6A). In view of these findings, it is likely that LGR5 may be the key molecule for maintaining normal CEC phenotypes.

Transformation of endothelial cells to fibroblastic cells is known as endothelial–mesenchymal transformation (MT) [33]. The interesting findings observed in the *LGR5*-transfected cells led us to further study whether or not the persistent expression of *LGR5* was able to block the MT process. The expression level of epithelial-MT (EMT)-related molecules (*Snail, Slug, Twist, and Collagen1*) [34] was examined using real-time PCR. Of great importance, the relative mRNA level of all EMT markers except Slug were lower in the *LGR5*-transfected cells than in the NT cells (Fig. 6D), suggesting that persistent *LGR5* expression blocked the MT process. We further examined which pathway regulates the endothelial-MT observed in CECs. Recent studies suggest that the Wnt/β-catenin signaling pathway plays an important role in EMT [34]. Therefore, the expression level of Wnt/β-catenin-related molecules was examined using western blot analysis. Worthy of note, the protein level of cytosolic (non membrane bound) β-catenin and phosphorylated low-density-lipoprotein receptor-related protein 6 (p-LRP6) was greatly decreased in the LGR5-transfected cells (Fig. 6F). We found that the expression of β-catenin shifted from the cell membrane to the cytoplasm and nucleus, which is well observed in the typical EMT process, in most of nontarget transfected CECs. In contrast, we could observe the expression of β-catenin in cell membrane of LGR5-transfected CECs (supplemental online Fig. 2). These findings indicated that persistent LGR5 expression inhibited the corneal endothelial-MT through the Wnt/β-catenin pathway.

RSPO1-Accelerated CEC Proliferation and Inhibited MT Through the Wnt Pathway

Previously, LGR5 was thought to be an orphan receptor of the G protein-coupled receptor superfamily, and its ligand was unknown. However, several recent reports demonstrated that RSPOs function as ligands of LGR5 to regulate Wnt/β-catenin signaling [35–37]. Interestingly, we discovered that *RSPO1*, *2*, *3*, and *4* mRNA were expressed in the corneal epithelium, stroma, and endothelium, and that *RSPO1*, *2*, and *3* mRNA were only expressed in the peripheral-region CECs (supplemental online Fig. 3A). To determine the function of RSPOs on CEC differentiation, we cultured the primary human CECs with or without human recombinant RSPOs. Worthy of note, 7 days after culture, only cultivated human CECs treated with RSPO1 [50 ng/ml] showed the compact, smaller-size, homogeneously hexagonal cells, whereas other RSPOs did not have an obvious effect on CEC differentiation in vitro (supplemental online Fig. 3B). To determine the function of RSPOs on donor CEC proliferation, we performed immunohistochemistry for Ki67. Most surprisingly and very interestingly, human donor CECs incubated with RSPO1 (50 ng/ml) for 48 hours at 37°C showed a dramatically increased level of $Ki67^+$ cell ratios as compared to other RSPOs (supplemental online Fig. 3C). In view of these findings, we think that among the RSPOs family, RSPO1 in particular may play an important role in the maintenance of CECs.

Finally, to further determine the effect of RSPO1 on CECs, we maintained the secondary culture of human CECs with or without RSPO1. Through culturing the CECs in both conditions, we clearly observed that the cultured cells with RSPO1 maintained their hexagonal morphology, whereas some of the cultured cells without RSPO1 still showed fibroblastic phenotypes (Fig. 6E). Moreover, the cell density of RSPO1-treated cells was elevated in comparison with that of the nontreated cells (Fig. 6E). To demonstrate which pathway regulates this type of corneal endothelial MT, we examined the expression level of Wnt/β-catenin-related molecules using western blot analysis. We performed the experiments twice, and the results were nearly identical; the protein level of cytosolic β-catenin and p-LRP6 in the LGR5-transfected cells treated with RSPO1 was decreased in comparison with that in the NT cells (Fig. 6F). Moreover, the protein levels of the RSPO1-treated NT and LGR5-transfected cells were more decreased as compared to the cells not treated with RSPO1 (Fig. 6F). These results suggested that the stimulation of cells overexpressing LGR5 with RSPO1 accelerates pLRP degradation and β-catenin turnover.

Discussion

Cornea tissue is extremely important, as most mammals acquire the majority of their external information through it. Recently, particular attention has been focused on CECs due to the fact that the corneal transplantation procedure is currently undergoing a paradigm shift from keratoplasty to endothelial keratoplasty. Therefore, both scientifically and clinically, to establish the next generation of novel therapy for treating cornea-related blindness worldwide, it is extremely important to understand the molecular mechanism of corneal endothelial stem/progenitor cells. However, very little is presently known about those molecular mechanisms.

It has been reported that the characteristics and proliferative potential of CECs are different between those located at the central region of the cornea and those located at the peripheral region of the cornea [38, 39], and a study has shown that the cornea has a higher density of endothelial cells in the peripheral region than in the central region [40]. Moreover, CECs from the peripheral region reportedly retain higher replication ability than those from the central region [12], and peripheral-region CECs contain more precursors and have a stronger self-renewal capacity than CECs in the central region [41]. He et al. recently identified a novel anatomic organization in the peripheral region of human corneal endothelium, suggesting a continuous slow centripetal migration of CECs from specific niches [15]. Thus, it is most likely that human corneal endothelial stem/progenitor cells are mainly distributed in the peripheral region. In fact, no stem/progenitor cell marker for CECs has thus-far been elucidated, and the results of this study demonstrate for the first time that CECs exhibit regional diversity with respect to LGR5 expression. In view of these findings and the unique expression pattern of LGR5 in CECs, LGR5 might represent a first marker for corneal endothelial stem-cell-containing populations.

It has been reported that cell size may distinguish keratinocyte stem cells from transient amplifying cells or differentiated cells [28]. In the epidermis, the response to phorbol esters of the smallest keratinocytes is different from that of other cells. Those keratinocytes also exhibited the highest clonogenicity. Even though CECs are different from ectoderm-derived keratinocytes, the average diameter of the $LGR5^+$ cells in this study was in fact smaller than that of the $LGR5^-$ cells. Based on these findings, and on the findings of the above-cited previous report regarding the size of peripheral CECs, it is possible that cell size might be a potential indicator of corneal endothelial stem/progenitor cells.

We found that LGR5 is a key molecule for maintaining the integrity of CECs and regulating normal cell phenotypes in vitro. We also found that isolated cells fractionated based on the intensity of their LGR5 expression could produce different cell populations with different properties. Only cells in

the LGR5$^+$ population exhibited exceptionally high proliferative potential, features associated with stem/progenitor cell populations. Based on these findings, the unique expression pattern and necessity in the in vitro condition, there is possibly a link between LGR5 and the function of corneal endothelial stem/progenitor cells.

Previous studies have indicated that high concentration of SHH caused a marked increase in retinal progenitor cell proliferation and a general increase in the accumulation of differentiated cells [29]. The findings of this present manuscript show that in the in vitro situation, the HH pathway is able to induce CEC proliferation, consistent with the findings of previous reports. HH is a family of secreted molecules that serve as morphogens during multiple aspects of development in a wide range of tissue types. HH is involved in the left-right asymmetry decision and anterior–posterior axis decision in limb pattern determination by regulating cell proliferation and survival. In CECs, there is regional variation of HH signal activity, and based on our findings, HH signaling might possibly control corneal endothelial morphogenesis.

RSPOs are a family of four cysteine-rich secreted proteins that were isolated as strong potentiators of Wnt/β-catenin signaling. A vast amount of information regarding the cell biological functions of RSPOs has emerged over the last several years, especially with respect to their role as ligands of the orphan receptors LGR 4/5/6. These updated and important findings led us to further study whether RSPOs may have an effect on the function of human CECs. As human CECs are mitotically inactive and are essentially nonregenerative in vivo, corneal endothelial loss due to disease or trauma is followed by a compensatory enlargement of the remaining endothelial cells. To the best of our knowledge, there are no reports regarding a useful inductive reagent or molecule to increase the level of human CEC proliferation and CEC density, although we previously developed the CECs culture protocol using Y-27632 [21, 22]. We examined the expression of RSPO1 in CECs and found that its protein level is quite low (data no shown), suggesting that external RSPO1, rather than internal RSPO1, plays a critical role in maintaining the CEC function. Moreover, although there was no expression of LGR5 in the cultured CECs, RSPO1 did have some effect on the condition of CECs in vitro. We do not precisely know the reason why, but from our results, we presume that the effect of RSPO1 on CECs might be of both an LGR5-dependent and -independent manner. The findings of this study show for the first time that CECs incubated with RSPO1 exhibited a dramatically increased level of cell proliferation and cell density, suggesting that it might represent a first candidate molecule for reconstructing the damaged cornea through topical application or for use as a culture reagent.

Several studies suggest that the Wnt/β-catenin pathway plays an important role in EMT and that activation of Wnt/β-catenin-dependent signaling modulates the expression of EMT-related genes [34]. However, previous reports have indicated that RSPOs potentiate Wnt/β-catenin signaling by actually functioning as a ligand of LGR5 [35–37]. The exact mechanism involved in this activation is still unclear and there are several conflicting findings as to whether LGR5 is a positive or negative regulator of the Wnt pathway [42–44]. One possible explanation is that the molecular mechanism depends on the tissues, organs, and the species of animal. The cornea is a unique avascular tissue, and its health is maintained by tears and aqueous humor. In contrast, the health of most other organs is maintained by vascular support, suggesting that the characteristics and mechanism of corneal cells are fundamentally different from the epithelial cells of other tissues. Thus, based on the findings of this study, RSPO1 dramatically accelerates CEC proliferation and inhibits corneal endothelial MT through the Wnt pathway.

Conclusion

In conclusion, the findings of this study are the first to demonstrate the function of LGR5 in human CECs (supplemental online Fig. 4). LGR5 has proven to be a powerful tool in identifying a multitude of stem/progenitor cell populations. Through the regulation of LGR5 through the HH and Wnt pathways, CEC integrity was well structured and maintained. In addition, the LGR5 ligand RSPO1 may exploit the novel substantial protocol to provide the efficient expansion of CECs, suggesting that RSPO1-based three dimensional culture and medical treatments hold promise for regenerative therapy, not only for the treatment of corneal dysfunctions, but also for a variety of severe general diseases.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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A Randomized, Multicenter Phase 3 Study Comparing 2% Rebamipide (OPC-12759) with 0.1% Sodium Hyaluronate in the Treatment of Dry Eye

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Objective: To investigate the efficacy of 2% rebamipide ophthalmic suspension compared with 0.1% sodium hyaluronate ophthalmic solution for the treatment of patients with dry eye.

Design: Randomized, multicenter, active-controlled parallel-group study.

Participants: One hundred eighty-eight patients with dry eye.

Methods: Following a 2-week screening period, patients were allocated randomly to receive 2% rebamipide or 0.1% sodium hyaluronate, administered as 1 drop in each eye 4 or 6 times daily, respectively, for 4 weeks.

Main Outcome Measures: There were 2 primary end points: changes in the fluorescein corneal staining (FCS) score to determine noninferiority of 2% rebamipide and changes in the lissamine green conjunctival staining (LGCS) score to determine superiority. Secondary objective end points were Schirmer's test results and tear film breakup time (TBUT). Secondary subjective end points were dry eye–related ocular symptoms (foreign body sensation, dryness, photophobia, eye pain, and blurred vision) score and the patients' overall treatment impression score.

Results: In the primary analysis, the mean change from baseline in FCS scores verified noninferiority, indicated significant improvement, and, in LGCS scores, verified the superiority of 2% rebamipide to 0.1% sodium hyaluronate. Values for the Schirmer's test and TBUT were comparable between the 2 groups. For 2 dry eye–related ocular symptoms—foreign body sensation and eye pain—2% rebamipide showed significant improvements over 0.1% sodium hyaluronate. Patients had a significantly more favorable impression of 2% rebamipide than of 0.1% sodium hyaluronate; 64.5% rated treatment as improved or markedly improved versus 34.7%, respectively. No serious adverse events were observed.

Conclusions: Administration of 2% rebamipide was effective in improving both the objective signs and subjective symptoms of dry eye. Those findings, in addition to the well-tolerated profile of 2% rebamipide, clearly show that it is an effective therapeutic method for dry eye.

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Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface.[1] Dry eye is one of the most common ophthalmologic problems,[2] and it is estimated that up to one-third of the population worldwide may be affected.[2–4] The effect on quality of life is substantial because of symptoms such as pain and irritation, which have a negative effect on ocular health, general health, and well-being and often disrupt daily activities.[2,5] Dry eye is caused by disease or disruption to components of the ocular surface[1,6] that help to maintain its integrity, driven by tear hyperosmolarity and tear film instability.[1] The tear film can be destabilized by decreased tear production or altered tear composition, damaging the ocular surface and resulting in inflammation and, ultimately, further tear film instability.[1]

Currently, tear supplementation with artificial tears is considered a mainstay treatment for cases of mild-to-moderate dry eye; however, frequent instillation often is required.[5] Sodium hyaluronate has shown some effectiveness in patients with dry eye.[7–9] Insertion of punctal plugs or permanent punctal occlusion also are options for cases of moderate or severe dry eye,[10] although a reduction in symptom relief over time has been reported.[11] Thus, treatment options are limited, especially for moderate-to-severe dry eye.

Recently, the role of ocular mucins has been attracting increased attention for the treatment of dry eye. The tear

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film currently is understood as being a meta-stable aqueous gel with a mucin gradient that decreases from the ocular surface to the undersurface of the outermost lipid layer.[12] Mucins are of 2 types, 1 type being secreted by goblet cells and the other type being expressed on the membranes of ocular surface epithelia.[6,13] In dry eye, reduced goblet cell density[14] and changes in mucin amount, distribution, and glycosylation have been reported,[15,16] and therapeutic improvements reportedly have occurred when mucin instillation is administered in patients with dry eye.[17]

Rebamipide (OPC-12759; Otsuka Pharmaceutical Co, Ltd, Tokyo, Japan) is a quinolinone derivative with mucin secretagogue activity,[18–20] and in Japan, rebamipide is marketed as an oral therapeutic drug of gastric mucosal disorders and gastritis under the trade name Mucosta in 2 forms: 100-mg tablets and 20% granules. When rebamipide was instilled in rabbit eyes, rebamipide increased the production of mucin-like substances and the number of periodic acid–Schiff-positive cells.[21,22] A recent study also reported a significant increase in a mucin-like glycoprotein and *MUC1* and *MUC4* gene expression after human corneal epithelial cells were incubated with rebamipide.[23] A phase 2 study in patients with dry eye showed that a 4-week treatment with 2% rebamipide ophthalmic suspension was significantly more effective than the placebo in terms of improving the fluorescein corneal staining (FCS) score, the lissamine green conjunctival staining (LGCS) score, and tear film breakup time (TBUT), as well as subjective symptoms (foreign body sensation, dryness, photophobia, eye pain, and blurred vision) and the patients' overall treatment impression.[24]

The objective of this study was to compare the efficacy of 2% rebamipide ophthalmic suspension with that of 0.1% sodium hyaluronate ophthalmic solution in patients with dry eye in terms of noninferiority in FCS score and superiority in LGCS score. This was a phase 3 trial designed to confirm the efficacy of 2% rebamipide for registration purposes. Sodium hyaluronate was selected as the active control because it is an indicated treatment for keratoconjunctival disorder accompanying dry eye in Japan and has demonstrated clinical efficacy.[7–9]

Materials and Methods

Study Design

This was a randomized, multicenter, active-controlled, parallel-group phase 3 trial conducted in 3 phases: screening, evaluation, and follow-up. As much as possible, the study was conducted under masked conditions for the investigators; the perfect masked conditions could not be accomplished because the instillation frequency and chemical properties differ between the rebamipide ophthalmic suspension and the sodium hyaluronate ophthalmic solution. During the initial 2-week screening period, the patients received preservative-free artificial tears (Soft Santear; Santen Pharmaceutical Co, Ltd, Osaka, Japan) 4 times daily, 1 drop per application. This screening period was performed to minimize the effects of any eye drops used before screening.

Eligible patients were allocated randomly to receive 2% rebamipide or 0.1% sodium hyaluronate. Central randomization was adopted for assigning patients to each group in a 1:1 ratio by using a dynamic allocation[25] of stratified centers, with or without Sjögren's syndrome and baseline FCS scores (4–6, 7–9, and 10–15).

Patients in the rebamipide group received 2% rebamipide ophthalmic suspension, 1 drop in each eye 4 times daily. Patients in the sodium hyaluronate group received 0.1% sodium hyaluronate ophthalmic solution (Hyalein Mini ophthalmic solution 0.1%; Santen Pharmaceutical Co, Ltd) 1 drop in each eye 6 times daily. For both groups, the total treatment period was 4 weeks, and examinations were conducted at week 2 and week 4 after the start of treatment. To monitor safety, follow-up examinations were conducted 2 weeks after the end of treatment.

The study was conducted in accordance with the ethical principles set forth in the Declaration of Helsinki and the Good Clinical Practice Guidelines. The study protocol and informed consent were reviewed and approved by the institutional review board before initiation. Written informed consent was obtained from each patient before the start of the study. The study was registered before patient enrollment (clinical trial identifier NCT00885079; accessed July 26, 2012).

Patients

Eligible patients were 20 years of age or older and had dry eye–related symptoms that were not fully relieved by conventional treatments (e.g., artificial tears), with symptoms present for more than 20 months before the screening examination. Other inclusion criteria were: (1) score of 2 or more for 1 or more dry eye–related ocular symptom(s), (2) an FCS score of 4 or more, (3) an LGCS score of 5 or more, (4) a no-anesthesia Schirmer's test value at 5 minutes of 5 mm or less, and (5) best-corrected visual acuity of 20/100 or better. These criteria needed to be met at both the screening and baseline examinations, with criteria 2 through 4 being met in the same eye.

Exclusion criteria included (1) anterior ocular disease (such as blepharitis or blepharospasm), (2) continued use of eye drops, (3) patients who had a punctal plug or had it removed within 3 months before the screening examination, and (4) patients who underwent an operation to the ocular surface within 12 months or intraocular surgery within 3 months before the screening period.

The following drugs or therapies were prohibited from the screening examination to the end of study treatment: rebamipide for gastric mucosal disorders and gastritis; any prescription or over-the-counter ophthalmic drugs (except Soft Santear during the screening period); contact lenses; and ocular surgery or any other treatment affecting the dynamics of tear fluid, including its nasolacrimal drainage process. The inclusion criteria, exclusion criteria, and prohibited drugs or therapies used in this study have been described previously[24] and were the same as those used for a phase 2 study.

Assessment of Outcome Measures

Efficacy Assessments. Efficacy was evaluated primarily with an objective measure and secondarily with objective and subjective measures. There were 2 primary objective end points, FCS and LGCS scores. Secondary objective end points were TBUT and the Schirmer's test value. Secondary subjective end points included dry eye–related ocular symptoms (foreign body sensation, dryness, photophobia, eye pain, and blurred vision) and the patient's overall treatment impression. All of these parameters were assessed at baseline, at week 2, at week 4, or at treatment discontinuation, except for the Schirmer's test assessed at baseline, at week 4, or at treatment discontinuation, and the patient's overall treatment impression was assessed at week 4 or at treatment discontinuation.

For FCS, 5 μl 2% fluorescein solution (provided by the sponsor) was instilled in the conjunctival sac as the patient blinked normally. Corneal staining was examined under standard illumination using a slit-lamp microscope with a cobalt blue filter. According to the National Eye Institute/Industry Workshop report, the cornea was divided into 5 fractions,[26] each fraction given a staining score from 0 through 3, and the total score then was calculated. The sponsor provided each investigator with a set of photographs of FCS and LGCS to ensure standardization when scoring.

For LGCS, 20 μl 1% lissamine green solution (provided by the sponsor) was instilled in the conjunctival sac, and the conjunctiva was divided into 6 fractions.[26] Conjunctival staining was evaluated under low illumination by slit-lamp microscopy and was scored from 0 through 3 for each fraction, then summed to calculate the total score.

For TBUT, 5 μl 2% fluorescein solution was instilled in the conjunctival sac, and TBUT was then evaluated by slit-lamp microscopy. The elapsed time from a normal blink to the first appearance of a dry spot in the tear film was measured 3 times.

The Schirmer's test was performed without anesthesia to measure tear volume as follows. A Schirmer's test strip was placed on the lower eyelid between eyelid conjunctiva and bulbar conjunctiva without touching the cornea. The tear volume then was measured for 5 minutes after the patient was instructed to close the eyelid lightly. The length in millimeters of tear fluid absorbed on the strip measured from the edge of the strip was recorded as tear volume.

Dry eye–related ocular symptoms, such as foreign body sensation, dryness, photophobia, eye pain, and blurred vision were examined by questioning each patient. These symptoms were scored from 0 through 4; a score of 0 indicated no symptoms and a score of 4 indicated very severe symptoms.

The patient's overall treatment impression was examined by questioning each patient and was scored from 1 through 7, a score of 1 being markedly improved compared with baseline and a score of 7 being markedly worsened compared with baseline.

Safety Assessments. The safety variable was the occurrence of adverse events, determined at various visits by means of physical signs and symptoms, external eye examination and slit-lamp microscopy, visual acuity, intraocular pressure, funduscopy, and clinical laboratory tests including hematology, biochemistry, and urinalysis.

Statistical Analyses

Sample size calculation was performed based on data from the previous trial.[24] Using the FCS score, by assuming the mean difference between the 2% rebamipide group and the 0.1% sodium hyaluronate group to be 0.7, with a standard deviation of 2.3, a significance level of 5%, and the noninferiority margin to be 0.4, the number of patients to verify noninferiority was calculated. The results showed that the power exceeded 80% when 85 patients were included per group. In addition, using the LGCS score, by assuming the mean difference between the 2% rebamipide group and the 0.1% sodium hyaluronate group to be 1.4, with a standard deviation of 3.1, and a significance level of 5%, the number of patients required to verify superiority was calculated. The results showed that the power exceeded 80% when 80 patients were included per group. On the basis of above results, the number of patients was set at 90 per group to allow for those who might be excluded from the efficacy analysis. Missing efficacy data, including those resulting from early study termination, were corrected by the last observation carried forward (LOCF) method. In the analysis of dry eye–related ocular symptoms, patients with a dry eye–related ocular symptom score of 0 at baseline were excluded. Missing safety data were treated as missing.

All patients who were enrolled in the study were included in the efficacy and safety analyses. A closed test procedure was used for multiplicity considerations of 2 primary end points. First, noninferiority was assessed in FCS. If the noninferiority was confirmed, the superiority was examined in LGCS. Noninferiority for change from baseline in the FCS score (LOCF) was determined by comparing the noninferiority margin (0.4) with the upper limit of the 95% confidence interval of the difference between the 2 treatment groups. Superiority was verified by comparing t test results for change from baseline in the LGCS score (LOCF) between the 2 treatment groups. Furthermore, an analysis of change from baseline of FCS and LGCS score at each visit and secondary end points was performed using the t test or Wilcoxon signed-rank test. The level of significance was 5% (2-sided).

The eye in which objective efficacy end points were analyzed was determined as follows: (1) if only 1 eye met the inclusion criteria, then this eye was used; (2) if both eyes met the inclusion criteria, the eye with the higher FCS baseline score was used; (3) if both eyes had the same FCS baseline score, then the eye with the higher LGCS baseline score was used; and (4) if both eyes had the same LGCS baseline score, the right eye was used.

Results

Participant Characteristics

A total of 188 patients were allocated randomly to receive 1 of the 2 treatments: 93 patients entered the 2% rebamipide group and 95 patients entered the 0.1% sodium hyaluronate group. In total, 96.8% of the patients completed the trial (Table 1). Patient characteristics across the treatment groups were comparable (Table 2). Of the total 188 participants, 163 were female (86.7%) and the mean age ± standard deviation was 56.6±17.4 years. Of the 188 patients, 34 patients (18.1%) had primary or secondary Sjögren's syndrome as the underlying cause of dry eye.

Table 1. Patient Disposition

	Total	2% Rebamipide	0.1% Sodium Hyaluronate
Randomized and treated	188	93	95
Completed	182 (96.8)	91 (97.8)	91 (95.8)
Discontinued	6 (3.2)	2 (2.2)	4 (4.2)
Occurrence of adverse events	4 (2.1)	1 (1.1)	3 (3.2)
Patient's wish	2 (1.1)	1 (1.1)	1 (1.1)

Data are presented as number (%).

Table 2. Demographics and Other Baseline Characteristics

	2% Rebamipide (n = 93)	0.1% Sodium Hyaluronate (n = 95)	*P* Value*
Gender			
Male	10 (10.8)	15 (15.8)	0.309
Female	83 (89.2)	80 (84.2)	
Age (yrs)			
20–49	29 (31.2)	35 (36.8)	0.713
50–64	26 (28.0)	24 (25.3)	
≥65	38 (40.9)	36 (37.9)	
Main cause or primary disease of dry eye			
Primary Sjögren's syndrome	11 (11.8)	9 (9.5)	0.766
Secondary Sjögren's syndrome	6 (6.5)	8 (8.4)	
Other systemic disease	0 (0)	0 (0)	
Ocular disease	0 (0)	0 (0)	
Menopause	1 (1.1)	0 (0)	
Unknown	75 (80.6)	78 (82.1)	
Fluorescein corneal staining score†			
4–6	47 (50.5)	47 (49.5)	0.985
7–9	32 (34.4)	33 (34.7)	
10–15	14 (15.1)	15 (15.8)	
Dry eye–related ocular symptom†			
Foreign body sensation	69 (74.2)	68 (71.6)	0.686
Dryness	76 (81.7)	87 (91.6)	0.046
Photophobia	62 (66.7)	53 (55.8)	0.126
Eye pain	50 (53.8)	58 (61.1)	0.312
Blurred vision	53 (57.0)	55 (57.9)	0.900

Data are presented as number (%) unless otherwise indicated.
*Chi-square test (Fisher exact test in the case of presence of cell with a frequency of lower than 5).
†At baseline.

Efficacy Evaluation

Primary End Points. At baseline, the mean FCS scores were 7.0 in both groups. The mean change from baseline to LOCF in FCS score was −3.7 for the 2% rebamipide group and −2.9 for the 0.1% sodium hyaluronate group (Fig 1A and Table 3). The 95% confidence interval of the difference between the 2 treatment groups was −1.47 to −0.24, and the upper limit was lower than the noninferiority margin of 0.4. Therefore, the noninferiority of 2% rebamipide to 0.1% sodium hyaluronate was verified. In addition, the 95% confidence interval did not include 0, indicating the significant improvement of 2% rebamipide to 0.1% sodium hyaluronate. At baseline, mean LGCS scores were similar between groups (9.8 for 2% rebamipide vs. 10.1 for 0.1% sodium hyaluronate). The mean change from baseline to LOCF in LGCS score was −4.5 for the 2% rebamipide group and −2.4 for the 0.1% sodium hyaluronate group (Fig 1B and Table 3), indicating superiority of 2% rebamipide over 0.1% sodium hyaluronate. At all estimations (week 2, week 4, and LOCF), the improvements in FCS and LGCS scores were significantly greater for 2% rebamipide than for 0.1% sodium hyaluronate.

In the 34 patients with Sjögren's syndrome (17 patients per each treatment group), there were no significant differences between the 2% rebamipide and 0.1% sodium hyaluronate groups in the change from baseline to LOCF in the FCS score (−3.4 vs. −2.5, respectively) and the LGCS score (−3.7 vs. −2.1, respectively). However, these scores in the 2% rebamipide group showed a tendency for better improvement compared with the 0.1% sodium hyaluronate group.

Secondary End Points. There were similar changes between treatment groups in the change from baseline to LOCF for Schirmer's test results or TBUT (Table 3). Changes from baseline to LOCF in the dry eye–related ocular symptoms of foreign body sensation and eye pain were significantly greater in the 2% rebamipide group than in the 0.1% sodium hyaluronate group (Table 3). There was no significant difference between groups for change from baseline to LOCF in dryness, photophobia, or blurred vision, although there were more improvements with 2% rebamipide.

The patients' overall treatment impression with 2% rebamipide was significantly more favorable than that with 0.1% sodium hyaluronate ($P<0.001$, Wilcoxon signed-rank test; Fig 2). Overall, 60 patients (64.5%) rated their symptoms as improved or markedly improved in the 2% rebamipide group, compared with 33 patients (34.7%) in the 0.1% sodium hyaluronate group.

Safety Evaluation

Adverse events were observed in 27 patients (29.0%) in the 2% rebamipide group and in 19 patients (20.0%) in the 0.1% sodium hyaluronate group. Adverse events observed in at least 2 patients are shown in Table 4. The most frequently observed adverse event was dysgeusia (bitter taste), which was observed only in the 2% rebamipide group (9 adverse events in 9 patients; 9.7%). All cases of dysgeusia reported in this study were judged to be treatment related. Dysgeusia and all eye disorders were mild in severity and resolved either with appropriate treatment or with no treatment. No deaths and no serious or severe adverse events were observed in this study. A total of 3 patients in the 0.1% sodium hyaluronate group and 1 patient in the 2% rebamipide group discontinued because of adverse events.

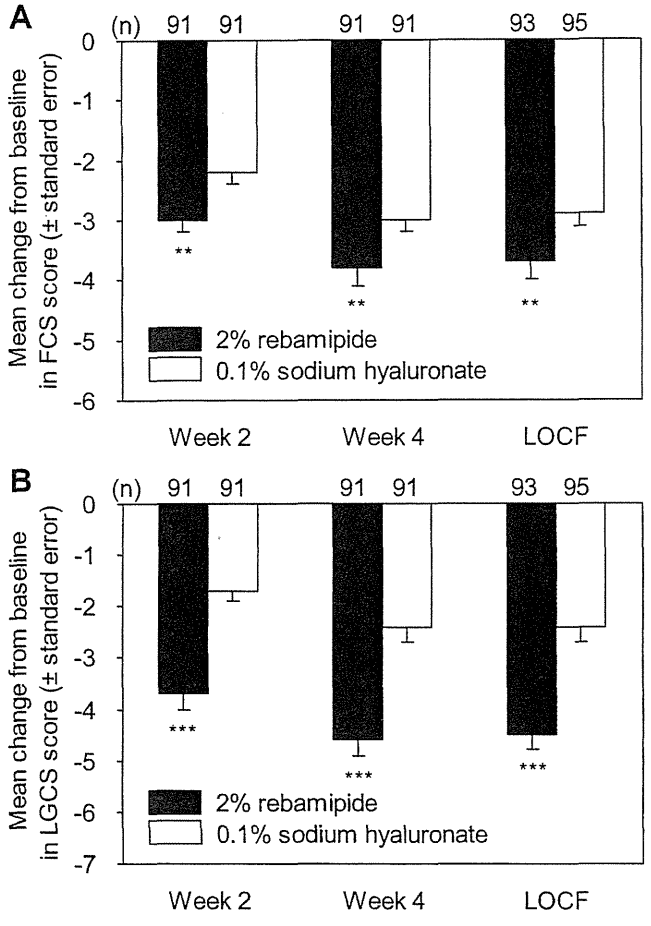

Figure 1. A, Change in fluorescein corneal staining (FCS) score from baseline to week 2, week 4, and last observation carried forward (LOCF); B, Change in lissamine green conjunctival staining (LGCS) score from baseline to week 2, week 4, and LOCF. **$P<0.01$, ***$P<0.001$ vs 0.1% sodium hyaluronate (t test).

Discussion

In this phase 3 trial, rebamipide demonstrated statistically significant efficacy improvements over sodium hyaluronate for the treatment of dry eye. The 4-week, 4-times daily ocular instillation of 2% rebamipide was effective at improving both the objective signs and the subjective symptoms of dry eye. Study data were obtained from a population representative of that seen in normal clinical practice, because dry eye commonly affects women who are middle-aged and older.[27,28] Results from this study support and confirm the data from the phase 2 trial, which reported significant benefits of 2% rebamipide over the placebo.[24] Furthermore, rebamipide again demonstrated its rapid onset of effect (2 weeks).

In the primary analysis, in relation to the change from baseline in both FCS and LGCS scores, 2% rebamipide clearly demonstrated a marked improvement. Such improvements in staining scores are important because they indicate an improvement in the ocular surface,[2] with FCS reflecting corneal epithelium integrity and LGCS reflecting conjunctival epithelium integrity. Staining with fluorescein and lissamine green are the standard methods used to demonstrate ocular surface damage.[2,29] Patients with Sjögren's syndrome may have a particularly severe form of dry eye. Subgroup analysis of the 34 patients with Sjögren's syndrome in this study showed a tendency for better improvement for 2% rebamipide over 0.1% sodium hyaluronate on the primary end points. This tendency suggests the potential use of 2% rebamipide for dry eye in patients with Sjögren's syndrome.

In addition to its benefits on objective measures, 2% rebamipide was more effective than 0.1% sodium hyaluronate on subjective outcomes, showing greater improvement in symptoms. Significantly greater improvements in foreign body sensation and eye pain were seen with 2% rebamipide compared with 0.1% sodium hyaluronate. The assessment of efficacy using subjective measures (symptoms) as well as objective measures (signs) is particularly important in patients with dry eye because it has been shown that there is poor correlation between symptoms and signs of dry eye; for instance, one study found that only 57% of symptomatic patients were shown to have objective signs of dry eye.[30] Improvements in symptoms are important, given the impact of dry eye on quality of life.[2,5]

Rebamipide has distinctive features compared with other drugs that are used in current therapies for dry eye. Cyclosporine, another ophthalmic solution used for the treatment of dry eye, showed significant improvement in FCS score, although efficacy was demonstrated only after 4 months.[31] Sodium hyaluronate also has shown effectiveness in patients with dry eye, with FCS scores demonstrating significant improvement at 4 weeks.[7] In addition, FCS scores also showed significant improvement at 2 weeks compared with baseline scores.[8] In the present study, rebamipide demonstrated better efficacy after 2 weeks of treatment compared with sodium hyaluronate. Cyclosporine has an anti-inflammatory and immunomodulatory mode of action, and sodium hyaluronate is a viscous material aimed at increasing tear retention and wound healing effects. In contrast, rebamipide has been shown to increase the number of periodic acid–Schiff-positive cells (goblet cells) in the conjunctiva[22] and the mucin level on the cornea and conjunctiva.[22,23] Because decreased mucin levels on the surface of the cornea and a decreased density of goblet cells have been observed in patients with dry eye,[32] the method of action of rebamipide is expected to be beneficial for this disease. With this mechanism in mind, rebamipide also is expected to be effective in patients with dry eye resulting from short

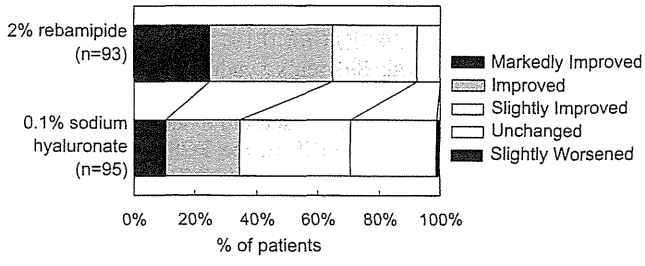

Figure 2. Patient's overall treatment impression by category. No patients responded worsened or markedly worsened in any treatment group.

Table 3. The Baseline and the Change from Baseline to Last Observation Carried Forward in Efficacy End Points

Efficacy End Points by Group	No.	Baseline, Mean±Standard Error	Change from Baseline to Last Observation Carried Forward		
			Mean±Standard Error	*95% Confidence Interval*	*P Value**
Fluorescein corneal staining					
2% Rebamipide	93	7.0±0.2	−3.7±0.3	−4.2 to −3.2	
0.1% Sodium hyaluronate	95	7.0±0.2	−2.9±0.2	−3.2 to −2.5	0.006
Lissamine green conjunctival staining					
2% Rebamipide	93	9.8±0.4	−4.5±0.3	−5.2 to −3.9	
0.1% Sodium hyaluronate	95	10.1±0.4	−2.4±0.3	−2.9 to −1.9	<0.001
Schirmer test					
2% Rebamipide	93	2.5±0.2	0.5±0.2	0.0–1.0	
0.1% Sodium hyaluronate	95	2.3±0.2	1.0±0.3	0.4–1.5	0.229
Tear film break-up time					
2% Rebamipide	93	2.7±0.1	0.8±0.1	0.5–1.1	
0.1% Sodium hyaluronate	95	2.5±0.1	0.6±0.1	0.3–0.8	0.218
Foreign body sensation					
2% Rebamipide	69	2.0±0.1	−1.4±0.1	−1.6 to −1.2	
0.1% Sodium hyaluronate	68	1.8±0.1	−1.0±0.1	−1.1 to −0.8	0.004
Dryness					
2% Rebamipide	76	2.4±0.1	−1.4±0.1	−1.7 to −1.2	
0.1% Sodium hyaluronate	87	2.6±0.1	−1.2±0.1	−1.4 to −1.0	0.139
Photophobia					
2% Rebamipide	62	1.8±0.1	−1.1±0.1	−1.3 to −0.9	
0.1% Sodium hyaluronate	53	1.8±0.1	−0.8±0.1	−1.1 to −0.6	0.127
Eye pain					
2% Rebamipide	50	1.9±0.1	−1.5±0.1	−1.7 to −1.2	
0.1% Sodium hyaluronate	58	1.9±0.1	−1.0±0.1	−1.2 to −0.7	0.014
Blurred vision					
2% Rebamipide	53	1.8±0.1	−1.1±0.1	−1.3 to −0.9	
0.1% Sodium hyaluronate	55	1.9±0.1	−0.9±0.1	−1.1 to −0.6	0.242

**t* test.

TBUT, because disturbance of ocular surface mucin is thought to be one of the main causes of tear film instability and the accompanying shorter TBUT.[1]

There were no significant safety problems associated with rebamipide treatment and the adverse event profile was consistent with previous studies. As in previous trials, the most frequent adverse event was dysgeusia, possibly caused by the bitter taste associated with the active ingredient. However, in Japan, of 10 047 patients with gastric ulcer and gastritis treated by oral formulation of OPC-12759, adverse reactions were reported in 54 patients (0.54%), mainly gastrointestinal symptoms such as constipation, flatulence, nausea, and diarrhea.[19]

Rebamipide ophthalmic suspension does not contain preservatives that can be detrimental to eye health. One of the most commonly used preservatives in ocular products is benzalkonium chloride, which destabilizes the tear film, disrupts the corneal epithelium, decreases the density of goblet cells, and causes conjunctival squamous metaplasia and apoptosis and damage to deeper ocular tissues.[33,34] Such adverse effects are clearly not ideal in a patient with dry eye, particularly if those patients must rely on such products over a long period of time. In fact, the use of preservative-free ocular products is recommended.[34,35] Thus, rebamipide may be expected to be less harmful even if it is used in the long-term.

The efficacy and safety of 2% rebamipide ophthalmic suspension in the short term (4 weeks) was demonstrated in both this study and the phase 2 study.[24] However, long-term treatment would be required for dry eye because it is often seen as a chronic disease. Further studies will be required to

Table 4. Incidence of Adverse Events Observed in at Least 2 Patients in Any Treatment Group

	2% Rebamipide (n = 93)	0.1% Sodium Hyaluronate (n = 95)
Patients with any adverse event	27 (29.0)	19 (20.0)
Ocular events		
Conjunctival hemorrhage	1 (1.1)	2 (2.1)
Eye irritation	0 (0.0)	2 (2.1)
Eye pain	0 (0.0)	3 (3.2)
Visual impairment	2 (2.2)	0 (0.0)
Eye pruritus	4 (4.3)	2 (2.1)
Nonocular events		
Nasopharyngitis	1 (1.1)	2 (2.1)
White blood cell count decreased	3 (3.2)	0 (0.0)
Dysgeusia (bitter taste)	9 (9.7)	0 (0.0)
Headache	2 (2.2)	0 (0.0)

Data are presented as number (%).

investigate whether the improvements reported with rebamipide are maintained in the longer term.

In conclusion, this study showed that a 4-week, 4-times daily treatment with 2% rebamipide was effective in improving the objective signs and subjective symptoms of dry eye. These results suggest that rebamipide may lead to improved treatment of corneal and conjunctival epithelial damage and improvement in symptoms in patients with dry eye. Such efficacy, in addition to the well-tolerated profile of rebamipide, makes it a potentially useful treatment option for dry eye.

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Footnotes and Financial Disclosures

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Classification of Secondary Corneal Amyloidosis and Involvement of Lactoferrin

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Purpose: To classify secondary corneal amyloidosis (SCA) by its clinical appearance, to analyze the demographics of the patients, and to determine the involvement of lactoferrin.

Design: Retrospective, observational, noncomparative, multicenter study.

Participants: Twenty-nine eyes of 29 patients diagnosed with SCA by corneal specialists at 9 ophthalmologic institutions in Japan were studied.

Methods: The clinical appearance of SCA was determined by slit-lamp biomicroscopy and was classified into 3 types. The demographics of the patients, for example, age, gender, and the duration of the basic disease (trichiasis, keratoconus, and unknown), were determined for each clinical type. Surgically excised tissues were stained with Congo red and antilactoferrin antibody. The postoperative prognosis also was determined.

Main Outcome Measures: Clinical appearance of the 3 types of SCA, along with the gender, age, and duration of the basic diseases were determined.

Results: Classification of SCA into 3 types based on clinical appearance found 21 cases with gelatinous drop-like dystrophy (GDLD)-like appearance (GDLD type), 3 cases with lattice corneal dystrophy (LCD)-like appearance (LCD type), and 5 cases with the combined type. Patients with the GDLD type were younger (average age: 40.9 years for the GDLD type, 74.3 years for the LCD type, and 46.8 years for the combined type), predominantly women (85.7% for the GDLD type, 33.3% for the LCD type, and 60% for the combined type), and had the basic disease over a longer time (average duration: 22.1 years for the GDLD type, 14.0 for the LCD type, and 11.4 for the combined type). The distribution of the basic diseases (trichiasis vs. keratoconus vs. unknown) was not significantly different for each type. Surgical treatments, for example, phototherapeutic keratectomy, lamellar keratoplasty, and simple keratectomy, resulted in a good resolution in all surgically treated cases. One subject dropped out of the study. Spontaneous resolution was seen in one subject after epilation of the cilia. Amorphous materials in the excised tissues showed positive staining results by Congo red and by antilactoferrin antibody.

Conclusions: Secondary corneal amyloidosis can be classified into 3 clinical types based on its clinical appearance. Larger numbers of females and lactoferrin expression were seen in all 3 types.

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Amyloidosis can develop as a primary or secondary condition in the cornea. Primary amyloidosis is divided into primary localized amyloidosis and primary systemic amyloidosis. Gelatinous drop-like dystrophy (GDLD) and lattice corneal dystrophy (LCD), with the exception of the LCD II type, are examples of primary localized amyloidosis. The LCD II type is a localized form of primary systemic amyloidosis. Localized secondary corneal amyloidosis (SCA) first was described by Stafford and Fine,[1] with its characteristics further defined in later studies.[2–7] Secondary corneal amyloidosis develops after chronic ocular inflammations or corneal disorders, for example, keratoconus, trachoma, phlyctenular keratitis, bullous keratopathy, interstitial keratitis, syphilis, trichiasis, and spheroidal degeneration.[8,9] Because there is a low incidence of this disease, most clinical studies simply have described a single case or, at most, only a few cases in each report.

To diagnose SCA accurately, a histologic examination using Congo red-stained tissues and observation by polarized light microscopy is required. From an ethical point of view, however, the excision of specimens from early-stage cases is not always possible. Therefore, to ensure that an early diagnosis of SCA can be made, it is necessary to establish a standard method that can be used to determine the clinical features of SCA.

It is known that the clinical appearance of SCA can be similar to that of primary corneal amyloidosis, such as GDLD and LCD. The *TACSTD2* gene has been identified as gene responsible for GDLD,[10] whereas the transforming growth factor-β-induced (*TGFBI*) and gelsolin genes are responsible for LCD.[11–14] However, it is still not known why the clinical appearance of SCA resembles primary corneal amyloidosis without the responsible gene mutation. Therefore, analysis of the demographics

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Table 1. Demographics of Each Clinical Type in SCA

Types (n = 29)	Sex (female dominancy)	Age (average: years old)	Duration (years)	Trichiasis: Keratoconus: Unknown
GDLD type (21 cases)	85.7%	40.9	22.1	12:6:3
LCD type (3 cases)	33.3%	74.3	14.0	2:1:0
Combined type (5 cases)	60%	46.8	11.4	3:1:1
Total (SD)	72.4%	45.5 (20.9)	19.6 (13.8)	

GDLD = gelatinous drop-like dystrophy; LCD = lattice corneal dystrophy; SCA = secondary corneal amyloidosis; SD = standard deviation.

of the patients with SCA may provide an answer as to why this occurs.

Lactoferrin was recognized as the precursor protein of SCA by the International Amyloid Committee and was named Alac.[15,16] Thereafter, it was hypothesized that the pathogenesis of SCA may be related to hormonal regulation, with the gender of the subject able to affect the clinical characteristics of SCA. To test this hypothesis, 29 cases of SCA from 9 ophthalmologic institutions in Japan were studied, with SCA classified based on its clinical appearance. The demographics of the patients with SCA then were analyzed.

Patients and Methods

This was a retrospective, observational, noncomparative, multicenter study. Twenty-nine eyes of 29 patients diagnosed with SCA were studied. No subjects demonstrated pathologic changes in the fellow eye, and family history of eye disease was negative in all cases. The examinations and diagnosis were made by corneal specialists at 9 ophthalmologic institutions in Japan. The gender, age, basic clinical findings, duration of symptoms and signs, clinical appearance as determined by slit-lamp examinations, and treatment and prognosis were analyzed.

For the histopathologic analysis, 11 specimens from 29 cases were obtained at keratoplasty, fixed in 10% formaldehyde overnight, and then embedded in paraffin. Then, 3-μm sections were cut and mounted on slides. After deparaffinizing and drying the slides, samples were stained with Congo red. Direct immunohistochemical methods were used to localize lactoferrin in the cornea, as has been described previously in detail.[16] Briefly, sections were incubated with 3% bovine serum albumin at room temperature for 30 minutes to block nonspecific binding. The slides then were incubated with horseradish peroxidase-conjugated rabbit immunoglobulin G fraction against human lactoferrin (no. 55242; Cappel, ICN Pharmaceuticals, Inc, Bryan, OH) for 30 minutes at room temperature. The sections were washed 3 times in phosphate-buffered saline for 10 minutes with 0.02% diaminobenzidine solution, dehydrated through an ethanol series (95% to 70%), and covered with a cover slip using mounting medium. The slides were examined with light and polarizing microscopy.

Informed consent was obtained from all patients. Examination procedures were reviewed by the Institutional Review Board of Ideta Eye Hospital, and the study was conducted in accordance with the tenets of the Declaration of Helsinki.

Results

Classification of Secondary Corneal Amyloidosis

The cases of SCA were classified into 3 types according to their clinical appearances (Fig 1). The GDLD type, which exhibits a clinical appearance similar to that of GDLD, had 1 or more milky-white soft masses on the corneal surface. The LCD type, which exhibits a clinical appearance similar to that of LCD, had fine linear branches that could be detected by scattered scleral illumination. When these types coexisted, for example, a milky-white mass surrounded by lattice-like lines, these corneas were classified as the combined type. Among the 29 cases, 21 were classified as the GDLD type, 3 were classified as the LCD type, and 5 were classified as the combined type. Table 1 summarizes the characteristics of all of the SCA types.

The GDLD-type patients were younger (average age: 40.9 years in the GDLD type, 74.3 years in the LCD type, and 46.8 years in the combined type) and predominantly women (85.7% in the GDLD type, 33.3% in the LCD type, and 60% in the combined type), with the basic diseases shown to be present for a longer time (average age: 22.1 years in the GDLD type, 14.0 years in the LCD type, and 11.4 years in the combined type). For all eyes, the average age of SCA patients was 45.4±20.9 years, with 21 (72.4%) of these 29 SCA cases found in women. No significant differences were noted for the distribution of the basic diseases (trichiasis vs. keratoconus vs. unknown) in each of the clinical types.

In all of the clinical types, the amyloid deposits were located at the corneal area that was inflamed or where the corneal epithelium was irritated continually. In the trichiasis cases, amyloid deposits were seen at the inferior part of the cornea where the eyelid cilia were present. All of the keratoconus cases had worn hard contact lenses for many years, and the amyloid deposits were seen where the contact lenses touched the corneal epithelium at the apex of the cornea. In cases that occurred after penetrating keratoplasty for keratoconus, the amyloid deposits were seen at the edge of the graft where the graft protruded slightly.[17] None of the pathologic regions were found at the limbus.

Prognosis of Secondary Corneal Amyloidosis

Superficial treatments, which included phototherapeutic keratectomy, keratectomy, or lamellar keratoplasty, were performed on 21 of 29 eyes. With a mean follow-up of 31 months, there were no severe recurrences, vessel invasions, infections, or postoperative complications in the surgically treated cases. One patient dropped out and was lost to follow-up, and there was a very slight recurrence in 7 patients. The other 8 cases were followed up conservatively with only lubrication, because the patients reported only a slight foreign body sensation.

Involvement of Lactoferrin in Secondary Corneal Amyloidosis

Histopathologic examination showed that the excised tissue in 11 cases consisted of amorphous eosinophilic material, with all of these tissues showing positive staining results by both Congo red and antilactoferrin antibody (Figs 2 and 3). This material was demonstrated by the apple-green birefringence typical of

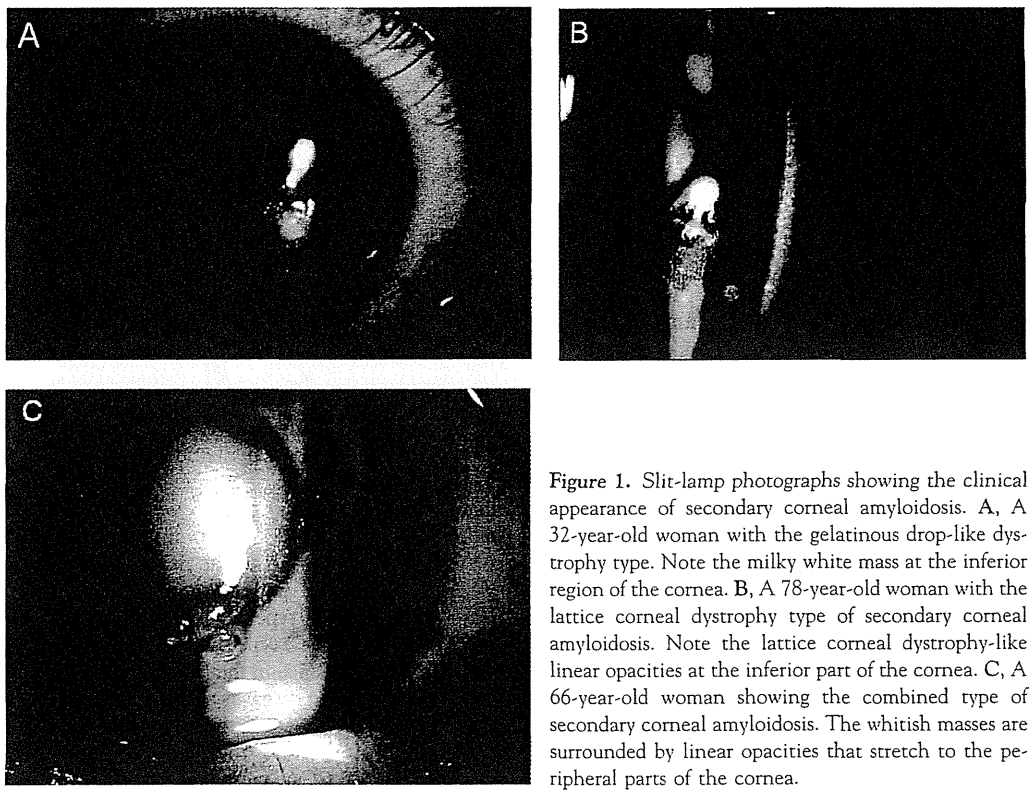

Figure 1. Slit-lamp photographs showing the clinical appearance of secondary corneal amyloidosis. A, A 32-year-old woman with the gelatinous drop-like dystrophy type. Note the milky white mass at the inferior region of the cornea. B, A 78-year-old woman with the lattice corneal dystrophy type of secondary corneal amyloidosis. Note the lattice corneal dystrophy-like linear opacities at the inferior part of the cornea. C, A 66-year-old woman showing the combined type of secondary corneal amyloidosis. The whitish masses are surrounded by linear opacities that stretch to the peripheral parts of the cornea.

amyloid deposits when observed under polarized light (Fig 4). The positive staining areas were located mainly between Bowman's layer and the corneal epithelium. Milky-white masses observed by slit-lamp examination appeared as amorphous material in the epithelial layer, with this material occasionally destroying Bowman's layer, the epithelial layer, or both. The material did not show positive staining with the anti-TGFBI antibody (data not shown).

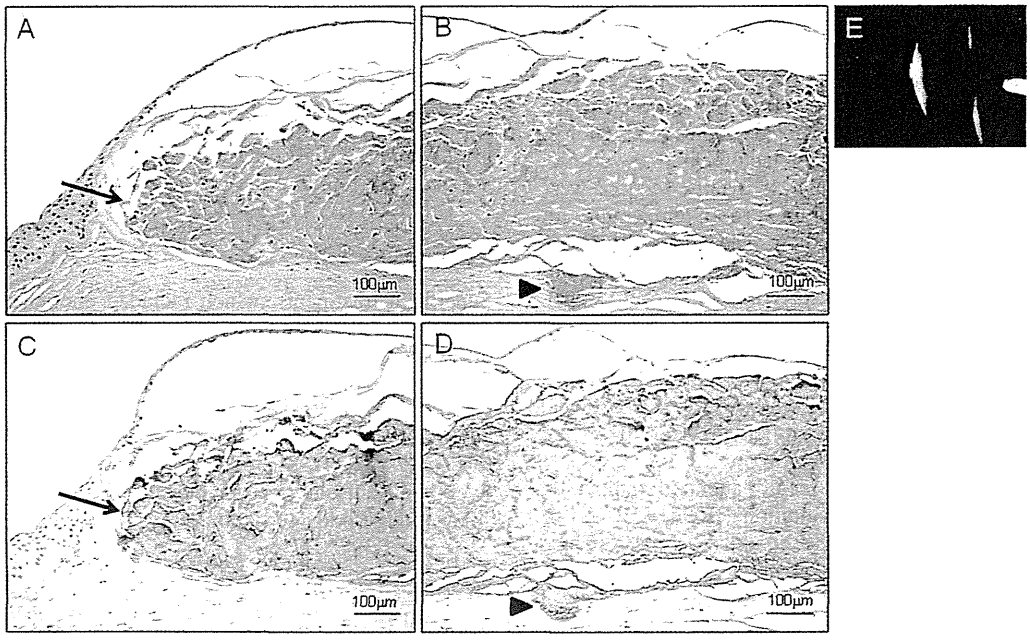

Figure 2. Images showing secondary corneal amyloidosis in a 51-year-old man caused by keratoconus. The tissue obtained during keratectomy was stained with (A, B) Congo red and (C, D) antilactoferrin antibody. A, C, Photomicrographs showing the peripheral region of secondary corneal amyloidosis. B, D, Photomicrographs showing the central region of the cornea. Amorphous eosinophilic material is present between the epithelial layer and Bowman layer (arrow) and shows positive staining results by Congo red and by antilactoferrin antibody. Amorphous materials protrude into the stroma (arrowhead) and totally destroyed the Bowman layer. Note that the epithelial layer is thin and atrophic. E, Slit-lamp photograph.

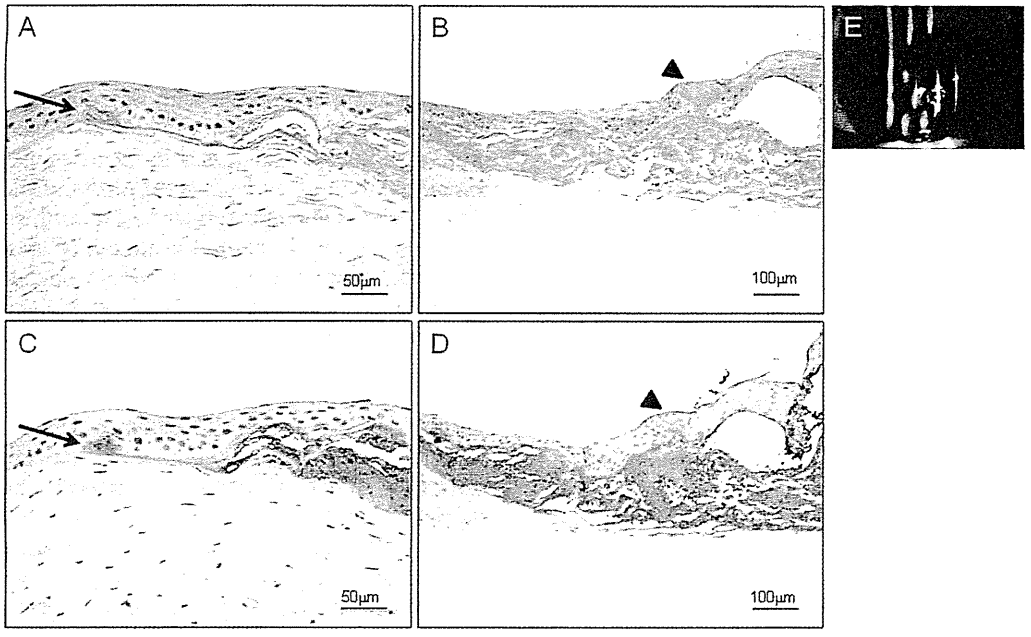

Figure 3. Findings in a 42-year-old man with secondary corneal amyloidosis associated with trichiasis. Amorphous eosinophilic material showed positive staining results by (A, B) Congo red and (C, D) antilactoferrin antibody. A, C, Photomicrographs showing high magnification. B, D, Photomicrographs showing low magnification. Note that the amorphous material is located between the epithelial layer and the Bowman layer (arrow) and the material occupies the epithelial layer (arrowhead). E, Slit-lamp photograph.

Discussion

This study identified characteristic patterns of disease in what is believed to be the first multicenter study of SCA. Because SCA is a rare disease, most of the previously published studies have reported on only a single case or, at most, a few cases at a time. Although a study by Hidayat and Risco[18] was able to examine 62 cases of SCA, unfor-

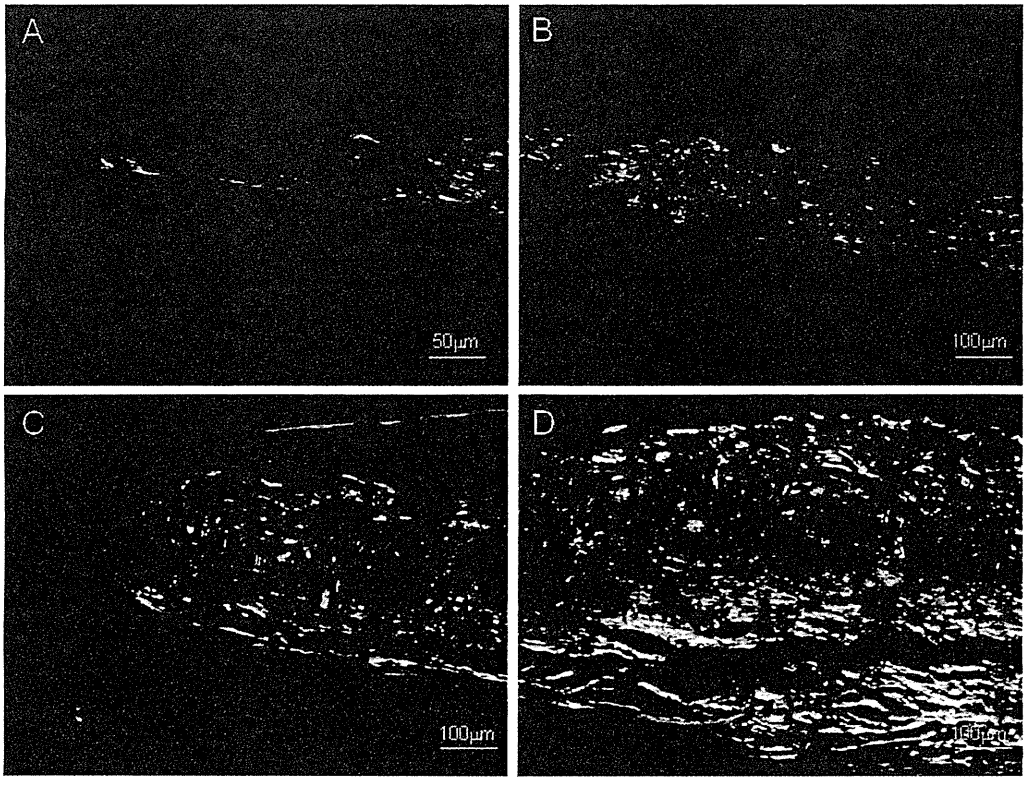

Figure 4. The findings of (A, B) Figure 2A, B and (C, D) Figure and 3A, B under the polarized microscopy. The deposits appear as apple-green birefringence when observed with polarized light.

tunately these cases were limited to those caused by trachoma and there was no classification of the clinical appearances for these patients. The present study collected data on 29 SCA cases that were caused by several basic diseases, with the diagnosis made by corneal specialists from 9 ophthalmologic clinics in Japan.

Secondary corneal amyloidosis can be classified into 3 clinical types based on its clinical appearance, namely, the GDLD type, the LCD type, and the combined type. Originally, GDLD and LCD were defined as belonging to the primary amyloidosis group. Although the original cause is different, SCA and these primary dystrophies (GDLD and LCD) most likely share common pathologic characteristics. Interestingly, patients with the GDLD type of SCA were younger, were predominantly women, and had a longer duration of the basic disease as compared with patients with the other 2 types. However, because the patients were not observed from the point of SCA onset, whether there was transition between these 3 types could not be determined.

These findings indicated a large number of women with SCA, especially in the GDLD-type group. In 2005, Ando et al[15] studied the polymorphism Glu561Asp in patients with SCA and proposed that lactoferrin was the precursor protein of SCA. In an extensive study by Araki-Sasaki et al,[16] this polymorphism was shown to be associated significantly with SCA. A further study also showed that SCA was stained only by the antilactoferrin antibody, and not by antibodies against transthyretin, κ, λ light chain, lysosome, AA, and keratin.[14] Results of in vitro experiments by Nilsson and Dobson[19] suggested that there was aggregation of lactoferrin to form amyloid material. In contrast, Suesskind et al[20] reported finding precipitation of keratoepithelin in eyes with SCA, which suggested that keratoepithelin was the precursor protein of SCA. Therefore, at present it has yet to be proven definitively that lactoferrin is the precursor protein of SCA.

Because Suesskind et al did not stain the specimens with antilactoferrin antibody, the possibility exists that lactoferrin could have played a role in the amyloid formation in their cases. To the contrary, all cases in the present study showed negative staining results for the anti-TGFBI antibody. A complete description of this finding will be shared in an upcoming report. Although preliminary evidence indicates that lactoferrin may be one of the key proteins in SCA, further studies will need to be performed to show definitively that lactoferrin is the precursor protein of SCA.

Klintworth et al[21] suggested that lactoferrin is derived from the tear film in GDLD and that the same may be true in SCA. Another possible origin of lactoferrin has been suggested to be the corneal epithelial cells, which can produce lactoferrin in response to exposure to sex hormones.[22,23] Some may question why a female predominance was seen for SCA but not for GDLD. The authors speculate that there may be differences in unrevealed pathologic events that occur during the very early phase, with these differences ultimately responsible for the variations that are seen for the female predominance in SCA and GDLD. Additionally, after the epithelial breakdown during the late phase, pathologic events such as amyloid extension with lactoferrin from tears very well may be a common event that occurs in both diseases.

Graute-Hernandez et al[24] reported finding a predominance of women patients with Salzmann nodular degeneration of the cornea. Farjo et al[25] also have reported that more than 90% of Salzmann nodular degeneration occurs in females. Stone et al[26] proposed that the increased expression of matrix metalloproteinase-2 and the nodules are the result of chronic epithelial remodeling and wound repair. Thus, SCA and Salzmann nodular degeneration have a common clinical background. Recently, Sundmacher[27] reanalyzed Salzmann's original paper and disclosed that the etiologic postulates were never substantiated by direct observation, but rather were proposed based on indirect histopathologic circumstantial evidence. He revealed that most Salzmann nodules occur without obvious preceding inflammation and discussed that dystrophy and degeneration should be investigated separately with regard to Salzmann degeneration. The entire histologic appearance in Salzmann nodular degeneration seems to be completely different from that seen in SCA, because oxytalan fibers have been identified in the nodular lesions of Salzmann nodular degeneration.[28] However, the pathologic mechanisms associated with SCA and Salzmann nodular degeneration seem to contain common elements to some extent, and this may result in the similarity of clinical features as gray or bluish elevated subepithelial nodules with predominancy in women.

The exact reason why there are 2 forms of SCA, that is, the GDLD type and the LCD type, is still unclear. One possibility is that the region where the amyloid develops may be involved in determining the ultimate clinical appearance. For example, deposits in the epithelial layer may lead to the GDLD type, whereas deposits in the stromal layer could lead to the LCD type. Another possibility is that the lattice appearance could be related to the corneal nerve fibers. Slit-lamp examinations have shown that the lattice appearance often resembles the pattern of myelinated nerve fibers. Although Klintworth[29] demonstrated that amyloid deposits were not associated with the nerve fibers in GDLD, it has been shown that amyloid accumulates in the nerve fibers in cases of familial amyloid polyneuropathy, with transthyretin acting as the precursor protein.[30,31] However, no corneal amyloid deposits were described in familial amyloid polyneuropathy.

A further possibility is that the total amount of aggregated lactoferrin can determine its clinical phenotype. Although the cause of the lattice line has not been determined, the predominance of a younger age and larger numbers of females in the GDLD type suggest that there is a greater production of lactoferrin, aggregated lactoferrin, or both that occurs in the GDLD type versus the LCD type. At present, unfortunately, there is insufficient evidence to prove definitively any of these possibilities. However, the authors speculate that the proportion of nonaggregated mutated precursor protein, aggregated precursor protein, and extended amyloid aggregation may be responsible for determining the ultimate clinical phenotypes seen in SCA. Further investigations will need to be undertaken to clarify why there are 2 amyloid deposit patterns in SCA.

Several studies[32–37] have demonstrated that chronic inflammatory stimulation can be a trigger for the amyloid deposits at the irritated region, for example, at the top of the protruded cornea where the hard contact lens is in contact most tightly with the keratoconic cornea. A previous study has reported that after penetrating keratoplasty was performed in 1 case, the contact lens made a tight contact at the edge of the graft because of astigmatism, with this subsequently leading to SCA in the region.[17] Although the exact mechanism that is involved with the transformation from irritation to amyloid formation has yet to be determined, this previous finding strongly indicates that mechanical irritation seems to be one of the key inducers of amyloid fibril formation during the first stage of inflammation.

In the present cases, there was good prognosis for the surgical treatment of SCAs, with no severe recurrences, vessel invasions, infections, or side effects noted after the procedure. The clinical self-resolution that occurred because of removal of the irritant, for example, extirpation of the eye lashes, suggests that the deposited amyloid may be metabolized. Additionally, the use of therapeutic soft contact lenses is useful in preventing GDLD recurrences after keratoplasty because of the downregulation of the cell metabolism. Thus, the therapeutic effects of SCL may be one method that can be used to prevent the development of SCA.

In summary, this study examined the clinical classifications and demographics of SCA patients. The results showed a predominance of women with SCA, especially the GDLD type, with all cases exhibiting positive staining results for the antilactoferrin antibody. If additional investigations can clarify the mechanism responsible for SCA development, this should provide clues to how to treat this disease successfully in the future.

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PLOS ONE

Inhibition of TGF-β Signaling Enables Human Corneal Endothelial Cell Expansion *In Vitro* for Use in Regenerative Medicine

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Abstract

Corneal endothelial dysfunctions occurring in patients with Fuchs' endothelial corneal dystrophy, pseudoexfoliation syndrome, corneal endotheliitis, and surgically induced corneal endothelial damage cause blindness due to the loss of endothelial function that maintains corneal transparency. Transplantation of cultivated corneal endothelial cells (CECs) has been researched to repair endothelial dysfunction in animal models, though the *in vitro* expansion of human CECs (HCECs) is a pivotal practical issue. In this study we established an optimum condition for the cultivation of HCECs. When exposed to culture conditions, both primate and human CECs showed two distinct phenotypes: contact-inhibited polygonal monolayer and fibroblastic phenotypes. The use of SB431542, a selective inhibitor of the transforming growth factor-beta (TGF-β) receptor, counteracted the fibroblastic phenotypes to the normal contact-inhibited monolayer, and these polygonal cells maintained endothelial physiological functions. Expression of ZO-1 and Na^+/K^+-ATPase maintained their subcellular localization at the plasma membrane. Furthermore, expression of type I collagen and fibronectin was greatly reduced. This present study may prove to be the substantial protocol to provide the efficient *in vitro* expansion of HCECs with an inhibitor to the TGF-β receptor, and may ultimately provide clinicians with a new therapeutic modality in regenerative medicine for the treatment of corneal endothelial dysfunctions.

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Introduction

Corneal endothelial dysfunction is a major cause of severe visual impairment leading to blindness due to the loss of endothelial function that maintains corneal transparency. Restoration to clear vision requires either full-thickness corneal transplantation or endothelial keratoplasty. Recently, highly effective surgical techniques to replace corneal endothelium [e.g., Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK)] have been developed [1–3] that are aimed at replacing penetrating keratoplasty for overcoming pathological dysfunctions of corneal endothelial tissue. At present, our group and several other research groups have focused on the establishment of new treatment methods suitable for a practical clinical intervention to repair corneal endothelial dysfunctions [4–9]. Since corneal endothelium is composed of a monolayer and is a structurally flexible cell sheet, corneal endothelial cells (CECs) have been cultured on substrates including collagen sheets, amniotic membrane, or human corneal stroma. Then the cultured CECs are transplanted as a cell sheet. However, these techniques require the use of an artificial or biological substrate that may introduce several problems such as substrate transparency, detachment of the cell sheet from the cornea, and technical difficulty of transplantation into the anterior chamber. In our effort to overcome those substrate-related problems, we previously demonstrated that the transplantation of cultivated CECs in combination with a Rho kinase (ROCK) inhibitor enhanced the adhesion of injected cells onto the recipient corneal tissue without the use of a substrate and successfully achieved the recovery of corneal transparency in two corneal-endothelial-dysfunction animal models (rabbit and primate) [10,11].

However, in the context of the clinical setting, another pivotal practical issue is the *in vitro* expansion of human CECs (HCECs). HCECs are vulnerable to morphological fibroblastic change under normal culture conditions. Although HCECs can be cultivated into a normal phenotype maintaining the contact-inhibited polygonal monolayer, they eventually undergo massive endothelial-mesenchymal transformation after long-term culture or subculture. Thus, cultivation of HCECs with normal physiological function is difficult, yet not impossible [12,13].

Epithelial mesenchymal transformation (EMT) has been well characterized in epithelial-to-mesenchymal transition, and transforming growth factor-beta (TGF-β) can initiate and maintain EMT in a variety of biological and pathological systems [14,15].

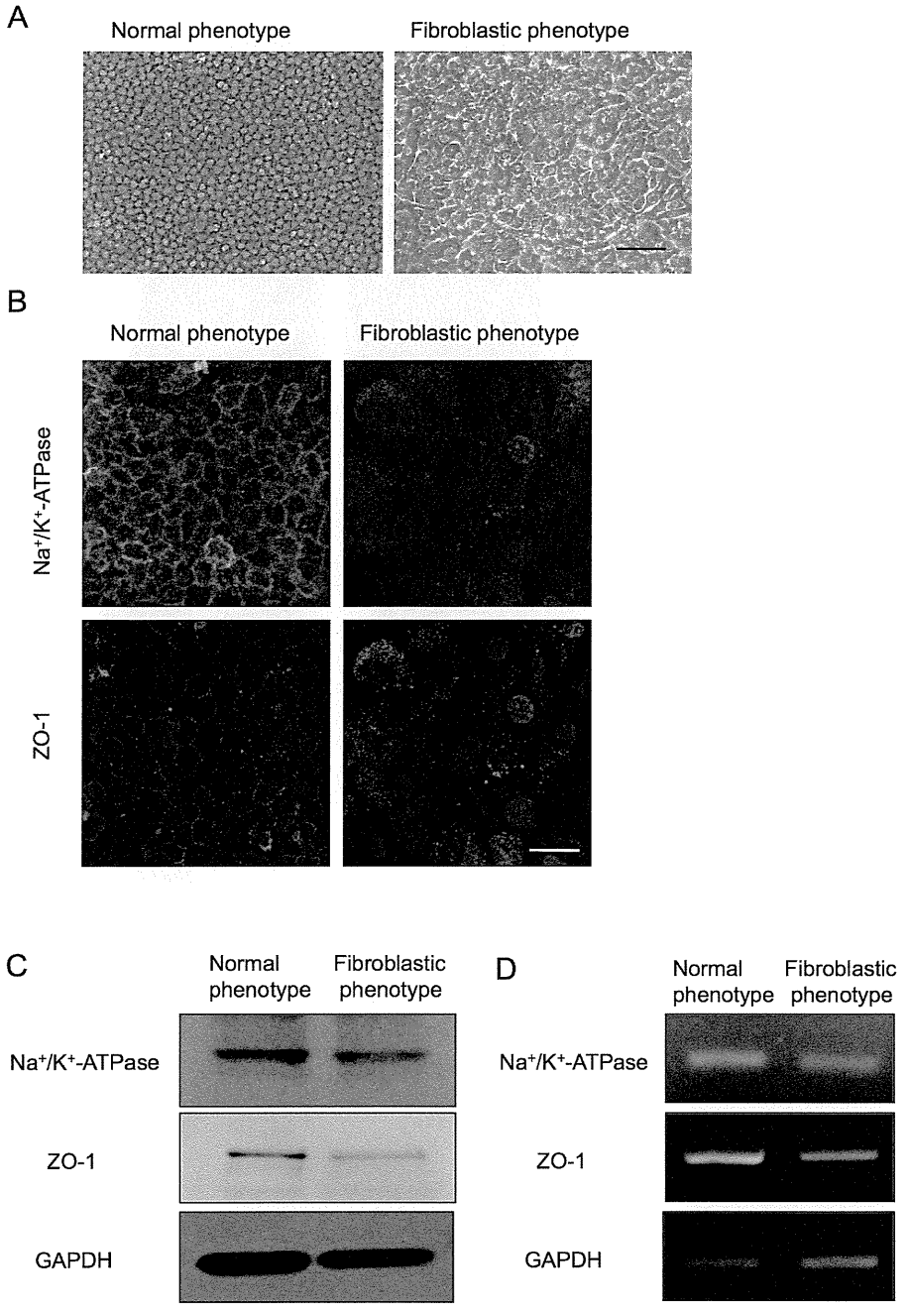

Figure 1. Primate corneal endothelial cells exhibit fibroblastic phenotype and lose functions during cell culture. (A) Cultivated primate CECs demonstrated two distinctive phenotypes; the cells maintained the characteristic polygonal cell morphology and contact-inhibited phenotype (normal phenotype) and the cells showed a fibroblastic cell shape with multi-layering (fibroblastic phenotype). Both phenotypes of the cultured CECs were primary cultured cells. Scale bar: 50 μm. The experiment was performed in triplicate. (B) Na^+/K^+-ATPase and ZO-1 at the plasma membrane was preserved in the normal phenotype, while fibroblastic phenotype completely lost the characteristic staining profile of Na^+/K^+-ATPase and ZO-1 at the plasma membrane. Scale bar: 100 μm. (C+D) Expression of the Na^+/K^+-ATPase and ZO-1 was higher in normal phenotypes than in the fibroblastic phenotypes at both the protein and mRNA levels. Samples were prepared in duplicate. Immunoblotting and semiquantitative PCR were performed in duplicate.
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The cellular activity of TGF-β is of particular interest in epithelial cells, as it inhibits the G1/S transition of the cell cycle in these cells. However, the same growth factor is the key signaling molecule for EMT, and the role of TGF-β as a key molecule in the development and progression of EMT is well studied [14–17]. Smad2/3 are signaling molecules downstream of cell-surface receptors for TGF-β in epithelial-to-mesenchymal transition [16,17]. Similar to epithelial cells, TGF-β inhibits the G1/S transition of the cell cycle in CECs [18,19], however, it is not known how TGF-β develops endothelial to mesenchymal transformation and maintains it in CECs. Endothelial-mesenchymal transformation is observed among corneal endothelial dysfunctions such as Fuchs' endothelial corneal dystrophy, pseudoexfoliation syndrome, corneal endotheliitis, surgically-induced corneal