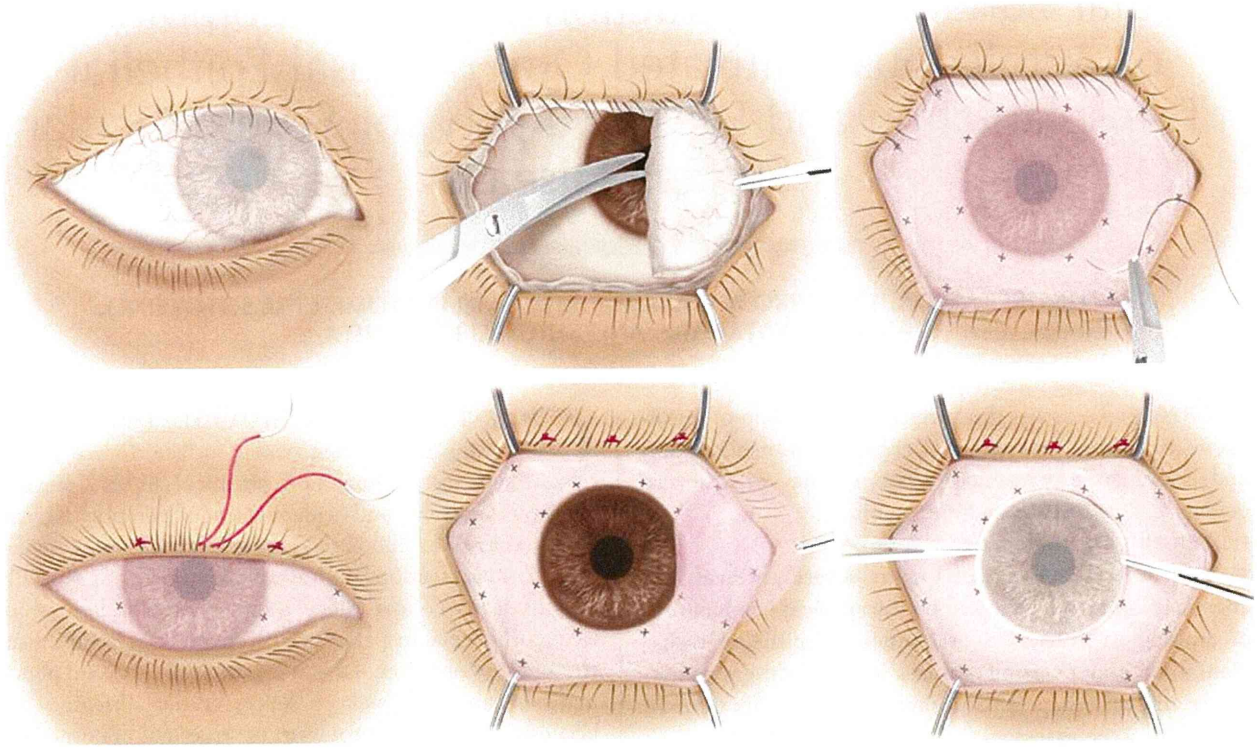

FIGURE 1. Surgical procedure used in the current study. (Top left) The eye manifested severe epithelial damage and entropion. (Top middle) The conjunctivalized tissue was completely removed by thin superficial keratectomy and peritectomy. (Top right) Transferred human amniotic membrane (AM) over the corneal surface. (Bottom left) A 6-0 nylon suture was passed through the eyelid from the tarsus side to the skin side. (Bottom middle) AM on the cornea was removed. (Bottom right) Finally, the autologous cultivated oral mucosal epithelial sheet was transferred onto the corneal surface.

mucosal epithelial transplantation and eyelid surgery, as we had experienced many previous cases where the ocular surface was severe damaged by cilia touching the surface because of entropion. In this present study, we report in detail for the first time the clinical outcome of using the surgical combination of autologous cultivated oral mucosal epithelial transplantation and eyelid surgery.

PATIENTS AND METHODS

THIS STUDY INVOLVED 3 EYES FROM 3 PATIENTS WITH total limbal deficiency that underwent autologous cultivated oral mucosal epithelial transplantation with everting sutures at Kyoto Prefectural University of Medicine, Kyoto, Japan, between August 2004 and October 2006. Their ages were 65 years (Case 1), 26 years (Case 2), and 32 years (Case 3). In Case 1, the primary reason for the patient's limbal deficiency was a severe, work-related chemical injury resulting from his right eye being exposed to the alkali of an aqueous-fluid solvent used for cleaning metal. He was found to have corneal edema and the manifested persistent epithelial defect (PED) involved disappearance of the palisades of Vogt and strong conjunctival hyperemia and chemosis with lower-eyelid entropion and symblepharon. The grading score for symblepharon severity was grade Ia 3+ (Kheirkhah score) and grade 2 (Sotozono score) according to the previous reports.[5,7] In Case 2, the primary reason for the patient's limbal deficiency was a severe thermal injury to his left eye attributable to its being hit by a bottle-rocket firework. He was found to have corneal opacity and the total corneal epithelial defect involved the disappearance of the palisades of Vogt with a large amount of hypopyon, conjunctivalization, entropion, symblepharon, and fibrovascular cicatrix to the upper fornix with the appearance of severe necrotic change in the conjunctiva and eyelid. The grading score for symblepharon severity was grade Ia 2+ (Kheirkhah score) and grade 1 (Sotozono score) according to the previous reports.[5,7] In Case 3, the primary reason for the patient's limbal deficiency was a severe thermal injury to his left eye attributable to its being hit by a bottle-rocket firework. He was found to have manifested almost-total PED involving the disappearance of the palisades of Vogt with conjunctivalization, entropion, and symblepharon, and the appearance of a severely scarred upper eyelid that involved fibrosis to the upper fornix. The grading score for symblepharon severity was grade Ia 2+ (Kheirkhah score) and grade 2 (Sotozono score) according to the previous reports.[5,7]

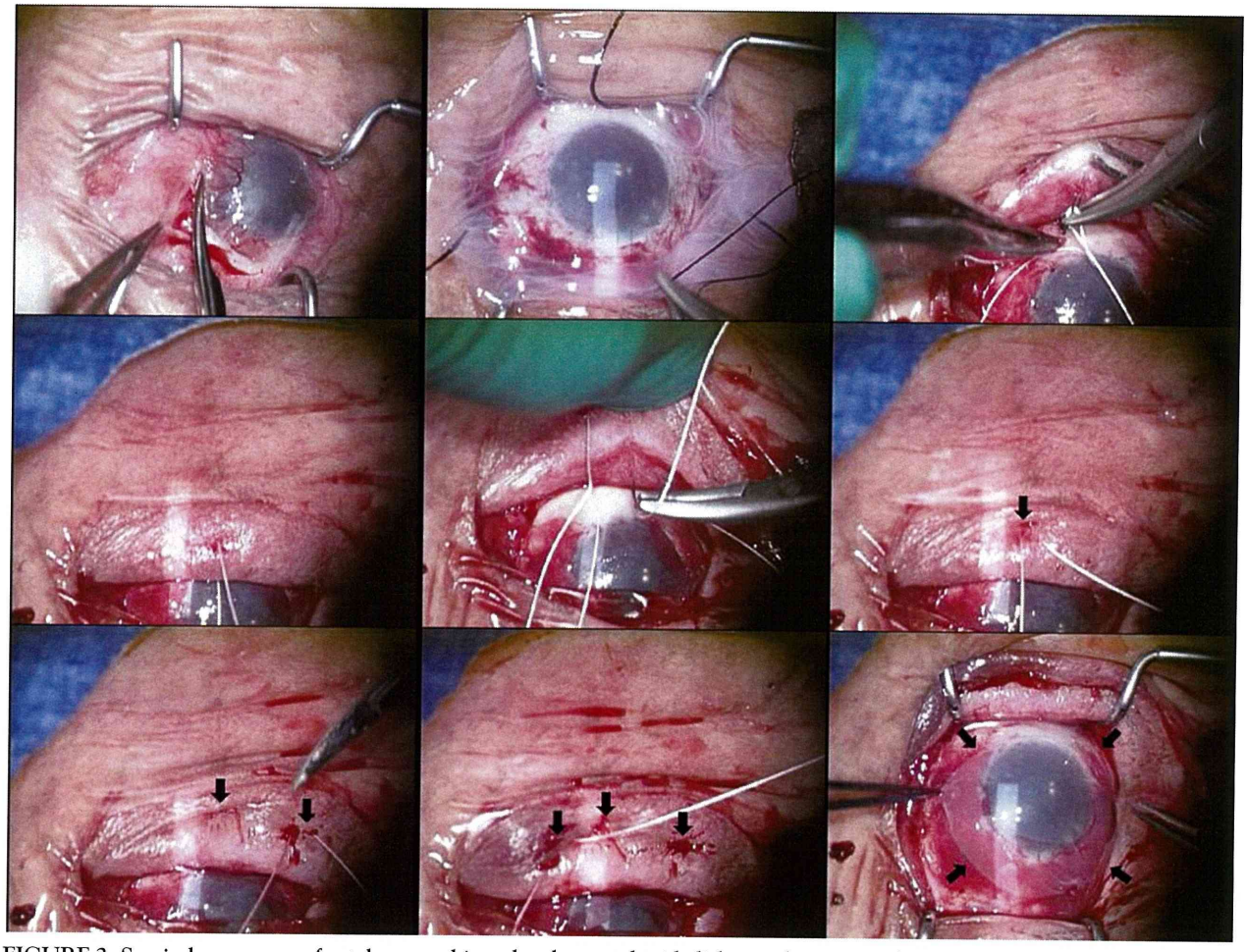

FIGURE 2. Surgical appearance of autologous cultivated oral mucosal epithelial transplantation and everting sutures in Case 1. (Top left) We completely removed the conjunctivalized tissue by thin superficial keratectomy and peritectomy. (Top center) Transferred human amniotic membrane (AM) was placed over the corneal surface. (Top right, Middle left) First, a 6-0 nylon suture was passed through the eyelid from the tarsus side to the skin side. (Middle center) The second needle of that same suture was then passed through in the same manner. (Middle right) Those 2 sutures were then tied off. (Bottom left and Bottom center) This procedure was repeated on both sides. (Bottom right) Finally, the autologous cultivated oral mucosal epithelial sheet was transferred onto the corneal surface.

All 3 patients were diagnosed as stem cell deficient on the basis of the disappearance of the palisades of Vogt. All 3 eyes manifested severe epithelial damage and entropion, and PED was induced in all 3 eyes because of cilia touching the ocular surface.

In this study, cultivated oral mucosal epithelial sheets were generated using the procedure previously reported.[21–23] Briefly, the presence of healthy oral mucosa in each patient was confirmed by a dentist. Human amniotic membrane was harvested at the time of caesarean section. Autologous oral epithelial cells, grown for 2 weeks on denuded AM and co-cultured with mitomycin C–inactivated 3T3 fibroblasts, were airlifted for 1 to 2 days.

The surgical procedure used in this study is illustrated in Figure 1.[21–23] Briefly, the conjunctivalized tissue of each patient was completely removed by performing a thin superficial keratectomy and peritectomy (Figures 1 and 2). Subconjunctival spaces were then treated with 0.04% mitomycin C for 5 minutes, followed by human AM being transplanted over the ocular surface and then sutured to the sclera. All 3 cases required an additional surgical procedure to address existing entropion of either the upper or lower eyelid. For this procedure, we selected everting sutures to connect the skin and tarsus when surgically reconstructing the abnormal eyelid with the existing destroyed palpebra structure that resulted from chemical or thermal injury. First, a 6-0 nylon suture was passed through the eyelid from the tarsus side to the skin side (Figures 1 and 2), followed by the second needle of that same suture being passed through in the same manner (Figures 1 and 2). The 2 ends of that suture were then tied off and implanted under the skin. This was repeated 2 or 3 times at the appropriate positions in relation to the first suture, with the number of additional sutures needed being determined by the severity of the entropion of each particular case

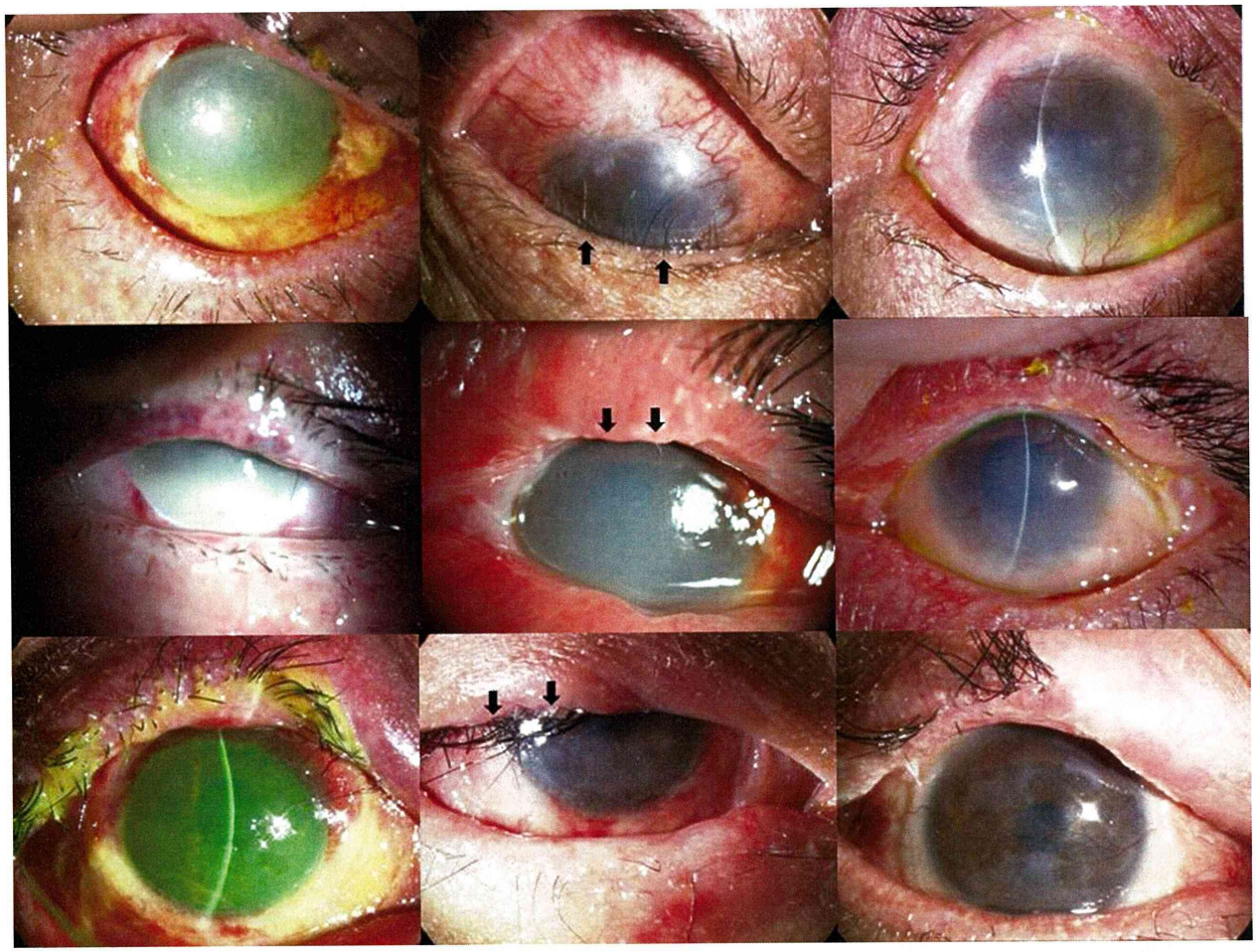

FIGURE 3. Clinical appearance before and after reconstruction using autologous cultivated oral mucosal epithelial transplantation and everting sutures for severe ocular surface disease. (Top left) Case 1 with alkali injury soon after arrival at our hospital. (Top center) Preoperatively, there was total conjunctivalization with symblepharon and lower-eyelid entropion. (Top right) Seven months after surgery, the cultivated oral mucosal epithelial sheet showed no epithelial defect and the ocular surface was stable. (Middle left) Case 2 with a fireworks-induced thermal injury soon after arrival at our hospital, manifesting severe epithelial damage with hypopyon, corneal opacity, ischemic conjunctival edema, and symblepharon. (Middle center) Preoperatively, there was upper-eyelid entropion with a severe scar and cilia had touched the corneal surface. (Middle right) After surgery, the cultivated oral mucosal epithelial sheet was stable with no recurrence of entropion. (Bottom left) Case 3 with a fireworks-induced thermal injury soon after arrival at our hospital, manifesting total corneal epithelial defect. (Bottom center) Preoperatively, there was persistent epithelial defect (PED), symblepharon, and scarred entropion of the upper eyelid. (Bottom right) Six weeks after surgery, the entropion relapsed and symblepharon also gradually occurred.

(Figures 1 and 2). Finally, the cultivated autologous oral mucosal epithelial sheet was transferred onto the corneal surface and then sutured onto the surface with 10-0 nylon (Figures 1 and 2). The ocular surface was then protected with a medical-use bandage contact lens. The 3 patients were followed up for 11, 29, and 50 months, respectively (mean follow-up period: 30 months).

CLINICAL RESULTS

IN ALL 3 CASES, WE WERE ABLE TO PERFORM THE OCULAR surface reconstruction using the combination of autologous cultivated oral mucosal epithelial transplantation and everting sutures without any complications during the operations. Postoperative follow-up showed that the simultaneous eyelid surgery caused no problems with the transplanted cultivated oral mucosal epithelial sheet. In addition, severe complications such as infection and inflammation were not observed in relation to the everting sutures. During the follow-up period, in 2 of the 3 eyes (Cases 1 and 2) the ocular surface and eyelid remained stable with no recurrence of entropion; however, in 1 eye (Case 3) there was a recurrence of entropion that induced an epithelial defect.

- **CASE 1:** Case 1 involved a 65-year-old man in the acute phase of alkali injury (Kinoshita grading score IIIb accord-

ing to the previously reported classification[24]) with severe corneal stromal opacity in July 2004. The patient was diagnosed as stem cell deficient on the basis of the disappearance of the palisades of Vogt and the manifested PED with marginal lower-eyelid entropion and symblepharon (grading Ia3+[7] and grading 2[5] according to the previously reported classifications) (Figure 3, Top left). The PED was caused by cilia touching the ocular surface (Figure 3, Top center). Preoperative visual acuity was 12/200, and autologous cultivated oral mucosal epithelial transplantation combined with everting sutures for the lower-eyelid entropion was performed. Postoperatively, the patient's ocular surface was stable with no recurrence of entropion (Figure 3, Top right). However, this case required penetrating keratoplasty 11 months after surgery because of the pre-existing corneal stromal opacity. The surviving oral mucosal epithelium then resulted in the formation of a stable ocular surface.

• CASE 2: Case 2 involved a 26-year-old man in the acute phase of thermal injury (Kinoshita grading score IIIb[24]) resulting from exposure to fireworks. The patient was diagnosed as stem cell deficient on the basis of disappearance of the palisades of Vogt and the manifested severe epithelial damage with hypopyon, conjunctivalization, entropion, and symblepharon (grading Ia2+[7] and grading 1[5] according to the previously reported classifications) (Figure 3, Middle left). Specifically, the patient's upper eyelid was entropion with a severe scar (Figure 3, Middle center). Autologous cultivated oral mucosal epithelial transplantation combined with everting sutures for the upper-eyelid entropion was performed. Postoperatively, after 29.1 months of follow-up, the patient's ocular surface was stable with no recurrence of entropion and the structure of the eyelid was maintained (Figure 3, Middle right).

• CASE 3: Case 3 involved a 32-year-old man in the acute phase of thermal injury (Kinoshita grading score IIIb[24]) resulting from exposure to fireworks. The patient was diagnosed as stem cell deficient on the basis of the disappearance of the palisades of Vogt and the manifested PED with conjunctivalization, entropion, and symblepharon (grading Ia2+[7] and grading 1[5] according to the previously reported classifications) (Figure 3, Bottom left). Specifically, the upper eyelid was entropion with a severe scar (Figure 3, Bottom center). Autologous cultivated oral mucosal epithelial transplantation combined with everting sutures for the upper-eyelid entropion was performed. Although a small epithelial defect occurred postoperatively, it eventually healed with the clinical use of a bandage contact lens. For this patient, the everting sutures were effective for a short period of time (Figure 3, Bottom right). However, after 6 weeks postoperatively, there was a recurrence of the entropion and a gradual recurrence of the symblepharon. Moreover, an epithelial defect appeared resulting from cilia touching the ocular surface. Though the ocular surface had been stable because of the clinical-use contact lens, after 15 months of follow-up, conjunctival reconstruction by autologous cultivated oral mucosal epithelial transplantation combined with upper-eyelid splitting was performed. After 50.7 months of follow-up, the surviving oral mucosal epithelium formed a stable ocular surface.

DISCUSSION

THIS STUDY PRESENTS A SIMULTANEOUS SURGICAL APproach to treat patients with severe limbal deficiency disorder. This surgical approach consists of a combination of autologous cultivated oral mucosal epithelial transplantation and everting sutures performed during 1 operation. The results of this study showed that everting sutures are safe to perform in combination with a cultivated epithelial sheet being transplanted onto the ocular surface. The 3 patients in this study were followed up for a mean period of 30 months, and our findings showed that their respective ocular surfaces and eyelid structures were successfully reconstructed by use of this combined surgical approach.

In general, both mild and moderate symblepharon can be corrected by AM transplantation alone when the remaining conjunctiva in the symblepharon is moved from the bulbar region to the tarsal region. However, all 3 patients presented in this study were diagnosed with total corneal epithelial stem cell deficiency and had manifested severe epithelial damage, symblepharon, and entropion. Therefore, the patients required reconstruction of not only the eyelid and conjunctival surface but also the corneal surface, by supplying it with epithelial stem cells. For this reason, we reconstructed the corneal surface by the transplantation of a cultivated oral mucosal epithelial sheet and reconstructed the conjunctival surface by the transplantation of human AM. The advantage of performing cultivated oral mucosal epithelial transplantation is that the transplanted epithelial sheet contains stem cells that help to reconstruct the corneal surface and maintain the ocular surface integrity.

This study involved 3 cases of burn injury that caused cicatricial entropion. Entropion is caused by a relative shortage of posterior lamella compared with the anterior lamella attributable to the cicatricial change in the tarsal-conjunctival layer. Conjunctival scarring by trauma or inflammation causes shortening of the fornices. There have been several reported approaches used for the reconstruction of the eyelid in patients with these types of severe OSD. However, the optimal surgical procedure is still an open question. Kemp and Collin[25] demonstrated that minimal and moderate entropion, such as minimal conjunctivalization of the eyelid margin, cilia touching the ocular surface, and thickening of the tarsal plate, can be treated by anterior lamellar repositioning and splitting the

eyelid margin. A case of severe eyelid disturbance requires rotation of the distal tarsal conjunctiva, such as a lamellar division. Previous reports have shown that if the tarsal-conjunctival layer does not advance enough by shortening of the conjunctival fornix, a hard-palate mucosal graft can be inserted between the 2 cut edges of the tarsus or as a spacer into the posterior eyelid lamella.[26–29] In this present study, these types of eyelid surgeries were not used, simply because they are more invasive to the damaged eyelid and strongly induce postoperative inflammation. Surgical methods for treating entropion such as the Jones method and the Hotz method are well known, but these methods are also surgically more invasive procedures than everting sutures. Moreover, everting sutures have less incidence of inflammation. Therefore, we considered the everting sutures safe to use in conjunction with the cultivated epithelial sheet on the ocular surface. However, since the everting sutures can result in an increased rate of recurrence, we explain this risk in detail to the patients and their immediate family prior to the operation.

Case 3 in this study unfortunately experienced postoperative recurrence of entropion and symblepharon. For this patient we were able to reconstruct the ocular surface with a second operation that involved eyelid splitting and autologous cultivated oral mucosal epithelial transplantation for the reconstruction of conjunctiva fornix. We now understand the importance of selecting the appropriate surgical procedure depending on the severity of the entropion and palpebral disorder, as we have learned that simple everting sutures are sometimes insufficient for eyelid reconstruction in the most severe cases.

The results of this study demonstrate that the combined surgical procedure of autologous cultivated oral mucosal epithelial transplantation and everting sutures is a useful approach for the treatment of severe ocular surface disorders with associated eyelid abnormality. Further study is required to develop new methods for eyelid surgery that lower the risk of complications following ocular surface reconstruction.

PUBLICATION OF THIS ARTICLE WAS 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), and 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 authors have no proprietary or commercial interest in any materials discussed in this article. Involved in design of the study (T.N., S.K.); conduct of the study (K.T., T.N.); collection, management, analysis, and interpretation of the data (K.T., T.N., T.I., C.S., A.W., S.K.); and preparation, review (T.N., S.K.), and approval of the manuscript (K.T., T.N., T.I., C.S., A.W., S.K.). In accordance with the guidelines set forth by the Kyoto Prefectural University of Medicine, this study was approved by the Human Studies Committee of Kyoto Prefectural University of Medicine, and proper informed consent was obtained from all patients prior to their involvement in the study.

Additionally, we thank Mr J. Bush for manuscript preparation.

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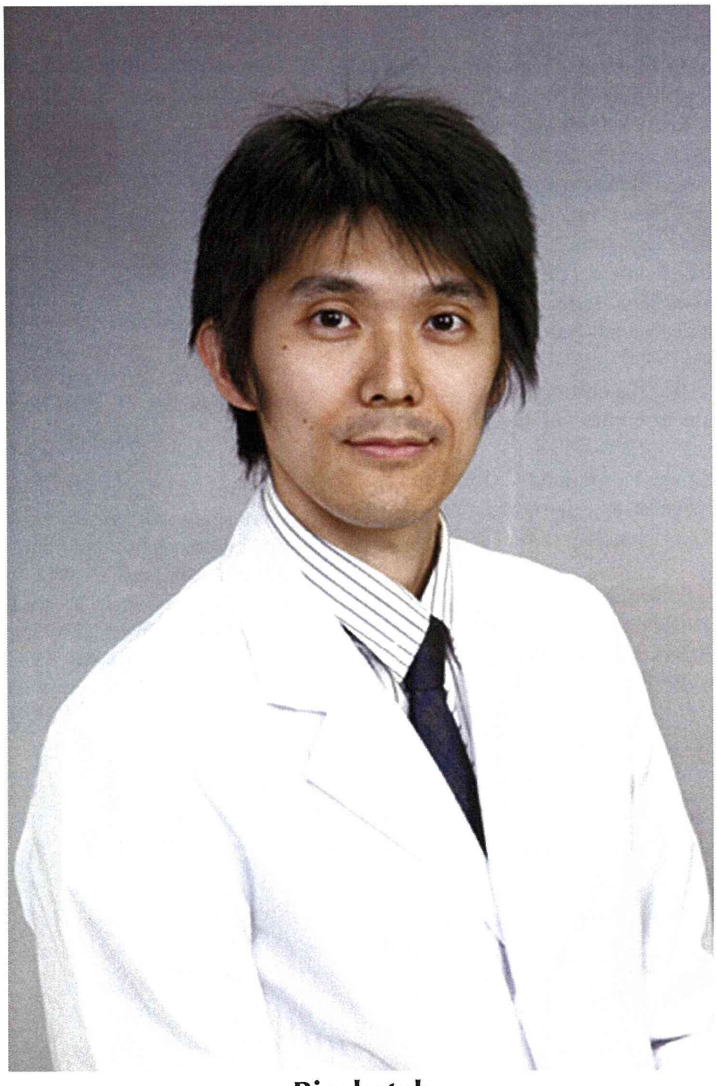

Biosketch

Kazunori Takeda, MD, is a clinical doctor of the Department of Ophthalmology at Kyoto Prefectural University of Medicine, Kyoto, Japan, where he received clinical training. He then spent 2 years as a staff surgeon at the Department of Ophthalmology, Maizuru Red Cross Hospital. Dr. Takeda's current interests include the treatment of ocular surface disorders through the use of regenerative medicine procedures.