Serum-Free Spheroid Culture of Mouse Corneal Keratocytes

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Purpose. To develop a serum-free mass culture system for mouse keratocytes.

Methods. Corneas of C57BL6/J mice were enzyme digested after the epithelium and endothelium were removed. Stromal cells were cultured in serum-free DMEM/F12 (1:1) containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and B27 supplement. Primary spheres were dissociated by trypsin and subcultured as suspended secondary spheres. Cells from postnatal day (P)6 to P10 spheres were subcultured onto plastic dishes or type I collagen gels for phenotype analysis. The expression of the keratocyte markers keratocan, aldehyde dehydrogenase (Aldh), and CD34, were analyzed by RT-PCR, and vimentin and α-smooth muscle actin (α-SMA) were examined by immunocytochemistry.

Results. Primary keratocytes formed spheres, which were cultured for over 12 passages. Suspended sphere cells expressed vimentin, keratocan, CD34, and lumican, but were negative for cytokeratin K12 (K12) and Pax6. Sphere cells subcultured on plastic exhibited a dendritic morphology characteristic of keratocytes, and maintained keratocan, Aldh, and CD34 expression in serum-free medium. Sphere cells subcultured with 10% serum became fibroblastic, and expressed α-SMA when stimulated by transforming growth factor (TGF)-β. α-SMA-positive cells demonstrated contractile properties on collagen gels, compatible with the myofibroblast phenotype.

Conclusions. The phenotype of mouse keratocytes can be maintained in vitro for more than 12 passages by the serum-free sphere culturing technique. (*Invest Ophthalmol Vis Sci.* 2005;46:1653–1658) DOI:10.1167/iovs.04-1405

The corneal stroma is characterized by a well-organized extracellular matrix consisting of a dense network of collagen fibrils and proteoglycans that are produced by keratocytes, the principal stromal mesenchymal cell of cranial neural crest origin.[1,2] In adult tissue, keratocytes are mitotically quiescent cells with a flat, dendritic morphology. Keratocytes form a three-dimensional network of cells through their extensive dendritic processes, linked via gap junctions,[3–10] and secrete collagens and keratan sulfate proteoglycans such as lumican, mimecan, and keratocan.[11–15] The corneal stroma is rich in total keratan sulfate proteoglycan content,[16] but contain relatively small amounts of dermatan sulfate proteoglycans.[17]

During corneal wound healing, the quiescent keratocytes are activated and transform into fibroblasts and/or myofibroblasts, losing their characteristic dendritic morphology. Keratan sulfate proteoglycans are downregulated,[11,18] whereas keratocytes proliferate and migrate to the site of injury, causing scar formation.[19–22] The conversion to myofibroblasts, characterized by intense expression of the contractile protein α-smooth muscle actin (α-SMA),[21,23,24] is induced by endogenous and exogenous transforming growth factor (TGF)-β.[25–27]

Ex vivo expansion of keratocytes is often performed to investigate keratocytes in vitro, and various culture techniques have been reported involving the use of plastic substrates. However, when cultured in serum-containing medium, collagenase-isolated keratocytes from bovine[28] and rabbit[27,29] corneas readily lose their in vivo quiescent phenotype and acquire a fibroblastic phenotype with altered physiological properties.[28,30,31] In the presence of 2% to 10% serum, keratan sulfate proteoglycan production is greatly reduced or absent in keratocyte-derived fibroblasts,[28,32,33] whereas production of dermatan sulfate proteoglycans is upregulated. Furthermore, TGF-β stimulation or culture at low densities[30] causes corneal fibroblasts to differentiate further into myofibroblasts with a more spread-out morphology.[26,29,32,34] Serum-free cultures have been reported to be effective in the maintenance of the dendritic morphology of keratocytes and the production of keratan sulfate proteoglycans.[27,28,30–33,35,36] However, the cultivation of a large quantity of cells by subculturing has been difficult.

In this report, we introduce our method for subculturing mouse corneal keratocytes in large quantities, using a modified version of a suspension culture method originally described for neural stem cells.[37–39] In our study, the sphere culture of keratocytes did not require serum, and the dendritic keratocyte phenotype was restored when subcultured on plastic substrate in serum-free medium.

Materials and Methods

Cell Culture

All animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Stromal cells were dissociated from adult C57BL6/J mice (7–8 weeks old) and then cultured as described previously[40] with modifications. In brief, cornea tissue was excised in Hanks' balanced salt solution (HBSS) supplemented with 10% fetal bovine serum (FBS) by circular incision outside the limbus. The iris, ciliary body, and Descemet's membrane including the endothelium were bluntly dissected from the cornea. The remaining stroma with epithelium was incubated in 5 mg/mL of Dispase II (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Loose epithelial sheets were then removed, and corneal stromal discs were cut into small segments and digested in 0.05% trypsin (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, followed by 78 U/mL

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TABLE 1. PCR Primers

Gene	Primer Sequence (5′–3′)	Product Size (bp)	GenBank Accession ID
Cytokeratin K12	Forward: TCCTCCTGCAGATTGACAACG	511	NM_010661
	Reverse: TTCCAGGGACGACTTCATGG		
Pax6	Forward: AGTTCTTCGCAACCTGGCTA	500	NM_013627
	Reverse: TGAAGCTGCTGCTGATAGGA		
Keratocan	Forward: AGGATGCCTTCATTCACGGAC	491	NM_008438
	Reverse: GCTCATTGTGGTGCTTATGGGG		
Lumican	Forward: TGCTGTCTCGGCTTCTCTGAAAG	567	NM_008524
	Reverse: AACATCCCCCACATTCCCAACC		
CD34	Forward: CCTTATTACACGGAGAATGGTGGAG	477	NM_133654
	Reverse: AAGAGGCGAGAGAGGAGAAATGGG		
Vimentin	Forward: GAACGGAAAGTGGAATCCTTGC	591	NM_011701
	Reverse: GGTTGGCAGAGGCAGAGAAATC		
Aldh	Forward: CTTCCAGCGGGTCATAAATCTG	528	NM_007436
	Reverse: AGCCAGCAAAACAAGTGTCAGG		
Gapd	Forward: GACCACAGTCCATGCCATCAC	453	NM_008084
	Reverse: TCCACCACCCTGTTGCTGTAG		

collagenase (Sigma-Aldrich) and 38 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C.

Stromal cells were mechanically dissociated into single cells, and cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 ng/mL of fibroblast growth factor 2 (FGF2, Sigma-Aldrich), B27 supplement (Invitrogen, Carlsbad, CA), and 10^3 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA) at a density of 5×10^5 cells/mL in a 37°C 5% CO_2 incubator. Initial culture was performed in 24-well plates or 35-mm dishes and then subcultured to 25-cm^2 culture flasks. The spheres were then further subcultured in 75 cm^2 culture flasks after 7 to 14 days, which was repeated every 7 to 14 days. Medium was changed every 5 to 7 days. All dishes and flasks used for sphere culture were polystyrene, noncoated vessels obtained from Asahi Techno Glass (Tokyo, Japan). Stromal sphere cells were examined by immunocytochemistry and RT-PCR. To allow cells to differentiate, cells dissociated from corneal spheres were cultured in serum-free or DMEM/F12 medium (10% FBS) supplemented with or without 2 ng/mL TGF-β (Sigma-Aldrich) for 4 days. Subcultured cells were stained by calcein-AM (Dojindo Laboratories, Tokyo, Japan), as described,[41] to visualize cell morphology. Primary stromal discs of mouse cornea were cultured in keratinocyte-serum free medium (K-SFM; Invitrogen) or DMEM/F12 with 10% FBS for 10 days (37°C, 5% CO_2), to identify any contamination by epithelial cells.

Immunocytochemistry

Immunocytochemistry was performed as described previously.[42] In brief, mouse corneal sphere cells and cells freshly isolated from mouse cornea were attached to glass slides by cytospin preparation (Auto Smear CF-120; Sakura, Tokyo, Japan) and then fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were incubated in blocking serum for 30 minutes and then incubated with primary antibodies for 60 minutes. Primary antibodies used were anti-cytokeratin K12 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pax6 (1:500, Chemicon International, Inc.), anti-vimentin (1:100, Santa Cruz Biotechnology), and anti-αSMA (1:200, Laboratory Vision, Fremont, CA). Immunoreactivity of primary antibodies was visualized with secondary antibodies conjugated with Cy3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

Reverse Transcription–Polymerase Chain Reaction

Sphere cells and freshly dissociated corneal cells were collected and immediately frozen in liquid N_2. cDNAs were synthesized with a cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) from total RNA also prepared with a kit (RNeasy; Qiagen, Hilden, Germany). Gene-specific primers used for cytokeratin K12 (K12), Pax6, vimentin, keratocan, lumican, CD34, aldehyde dehydrogenase (Aldh), and Gapd are shown in Table 1. PCR was then performed (GeneAmp 9700; Applied Biosystems, Foster City, CA). The PCR products were analyzed by agarose gel electrophoresis.

Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as described previously,[43–47] with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmaterix Type I-A; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 10-fold concentrated DMEM/F12 medium and 50 mM NaOH containing 260 mM $NaHCO_3$ and 200 mM HEPES at a proportion of 8:1:1 (vol/vol/vol) at 4°C. Then a 0.2-mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2×10^5 cells /mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO_2 incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved.

Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at $P < 0.01$.

RESULTS

Sphere Formation from Stromal Cells

More than five mice were used to prepare corneal stromal cells in each experiment. From 10 corneas, $1.32 \pm 0.16 \times 10^4$ cells ($n = 3$) were isolated, and subcultured cells proliferated into spheres, to yield an average of $7.97 \pm 0.35 \times 10^7$ cells per 75 cm^2 flask ($n = 6$) after four passages (P4). Sphere cells were propagated for >12 passages through 5 months without loss of viability. To avoid contamination of epithelial and endothelial cells, stromal discs were carefully prepared as described in the Materials and Methods sections. Dissociated cells from mouse stromal discs formed spheres when cultured in serum-free

medium containing EGF and FGF2 (Fig. 1A, left). To exclude the possibility that spheres may have originated from contaminating epithelial cells, we first performed primary cultures of mouse corneal discs, with or without dispase treatment, followed by epithelium separation. K-SFM with low Ca^{2+} was used to examine epithelial expansion.[48,49] When untreated discs were cultured, migration of epithelial and stromal cells was observed in K-SFM and in DMEM/F12 containing 10% FBS, respectively (Fig. 1B, left). There were no epithelial cells migrating from dispase-treated discs in both media, whereas fibroblasts migrated from the discs in DMEM/F12 with serum (Fig. 1B, right). We further cultured dissociated epithelial cells under conditions that allowed stromal spheres to form by 14 days. As a result, no spheres were observed in the epithelial cell culture (Fig. 1A, right). To demonstrate whether the spheres were hollow or solid, confocal microscopy of 4′,6′-diamino-2-phenylindole (DAPI)-stained spheres was performed. Imaging in different focal planes showed that the inside of spheres was filled with cells, not hollow (Fig. 1C).

We then examined the expression of epithelial and stromal markers in primary and subcultured spheres (P10) by RT-PCR. Stromal markers examined were the proteoglycans, keratocan, and lumican,[13,14,50,51] as well as CD34, which was recently reported to be expressed in keratocytes.[52–54] As shown in Figures 2A and 2B, in addition to the mesenchymal intermediate filament vimentin, the expression of the genes described earlier were detected in the stromal spheres. On the contrary, K12 and Pax6, both of which are expressed in corneal epithelium,[40,42,55–58] were not detected in sphere cells (Fig. 2A).

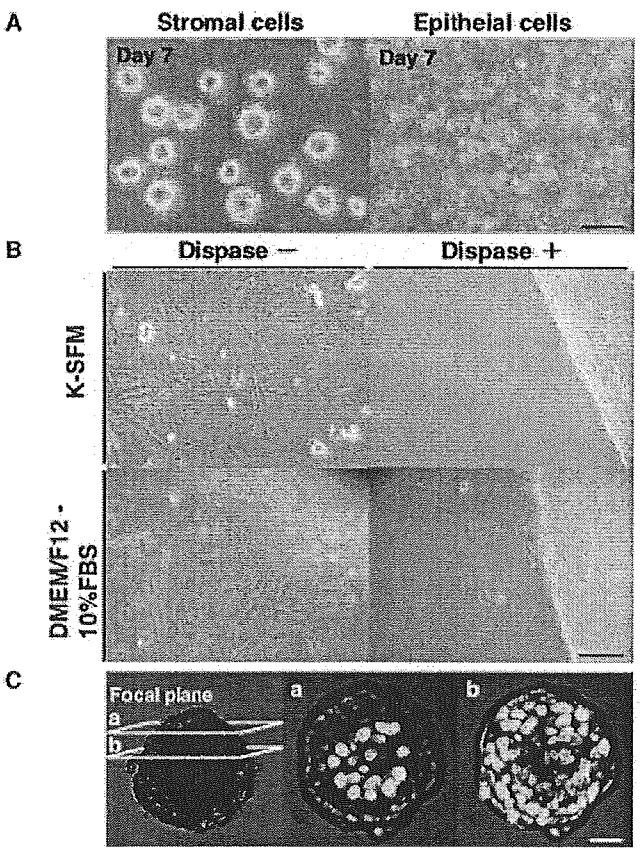

FIGURE 1. Sphere cells derived from the mouse corneal stroma. (A) Mouse corneal stroma and epithelium were separated by dispase treatment. Cells were cultured in DMEM/F12 supplemented with EGF and FGF2. After 7days' culture, spheres formed from stromal cells, but not from epithelial cells. (B) Mouse corneal discs were cultured in K-SFM or DMEM/F12 with 10% FBS. Epithelial cells migrated from intact corneal discs in K-SFM (*top left*) but not from dispase-treated (denuded) discs (*top right*). Expanding fibroblastic cells were still observed after dispase treatment. (C) Confocal images of the sphere in two different focal planes, a and b, as shown schematically (*left*). *Blue*: DAPI-stained nuclei. Scale bar: (A) 50 μm; (B) 100 μm; (C) 10 μm.

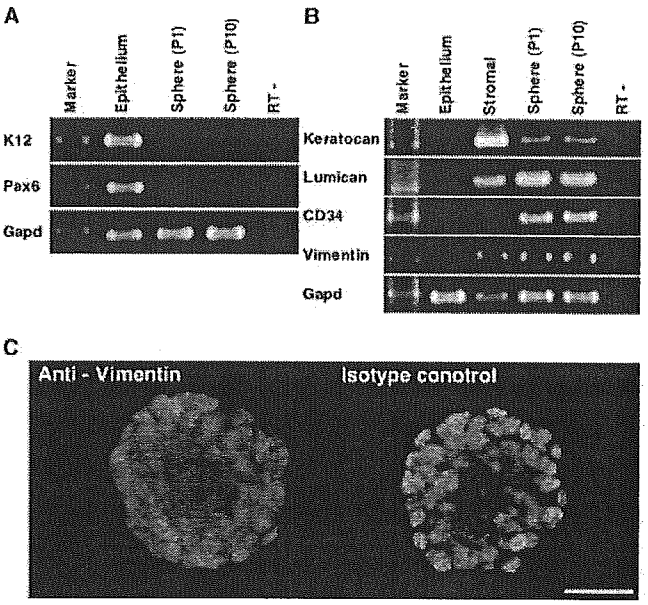

FIGURE 2. Sphere cells express keratocyte markers. (A, B) Total RNA was prepared from epithelial, stromal, and sphere cells. RT-PCR was performed with gene-specific primers. The epithelial markers K12 and Pax6 were not detected in the spheres (A). In contrast, the keratocyte markers keratocan, lumican, and CD34 were detected (B). Immunocytochemical analysis showed expression of the mesenchymal marker, vimentin, in spheres (C). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

Immunocytochemical analysis of spheres did not detect K12 and Pax6 expression (not shown), whereas vimentin staining was positive (Fig. 2C). These results show that sphere cells were of stromal, not epithelial, origin.

Characteristics of Sphere Cells

Sphere cells plated on collagen I-coated dishes in serum-free medium exhibited a dendritic morphology consistent with keratocytes (Fig. 3A).[3–10] RT-PCR showed that expression of keratocan and Aldh were retained under these conditions (Fig. 4). In contrast, the morphology of sphere cells subcultured in 10% serum were fibroblastic, and the expression of these genes was not detected (Fig. 4). Corneal sphere cells further differentiated to express α-SMA after exposure to TGF-β, which is consistent with the myofibroblast phenotype (Figs. 3C, 3D). Furthermore, when cells were subcultured on collagen gels in the presence of TGF-β, fibroblast-mediated gel contraction was observed (Fig. 5). Without TGF-β, contraction to 68.5% ± 1.75% of the original gel thickness was observed, whereas contraction was enhanced to 50.2% ± 3.96% or 29.4% ± 1.96% of the original thickness in the presence of 0.1 ng/mL or 1 ng of TGF-β, respectively ($P < 0.01$). TGF-β-dependent contraction was reduced to control levels when anti-TGF-β antibody was added to the medium.

Discussion

We successfully isolated and subcultured sphere-forming cells from the mouse corneal stroma, yielding a multifold increase in available cells for further experiments. Zhao et al.[40] have re-

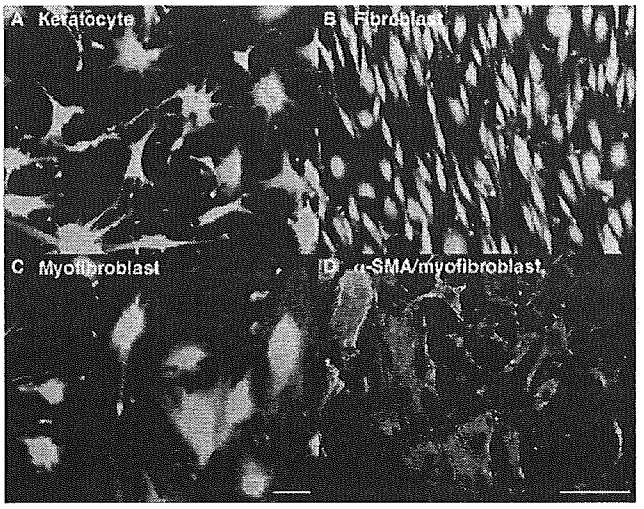

FIGURE 3. Phenotype of corneal stromal sphere cells stained by calcein-AM. Dissociated sphere cells were dendritic in SFM (A) and fibroblastic in adherent culture with medium containing 10% FBS (B). In the presence of TGF-β, morphology of adherent cells became myofibroblastic (C), and the cells expressed α-SMA, detected by immunocytochemistry (D, *green*). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

ported that cells present in limbus-derived spheres are derived from the limbal epithelium but not the stroma. However, the corneal sphere cells that we isolated did not express the epithelial markers K12 or Pax6 throughout the study, and furthermore, exhibited properties of corneal keratocytes when subcultured in serum-free medium. The morphology of the subcultured cells as shown in Figure 3 was similar to that of keratocytes in situ, and together with the expression of keratocan, lumican, Aldh, and CD34 in the subcultured cells, the

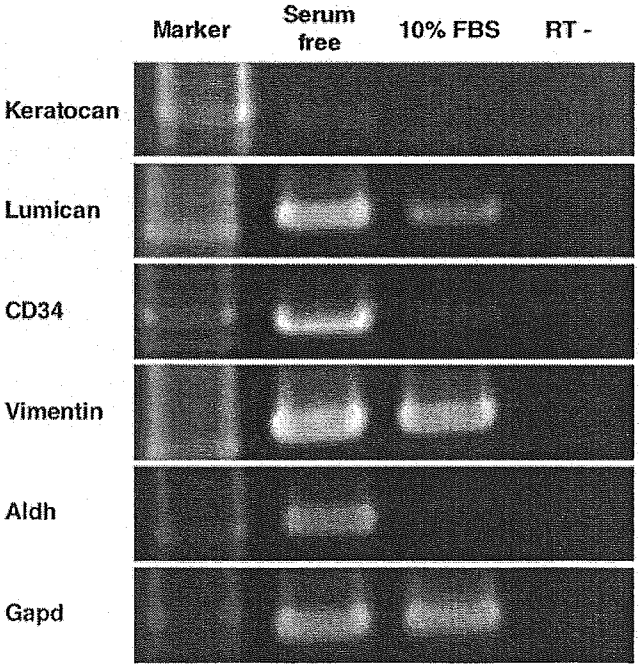

FIGURE 4. RT-PCR analysis of keratocyte markers expressed in sphere cells subcultured on plastic. Keratocan and Aldh were expressed only in cells in SFM, whereas lumican, CD34, and vimentin were also detected in cells cultured in the presence of 10% FBS.

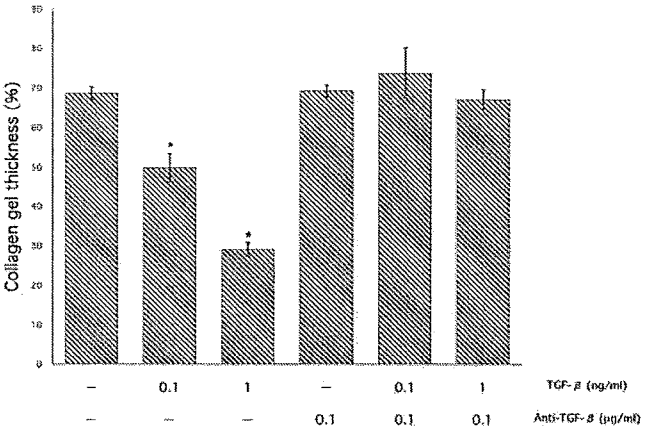

FIGURE 5. Collagen gel contraction assay of fibroblasts. Mouse corneal spheres were allowed to differentiate in 10% serum-containing medium. Dose-dependent TGF-β-induced collagen gel contraction was observed, which was inhibited by an anti-TGF-β antibody ($^*P < 0.01$).

collective evidence shows that these cells were of keratocyte origin. Although most genes were expressed during sphere cultures and maintained after adhesion to plastic dishes, keratocan, and Aldh were exclusively expressed in the keratocyte phenotype in serum-free medium (Fig. 4). Although the biological role of Aldh is not known, abundant expression of the water-soluble enzyme was shown to be expressed in the keratocyte phenotype, but not by the fibroblasts or myofibroblasts.[32,59]

Plated sphere cells can further be induced to differentiate into the fibroblast and myofibroblast phenotypes. Sphere cells seeded onto plastic in the presence of 10% serum exhibited the morphology and properties of stromal fibroblasts.[28] The transition to α-SMA-positive myofibroblasts by exposure to TGF-β, causing collagen gel contraction (Fig. 5), is also a functional property of stromal fibroblast primary cultures.[29,43,60] Therefore, subcultured sphere cells can be conditioned to express all three known phenotypes of keratocytes after expansion by sphere culture. Berryhill et al.[36] reported that the fibroblast phenotype can be partially restored to the keratocyte phenotype in terms of extracellular matrix production and morphology. However, biological functions, including Aldh activity, were not restored, suggesting that reversal to keratocyte phenotype after mass culture in serum-containing medium is not practical. Espana et al.[41] have reported the use of amniotic membrane (AM) as a substrate for keratocyte cultures in the presence of serum. They have shown that even with the use of serum, primary keratocytes maintained dendritic morphology on AM. The expression of keratocan and lumican was also present for up to five passages, which is a significant improvement over previous reports using artificial substrates. Still, the scarcity of keratocytes in tissue usually necessitates the use of human tissue or cells from larger animals, such as cows,[28,36] rabbits,[30] and rhesus monkeys.[61] Biochemical and molecular analysis of such cells are difficult due to the lack of available antibodies and genomic information.

During subcultures of spheres, cells that failed to form spheres were found attached to the dish as nondividing, fibroblast-like cells (data not shown). Although these cells may have had low viability, there may be a selection process that allows only cells with high growth potential to propagate as spheres. Once secondary spheres are successfully initiated, subsequent passages continue to produce spheres for at least 12 passages, the longest that we observed. To our surprise, cells from later passages continued to show the keratocyte phenotype when subcultured on plastic, suggesting the possible presence of

committed progenitor cells during the sphere culture stage. Many aspects of the keratocyte are still not understood, and the availability of cells from the mouse cornea should be a powerful tool in studying the biology of these cells.

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Expression of Keratinocyte Transglutaminase in Cornea of Vitamin A–Deficient Rats

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ABSTRACT *Purpose*: To determine the role played by keratinocyte transglutaminase (TG1, TG_K) in the abnormal keratinization of the cornea. *Methods*: Vitamin A–deficient rats were produced as a model of severe dry eyes, and the expression of the mRNA and the enzyme activity of TG1 were examined in the corneas. The envelope proteins and keratins of cornified cells were also examined immunohistochemically. *Results*: The expression and enzyme activity of TG1 mRNA on the ocular surface were significantly upregulated as the vitamin A deficiency developed. As the TG1 expression was upregulated, involucrin, loricrin, and keratin 10 began to be expressed on the epithelial cells of the cornea. *Conclusions*: Upregulation of TG1 expression followed by the appearance of the envelope proteins and keratin10 in cornified cells indicated that TG1 is involved in the abnormal keratinization of the cornea.

KEYWORDS cornea; keratin; keratinization; keratinocyte transglutaminase; vitamin A deficiency

INTRODUCTION

For the cornea to maintain its optical transparency, the corneal epithelial cells need to proliferate and differentiate continuously throughout life. Clinically, severe dry eye diseases, such as ocular cicatricial pemphigoid and Stevens-Johnson syndrome, are accompanied by a high degree of keratinization of the corneal epithelial cells. In these diseases, the corneal transparency can be profoundly compromised because of abnormal epithelial differentiation and loss of limbal stem cells.

New surgical approaches, including amniotic membrane transplantation accompanied with limbal transplantation and cultured corneal epithelial cell-sheet transplantation, have been introduced with promising results.[1,2] However, difficulties still remain in treating patients with severe dry eye. A better understanding of the mechanism of keratinization is required to overcome these difficulties.

Transglutaminase (R-glutaminylpeptide: amine γ-glutamyltransferase; EC 2.3.2.13) is a calcium-dependent enzyme, and it acts by forming covalent bonds between peptides by catalyzing the reaction that forms ε-(γ-glutamyl)-lysine from lysine residue and glutamine.[3,4] At present, there are 10 known forms of transglutaminase in the tissues and fluids of mammals, and the genes and amino acid sequences have been determined for all of them.[5–12]

It has been well established that keratinocyte transglutaminase (TG1, TG_K) plays a role in the formation of the horny layer of the epidermis.[10,11,13,14]

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It has also been known for a long time that vitamin A plays an important role in cell proliferation and differentiation.[15–18] Relevant to this study, vitamin A deficiency is known to cause a high degree of keratinization in mucocutaneous tissue (e.g., the trachea, digestive and urinary tracts, and ocular surfaces).[16,19] Thus, vitamin A–deficient animal should be a good model to use to analyze the mechanism of keratinization of the corneal epithelium.

The purpose of this study was to track the progress of keratinization of the corneal epithelial cells in rats fed with a vitamin A–deficient diet and to explore the relationship between the resulting TG1 expression and keratinization.

MATERIALS AND METHODS

Vitamin A–Deficient Rats

The experiments were carried out in compliance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Three-week-old Sprague-Dawley (SD) rats were fed a vitamin A–deficient diet (Oriental Enzyme, Tokyo, Japan) to develop vitamin A deficiency. Other rats were fed a normal diet and acted as controls. The ocular surfaces of the vitamin A–deficient and control rats were examined by slit-lamp biomicroscopy every 3 weeks for the first 18 weeks.

Ten animals were sacrificed at 3, 6, 9, 12, 15, and 18 weeks after beginning the vitamin A–deficient diet. Five corneas were pooled for the measurement of the TG1 enzyme activity, and five other corneas were stored at −80°C for Northern blot analysis. The other eyes were enucleated, and the whole eye was embedded in paraffin for histology and immunohistochemistry. The control rats were processed in the same way.

After the 12th week, a subgroup of the vitamin A–deficient rats (20 rats) was given 50 μl/eye of palmitic acid retinal eye drops 3 times per day (2000 IU/ml; Eisai, Tokyo, Japan). After the retinal drops, these rats were examined every week not only for the ocular maladies but also for the enzyme activities of TG1 (see below).

Measurement of all-*trans* Retinal Serum Concentration

Blood samples were taken from the experimental animals. After separating the plasma by centrifugation (3000 rpm for 10 min), the all-*trans* retinal concentration in the plasma was determined by high-performance liquid chromatography (HPLC) following Furr's method.[20]

Histological and Immunological Examinations

Paraffin-embedded eyes from control and vitamin A–deficient rats were sectioned at 5 μm. The sections were stained by either hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS).

The primary antibodies for immunohistochemical staining were mouse monoclonal anti-keratin 10 Ab-2 (Neo Markers, Fremont, CA, USA); mouse monoclonal anti-keratin 14 Ab-1 (Neo Markers); rabbit polyclonal anti-keratin 12 K12n[21] (generous gift from Dr. Kao); rabbit polyclonal anti-involucrin (Covance, Richmond, CA, USA); and rabbit polyclonal anti-loricrin (Covance).

All immunohistochemical stainings were carried out using HISTOFINE SIMPLE STAIN MAX-PO (NICHIREI, Tokyo, Japan), according to the manufacturer's protocols. The sections were then developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. For control, sections were treated with normal mouse or rabbit immunoglobulin G (IgG), and no positive staining was detected for any of these antibodies.

Preparation of Rat TG1 Probes

Total RNAs were isolated from the skin of SD rats using TRIZOL REAGENT (Life Technologies, Rockville, MD, USA), and reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Reverse Transcriptase XL TAKARA kit and TAKARA Taq (TAKARA, Ohtsu, Japan) with rat TG1 primer pair (sense; 5′-CATCCTCTTCAATCCCTGGTG-3′ and antisense; 5′-GCAAAGACCCAGCATTGGCC-3′). The PCR fragments were subcloned into pBluescript SKII (Stratagene, LaJolla, CA, USA) and sequenced completely in both directions by Sawady Technology (Tokyo, Japan). The subcloned TG1 cDNAs were used for the synthesis of probes for Northern blot hybridization.

Northern Blot Analysis

To determine the level of TG1 mRNA in the corneas, 5 corneas from the controls and from rats at 3, 6, 9, 12,

and 15 weeks after vitamin A depletion were pooled, and total RNAs were isolated. Ten micrograms of total RNAs were subjected to agarose gel electrophoresis and then transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA, USA). The membrane was hybridized with ^{32}P-labeled rat TG 1 cDNA probes using Quick Hyb Hybridization Solution (Stratagene) according to the manufacturer's protocols. The hybridization signals were detected by BAStation (Fuji film, Tokyo, Japan). The hybridized membrane was then stripped and hybridized with ^{32}P-labeled rat G3PDH cDNA probes. The expression level of TG1 mRNA was designated relative to that of G3PDH mRNA in the same sample using NIH Image.

Measurement of TG1 Enzyme Activity

To measure the TG1 enzymatic activity in the cornea, the methods of Lee *et al.* and Piacentini *et al.* were used.[20,22] Five corneas from the controls, and from rats at 3, 6, 9, 12, and 15 weeks after the beginning of the vitamin A–deficient diets were pooled and homogenized in a buffer (10 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, 0.5 mM EDTA) and centrifuged (100,000 × g) at 4°C for 1 hr. The supernatants were adjusted to 0.5 mg of total protein, then placed in incubation buffer (50 mM Tris-HCl, pH 8.3, 5 mM CaCl2, 10 mM DTT, 30 mM NaCl, 2.5 mg/ml N-N′-dimethylcasein, 0.2 mM putrescine) and 1 μCi [1.4(n)-3H] putrescine dihydrochloride (Amersham, Pharmacia Biotech, Tokyo, Japan) at 30°C for 20 min. After the incubation, the samples were applied onto GF/A filter paper (Whatman, Kent, UK) to measure specific enzyme activity of TG1.

RESULTS

Clinical Observations

The clinical appearance of the ocular surfaces of the vitamin A–deficient rat was within the normal variations up to the sixth week by slit-lamp examinations (Fig. 1A). However at the ninth week, they began to show punctate keratopathy (Fig. 1B) and thereafter developed xerophthalmic changes such as debris- and

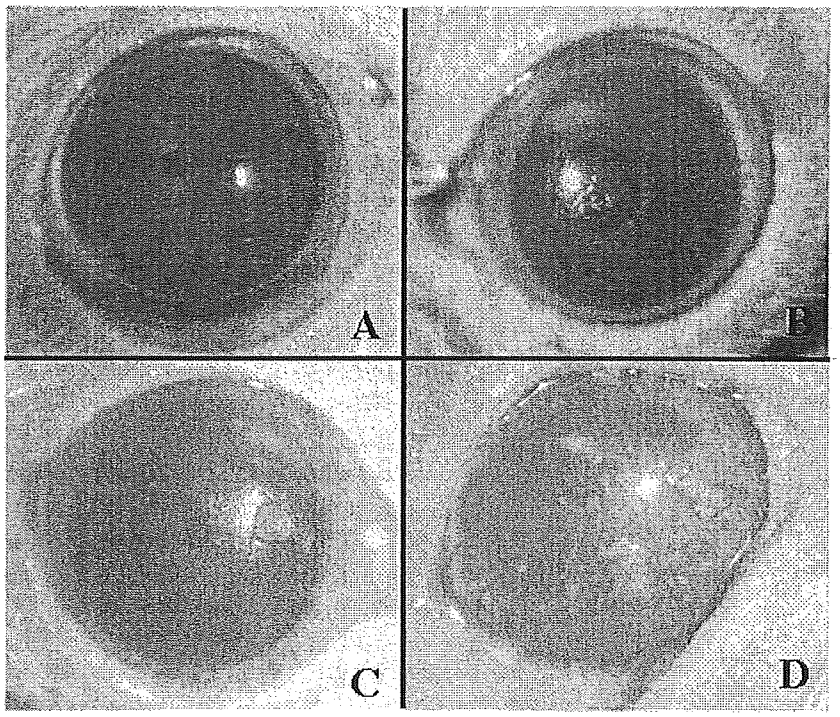


FIGURE 1 Clinical observation of the ocular surface of vitamin A–deficient rats. (A) After 6 weeks of systemic vitamin A–deficiency, the cornea and conjunctiva appear normal. (B) After 9 weeks of systemic vitamin A deficiency, punctate epithelial keratopathy with lusterless, peau d'orange appearance can be seen. (C) After 12 weeks of systemic vitamin A deficiency, keratinization with early plaque formation and corneal neovascularization can be observed. (D) After 18 weeks of systemic vitamin A deficiency, keratomalacia or advanced keratinization of the epithelia with stromal edema can be observed in some experimental animals.

TABLE 1 Clinical Scores of the Ocular Surface in Vitamin A-Deficient rats

	Weeks					
	3	6	9	12	15	18
Keratoconjunctivitis sicca	−	−	+	+	+	+
Debris	−	−	+	+	+	+
Bitot spot	−	−	−	−	+	+
Corneal neovascularization	−	−	−	+	++	++

For corneal neovasclarization: (+) peripheral neovascularization; and (++) central corneal neovascularization.

filament-like secreta. Corneal neovasculalization was observed at 12 weeks (Fig. 1C), and at week 15, Bitot spots were present in the bulbar conjunctiva, and the blood vessels had extended to the center of the cornea. In week 18, the corneal opacification, indicative of keratomalacia, was quite significant (Fig. 1D; Table 1).

The serum concentration of retinol in the control rats was 316 ± 48 ng/ml (mean ± standard error of the mean). The level in the vitamin A-deficient rats at week 6 was 346 ± 34 ng/ml, but by week 9 the value had dropped markedly to 22 ± 9 ng/ml, and thereafter only a trace amount was found (Fig. 2).

Histological Observations

Histological examinations at 9 weeks showed that the number of conjunctival goblet cells was decreased (Fig. 3D), and the corneal epithelium was thin (Fig. 3C). At week 12 and beyond, the nuclei of the corneal epithelial cells were more flattened (Fig. 3E), and goblet cells were not observed in the conjunctival epithelium (Fig. 3F). In addition, an eosinophilic keratinized layer had developed on the surface of both the corneal and conjunctival epithelium (Figs. 3E and 3F).

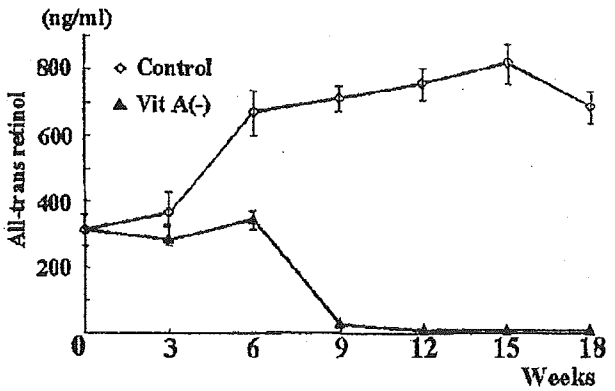


FIGURE 2 The serum concentration of all-*trans* retinol in vitamin A-deficient and age-matched control rats. The serum concentration of all-*trans* retinol in normal rats is elevated up to 9 weeks of age and then plateaued. However, the serum concentration of all-*trans* retinol decreases to trace amounts after 9 weeks of systemic vitamin A deficiency. Data are shown as average ± standard error of the means. At 0 weeks, the rats were 3-weeks-of-age and the vitamin A-deficient diet was started. □, control rats; ▲, vitamin A-deficient rats.

Changes in TG1

Northern blot analysis showed that the level of TG1 mRNA expression was low in normal rat corneas. In the vitamin A-deficient rats, the TG1 mRNA expression in the cornea began to be upregulate by 2.7 times at 6 weeks, 11 times at 9 weeks, and 26 times at 12 weeks and beyond (Fig. 4A).

As the expression of TG1 mRNA was upregulated, the TG1 enzyme activity increased in the vitamin A-deficient rat corneas by as much as 18 times at 9 weeks (Fig. 4B).

Other Immunohistochelical Studies

Involucrin and Loricrin

Immunohistochemical staining for involucrin and loricrin, substrates of TG1, was performed to assess the enzyme activity of TG1. Involucrin and loricrin immunoreactivities were detected in minimal levels in normal rat corneas (Figs. 5A and 5C). However, both involucrin and loricrin were observed on keratinized epithelial surfaces of normal rat epidermis (data not shown). In the vitamin A-deficient rat corneas, involucrin immunoreactivity began to be observed as early as 9 weeks and definitely at 12 weeks and later. The immunopositivity was observed in all cell layers except the basal cells of vitamin A-deficient rat corneas (Fig. 5B).

In the cornea of the vitamin A-deficient rats, loricrin immunoreactivity was observed in the nuclei of all cell layers (Fig. 5D). Interestingly, loricrin immunoreactivity was detected in the nuclei of the basal epithelial cells in the vitamin A-deficient rat epidermis (data not shown).

Keratin10, 14, and 12

Keratin 10 was not detected in normal rat corneas, but immunoreactivity for keratin 10 was strongly observed on the corneal epithelium, except for the basal cells, in the vitamin A-deficient rats (Figs. 6A and 6B). Keratin 14 immunoreactivity was detected in basal cells

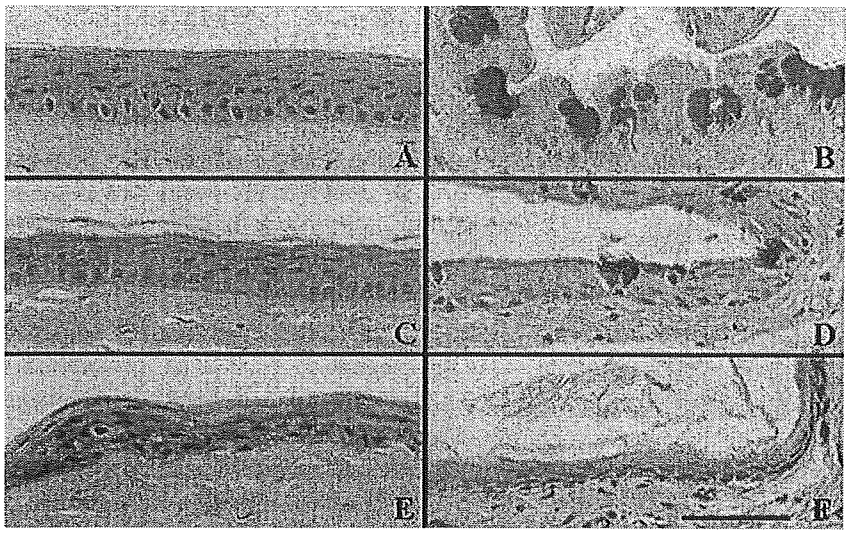


FIGURE 3 Histological observation of the ocular surface of vitamin A–deficient rats. The corneas from normal (A), 9 week (C), and 15 week (E) vitamin A deficient rats were stained by hematoxylin and eosin (H&E), and the conjunctivas from normal (B), 9 weeks (D), and 15 week (F) vitamin A–deficiency, rats were stained by periodic acid–Schiff (PAS). At 9 weeks of vitamin A deficiency, rats showed an irregular and a slightly keratinized surface of corneal epithelium, and the number of goblet cells was decreased in the conjunctival epithelium. At 15 weeks, an eosinophilic keratinized layer and irregularity had developed on the corneal epithelium and the nuclei were flattened and no goblet cell observed. Bar, 50 μm.

and some wing cells of normal rat corneal epithelium. In vitamin A–deficient rats, its expression was reduced but was observed in all layers of the corneal epithelium (Figs. 6E and 6F). Keratin 12 immunoreactivity was detected in vitamin A–deficient rat corneal epithelium as well as normal rat cornea (Figs. 6C and 6D).

Effect of Topical Vitamin A (Palmitic Acid Retinal)

At 12 weeks and beyond, a subgroup of the vitamin A–deficient rats were treated with vitamin A eye drops. After 1 week of treatment, the superficial punctate keratopathy was still observed, but it was not present by the second week. Then, at 4 weeks after treatment, no xerotic changes were observed by slit-lamp examination (Fig. 7B). In addition, by the end of 1 week of treatment, serum all-*trans* retinol had improved to 110 ± 16 mg/ml and was maintained at this level thereafter (data not shown).

In agreement with the clinical observations, the enzyme activity of TG1 quickly returned to the normal range by the end of the first week of treatment (Fig. 7C).

DISCUSSION

It has been well documented that vitamin A–deficient animals develop xerophthalmia and are suitable models to investigate the abnormal keratinization of ocular surface epithelia.[17,23] Our results showed that the TG1 enzyme activity and TG1 mRNA expression were significantly upregulated after vitamin A depletion in the serum, although the TG1 enzyme upregulation stopped after 9 weeks. Gipson *et al.* mentioned separate control mechanisms of message and protein levels of transglutaminase in human keratinocytes.[24] At 9 weeks, the TG1 enzyme activity was already upregulated as much as 18 times, thus the enzyme activity may reach a plateau, and a control mechanism could regulate post-translational level. At about the time that TG1 began to be upregulated in the corneal epithelium, the vitamin A–deficient rats began to develop xerophthalmic changes by both clinical and histopathological observations.

At the same time, involucrin and loricrin, which are the cornified envelope precursor proteins, also began to be expressed in the corneal epithelium of the vitamin A–deficient rats. The cornified cell envelope (CE) is a specialized structure that is formed during terminal differentiation of keratinized stratified epithelia in which TG1 plays a major role in cross-linking.[25,26] Our findings indicated that TG1 is responsible for abnormal keratinization of corneal epithelium in the vitamin A–deficient rats.

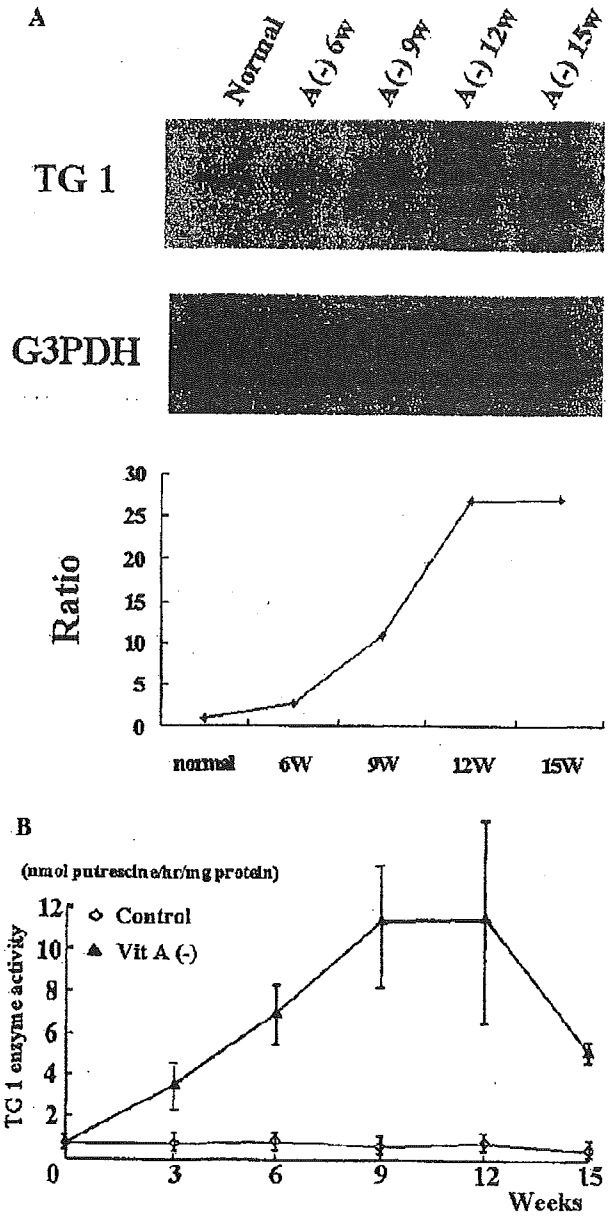


FIGURE 4 Change of TG1 in vitamin A–deficient rat corneas. (A) Northern blot analysis for TG1 mRNA expression in normal and vitamin A–deficient rat corneas. Quantification of TG1 mRNA content is showed on the bottom. Image analysis of TG1 mRNA signals, normalized to G3PDH, shows that the TG1 mRNA expressions is upregulated 2.7 times at 6 weeks, 11 times at 9 weeks, and 26 times at 12 weeks and beyond. TG1 mRNA expression in the normal cornea showed the same levels at all ages examined. The result of normal cornea shows 9-week-old rat corneas as representative of the cornea of rats on normal diet. (B) Enzyme activities of TG1 in vitamin A–deficient and age-matched normal rat corneas. The TG1 enzyme activities in normal rat corneas is at trace level in 3-week-old rat and the level does not change up to 15 weeks of age. The TG1 enzyme activities in vitamin A–deficient rat corneas are elevated by as much as 18 times at 9 weeks of vitamin A deficiency. Data are shown as average ± standard error of the means. At 0 week, the age of the rats was 3 weeks and the vitamin A–deficient diet was started. □, control rats, ▲; vitamin A–deficient rats.

A number of keratins are known to be differentiation markers of the epithelium.[27,28] Our immunohistochemical results on the expression of keratin 10 and 14 were in good agreement with the reports on vitamin A–deficient animals.[29] The presence of keratin 12 clearly demonstrated that the corneal epithelia of vitamin A–deficient rats still maintained corneal specificity, although they exhibited epidermal-like characteristics.

It has been shown that the transglutaminases catalyze permanent covalent attachments of the keratin intermediate filaments to the cell envelope, such as involucrin and loricrin.[30] The upregulated expression of TG1, keratinization specific keratins, and CE proteins in the corneal epithelium of vitamin A–deficient rats suggest that TG1 may be acting to cross-link keratins and CE proteins for keratinization.

The close association of TG1 expression and vitamin A was further supported by the experiments in which the xerotic changes of the ocular surface of vitamin A–deficient animals were readily resolved when the TG1 activity in the corneal tissue normalized soon after topical instillation of vitamin A was started. It is highly likely that vitamin A controls the gene expression of TG1, and the upregulated TG1 catalyzes the polymerization of keratins and other proteins involved in keratinization.

The existence of TG1 in the conjunctival epithelial surface has been documented in specimens obtained from patient with cicatrizing ocular surface diseases.[31,32] Nakamura *et al.* suggested that an upregulation of TG1 was involved in the pathological keratinization process on the ocular surface.[31] Serial results suggest that the increase of TG1 activity is strongly related to the keratinization of the ocular surface epithelia.[31–33]

For the effective treatment of xerophthalmia and the maintenance of the healthy ocular surface, it is essential to suppress TG1 gene expression. Although retinoic acid has been applied experimentally and clinically for the treatment of xerophthalmia, its effect has at times been inconsistent, suggesting the existence of other mechanisms to regulate TG1 gene expression. In fact, several *in vitro* studies have demonstrated that interferon-gamma (IFN-γ) stimulates TG1 expression, and its expression is antagonized by TGF-β as well as retinoic acid.[34–36] As $CD4^+$ T cells derived from vitamin A–deficient mice have been shown to overexpress IFN-γ,[36] IFN-γ might be responsible for

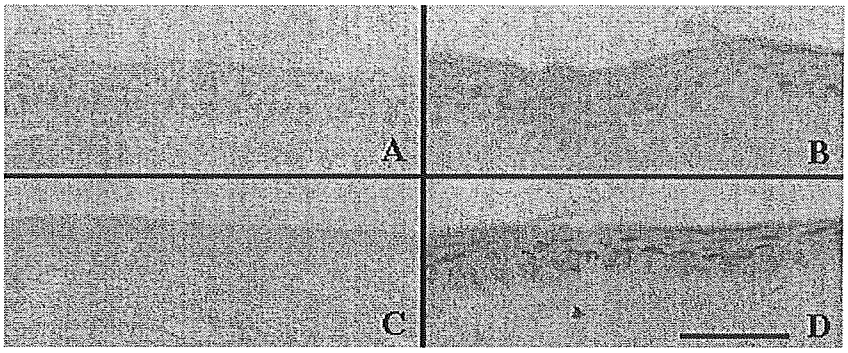


FIGURE 5 Immunohistochemical staining for involucrin and loricrin in normal and 15-week vitamin A–deficient rat corneas. Normal rat: Involucrin immunoreactivities are barely observed in normal corneas (A). Loricrin immunoreactivities are also barely observed in normal corneas (C). Vitamin A–deficient rats: Involucrin is observed in all cell layers except the basal cells of vitamin A–deficient rat corneas (B). Loricrin is observed in nuclei and perinuclei in all cell layers of vitamin A–deficient rat corneas (D). Bar, 50 μm.

regulating TG1 expression in vitamin A–deficient rats. In addition, it has been demonstrated that IFN-γ could be involved in keratinization of the ocular surface epithelia in patients with Sjögren syndrome.[37] The balance between IFN-γ and TGF-β/retinoic acid could determine the TG1 expression level of the ocular surface.

In summary, our findings suggest that an upregulation of TG1 expression is related to the abnormal keratinization of the corneal epithelium. It is essential to clarify the mechanism of TG1 gene regulation in more detail for better control of the abnormal keratinization of cornea. Such investigations are underway in our laboratory.

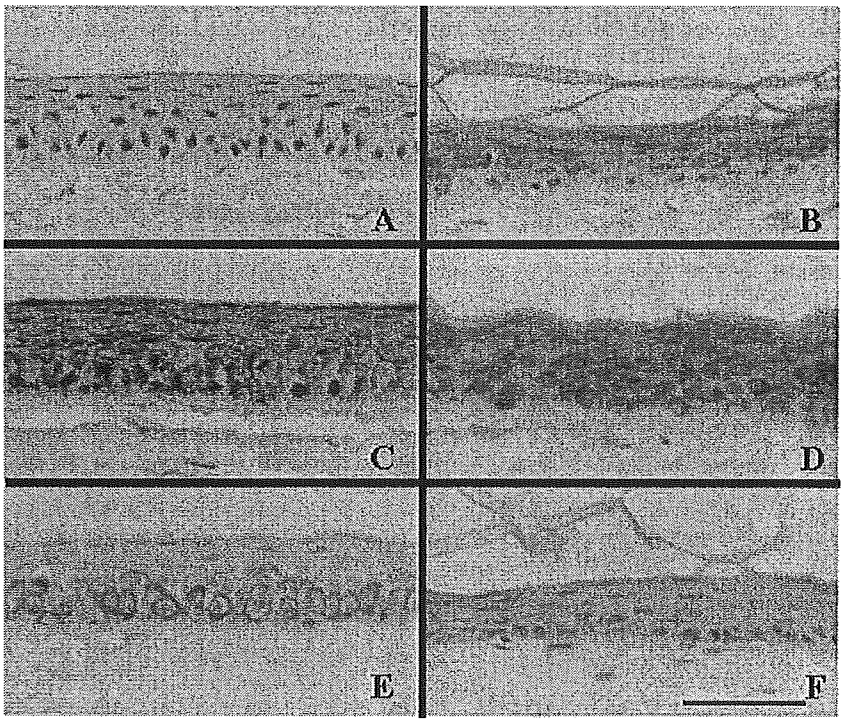
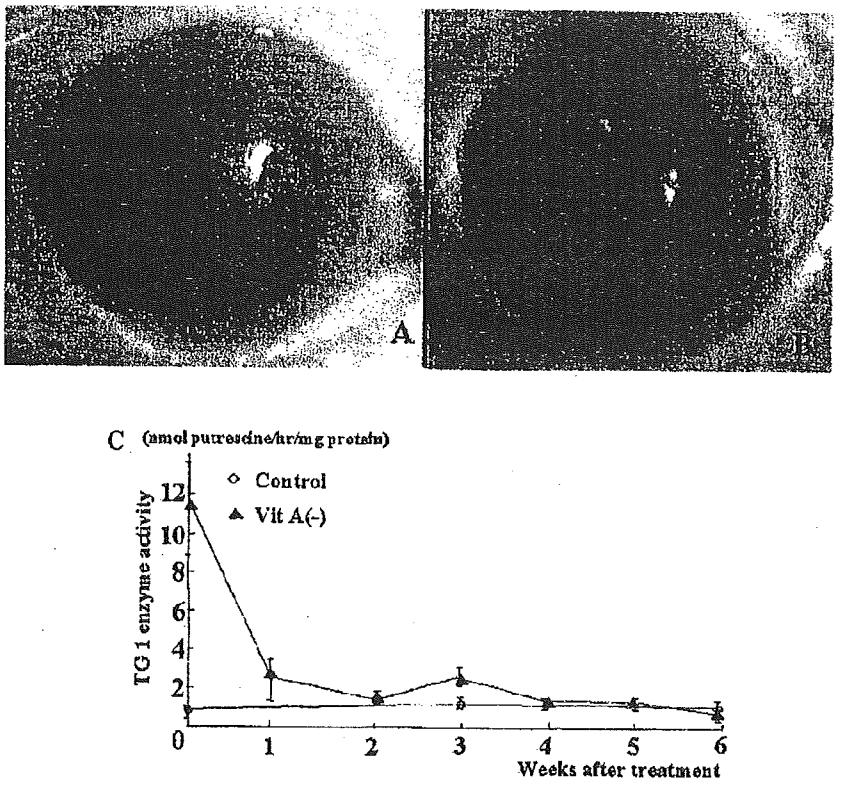


FIGURE 6 Immunohistochemical staining for keratin 10, 12, and 14 in normal and 15-week vitamin A–deficient rat corneas. Kerain 10: Kerain 10: is not observed in normal cornea (A), but strong expression can be seen in all cell layers except basal cell layer of vitamin A–deficient rat corneas (B). Keratin 12: Keratin 12 is observed in all cell layers of normal and vitamin A–deficient rat corneas (C and D). Keratin 14: Keratin 14 is strongly observed in the basal cells and some wing cells of normal rat corneas (E), and its expression is weakly seen in all cell layers of vitamin A–deficient rat corneas (F). Bar, 50 μm.



IGURE 7 The effect of topical palmitic acid retinol instillation on the corneas of the vitamin A–deficient rats. Clinical observation: eratinization and neovascularization is still observed at 12-week vitamin A–deficient rat corneas (A). The keratinization is not present fter 4 weeks of topical palmitic acid retinol instillation on the corneas (B). (C) TG1 enzyme activity: upregulated TG1 enzyme activity in itamin A–deficient rat corneas decreases sharply as early as 1 week after topical palmitic acid retinol instillation. Data shown are the verages ± standard error of the means. At 0 weeks, the rats had been on the systemic vitamin A–deficient diet for 12 weeks, and the ›pical palmitic acid retinol instillation was started. □, age matched control rats; ▲, vitamin A–deficient rats.

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Transient keratectasia caused by intraocular pressure elevation after laser in situ keratomileusis

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Transient keratectasia developed in association with a marked intraocular pressure (IOP) elevation in a 26-year-old man who had laser in situ keratomileusis (LASIK) for myopia of −9.0 diopters. The keratectasia subsided promptly after the IOP was normalized by an intravenous administration of mannitol. Keratectasia did not recur. Intraocular pressure elevation can cause keratectasia in a structurally compromised cornea after LASIK.

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Iatrogenic keratectasia after laser in situ keratomileusis (LASIK) has been reported.[1,2] Keratectasia is usually an irreversible, often progressive complication requiring use of hard contact lenses or penetrating keratoplasty. We report a case of transient keratectasia associated with marked elevation of intraocular pressure (IOP). The keratectasia subsided promptly after the IOP was lowered.

Case Report

Laser in situ keratomileusis was performed at a local eye clinic in both eyes of a healthy 26-year-old man with myopic astigmatism. The preoperative best spectacle-corrected visual acuity (BSCVA) was 20/10 with −9.5 +1.5 × 90 in the right eye and 20/15 with −9.5 +1.5 × 90 in the left eye. The keratoconus screening test using Placido disk videokeratography (TMS-2, Computed Anatomy Inc.) was negative in both eyes.

Surgery was performed with the MK-2000 microkeratome (Nidek) and the EC-5000 excimer laser (Nidek). During the postoperative follow-up, the best corrected visual acuity in the left eye fluctuated between 20/50 and 20/25. Moderate striae and slight opacity were observed in the corneal flap. A topical steroid was applied in the left eye for several months. Since striae and corneal opacity did not improve, the patient was referred to us at 10 months. The IOP had not been measured before referral.

At the initial examination, the uncorrected visual acuity was 20/100 in the left eye. The BSCVA was 20/80 with plano −1.5 × 120. The IOP was 40 mm Hg in the left eye measured with the Goldmann applanation tonometer. Slitlamp examination showed macro- and microstriae in the flap and mild corneal opacification. There was no corneal edema. Intravenous mannitol (400 mL) was given in a 20% solution over 1 hour to reduce the IOP. When treatment was completed, the IOP was 15 mm Hg and the BSCVA was 20/25 with −0.5 +1.5 × 20.

Posterior elevation maps obtained with a scanning-slit corneal topography system (Orbscan II, Bausch & Lomb) before and after intravenous mannitol was administered are shown in Figure 1. Anterior protrusion of the posterior corneal surface was seen before mannitol was used; this markedly improved after the IOP was normalized. Changes in the posterior corneal elevation were evaluated at the center of the difference map generated from the 2 elevation maps. For surface alignment in the difference map, a 3.0 mm peripheral annular fit zone from 7.0 to 10.0 mm in diameter was used.[3,4] The color scale is

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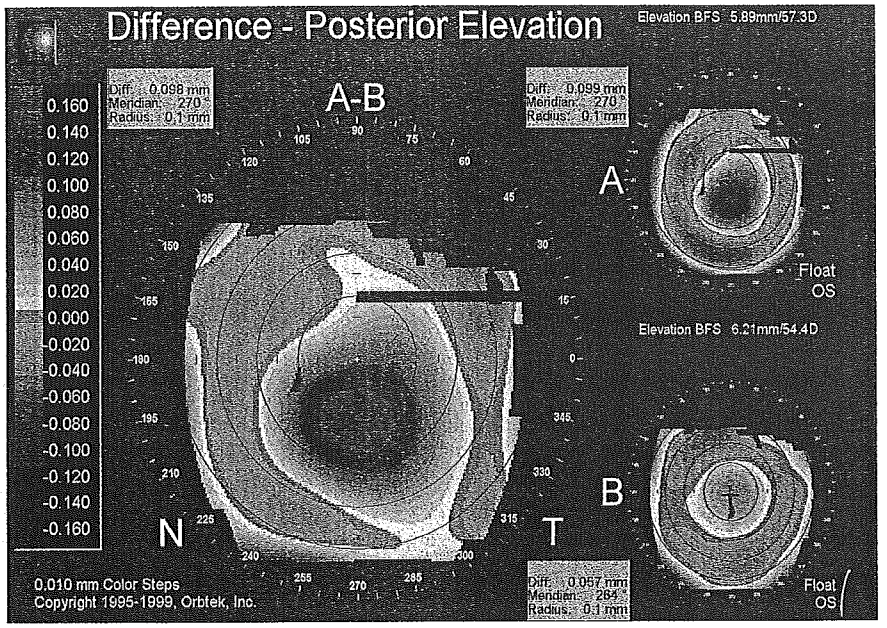

Figure 1. A difference map (*A-B*) generated from the 2 posterior corneal elevation maps in the eye after LASIK. Reduction of the IOP improved the forward shift of the posterior corneal surface by 98 μm. The posterior elevation maps show the anterior protrusion of the posterior corneal surface before intravenous mannitol was administered (*A*) and the marked improvement after the IOP was normalized by treatment (*B*). The color scale is 10 μm, which does not exaggerate the elevation changes.[7]

10 μm, which does not exaggerate the elevation changes.[5] Reduction of the IOP improved the forward shift of the posterior corneal surface by 98 μm.

Topical steroid use was discontinued, and topical application of a β-blocker was initiated. The IOP stabilized thereafter, and keratectasia has not recurred. The 12-month follow-up showed no corneal ectasia on the posterior elevation map, and the BSCVA remained 20/20 with plano +1.0 × 180.

Discussion

Eyes with thin corneas, high IOP, and high myopia requiring greater laser ablation are reportedly predisposed to an anterior shift of the cornea after LASIK.[4] In this case, the keratectasia seemed to be attributable to the IOP elevation since reduction of the IOP resulted in prompt improvement of the ectasia and the complication did not recur after the IOP was normalized. Because the IOP was measured with the Goldmann applanation tonometer, which reportedly underestimates IOP after keratorefractive surgery,[6,7] the true IOP was probably higher than the readings. Increases in the internal pressure may have expanded and distended the cornea, which had been structurally modified by the previous keratorefractive surgery, leading to forward movement and stretching. The contralateral eye had a similar amount of myopic correction, but the postoperative course was uneventful and no sign of iatrogenic keratectasia was seen, possibly because no IOP elevation occurred.

There may be concern about the effect of IOP elevation on scanning-slit corneal topography measurements. To address this question, topographical measurements were performed in an eye that was treated with intravenous mannitol for elevated IOP, as in the case presented. The patient was a 38-year-old woman with Posner-Schlossman syndrome with no other disease or history of ocular surgery. The mannitol treatment decreased the IOP from 46 mm Hg to 16 mm Hg. As shown in Figure 2, the posterior corneal elevation maps before and after mannitol treatment showed little difference, and the central corneal thickness was 575 μm and 557 μm before and after administration of the osmotic agent, respectively.

After LASIK in our case, marked elevation of the IOP adversely affected the structurally compromised cornea, leading to transient keratectasia. The keratectasia was reversible, and its recurrence was prevented by treatment. Intraocular pressure elevation, which may be caused by steroid application, can be a risk factor for iatrogenic keratectasia after keratorefractive surgery.

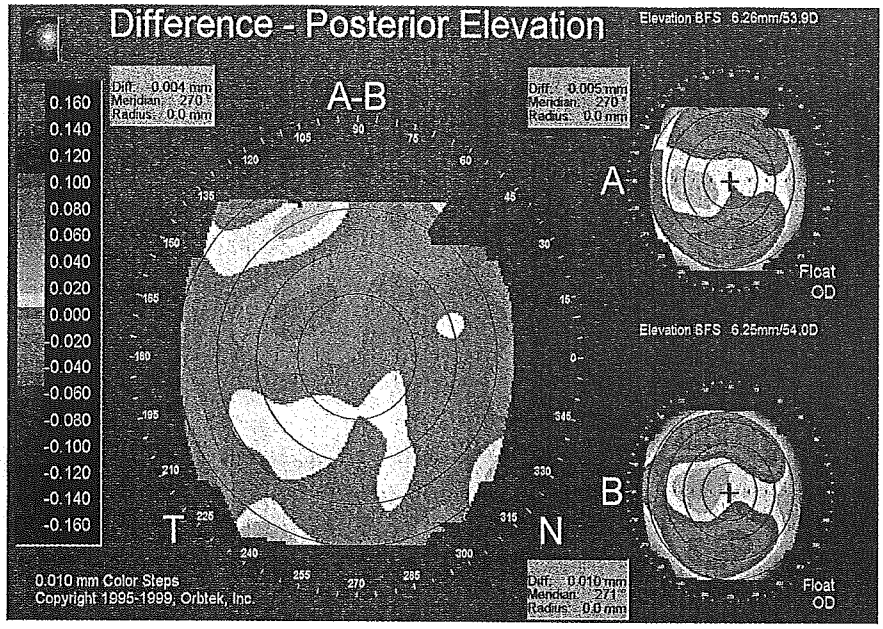

Figure 2. A difference map (*A-B*) generated from 2 posterior corneal elevation maps in the eye with Posner-Schlossman syndrome that was treated for elevated IOP. The posterior elevation maps before (*A*) and after (*B*) intravenous mannitol was administered show the reduction of IOP had little effect on the posterior corneal elevation.

The condition might be irreversible if undiagnosed and untreated.

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Prevalence of *Staphylococcus epidermidis* Strains With Biofilm-Forming Ability in Isolates From Conjunctiva and Facial Skin

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• PURPOSE: To compare the prevalence of biofilm-forming strains of *Staphylococcus epidermidis* in the conjunctival and facial skin microflora.
• DESIGN: Experimental study.
• METHODS: The prevalence of biofilm-forming ability of 10 *S epidermidis* strains obtained from the conjunctival sac of healthy volunteers was compared with 40 strains obtained from the facial skin of healthy volunteers. The ability to form biofilm was determined by the presence of the *icaA* gene, and the production of biofilm was examined by qualitative (Congo red agar [CRA]) and quantitative (microtiter plate) assays. Additionally, the prevalence of 36 *S epidermidis* strains obtained from the conjunctival sac of precataract patients to form biofilm was investigated.
• RESULTS: The *icaA* gene was detected in 60% of the isolates from the conjunctival sac of volunteers and 15% of those from the facial skin. Fifty percent of the isolates from the conjunctiva of volunteers and 5% from the facial skin were CRA positive. Biofilm production was significantly greater in isolates from the conjunctiva of volunteers. Of the nine pairs of isolates found in the same volunteers, six conjunctival sac isolates were positive for the *icaA* gene with biofilm-forming ability except one strain, whereas only one of the facial skin isolates was positive for the *icaA* gene and none exhibited biofilm-forming phenotype. Sixty-nine percent and 44% of the isolates from the conjunctival sac of precataract patients were positive for *icaA* gene and CRA test, respectively.
• CONCLUSIONS: The prevalence of biofilm-forming *S epidermidis* isolates is higher in the conjunctival sac than the facial skin. (Am J Ophthalmol 2005;140: 844–850. © 2005 by Elsevier Inc. All rights reserved.)

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STAPHYLOCOCCUS EPIDERMIDIS, ALONG WITH *Corynebacterium* species and *Propionibacterium acnes*, comprise the major microflora of the conjunctiva sac, eyelids, and meibomian glands, and is the most common cause of postoperative suppurative endophthalmitis.[1] It is generally accepted that *S epidermidis* enters the eye during and after intraocular surgery to cause this disorder. However, the factors contributing to the virulence of *S epidermidis* is not well understood.[2]

It has been suggested that the ability to form biofilms on polymer surfaces greatly contributes to the virulence of *S epidermidis*.[3–5] This ability depends on the production of polysaccharide intercellular adhesion (PIA) molecules, encoded by the intercellular adhesion (*ica*) locus including the *icaA* gene, *icaB* gene, *icaC* gene, and *icaD* gene.[6–8] Recently, Pinna and associates[9] showed that *S epidermidis* adhered more firmly to the AcrySof intraocular lenses (IOLs) when the bacterial DNA contained the *icaA* gene. In addition, Kodjikian and associates[10] showed that *S epidermidis* strains carrying the *icaA* gene appeared to be anchored to the surface of the IOLs by a biofilm in the first phase of adhesion. Thus, a good possibility is that *S epidermidis* is introduced into the eye together with an implanted IOL, and that the *icaA* gene plays an important role in the virulence of this organism. However, the prevalence of biofilm-forming strains of *S epidermidis* in the conjunctival microflora has not yet been determined. We therefore sought to determine the prevalence of *S epidermidis* strains with biofilm-forming ability in the conjunctival sac compared with that on the facial skin.

METHODS

THE PREVALENCE OF *S EPIDERMIDIS* STRAINS THAT CAN form a biofilm layer was determined from strains obtained from the conjunctiva and facial skin of healthy volunteers.

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The genotype and phenotype of these strains were determined. The isolates from the conjunctiva of patients collected before cataract surgery were also investigated. The findings of the culture conditions between volunteer and patient groups were compared.

Informed consent was obtained from all volunteers and patients after an explanation of the nature and possible consequences of the study. The conduct of the research conformed to the tenets of the Declaration of Helsinki.

Swabs of the conjunctiva and facial skin of 47 healthy volunteers (mean ± SD age, 72 ± 9 years; 10 men, 37 women) were cultured. Swabs of the conjunctiva of 89 patients (70 ± 11 years; 29 men, 60 women) before cataract surgery were also cultured. The volunteers and patients had not used antibiotic eyedrops and systemic antibiotics, and their conjunctivas and facial skin were not inflamed. In addition, the sex composition of each group was investigated, as was the medication being taken by the volunteers and patients that could influence the culture-positive rate of *Staphylococcus* species.

Conjunctival and skin isolates were plated on Mannitol salt agar (Eiken Chemical, Tokyo, Japan) and incubated at 37°C in air for 48 hours for selection of staphylococci. The identification of the staphylococci strain was made by their biochemical properties with the Staphyogram system (Wako Pure Chemical Industries, Osaka, Japan). Isolates were stored in tryptic soy broth (TSB; Becton Dickinson, Sparks, Maryland) with glycerol (15% vol/vol) at −80°C. The biofilm-forming ability of these strains was investigated. Two reference strains of *S epidermidis* were also studied: the biofilm-forming GTC 1836 strain (Gifu Type Culture Collection; ATCC35984, American Type Culture Collection), and the biofilm-negative GTC289^T strain (ATCC14990).

Chromosomal DNA was extracted from the *S epidermidis* isolates with the UltraClean Microbial DNA Isolation Kit (MO BIO, Salana Beach, California). An 814-bp segment of the *icaA* gene was amplified by polymerase chain reaction (PCR). The PCR conditions and primers were based on those described by Ziebuhr and associates.[11] Briefly, the targeted gene was amplified with EX Taq polymerase (Takara, Tokyo, Japan). The primers used were *icaA*-F (5′-GACCTCGAAGTCAATAGAGGT-3′) and *icaA*-R (5′-CCCAGTATAACGTTGGTACC-3′) (Invitrogen, Tokyo, Japan). The PCR cycling conditions were as follows: 30 cycles of denaturation for 1 minute at 95°C, elongation for 1.5 minutes at 72°C, and annealing for 30 seconds at 50°C. The PCR amplification products were separated and identified by 1.5% agarose gel electrophoresis.

The morphology of the colonies and their phenotypic changes were studied on Congo red agar (CRA) cultures as described by Arciola and associates.[12] The CRA was composed of 37 g/l of brain-heart infusion broth (Becton Dickinson), 36 g/l of sucrose (Sigma Chemical Company, St Louis, Missouri), 15 g/l of agar (Becton Dickinson), and 0.8 g/l of Congo red (Sigma).

Each isolate and reference strain was inoculated onto a plate and incubated for 24 hours at 37°C, followed by overnight incubation at room temperature. PIA-positive strains appeared as black colonies, and PIA-negative strains were red.[12]

The degree of biofilm production was determined by a microtiter plate assay as previously described,[13] but safranin was used instead of crystal violet. Bacteria were inoculated into 10 ml of TSB with 0.25% glucose (Wako Pure Chemical Industries) and incubated overnight with shaking at 37°C in a water bath. This solution was diluted 1:100 in TSB with glucose, and 200 µl was dispensed into 96-well polystyrene microtiter plates. The plates were incubated overnight at 37°C under aerobic conditions. The plates were then washed three times with distilled water and stained with 0.2% safranin (Wako Pure Chemical Industries). The quantity of the adherent biofilm was determined by the optical density (OD) at 490 nm. A well with sterile TSB served as control, and its OD was subtracted from that of the experimental strains.

Differences between strains from the conjunctiva and from facial skin of volunteers carrying the *icaA* gene and CRA positivity were compared by the χ^2 test. The mean ODs of the microtiter plate assay were compared between strains from the conjunctiva and from facial skin in volunteers by analysis of Welch's unpaired *t* test. Differences between characteristics that could influence culture-positive rate were compared by the χ^2 test. For all tests, $P < .05$ was considered significant.

RESULTS

FIFTEEN STRAINS OF *STAPHYLOCOCCUS* WERE ISOLATED from the conjunctiva and 43 from the facial skin of 41 of the 47 volunteers. The 15 isolates from the conjunctiva were identified as *S epidermidis* in 10 volunteers, as *S aureus* in two, and as coagulase-negative *Staphylococcus* in three. The 43 strains from the facial skin were identified as *S epidermidis* in 40, *S aureus* in two, and coagulase-negative *Staphylococcus* in one. In 30 volunteers, *S epidermidis* was isolated from only the facial skin, and in nine volunteers, *S epidermidis* was isolated from both conjunctiva and facial skin. In one volunteer, one specimen of *S epidermidis* was isolated from only the conjunctiva.

A total of 47 strains were isolated from the conjunctiva of 44 of the 89 precataract patients. They were identified as *S epidermidis* in 36, *S aureus* in seven, and coagulase-negative *staphylococcus* in four. The 36 *S epidermidis* strains isolated from the conjunctiva of 36 patients (70 ± 9 years; 17 men, 19 women) of the 89 precataract patients, and 36 isolates from the conjunctiva of patients, 10 isolates from the conjunctiva of volunteers, and 40 isolates from facial skin of volunteers were investigated in more detail.