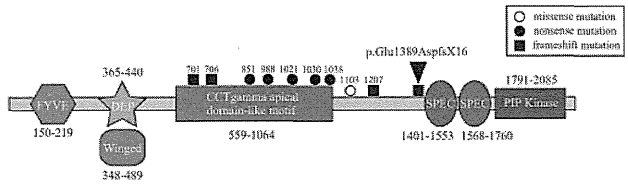

Figure 3. Schematic representation of the domain structure of the *PIKFYVE* gene. Mutations reported thus far are depicted with amino acid numbers and effects on the protein sequence (empty circle: missense, solid circle: nonsense, solid square: frameshift).

alteration and have a significant impact on the function of the *PIKFYVE* protein. A homology search of its amino acid sequence identified six putative functional domains in the *PIKFYVE* protein [15,16], including a zinc-finger-containing phosphoinositide kinase (FYVE) located at the 150–219 amino acid region, Disheveled, EGL-10, plextrin homology domain (DEP) of unknown function located at the 365–440 amino acid region, a cytosolic chaperonin CCT gamma apical domain-like motif located at the 667–843 amino acid region, a common kinase core motif found in the type IIβ phosphatidylinositol-4-phosphate 5-kinase (PIP kinase) located at the 1791–2085 amino acid region, a small β-sheet "winged helix " DNA/RNA-binding motif (Winged) located at the 348–489 amino acid region, and two spectrin repeats (SPEC) located at the 1490–1538 and 1679–1729 amino acid regions (Figure 3). Our mutation may lead to a truncation of the *PIKFYVE* protein lacking the two spectrin repeats and phosphatidylinositol-4-phosphate 5-kinase core domains. Thus, the mutation might imply the necessity of at least each of the two domains for completing the function of the *PIKFYVE* protein, at least in corneal keratocytes. The fact that the mutation was not found in any of the examined 96 normal Japanese volunteers, along with the fact that the phenotype well cosegregated with genotype in our pedigree, supports the pathogenicity of the mutation.

There are several genetically modified model organisms for the *PIKFYVE* gene. In *C. elegans* [17] and *Drosophila* [18], several mutant lines were generated for the *PIKFYVE* gene with the ethyl methanesulfonate mutagenesis technique. In both organisms, mutant lines harboring loss-of-function mutations of the gene displayed numerous vacuoles in their cells and died at the embryonic or pupal stages. However, partial loss-of-function situation in the worm model displayed growth retardation. In mice, although no knockout models have been established for the gene, knockout lines were generated for the *Sac3* [19] and *ArPIKfyve* [20] genes, both of which are functionally associated with the *PIKFYVE* gene.

Table 2. List of mutations thus far reported within the *PIKFYVE* gene in FCD patients.

Region	Nucleotide change	Amino acid change	Original description	Report
Exon 16	c.2098delA	p.Asn701ThrfsX7	2256delA	Li et al. [15]
Exon 16	c.2116_2117delCT	p.Leu706ValfsX6	2274delCT	Li et al. [15]
Intron 19	c.3619 −1G>C	p.Val1207AlafsX11	IVS19–1G→C,intron 19	Li et al. [15]
Exon 19	c.2551C>T	p.Arg851X	R851X	Li et al. [15]
Exon 19	c.2962C>T	p.Gln988X	Q988X	Li et al. [15]
Exon 19	c.3088G>T	p.Glu1030X	E1030X	Li et al. [15]
Exon 19	c.3112C>T	p.Arg1038X	R1038X	Li et al. [15]
Exon 19	c.3308A>G	p.Lys1103Arg	K1103R	Li et al. [15]
Exon 19	c.2902_2905delCCTT	p.Asp1021ThrfsX28	c.3060–3063delCCTT	Kotoulas et al. [13]
Exon 24	c.4166_4169delAAGT	p.Glu1389AspfsX16	p.Glu1389AspfsX16	This report

The notation convention of the nucleotide and amino acid changes follows the nomenclature guidelines of human genome variation society (HGVS). Note that there is a confusing situation in that the numbers of the exons and the intron of the *PIKFYVE* mutations listed in IC3D [21] appear to be incorrect, while those originally described by Li et al. [15] are correct. There is also another confusing situation in that the nucleotide number of the mutation reported by Li et al. and Kotoulas et al. [13] appears to be of mRNA, not of the coding sequence. We corrected this nucleotide number so that it is now in accordance with the nomenclature convention of the HGVS.

In both knockout lines, neurologic defects and juvenile or perinatal death was seen. Thus, complete loss of function of the *PIKFYVE* gene may lead to death in many organisms, possibly also in humans, which may account for the fact that all of the mutations thus far reported within the *PIKFYVE* gene, including the mutation shown in this report, are heterozygous.

No mutations have been previously reported for this disease in the Japanese population. In addition, even on a global scale, only two studies have been conducted to report nine mutations within the *PIKFYVE* gene in patients with FCD [13,15] (Table 2). This may be mostly due to the quite faint corneal phenotype with almost no disturbance in visual function in patients with this disease, even when those patients are older. We theorize that most ophthalmologists may fail to notice the subtle opacities associated with this corneal dystrophy. Moreover, there is a good chance that most patients with this disease might never visit an eye clinic complaining of symptoms associated with this disease. We imagine that the prevalence of patients with FCD might be much more common than has been recognized in previous reports, and potentially may exist in many countries.

In summary, we show here that a novel mutation (p.Glu1389AspfsX16) causing the truncation of the *PIKFYVE* protein causes fleck corneal dystrophy in the Japanese population. We hope that this study will contribute to future investigations focusing on understanding the biochemical properties and physiologic significance of the *PIKFYVE* gene as well as elucidating the molecular pathogenesis of FCD.

ACKNOWLEDGMENTS

This work was supported in part by grant-in-aids (#23390404, #24592675) from the Japanese Ministry of Education, Science, Culture and Sports and by a grant (H23-Nanchi-Ippan-084) from Japanese Ministry of Health, Labour and Welfare. The authors wish to thank John Bush for his assistance in reviewing this manuscript. The authors also wish to thank the staff members at Tokai Eye Clinic for their excellent clinical support, as well as the case report patient and her family members for their participation in this study. There is no commercial interest with regard to this manuscript. There is no prior presentation regarding this manuscript.

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ESTABLISHMENT OF A HUMAN CONJUNCTIVAL EPITHELIAL CELL LINE LACKING THE FUNCTIONAL *TACSTD2* GENE (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

By Shigeru Kinoshita MD PhD, Satoshi Kawasaki MD PhD, Koji Kitazawa MD, and Katsuhiko Shinomiya MD PhD

ABSTRACT

Purpose: To report the establishment of a human conjunctival epithelial cell line lacking the functional tumor-associated calcium signal transducer 2 (*TACSTD2*) gene to be used as an in vitro model of gelatinous drop-like corneal dystrophy (GDLD), a rare disease in which the corneal epithelial barrier function is significantly compromized by the loss of function mutation of the *TACSTD2* gene.

Methods: A small piece of conjunctival tissue was obtained from a GDLD patient. The conjunctival epithelial cells were enzymatically separated and dissociated from the tissue and immortalized by the lentiviral introduction of the SV40 large T antigen and human telomerase reverse transcriptase (*hTERT*) genes. Population doubling, protein expression, and transepithelial resistance (TER) analyses were performed to assess the appropriateness of the established cell line as an in vitro model for GDLD.

Results: The life span of the established cell line was found to be significantly elongated compared to nontransfected conjunctival epithelial cells. The SV40 large T antigen and *hTERT* genes were stably expressed in the established cell line. The protein expression level of the tight junction–related proteins was significantly low compared to the immortalized normal conjunctival epithelial cell line. TER of the established cell line was found to be significantly low compared to the immortalized normal conjunctival epithelial cell line.

Conclusions: Our conjunctival epithelial cell line was successfully immortalized and well mimicked several features of GDLD corneas. This cell line may be useful for the elucidation of the pathogenesis of GDLD and for the development of novel treatments for GDLD.

Trans Am Ophthalmol Soc 2012;110:166-177

INTRODUCTION

PURPOSE OF THIS THESIS

In some human body tissues, epithelial cells are exposed to the external environment, which is sometimes bacteria-rich and not consistent in relation to various kinds of attributes, such as temperature, osmotic strength, and pH. For the maintenance of a constant internal environment in the human body, epithelial cells play an important role: they form a definitive border between the external environment and the body by inhibiting outside fluids from permeating into the body as well as by inhibiting internal body fluids from permeating out through external tissue layers. Of special note is that in skin epidermis, the epithelial barrier function is important for protecting the body from dehydration and for acting as a defense against bacterial invasion into the body. The epithelial barrier function is mainly produced through the existence of a special "tight junction" cellular structure that is composed of several functional proteins such as claudin (CLDN), occludin (OCLN), and zonula occludens-1 (ZO-1), also known as tight junction protein 1 (TJP1).[1]

The ocular surface is composed of two similar, but different, types of epithelia—conjunctival and corneal. In the cornea, the epithelial barrier function is known to be essential for good vision. When the tight junction of corneal epithelial cells is compromised, amyloid deposition sometimes occurs at the subepithelial region of the cornea, possibly because of an excessive permeation of tear fluid into the corneal tissue. The causes of a compromised epithelial barrier function in the cornea include trichiasis, keratoconus, and the loss of function mutation of the tumor-associated calcium signal transducer 2 (*TACSTD2*) gene. The *TACSTD2* gene has been reported to be essential for the proper formation of the tight junction,[2] and the loss of *TACSTD2* gene expression reportedly leads to gelatinous drop-like corneal dystrophy (GDLD; Online Mendelian Inheritance in Man [OMIM] 204870).[3] However, the findings of a previous report implied the existence of another responsible gene for this disease.[4] In such situations, visual acuity is significantly decreased because of irregular astigmatism, which can be treated only through the replacement of corneal tissue.

The purpose of this thesis is to review previous studies, both from our group and from others, regarding GDLD, as well as to report our new data pertaining to the establishment of an immortalized conjunctival epithelial cell line that was derived from a GDLD patient. The established conjunctival epithelial cell line lacking the functional *TACSTD2* gene may be useful for the assessment of potential novel treatments for GDLD, such as the administration of a proteasome inhibitor onto the cornea.

PREVIOUS REPORTS ON GDLD

GDLD is an uncommon autosomal recessive disease that is characterized by bilateral corneal amyloidosis.[5] Although this disease is still quite rare in many countries, it is relatively common in Japan, with an estimated prevalence rate of 1 in 31,546 based on the frequency of parental consanguinity (Fukjiki K, et al. Seventh International Congress on Human Genetics 1986;248-249; Abstract).[6] In the first decade of life of GDLD patients, subepithelial nodular amyloid depositions appear and result in severe photophobia, excessive tearing, and foreign body sensation.[7,8] As the age of those patients progresses, the amyloid depositions typically enlarge, increase in number, coalesce, and exhibit a mulberry-like appearance, thus leading to severe bilateral vision loss that usually begins

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within the third decade of life. The clinical phenotype of GDLD is known to significantly vary among individuals with the disease, and, in fact, four unique corneal phenotypes for GDLD have previously been reported (Figure 1).[9]

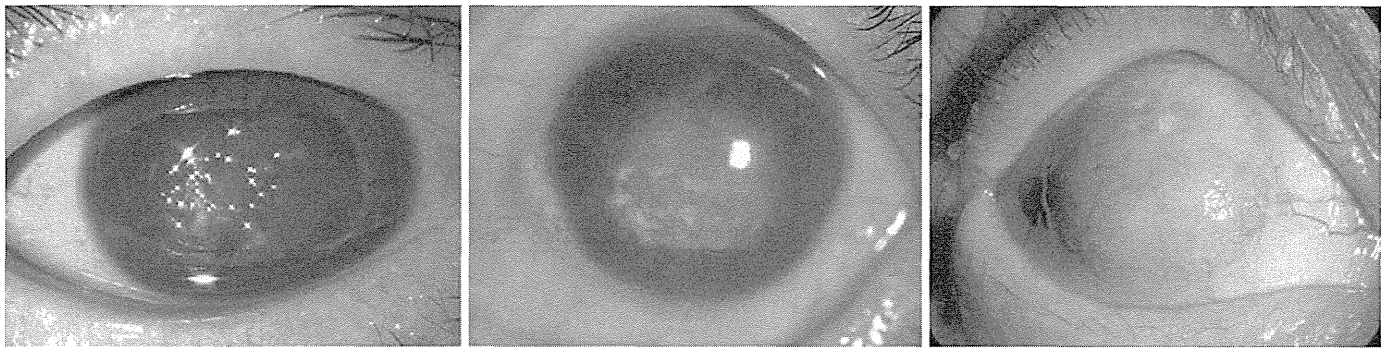

FIGURE 1

Corneal clinical phenotypes for gelatinous drop-like corneal dystrophy: (GDLD) mulberry type (left), band keratopathy type (middle), and kumquat-like type (right). Reprinted with permission from *Developments in Ophthalmology*.[36] All rights reserved.

The subepithelial amyloid deposition was discovered to be derived from lactoferrin by Klintworth and associates[10]; however, they found no mutation within the lactoferrin gene of GDLD patients.[11] The responsible genomic region for GDLD was identified by Tsujikawa and associates[12] in 1998 by the use of a linkage analysis technique, which was further narrowed to 2.6 centimorgans by a subsequent haplotype analysis. The following year, that group finally identified the causative gene for GDLD by sequencing of the genes that were included within the narrowed critical genomic region.[3] The responsible gene for GDLD was identified as the *TACSTD2* gene, which had already been reported as a transmembrane protein with potential cancer-promoting activity.[13,14] However, the findings of a previous report implied the existence of another responsible gene for this disease.[4] After this discovery, a large number of mutation studies were conducted, resulting in the discovery of 26 unique mutations in GDLD patients from different countries and different ethnic backgrounds.[15-19]

In patients with GDLD, the epithelial barrier function is significantly decreased (Figure 2). Quantock and associates[20] reported an increased permeation of horseradish peroxidase, which was used as a molecular tracer with a molecular weight of 44 kDa, in a GDLD cornea (Figure 3). Our group demonstrated that in the cornea of GDLD patients, fluorescein permeation was significantly increased, possibly due to an excessive desquamation and loosened intercellular space of epithelial cells (Figure 4).[21] We also reported the decreased expression of tight junction–related proteins CLDN 1, TJP1, and OCLN in the epithelium of GDLD patients (Figure 5).[22] Moreover, we reported that the *TACSTD2* gene is essential for the formation of the tight junction by regulating the subcellular localization of tight junction–related proteins such as CLDN 1 and 7.[2] We also proposed the possibility of an excessive protein degradation of CLDN 1 and 7 in the epithelial cells of GDLD corneas, possibly through the ubiquitin-proteasome system (Figure 6).

From these lines of evidence reported by us and the other groups mentioned above, the pathological sequence that occurs in GDLD corneas appears to be (1) a loss of function mutation of the *TACSTD2* gene, (2) excessive protein degradation of CLDN 1 and 7, (3) the failure of the maturation process of the tight junction, (4) an increased tear permeation into corneal stroma through the loosened epithelial barrier of the cornea, and finally (5) transformation of the permeated lactoferrin to amyloid deposition by mechanisms that are still undefined.

METHODS

ETHICAL ISSUES

All experimental procedures were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine (accepted January 15, 2010, with an approval number: C-660). Prior informed consent was obtained from all patients after a detailed explanation of the study protocols, and this study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

BIOHAZARD

For the production and the use of the lentivirus vector, we used a P2-level biohazard room after obtaining permission from the Institutional Review Board for Studies in Gene Recombination of Kyoto Prefectural University of Medicine.

OLIGOMERS

All oligomers used in this study were synthesized by Life Technologies Corporation (Carlsbad, California).

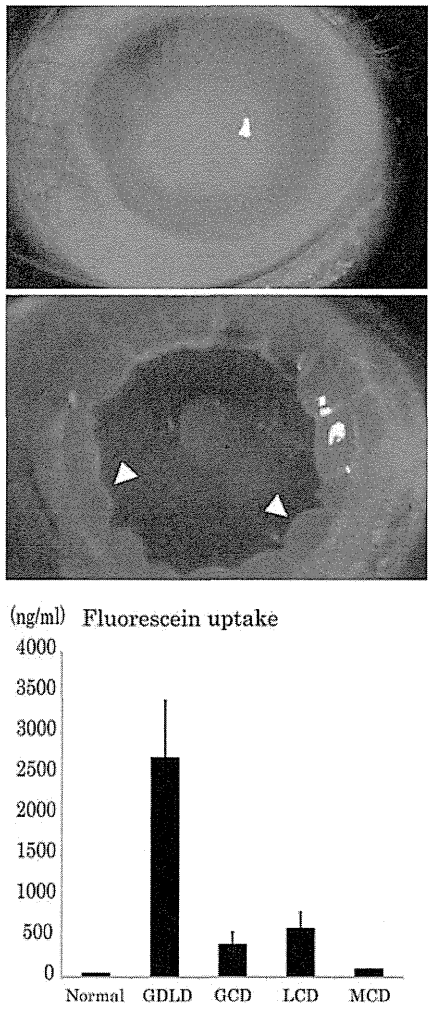

FIGURE 2

Epithelial barrier function is significantly decreased in cornea with gelatinous drop-like corneal dystrophy (GDLD). Top, Slit-lamp microscopy photograph of the hyperfluorescence of the cornea observed in a GDLD patient. Middle, Slit-lamp microscopy photograph demonstrating a GDLD cornea that underwent keratoplasty. Triangles indicate the boundary between the host corneal epithelium and the donor corneal epithelium. Note that the host corneal epithelium demonstrates hyperfluorescence, but that of the donor cornea does not. Bottom, Bar graph demonstrating fluorescein uptake among several corneal dystrophies. GCD, granular corneal dystrophy; LCD, lattice corneal dystrophy; MCD, macular corneal dystrophy. Reprinted with permission from *Developments in Ophthalmology*.[36] All rights reserved.

FIGURE 3

Electron microscopy image showing decreased epithelial barrier function in a cornea with GDLD. Permeated horseradish peroxidase is demonstrated through the loosened epithelial barrier. Horseradish peroxidase is visible as an electron-dense tracer in a degenerated superficial epithelial cell (asterisk), and is seen penetrating beneath the epithelial surface (arrows). Reprinted with permission from *Cornea*.[20] All rights reserved.

ANTIBODIES

All antibodies were raised against human antigens. The primary antibodies used in this study included anti-SV40 large T antigen (mouse monoclonal [MM]; Abcam plc, Cambridge, United Kingdom), anti-human telomerase reverse transcription (anti-hTERT) (MM; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), anti-CLDN1 (MM, clone 1C5-D9; Abnova Corporation, Taipei, Taiwan), CLDN4 (MM, clone 3E2C1; Zymed Laboratories, Inc., South San Francisco, California), CLDN7 (MM, clone 5D10F3; Zymed Laboratories), TACSTD2 (MM, clone 77220 or goat polyclonal; R&D Systems, Inc., Minneapolis,

Minnesota), TJP1 (MM, clone ZO1-1A12; Life Technologies), and OCLN (goat polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, California). For an isotype control, normal mouse IgG_1 (Dako Denmark A/S, Glostrup, Denmark), normal mouse IgG2a (Ancell Corporation, Bayport, Minnesota), or normal goat IgG (Santa Cruz Biotechnology) was used.

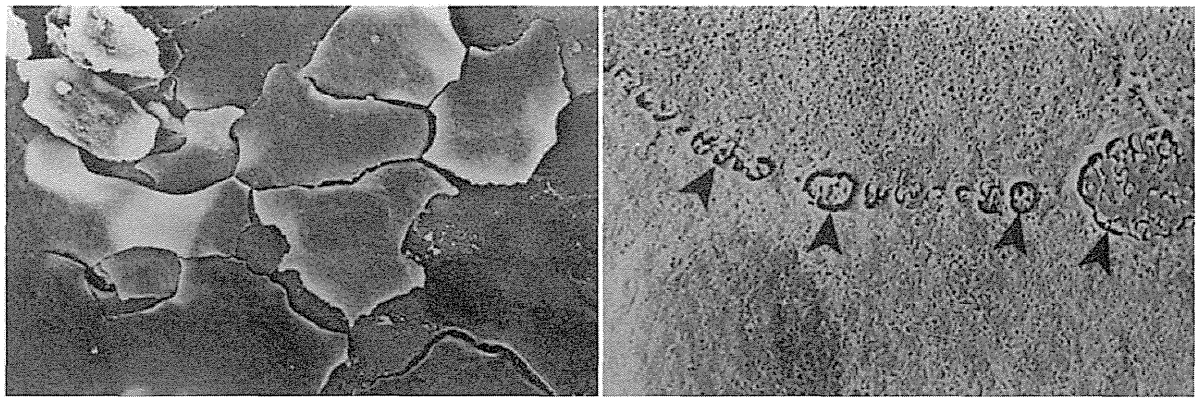

FIGURE 4

Electron micrographs showing excessive desquamation of superficial corneal epithelial cells of cornea (left) and enlarged intercellular space of corneal epithelium (right) in a patient with GDLD. Reprinted with permission from *Cornea*.[21] All rights reserved.

TISSUE PREPARATION AND CELL CULTURE

A small sample (2 mm × 2 mm) of conjunctival tissue was obtained from a normal subject and from a GDLD patient at the time of cataract surgery. The conjunctival tissues were then soaked overnight in 1000 PU/mL of dispase (Dispase I; Sanko Junyaku Co, Ltd., Tokyo, Japan) at 4°C. Next, the epithelial sheet was peeled from the underlying stroma and dissociated by the treatment of 0.05% trypsin at 37°C for 5 minutes. The dissociated conjunctival epithelial cells were then cultured in a growth-facilitating medium (CnT-20; CELLnTEC, Bern, Switzerland). Supplemented hormonal epithelial medium (SHEM), composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplemented with 10% fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, 5 μg/mL insulin, 0.1 μg/mL cholera toxin, and a mixture of antibiotic and antimycotic (Anti-Anti; Life Technologies), was also used for the specific purpose of measuring the transepithelial resistance. HCE-T and HeLa cells were cultured in the SHEM medium and DMEM medium supplemented with 10% FBS and a mixture of antibiotic and antimycotic (Anti-Anti), respectively.

DIRECT SEQUENCING

Genomic DNA was extracted from the peripheral blood of the GDLD patient using a commercially available kit (DNeasy Blood & Tissue Kit; QIAGEN GmbH, Hilden, Germany). Sequencing analysis was performed using a commercially available kit (BigDye3.1; Applied Biosystems, Inc., Foster City, California). Polymerase chain reaction (PCR) was performed with a primer pair against the *TACSTD2* gene (TACSTD2_seq_F; 5′-CCT GCA GAC CAT CCC AGA C-3′, TACSTD2_seq_R; 5′-CAG GAA GCG TGA CTC ACT TG-3′) that fully covered the coding sequence of this gene. The PCR product was bidirectionally sequenced in a 20 μL reaction buffer containing a 2× sequencing mixture and either of the above two primers. After purification with a commercially available kit, the sequence products were electrophoresed on an automated capillary sequencer (Genetic Analyzer 3130*xl*; Applied Biosystems).

LENTIVIRAL INTRODUCTION OF SV40 LARGE T ANTIGEN AND hTERT

RNA was extracted from immortalized human corneal epithelial cells (HCE-T, RCB1384, RIKEN Cell Bank, Ibaraki, Japan) and reverse transcribed. Using the cDNA, reverse transcription PCR (RT-PCR) was performed to amplify the coding sequence of the SV40 large T antigen (SV40_LTEG_exp_F; 5′-GGC GCC ATG GAT AAA GTT TTA AAC AGA GAG GA-3′, SV40_LTEG_exp_R; 5′-TTA TGT TTC AGG TTC AGG GGG AG-3′) and *hTERT* (hTERT_exp_F; 5′-ACC CCC GCG ATG CCG CGC GCT CCC-3′, hTERT_exp_R; 5′-GGG TGA GGT GAG GTG TCA CCA ACA AG-3′) genes. The amplified products were cloned into a commercial lentiviral vector (pLenti6.3/V5-TOPO; Life Technologies) and were validated by sequencing analysis using a primer pair (CMV_seq_F; 5′-CGC AAA TGG GCG GTA GGC GTG-3′, V5_seq_R; 5′-ACC GAG GAG AGG GTT AGG GAT-3′). The lentiviral vectors were transfected to 293T cells along with a mixture of 3 packaging plasmids (ViraPower Packaging Mix; Life Technologies Corporation) using a commercial transfection reagent (FuGENE HD, Promega Corporation, Madison, Wisconsin). Supernatant of the culture medium for the 293T cells was harvested and stored in a −80°C freezer until use. The virus-containing supernatant was added onto the culture of the conjunctival epithelial cells after they were treated with 5 μg/mL of polybrene.

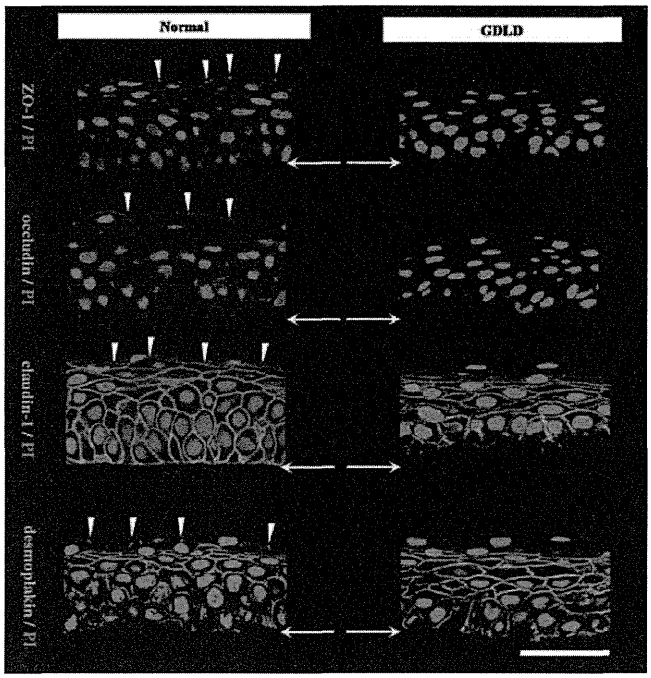

FIGURE 5

Results of immunostaining analysis show that the expression of the tight junction–related proteins, zonula occludens-1 (ZO-1) (top), occuludin (OCLN) (middle upper), claudin 1 (CLDN1) (middle lower), as well as desmosome protein desmoplakin (bottom), was significantly attenuated in cornea with gelatinous drop-like corneal dystrophy (GDLD) (right) compared to normal cornea (left). Arrowheads indicate the apical expression of these tight junction–related proteins in normal epithelium. Arrows indicate the basement of corneal epithelium. Bar = 50 μm. Reprinted with permission from *Investigative Ophthalmology & Visual Science.*[22] All rights reserved.

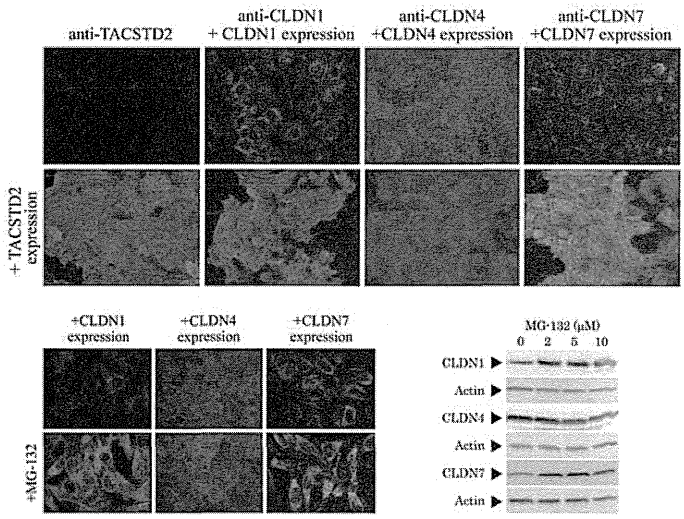

FIGURE 6

Data indicating that the tumor-associated calcium signal transducer 2 (*TACSTD2*) protein may protect CLDN1 and 7 proteins from protein degradation by the ubiquitin-proteasome system. Top, HeLa cells were introduced with CLDN1, 4, or 7 genes with or without the *TACSTD2* gene. Introduction of the *TACSTD2* gene significantly enhanced the expression of CLDN1 and 7, but not of CLDN4. Bottom left, HeLa cells introduced with CLDN1, 4, or 7 genes were treated with the proteasome inhibitor MG-132. The MG-132 treatment significantly enhanced the expression of CLDN1 and 7, but not of CLDN4. Bottom right, Immunostaining analysis data was confirmed by Western blotting analysis. Reprinted with permission from *American Journal of Pathology.*[2] All rights reserved.

hTERT GENES

RNA was extracted from immortalized human corneal epithelial cells (HCE-T, RCB1384; RIKEN Cell Bank) and reverse transcribed. Using the cDNA, RT-PCR was performed to amplify the coding sequence of the SV40 large T antigen (SV40_LTEG_exp_F; 5′-GGC GCC ATG GAT AAA GTT TTA AAC AGA GAG GA-3′, SV40_LTEG_exp_R; 5′-TTA TGT TTC AGG TTC AGG GGG AG-3′) and *hTERT* (hTERT_exp_F; 5′-ACC CCC GCG ATG CCG CGC GCT CCC-3′, hTERT_exp_R; 5′-GGG TGA GGT GAG GTG TCA CCA ACA AG-3′) genes. The amplified products were then cloned into a commercial lentiviral vector (pLenti6.3/V5-TOPO; Life Technologies) and validated by sequencing analysis using a primer pair (CMV_seq_F; 5′-CGC AAA TGG GCG GTA GGC GTG-3′, V5_seq_R; 5′-ACC GAG GAG AGG GTT AGG GAT-3′). The lentiviral vectors were then transfected to 293T cells along with a mixture of 3 packaging plasmids (ViraPower Packaging Mix; Life Technologies) using a commercial transfection reagent (FuGENE HD; Promega). The supernatant of the culture medium for the 293T cells was extracted and stored in a –80°C freezer until use. The virus-containing supernatant was added onto the culture of the conjunctival epithelial cells after they were treated with 5 μg/mL of polybrene.

POPULATION DOUBLING (PD) ANALYSIS

Cell-growth kinetics was analyzed by PD analysis according to the standard procedure. Briefly, the conjunctival epithelial cells were seeded at 2×10^4 to 1×10^5 to a T25 plastic flask. The following day, cells that failed to reattach were collected and counted. Thereafter, those cells were fed every 2 days and harvested in 3 to 5 days while their growth was still in a mid-log phase, where cell confluency is

roughly less than 70%. Incremental PD per passage was calculated using a formula $\log_2$ (Ch/[Cs-Cc]), where *Ch* corresponds to the number of harvested cells, *Cs* to the number of seeded cells, and *Cc* to the number of collected cells.

COLONY-FORMING ASSAY

Conjunctival epithelial cells, 1×10^2 to 1×10^4, with or without the gene transfection, were seeded onto 6-well culture plates with mitomycin-C-treated 3T3 cells. The cells were then maintained in a growth-promoting medium (2:1 mixture of CnT-20 and SHEM). After some cell colonies appeared and grew to the size where they could be detected with the naked eye, the cells were fixed by formaldehyde, stained with 1% rhodamine B, and then photographed.

TELOMERIC REPEAT AMPLIFICATION PROTOCOL (TRAP) ASSAY

TRAP assay was performed according to the previous reports, yet with minor modifications. Briefly, 2×10^5 cells were lysed in a lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 1 × protease inhibitor cocktail (Nakarai, Kyoto, Japan), and 10% glycerol, and then incubated on ice for 30 minutes and centrifuged. The supernatant of the lysate was added to a reaction mixture containing 1 × TRAP reaction buffer (20 mM pH 8.3 Tris-HCl, 1.5 mM $MgCl^2$, 68 mM KCl, 0.05% Tween 20, 1 mM EGTA), 50 μM dNTP mix, and 344 nM TS primer (5′-AAT CCG TCG AGC AGA GTT-3′), and then incubated at room temperature for 30 minutes to allow the TS primer to elongate by the telomerase activity contained in the lysate. After purification by phenol-chloroform extraction and ethanol precipitation, the reaction mixture was amplified by PCR in a buffer containing 1 × ExTaq buffer, 0.2 mM dNTP, and 0.025 U/μL Taq polymerase (ExTaq Hot Start Version; Takara Bio Inc., Otsu, Japan) using a 344 nM primer pair (TS primer and CX primer; 5′-CCC TTA CCC TTA CCC TTA CCC TAA) with a 3-step thermal-cycle condition (95°C for 30 seconds, 50°C for 40 seconds, and 72°C for 45 seconds) for 35 cycles. The PCR products were then electrophoresed on a 10% nondenaturing acrylamide gel and stained with a gel-staining fluorescence solution (SYBR Green I; Takara Bio) and photographed.

IMMUNOSTAINING ANALYSIS

Cells grown on a commercially available culture-glass slide (Nunc Lab-Tek Chamber Slide System; Thermo Fisher Scientific, Inc., Rochester, New York) were fixed with Zamboni's fixative or 95% ice-cold ethanol, blocked with 1% skim milk, incubated overnight with a primary antibody at 4°C, washed, incubated with a secondary antibody (Alexa Fluor 488-labeled anti-mouse or anti-goat IgG; Life Technologies) at room temperature for 1 hour, washed again, counterstained with propidium iodide, mounted, covered with coverslips, and photographed by use of a fluorescence microscope (AX70 TRF; Olympus Corporation, Tokyo, Japan) or a confocal laser scanning microscope (TCS-SP2; Leica Microsystems GmbH, Wetzlar, Germany).

WESTERN BLOTTING ANALYSIS

Proteins were separated on a commercially available 4% to 20% gradient SDS-polyacrylamide gel (Mini-PROTEAN TGX; Bio-Rad Laboratories, Hercules, California) and transferred to a PVDF membrane (Trans-Blot Turbo Transfer Pack; Bio-Rad Laboratories). The membrane was then blocked in a TBS-T buffer containing 1% skim milk, incubated overnight with primary antibodies at 4°C, washed, incubated with a horseradish peroxidase–conjugated secondary antibody at room temperature for 1 hour, and then washed again. A chemiluminescent reagent (ECL Advance Western Blotting Detection Kit; GE Healthcare, Little Chalfont, United Kingdom) was then applied onto the membrane and its luminescent signal was detected by a chilled charge-coupled device digital imaging camera (LAS-3000UVmini; Fujifilm Corporation, Tokyo, Japan).

MEASUREMENT OF TRANSEPITHELIAL RESISTANCE (TER)

For the analysis of epithelial barrier function, transepithelial migration of a labeled tracer or a measuring of resistance between the apical side and basal side of epithelium is generally used. We previously demonstrated that *TACSTD2*-knocked-down corneal epithelial cells exhibited significantly decreased epithelial barrier function by measuring TER. Thus, we measured TER for the immortalized normal and diseased corneal epithelial cells. When the TER value is high, the epithelial barrier function is estimated to be high.

Epithelial cells were cultured in a 12-well Transwell (12-mm Transwell with 0.4-μm Pore Polyester Membrane Insert; Corning, Inc., Corning, New York) culture filter. Once the cells had reached 100% confluence, the culture medium was switched to a high calcium media (SHEM or a 1:1 mixture of CnT-20 and SHEM) to promote barrier function. Resistance between the upper and lower chambers of the Transwell filter was measured with the use of a volt-ohm meter (EVOM; World Precision Instruments, Inc., Sarasota, Florida), and the TER was then calculated by multiplying the measured resistance (ohms) by the growth area of the Transwell filter (1.12 cm^2). The background resistance due to the filter alone was subtracted from each of the obtained data.

RESULTS

Sequencing analysis of the *TACSTD2* gene revealed that the GDLD patient (Figure 7, top) who underwent cataract surgery bears a biallelic c.352C>T mutation, which may produce a p.Gln118x nonsense mutation (Figure 7, middle right). The p.Gln118x mutation is the most prevalent type of GDLD mutation in Japan and produces a truncated form of the TACSTD2 protein lacking the C-terminal transmembrane domain of the protein and is thus considered to be nonfunctional (Figure 7, bottom).

The lentiviral vectors harboring the coding sequence of the SV40 large T antigen and *hTERT* genes (Figure 8, top) were

successfully constructed and were cotransfected to the conjunctival epithelial cells of the GDLD patient. The cell proliferation kinetics of the conjunctival epithelial cells, with or without the gene transfection, was analyzed by PD analysis. The transfected conjunctival epithelial cells continued to proliferate even after the cumulative PDs during our culture process exceeded 35 PDs, whereas the conjunctival epithelial cells without the gene transfection stopped proliferating when their cumulative PDs during our culture process reached 12 PDs (Figure 8, middle left). The colony-forming assay revealed that the transfected conjunctival epithelial cells contained many cells with a colony-forming ability, whereas those without the gene introduction had only a few cells possessing a colony-forming ability (Figure 8, middle right). Most of the gene-introduced conjunctival epithelial cells were small in size with a high nucleus to cytoplasm ratio, whereas most of the cells without the gene introduction looked flattened and were large in size with a low nucleus to cytoplasm ratio at their final stage of culture (Figure 8, bottom).

Immunostaining and Western blotting analyses revealed that the immortalized conjunctival epithelial cells from the GDLD patient expressed the SV40 large T antigen and *hTERT* genes in the nucleus or cytoplasm (Figure 9, top and middle left). TRAP assay revealed that the transfected conjunctival epithelial cells possessed functional telomerase activity at nearly the same level as HeLa or HCE-T cells (Figure 9, bottom right).

The epithelial barrier function was measured by TER (Figure 10, top). Since the epithelial barrier function is strongly affected by the content of the culture media, we tested 3 different types of culture media, including low-calcium (0.07 mM) serum-free medium (Medium 1, CnT-20), high-calcium (1 mM) serum-containing (10%) medium (Medium 2, SHEM), and a 1:1 mixture of these 2 media (Medium 3) with moderate calcium ion strength (0.54 mM). The immortalized normal conjunctival epithelial cells exhibited significantly high TER after the switch of the culture media from Medium 1 to Medium 2 (Figure 10, bottom left) or 3 (Figure 10, bottom right), indicating that switching of culture media from low-calcium to high-calcium might promote tight junction formation, leading to higher resistance between the basal and apical sides of epithelium. On the other hand, the immortalized conjunctival epithelial cells derived from the GDLD patient exhibited significantly lower TER, even after switching the culture media from low-calcium to high-calcium.

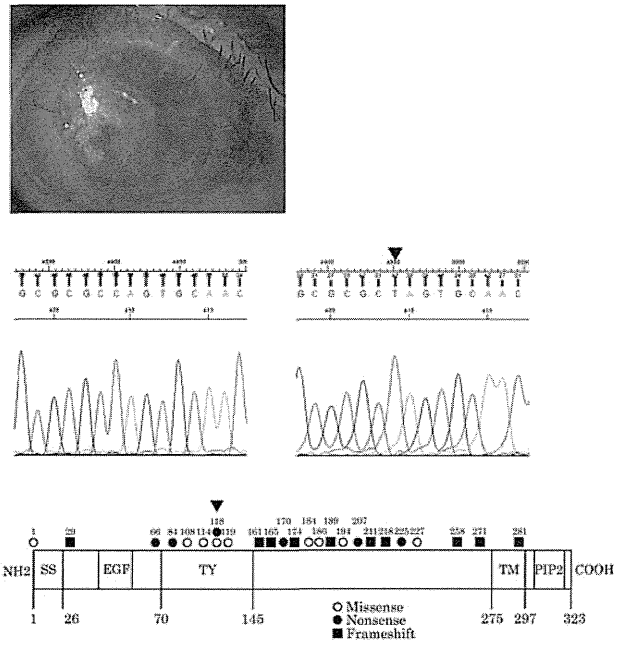

FIGURE 7

Clinical and genetic information for the gelatinous drop-like corneal dystrophy (GDLD) patient whose conjunctival tissue was obtained for the establishment of our immortalized conjunctival epithelial cell line. Top, Slit-lamp microscopy image demonstrating the clinical phenotype of the GDLD patient. Middle, Electropherogram data of the *TACSTD2* gene from a normal volunteer (left) and the GDLD patient (right). Triangle denotes the c.352C>T mutation, which may produce a p.Gln118x mutation within the *TACSTD2* gene. Bottom, Domain structure of the TACSTD2 protein with mutations thus far reported for the *TACSTD2* gene. SS, signal sequence; EGF, epidermal growth factor–like repeat; TY, thyloglobulin repeat; TM, transmembrane domain; PIP2, PIP2-binding sequence. Note that the TM domain

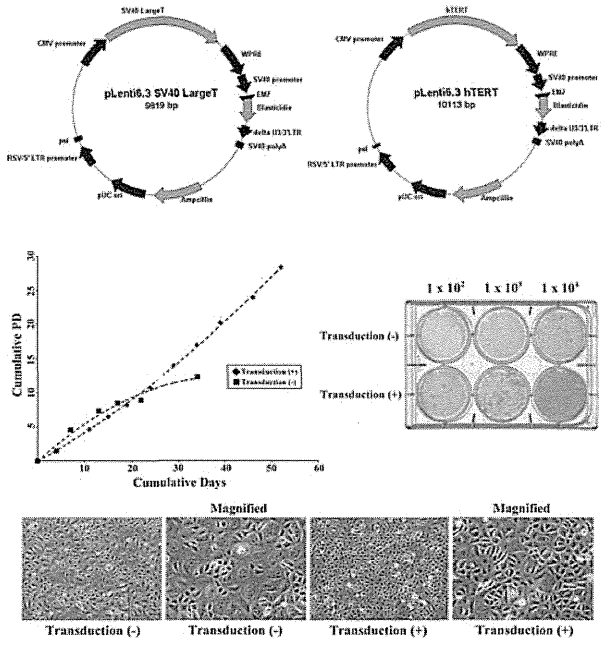

FIGURE 8

Lentiviral introduction of SV40 large T antigen and human telomerase reverse transcriptase (*hTERT*) genes significantly elongated the life span of the conjunctival epithelial cells from a gelatinous drop-like corneal dystrophy (GDLD) patient. Top, Plasmid map of the lentiviral vector harboring the expression cassette for the SV40 large T antigen (left) and *hTERT* (right) genes. Middle left, Scatter diagram showing the population doubling analysis data of transfected or nontransfected GDLD conjunctival epithelial cells fitted by quadratic curve. Note that the fitted curve of the transduced cells curves downward, whereas that of the nontransduced cells curves upward. Middle right, Results of colony-forming assay. Bottom, Cell shape of conjunctival epithelial cells of the GDLD patient with or

is located near the C-terminus of this protein; thus the p.Gln118x nonsense mutation (indicated by a triangle) may produce a truncated form of this protein.

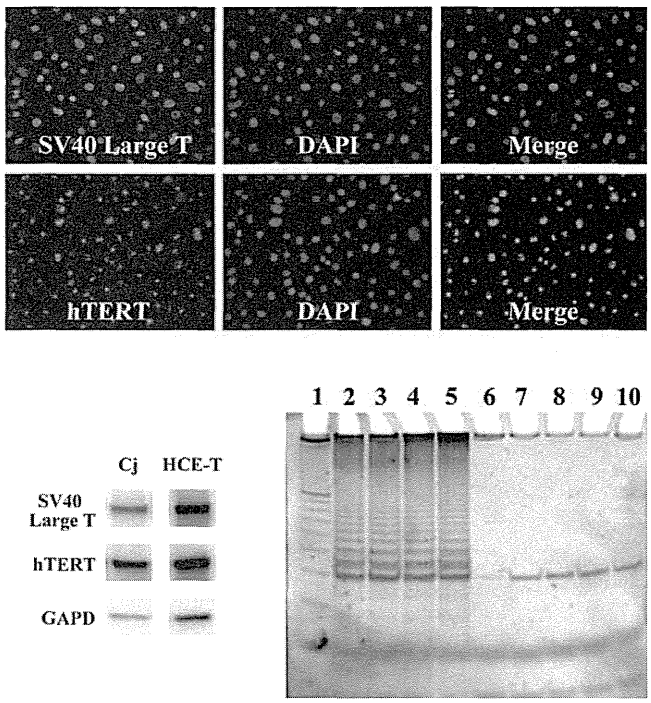

FIGURE 9

Expression of SV40 large T antigen and telomerase in the immortalized conjunctival epithelial cells from a gelatinous drop-like corneal dystrophy (GDLD) patient. Results of immunostaining (top) and Western blotting (bottom left) analyses for the expression of the SV40 large T antigen and *hTERT* genes in the immortalized conjunctival (Cj) epithelial cells from a GDLD patient and immortalized human corneal epithelial cells (HCE-T). Lysate of HCE-T cells was used in the Western blotting analysis (bottom left) as a positive control. Bottom right, Results of telomeric repeat amplification protocol (TRAP) assay show the expression of functional telomerase. The ladder pattern seen at lanes 2, 3, 4, and 5 indicates the existence of telomerase activity. 1: size marker, 2: HCE-T cells, 3: HeLa cells, 4: immortalized human normal conjunctival epithelial cells, 5: immortalized human GDLD conjunctival epithelial cells, 6: heat inactivated HCE-T cells, 7: heat-inactivated HeLa cells, 8: heat-inactivated immortalized human normal conjunctival epithelial cells, 9: heat-inactivated immortalized human GDLD conjunctival epithelial cells and 10: buffer only.

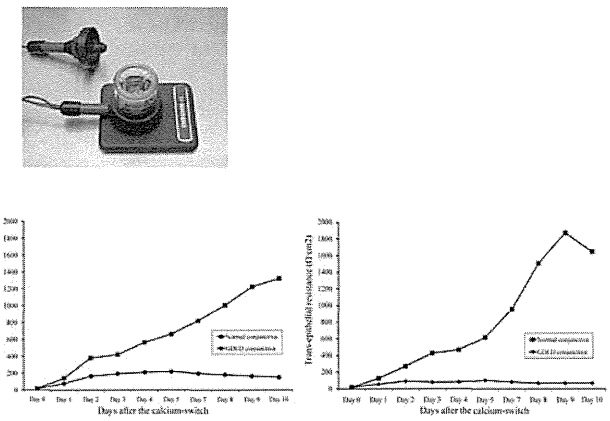

FIGURE 10

Decreased epithelial barrier function in the immortalized conjunctival epithelial cells from a gelatinous drop-like corneal dystrophy (GDLD) patient. Epithelial barrier function was investigated using a commercial voltmeter (top). Epithelial barrier function was found to be significantly low in the immortalized conjunctival epithelial cells from the GDLD patient both in high (1 mM, bottom left) and middle (0.54 mM, bottom right) calcium media.

As expected from the above mutation data, virtually no *TACSTD2* expression was found in the immortalized conjunctival epithelial cells from the GDLD patient (Figure 11, top). The findings of our recently published reports indicated a decreased expression of some tight junction–related proteins in the *TACSTD2*-knocked-down HCE-T cells.[2] Western blotting analyses clearly demonstrated that the expression level was significantly decreased in CLDN1 and 7 proteins but almost unchanged in CLDN4, OCLN, and TJP1 as compared to immortalized normal conjunctival epithelial cells (Figure 11, bottom), which is fairly consistent with our

previous data.

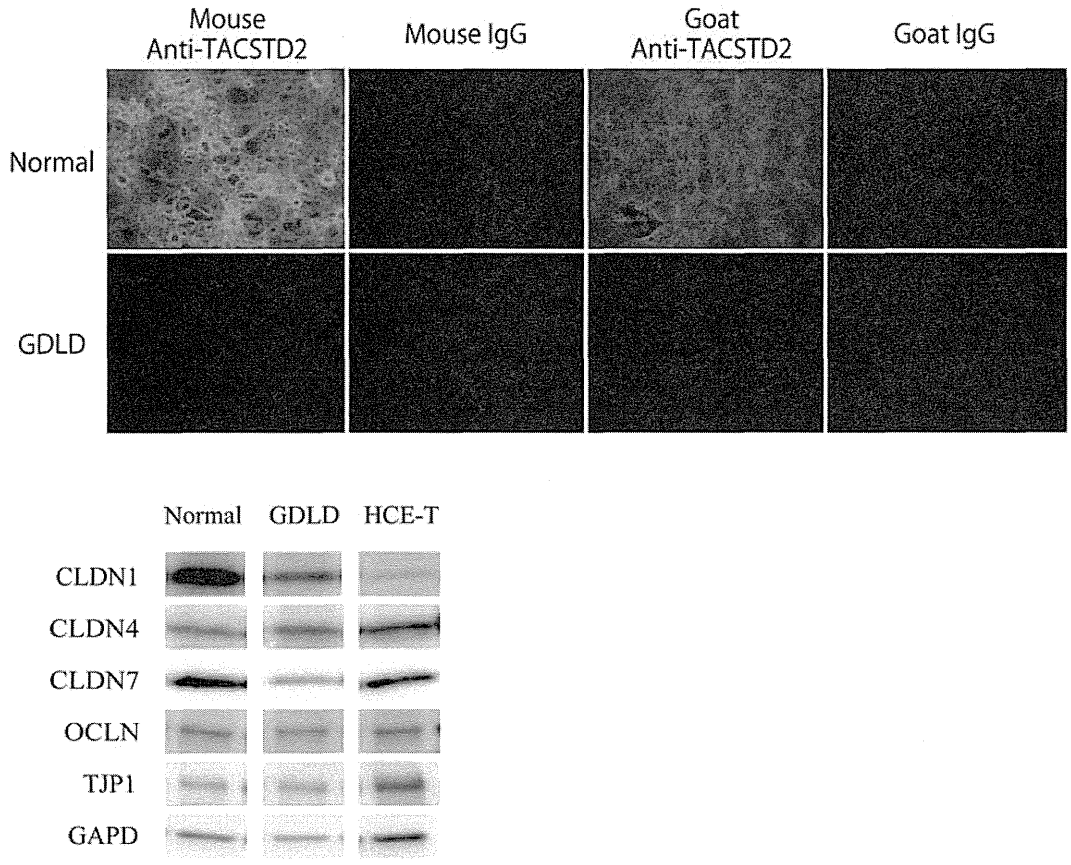

FIGURE 11

Expression of the *TACSTD2* and tight junction–related proteins in the immortalized conjunctival epithelial cells from a gelatinous drop-like corneal dystrophy (GDLD) patient. Top, Results of immunostaining analysis for the expression of the *TACSTD2* protein in the immortalized normal and GDLD conjunctival epithelial cells. Bottom, Protein expression of tight junction–related proteins CLDN1, CLDN4, CLDN7, OCLN, and tight junction protein-1 (TJP1) in the immortalized normal and GDLD conjunctival epithelial cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) was investigated as a loading control. HCE-T cells were also used as a positive control.

DISCUSSION

In this study, we established a conjunctival epithelial cell line from a GDLD patient that lacked the functional *TACSTD2* gene. The cells exhibited a prolonged life span and stable expression of the SV40 large T antigen and the *hTERT* genes. In addition, the cells demonstrated significantly decreased epithelial barrier function along with a decreased expression of the tight junction–related proteins. These results indicate that our established conjunctival epithelial cells were successfully immortalized and well mimicked several features of GDLD corneas, including decreased epithelial barrier function and decreased expression of CLDN1 and 7 proteins.

There are several methods in which to achieve cell immortality. The SV40 large T antigen has previously been employed for the immortalization of various types of cells from various kinds of animals. This gene is known to bind to retinoblastoma 1 (RB1) and p53 (TP53) proteins and inhibit their tumor-suppressing activity,[23] thereby allowing cells to proliferate infinitely in spite of the existence of some cyclin-dependent kinase inhibitors, such as p16 or p21. Other than the SV40 large T antigen gene, human papillomavirus (HPV) E6 and E7 genes have been used for the immortalization process,[24,25] and they respectively bind to and inhibit TP53 and RB1 proteins.[26,27] The *hTERT* gene is a reverse transcriptase subunit of telomerase that elongates the telomere of the linear chromosome of

eukaryotic cells, which is essential for cell surival.[28] Since this gene is generally silenced in most human cells, even in their related stem cells, introduction of the SV40 large T antigen gene or HPV E6 and E7 genes appears to be insufficient for cell immortalization, even though some cell lines have been successfully immortalized without the introduction of the *hTERT* gene,[29] possibly because of the spontaneous activation of the endogenous *hTERT* gene. In this present study, we introduced the *hTERT* gene in addition to the SV40 large T antigen gene in order to facilitate the efficacy of the immortalization process, because our initial number of cells was quite limited.

Our established cells were not corneal, but conjunctival epithelial cells from a GDLD patient. As an in vitro model for GDLD, immortalized corneal epithelial cells from a GDLD patient appear to be ideal. Corneal epithelial stem cells are believed to reside preferentially at the limbus,[30] yet the number of those cells appears to be quite limited compared to the number of conjunctival stem cells. Since the resection of even a small piece of limbal tissue may produce a potential risk for limbal deficiency, we alternatively obtained a conjunctival tissue sample from the GDLD patient. As we have shown in our recent report, the subtype-expression pattern in CLDN proteins is quite similar in corneal and conjunctival epithelia.[31] In addition, the immortalized GDLD conjunctival epithelial cells exhibited significantly lower epithelial barrier function as well as the significantly decreased expression of the tight junction–related proteins compared to immortalized normal conjunctival epithelial cells, which are findings that are fairly consistent with our previous observation in GDLD corneas.[2] Thus, we believe that our established immortalized conjunctival epithelial cell line derived from a GDLD patient well mimics the disease situation of GDLD corneas and is useful as an in vitro model for GDLD corneal epithelial cells.

Of particular interest is the relationship between an impaired ocular surface epithelial barrier function and the corneal pathological clinical appearance. One could easily assume that lactoferrin and other molecules in tear fluids can easily penetrate and deposit into the corneal stroma due to the impaired epithelial barrier function caused by GDLD. This microscopic accumulation may be an initial catalyst for inducing amyloid deposits in the corneal stroma. A similar event may occur in patients with secondary corneal amyloidosis. In such cases, lactoferrin can penetrate into the corneal stroma through a regionally damaged epithelial layer caused by trichiasis in the lower eyelid.[32] Furthermore, the lactoferrin itself may spontaneously develop inflammation due to a polymorphism of this molecule in these patients. A similar pathological event may occur in patients with climatic droplet keratopathy, which is seen mostly in countries adjacent to the Red Sea including Saudi Arabia, arctic keratopathy observed in the Labrador region of Northern Canada and other areas of the Arctic, and so-called spheroid degeneration.[33-35] In these patients, the epithelial barrier function may be heavily damaged due to severe dryness of the ocular surface and excessive exposure to ultraviolet light. In summary, it is surmised that sustained severe damage of the ocular surface epithelial barrier could cause an accumulation of many proteins in tear fluids, resulting in corneal subclinical inflammation and amyloid and spheroid deposits.

Current treatments for GDLD are corneal transplantation and superficial keratectomy.[36] However, since the epithelial cells of the transplanted corneal graft are gradually replaced by the patient's pathological corneal epithelial cells, amyloid deposition may recur within several years after the surgery.[37] Accordingly, repeated corneal transplantations are frequently performed in most GDLD patients. Therefore, the development of novel effective treatments beyond the currently existing ones is still an unmet need for GDLD patients. We hope that our established conjunctival epithelial cell line lacking a functional *TACSTD2* gene will work as a good in vitro model for GDLD corneas and will contribute to the future development of novel effective treatments for patients with GDLD.

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

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ABSTRACT

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

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

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Patients

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

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

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

Histological and immunohistochemical analysis

Preparation of the sections and H&E staining

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

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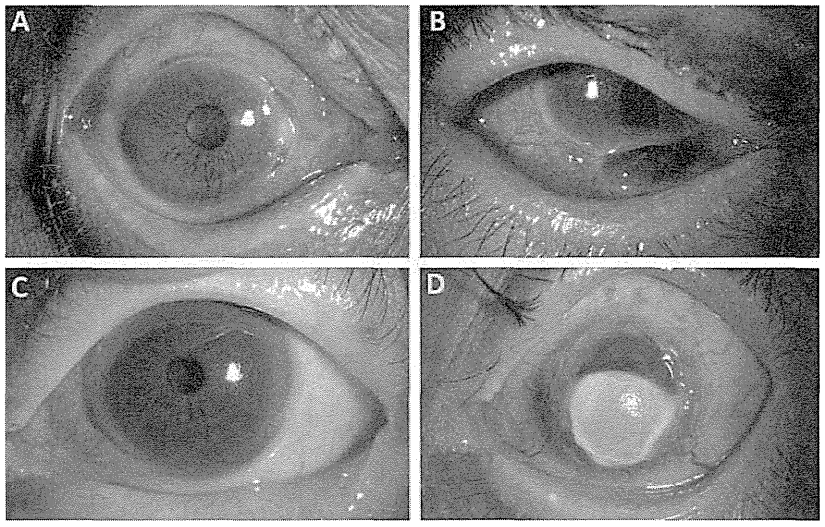

Figure 1 Macroscopic images of the patients' ocular surface. (A) Case 1, the right eye of a 60-year-old man. (B) Case 2, the right eye of a 56-year-old man. (C) Case 3, the left eye of an 82-year-old woman. (D) Case 4, the left eye of a 64-year-old woman. This figure is only reproduced in colour in the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

Immunohistochemical staining

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

Analysis

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

RESULTS

H&E staining

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

Immunohistchemical staining

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

Table 1 List of primary antibodies

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

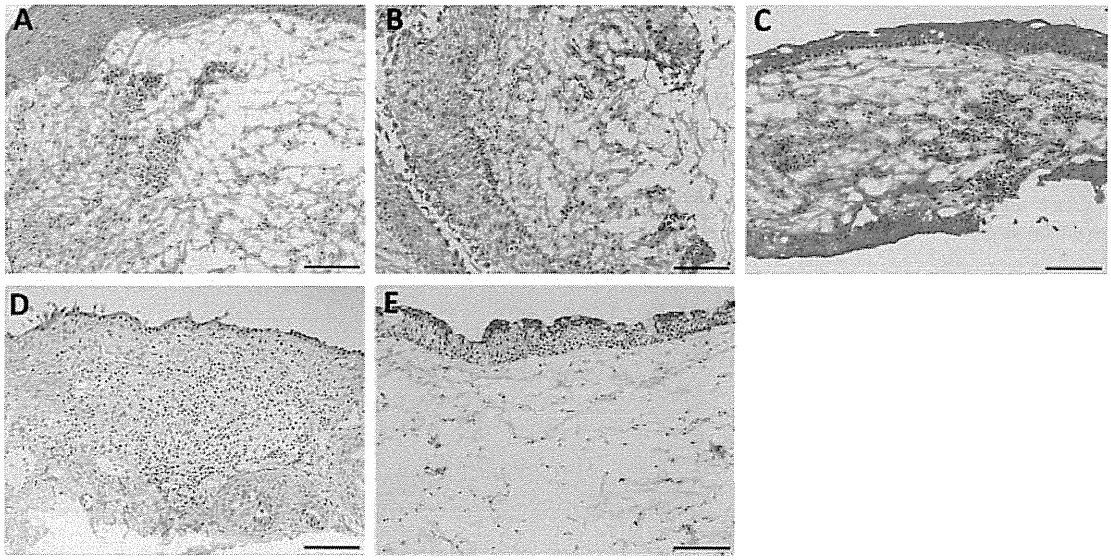

Figure 2 H&E staining images. (A) Case 1, (B) case 2, (C) case 3, (D) case 4 and (E) control with conjunctivochalasis (CCh). Some case-specific differences of grade can be seen, and small to slight large-sized round-shaped cells infiltrate into the submucosal connective tissue of the conjunctiva in all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

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

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

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

Table 2 Results of immunohistochemistry

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

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

DISCUSSION

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

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

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

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

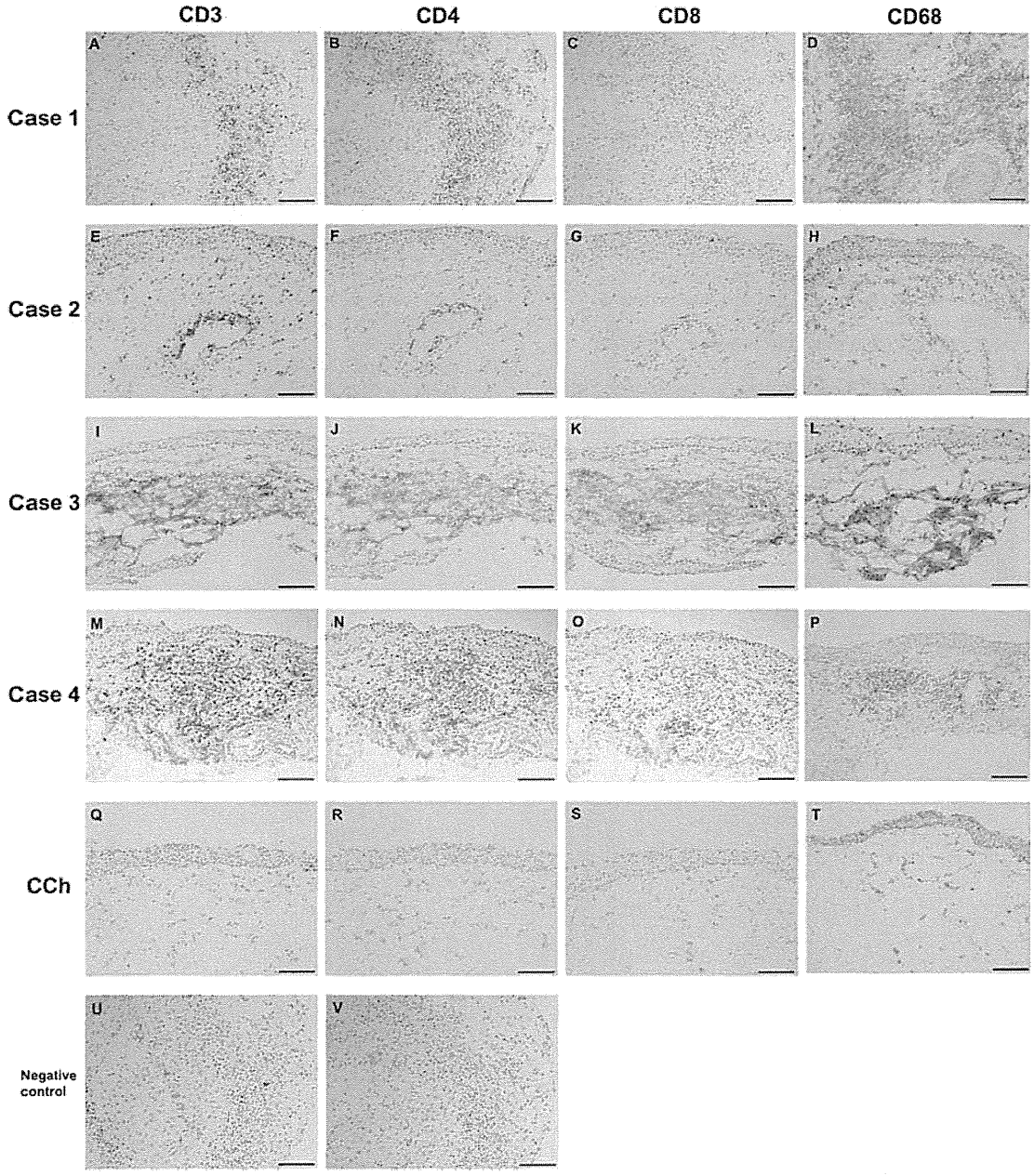

Figure 3 Immunohistochemical staining images (CD3, CD4, CD8 and CD68). (A–D and U, V) Case 1, (E–H) case 2, (I–L) case 3, (M–P) case 4, (Q–T) control with conjunctivochalasis (CCh). (A, E, I, M) Submucosal infiltrating cells show positive reactivity with CD3 in all cases. (B, F, J, N) Some CD3-positive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

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

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

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

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by Kyoto Prefectural University of Medicine.

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