Characterization of Corneal Pannus Removed from Patients with Total Limbal Stem Cell Deficiency

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PURPOSE. To determine the epithelial lineage of origin in corneal pannus tissue surgically removed from patients with total limbal stem cell (SC) deficiency.

METHODS. The lineage of origin of the entire conjunctivalized pannus removed from eight corneas with a diagnosis of total limbal SC deficiency was characterized by anti-keratin (K)-3 and anti-K19 monoclonal antibodies. The protein and mRNA of epithelial outgrowth from segments of five such pannus specimens were analyzed by Western blot and reverse transcription–polymerase chain reaction, respectively.

RESULTS. Cross sections of all eight specimens showed a stratified epithelium with goblet cells expressing mucin (MUC)-5AC, and a stroma showing blood vessels and inflammatory cell infiltrates. Immunostaining showed full-thickness expression of K19 in the entire pannus of all eight specimens. Expression of K3 was negative in seven patients, but was sporadically positive in a patient with Stevens-Johnson syndrome. In culture, all five pannus specimens generated a compact, small epithelial cell outgrowth, and except for one, reached confluence in 2 to 3 weeks. The K3/K12 pair was expressed by extracts of cell outgrowth from the control limbal epithelial explant, but not in all five pannus specimens. A 60-kDa band of ΔNp63 was expressed in the control specimen and in all five pannus specimens. Cell outgrowth expressed K3 transcript in three, but none showed K12 transcript.

CONCLUSIONS. The resultant epithelial phenotype of the pannus tissue was not corneal, as evidenced by the negative staining to cornea-specific K12 mRNA and protein, but was conjunctival, as evidenced by the presence of goblet cells, the weak expression of K3, and the strong expression of K19. The abundant expression of ΔNp63 in such conjunctiva-derived epithelium in eyes with total limbal SC deficiency raises doubts as to its validity as a limbal SC marker. (Invest Ophthalmol Vis Sci. 2004;45:2961–2966) DOI:10.1167/iovs.03-1397

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Corneal diseases with total limbal stem cell (SC) deficiency are some of the most challenging clinical entities.1–4 These diseases completely destroy limbal epithelial SCs, or their surrounding environment, or a combination of both.5 Histopathologically, corneas with total limbal SC deficiency are characterized by conjunctival ingrowth (conjunctivalization), vascularization, and chronic inflammation.6–8 To confirm limbal SC deficiency, ophthalmologists look for such clinical signs as the loss of the palisades of Vogt9 and late fluorescein staining.9 However, a definite diagnosis relies on the detection of conjunctivalization, which is verified by the presence of goblet cells or the expression of certain types of keratins10–11 in specimens obtained by impression cytology.

Impression cytology is useful as a relatively noninvasive diagnostic tool and has been successfully applied to detect conjunctival goblet cells as an index of conjunctivalization.5 Nevertheless, impression cytology cannot always remove all basal epithelial cells, which may pose a problem in assessing limbal SC deficiency. Therefore, we undertook immunohistochemical and biochemical characterization of several keratins and the protein ΔNp63 in the corneal pannus with presumed conjunctivalization excised surgically from patients with total limbal SC deficiency and in the epithelial cells expanded from these tissues. The rationale is based on the fact that the keratin (K)-3/K12 pair is expressed throughout the full thickness of the human corneal epithelium, but is only suprabasally expressed in the limbal epithelium and is negative in the conjunctival epithelium.12–14 In contrast, expression of K19 is negative in the corneal epithelium but positive in the epithelium of limbus and conjunctiva.15 Besides differences in keratin expression, the proliferative potential may also vary among limbal, corneal, and conjunctival epithelial cells. The limbal region is known to harbor the SC population, which has a high proliferative potential necessary to maintain the corneal epithelial integrity.16–18 Recently, it has been reported that the limbal epithelial basal cells are the only population expressing the protein ΔNp63 in the ocular surface.19 This protein, expressed in the nuclei of basal cells of different epithelia, has been regarded as a positive SC marker for the epidermis and the limbal epithelium.

MATERIALS AND METHODS

Patients
Written informed consent was obtained from all patients or, in the case of minors, from their parents after explanation of the nature of the study. The research adhered to the tenets of the Declaration of Helsinki. Institutional Review Board approval was also obtained, and the study was in compliance with the Health Insurance Portability and Accountability Act (HIPAA). Eight patients with a clinical diagnosis of total limbal SC deficiency and confirmed by impression cytology in six cases were included. In two cases, an obvious clinical diagnosis of total limbal SC deficiency avoided cytological confirmation. The etiology included aniridia (n = 2), chemical burn (n = 3), Stevens-Johnson syndrome (n = 1), and idiopathic limbal SC deficiency (n = 2)20 (Table 1). All patients had not undergone prior ocular surface surgery and had
undergone keratolimbal allograft and amniotic membrane transplantation by one surgeon (SCGT) at the Ocular Surface Center, as previously described (for review, see Refs. 21, 22). All pannus specimens were immediately excised and placed in a sterile medium for cell culturing.

**Immunofluorescence Staining**

A normal section comprising corneal, limbus, and conjunctival epithelium was used as the control. Multiple 5-μm-thick frozen sections were subsequently prepared on gelatin-coated slides and fixed in cold acetone for 10 minutes at −20°C. Immunofluorescence staining was performed as previously described.²³ The following antibodies at the mentioned dilution were used to detect goblet cell mucin (MUC)-5AC (BA17 clone, 1:70; Dako, Carpinteria, CA). Binding with the primary antibody was detected by a goat anti-mouse IgG and IgM antibody (Vector Laboratories, Burlingame, CA) and propidium iodide (Sigma-Aldrich, St. Louis, MO) and were analyzed with an epifluorescence microscope (Te-2000u Eclipse; Nikon, Tokyo, Japan).

**Tissue Culture**

Small pieces (1 × 1 mm), cut from different regions of the pannus removed from five patients, were incubated in 15 mg/mL dispase II (Roche, Indianapolis, IN) at 37°C for 20 minutes, seeded in a 60-mm plastic dish (Falcon, Franklin Lakes, NJ), and cultured in supplemented hormonal epithelial medium (SHEM) medium, which was made of an equal volume of HEPES-buffered DMEM and Ham’s F12, containing bicarbonate, 5% fetal bovine serum (FBS), 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B (all from Invitrogen-Gibco), and propidium iodide, 0.5 μg/mL hydrocortisone, and 30 ng/mL cholecalciferol A subunit (all from Sigma-Aldrich). Cells were cultured until the outgrowth reached confluence.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was extracted (Trizol; Invitrogen-Gibco) from two 8-mm central corneal buttons that had been minced with a blade and sonicated at 6000 rpm (Tissue Tearor; BioSpec Products Inc., Bartlesville, OK) as a positive control. Total RNA was similarly extracted from cells cultured on plastic. Total RNA equivalent to 1 × 10⁵ cultured cells or one corneal button was subjected to RT-PCR based on a protocol recommended by Promega (Madison, WI). The final concentration of RT reaction was 10 mM Tris-HCl (pH 9.0 at 25°C), 5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 1 μM each dNTP, 1 μM recombinant RNase inhibitor (5 U AMV reverse transcriptase, 0.5 μg Oligo(dT)₁₅ primer, and total RNA in a total volume of 20 μL. The reaction was kept at 42°C for 60 minutes. One tenth of total RT product was used for subsequent PCR with the full concentration of PCR reaction being 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM Mg(OAc)₂, and 1.25 U Taq DNA polymerase in a total volume of 50 μL, using the following primers: K5, sense 5’-CAG AGA TAG AGG GTG TCA AGA AG-3’, antisense, 5’-AGG TGG GAG AAC TTG ATG -3’; and K12, sense 5’-GAG TTC CCA AGC TTC CGG GTG GCC-3’, and antisense, 5’-CAT TAG TTC TCC AAT TTC CTG AAG-3’. (Table 2). The PCR mixture was first denatured at 94°C for 5 minutes and then amplified for 30 cycles (94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute), with a programmable thermal controller (model PTC-100; MJ Research, Inc., Watertown, MA). After amplification, 15 μL of each PCR product and 3 μL of 6× loading buffer were mixed and electrohoresed on a 1.5% agarose gel in 0.5× Tris-borate/EDTA (TBE), containing 0.5 μg/mL ethidium bromide. Gels were photographed and scanned.

**Table 1. Clinical Characteristics and Laboratory Investigation of Studied Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Immunofluorescence Staining</th>
<th>Cultured Cells</th>
<th>Western Blot</th>
<th>RT-PCR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>K3</td>
<td>K19</td>
<td>K3/K12</td>
<td>P63</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>Aniridia</td>
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<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>2</td>
<td>41</td>
<td>Aniridia</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>3</td>
<td>51</td>
<td>C. burn</td>
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<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>C. burn</td>
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<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
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<td>−</td>
<td>+</td>
<td>−</td>
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<td>6</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
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<td>Idiopathic</td>
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<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
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<td>7</td>
<td>SJS</td>
<td>sporad</td>
<td></td>
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**Table 2. RT-PCR Primer Sequences**

<table>
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<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>cDNA Location</th>
<th>Size of PCR Product</th>
<th>GenBank Accession Number</th>
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<td>Keratin 3 Sense</td>
<td>CAGAGATCGAAGGTTGTCAGAAAG</td>
<td>(1289–1311)</td>
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<tr>
<td>Antisense</td>
<td>AAGACTGAGGAAACTTGAAGCTG</td>
<td>(1861–1859)</td>
<td>451 bp</td>
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<td>Keratin 12 Sense</td>
<td>GAGTCCAAAAGCTCCTGGGGTTGGG</td>
<td>(832–854)</td>
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<tr>
<td>Antisense</td>
<td>CATTAATTCCTGAATTTGCTGGAAAC</td>
<td>(1506–1483)</td>
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<td>GAPDH Sense</td>
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<td>(60–82)</td>
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<tr>
<td>Antisense</td>
<td>GGGGCGATCAAGGGCCACAGTTG</td>
<td>(654–633)</td>
<td>575 bp</td>
<td>BC029618</td>
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</table>
Western Blot Analysis

Proteins of confluent cultures were obtained from pannus of five patients (Table 1) by RNA extraction (Trizol; Invitrogen-Gibco) and precipitated by centrifugation at 12,000g in 100% isopropyl alcohol. Epithelial cells obtained from the human limbus were used as a positive control. Prestained broadband SDS-PAGE standard and protein samples were dissolved into 1× SDS loading buffer. Ten micrograms of total proteins were electrophoresed in a 4% to 15% gradient polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred to a nitrocellulose membrane, followed by 1-hour blocking with 5% low-fat dry milk in Tween-TRIS-buffered saline (TTBS). Membranes were incubated for 1 hour at room temperature with primary antibody against ΔNp63 (4A4 clone; 1:300 dilution; Dako), K3 (1:1000 dilution), K12 (1:500; a kind gift of Winston Kao, University of Cincinnati, Cincinnati, OH), and β-actin (1:3000 dilution; Sigma-Aldrich). Membranes were subsequently incubated with 1:50 diluted avidin-biotin complex reagent (ABC; Vectastain Elite, Vector Laboratories), conjugated with peroxidase for 50 minutes, and developed in diaminobenzidine (Dako).

RESULTS

The demographic data and etiology of total limbal SC deficiency of eight patients are summarized in Table 1. Pannus tissue was dissected and removed as an entire sheet of epithelial and stromal components in six patients (examples shown in Fig. 1). In the remaining two patients, complete pannus tissue excision did not produce a single piece of pannus tissue.

Impression cytology specimens demonstrated the presence of PAS-positive goblet cells in all six patients, with presence of abundant inflammatory cells in the case of one patient with Stevens-Johnson syndrome (Fig. 1A, 1C, insets). After hematoxylin staining, the cross section of excised pannus tissue always consisted of an epithelium with variable degrees of epithelial stratification (Figs. 2A, 2B) and variable amounts of the underlying stroma, which had blood vessels and pronounced inflammatory cell infiltration (Figs. 2A, 2B, arrowheads). Because the morphology of the goblet cell was clearly recognized by hematoxylin staining, even when followed by PAS staining, we examined the same section by immunostaining with a monoclonal antibody (AM3) against goblet cell MUC5AC, and confirmed the presence of conjunctival goblet cells in all eight pannus specimens (Figs. 2C, 2D).

Immunofluorescence staining with monoclonal antibodies showed continuous full-thickness expression of K19 in all eight pannus specimens (Figs. 3A, 3C). In contrast, expression of K3 was negative in seven patients (Fig. 3B), but was only sporadically positive in the superficial cells in the patient with Stevens-Johnson syndrome (Fig. 3D). ΔNp63 expression was found in basal and suprabasal layers (data not shown).

A segment was cut from the entire pannus (Figs. 4A, 4B) for culturing. Four days later, epithelial cells migrated onto the

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)
plastic surface (Fig. 4C) and became confluent in 2 to 3 weeks (Fig. 4D) in all five explants except one. Such epithelial outgrowth was composed of a monolayer of small and compact cells without fibroblast contamination (Fig. 4D).

To determine more about the origin of epithelial lineage, RT-PCR analysis of RNA extracted from expanded cells from the pannus tissue was performed. The control corneal epithelium scraped from the corneal button expressed both K3 and K12 transcripts (Fig. 5). K3 transcript was expressed in three of five patients. Nevertheless, expression of K12 transcript was absent in all five specimens. To confirm further the changes in keratin expression at the translational level and to determine whether a proliferation marker (ΔNp63) was also expressed, we performed Western blot analysis. As shown in Figure 6, proteins extracted from cells expanded from all five excised panni and in the control culture of the limbal epithelium showed a positive 60-kDa band of ΔNp63 (also see Table 1). In contrast, proteins extracted from expanded pannus cells did not express K3 or K12, whereas the control cells did (Fig. 6).

**DISCUSSION**

In eight patients with clinical diagnoses of aniridia, chemical burn, idiopathic limbal SC deficiency, and Stevens-Johnson syndrome, we used a battery of tests to confirm that epithelial cells in the corneal pannus tissue are not derived from the cornea, but instead are of conjunctival derivation.

The hallmark of limbal SC deficiency (i.e., conjunctivalization, refers to the process of conjunctival epithelial ingrowth onto the corneal surface). Traditionally, the diagnosis of conjunctivalization of the corneal surface relies on the detection of conjunctival epithelial goblet cells on impression cytology specimens. Despite the advantage of being a noninvasive procedure, impression cytology cannot obtain a full-thickness epithelial sample or rule out limbal SC deficiency when conjunctival goblet cells are lost in the process of squamous metaplasia. Furthermore, even if conjunctivalization is detected by impression cytology, this diagnostic criterion has not been validated by thoroughly investigating the full thickness of an entire corneal pannus.

When the cross sections of these pannus tissues were first examined by hematoxylin staining, we found, as expected, goblet cells in all eight specimens. This finding supports results obtained by impression cytology. In addition, we noted that conjunctivalization was accompanied by stromal vascularization and inflammatory cell infiltration in all specimens, supporting the notion that inflammation plays a major role in the development or maintenance of total limbal SC deficiency. To confirm that there was no corneal epithelial lineage left in the midst of conjunctival epithelium or in some focal areas of the cornea, we immunostained with anti-K3 and anti-K19 antibodies. Our results showed indeed K3 was absent in seven of eight specimens, but not K3 and K12, was expressed by epithelial cells expanded from five pannus specimens.

![Figure 4. Characterization of epithelial outgrowth from pannus explant.](image)

![Figure 5. RT-PCR analysis of K3 and K12 transcripts.](image)

![Figure 6. Western blot analysis of K3, K12, and ΔNp63.](image)
all eight specimens. The pattern of K19 expression resembled that in normal conjunctival epithelium during fetal and postnatal development.27

Because selective cross sections of pannus tissues may not represent the entire pannus, we studied the epithelial outgrowth from five pannus specimens with the more sensitive RT-PCR and Western blot assays. Western blot analysis did not detect the cornea-specific markers K5 and K12, suggesting that cells expanded from pannus did not contain cells of corneal derivation. Although RT-PCR showed a weak expression of K5 transcript in patients 1, 4, and 7, it did not detect any K12 transcript in all five patients. It has been reported that K3 is expressed by noncorneal tissues, including bulbar conjunctiva,15 nose, and oral mucosa.28,29 In contrast, K12 is found only in the corneal epithelium.14,30–31 Therefore, based on the absolute absence of K12 protein and transcript, we conclude that pannus epithelial cells are not derived from the cornea. Our result is consistent with that reported by Elder et al.,15 who used immunostaining to determine the epithelial cell derivation in pannus specimens obtained from patients with different ocular surface diseases. Most of their pannus tissues expressed K19 but not K5, but some expressed both K3 and K19. The latter finding may be because some of their patients did not have total limbal SC deficiency. Aniridia has been recognized as a hereditary example of limbal SC deficiency based on immunostylogy findings of goblet cells on the corneal surface.3,8 In addition, abnormal expression of PKC isoforms has been reported.32 In transgenic Pax6+/− mice, a model of human aniridia, conjunctival goblet cells are found on the corneal surface.29 For the first time, our study revealed more detailed histologic and biochemical changes of abnormal pannus in aniridia, especially regarding the expression of the K5/K12 pair.

The absence of corneal cell lineage is compatible with the diagnosis of limbal SC deficiency, and hence we speculated that the expression of ΔNp65, a positive SC marker expressed exclusively by the limbal SCs and not by the conjunctival or corneal cells,19 should be absent. To our surprise, our Western blot data showed the presence of ΔNp65 in the conjunctivalized epithelium of all patients with total limbal SC deficiency. The expression of ΔNp65 has also been reported in the conjunctivalized epithelium of murine and rat models with limbal SC deficiency.34 Taken together, these findings suggest that ΔNp65 is expressed by differentiated conjunctival epithelial cells and raises the doubt that ΔNp65 is only a bona fide marker for epithelial SC. Recent reports suggest that there may be a broader role of ΔNp65 based on the finding that ΔNp65 expression is not specific to epithelial stemness, but is essential for proper epithelial stratification.35,36 Given the fact that all pannus epithelia were stratified, we were not surprised to note the expression of ΔNp65 in these specimens.

An accurate diagnosis and characterization of the pannus tissue, as shown in this study, validates the clinical utility of impression cytology, confirms that the process of conjunctivalization is the hallmark of total limbal SC deficiency, and justifies the transplantation of limbal epithelial SCs as the only way to restore a normal corneal surface.

References


