

Keratin 13 Immunostaining in Corneal Impression Cytology for the Diagnosis of Limbal Stem Cell Deficiency

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PURPOSE. The aim of this study was to develop a validated, reliable, and minimally invasive technique for diagnosing limbal stem cell deficiency (LSCD) by immunocytochemical detection of conjunctival and corneal keratins on epithelial cells collected by impression cytology (IC).

METHODS. After validation of labeling techniques on a cohort of 10 healthy control patients, keratins K12, K13, and K19 were labeled on corneal IC of 10 eyes suspected of LSCD. Positive scores for the conjunctival markers K13/K19, coupled with the rarity of the corneal marker K12, were diagnostic proof of LSCD.

RESULTS. IC is a reliable and noninvasive technique for collecting epithelial cells. The labeling validation phase has permitted K3 labeling to be eliminated due to lack of corneal specificity. Among patients with LSCD, nine samples were diagnosed with LSCD (K13+/K19+), which was severe (K12−) in eight cases and mild (K12+) in one case. One sample could not be analyzed due to lack of cells.

CONCLUSIONS. K13 has shown to be a new marker of conjunctival differentiation. The immunocytochemical search for the K13/K19 couple by corneal IC provides a simple and reliable method for diagnosing LSCD, whereas the level of K12 could provide a score of disease severity. On the other hand, the authors question the corneal specificity of K3 as conventionally established. (*Invest Ophthalmol Vis Sci.* 2011;52:9411–9415) DOI:10.1167/iovs.10-7049

The limbus, specific source of stem cells for the corneal epithelium, is an anatomic and functional boundary between the conjunctiva and cornea. Limbal stem cell deficiency syndrome (LSCD), which is often attributed to trauma and damage to the stem cells and their niche, is characterized by dystrophy of the corneal epithelium followed by invasion of the corneal surface by a vascularized epithelium with conjunctival differentiation, a vector of immune cells.¹ Thus, it is a major risk factor for graft rejection.

Diagnosis of the disease has long been based on clinical examination, associating pathologic conditions thought to involve stem cell deficiencies to classic biomicroscopic findings such as chronic inflammatory condition, poor corneal epithe-

lial healing, replacement of the corneal epithelium with conjunctiva (conjunctivalization), and corneal scarring that contribute to severe visual loss. In recent years, bioengineered corneal epithelial equivalents, developed from the ex vivo expansion of limbal stem cells, have been used to treat severe limbal stem cell deficiency, with promising results.^{2,3} To justify these expensive treatments, health service regulators require a reliable paraclinical diagnostic technique for this disease.

The epithelial cells (ECs) of the ocular surface are characterized by markers with high tissue specificity, the keratin antigens of intermediate filaments. The current dogma is that the expression of K3/12 (K3 basic keratin of 64,000 Da; K12 acidic keratin of 55,000 Da) is thought to be a hallmark of corneal ECs.^{4–9} However, the corneal specificity of K3 has recently been questioned, strongly suggesting that the actual expression patterns of K3/12 in the ocular surface epithelium are not as clear cut as the current dogma suggests.^{10–14}

The superficial layers of the conjunctival epithelium are characterized by specific keratins of nonkeratinized stratified epithelia (K4/K13), simple epithelia (K8/K19), and glandular epithelia (K7).^{15–17} The diagnosis of LSCD has long relied on the detection of goblet cells after impression cytology (IC) of the corneal epithelium, either by periodic acid–Schiff staining, or by more specific techniques, such as Mucin 5AC staining.^{14,18,19} However, the density of conjunctival goblet cells can decrease by up to 99% under pathologic situations,^{20–23} and at the early stages of LSCD, goblet cells may not even be detectable in the cornea, whereas there are already recurrent erosions with delayed corneal healing and neovascularization.²⁴ Thus, the absence of goblet cells within the corneal epithelium does not exclude the diagnosis of LSCD.²⁵

The aim of this study was to develop a validated, reliable, and minimally invasive technique for diagnosing LSCD by immunocytochemical detection of conjunctival and corneal keratins on epithelial cells collected by IC.

In this study, we validated the K12, K13, and K19 labeling techniques and refuted the K3 marker (most commonly used for the diagnosis of LSCD) on tissue and cells collected by IC and used these markers for the diagnosis of LSCD. Our hypothesis was that the expression of differentiation markers of the conjunctival epithelium by IC of the corneal epithelium was diagnostic proof of LSCD. The presence of differentiation markers of the corneal epithelium would make it possible to quantify the severity of the disease.

MATERIALS AND METHODS

Human Samples

This prospective and monocentric study followed the tenets of the Declaration of Helsinki and was approved by the institutional review board.

The inclusion criteria for healthy patients were normal visual acuity and biomicroscopic examination. Pathologic patients enrolled should

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TABLE 1. Primary Antibodies Used during the Study

Antigen	Clone	Type	Host	Immunogen	Manufacturer	Dilution
K3/K76	AE5	Monoclonal	Mouse	Human K3	Progen, Biotechnik GmbH, Heidelberg, Germany	1/100
K12	12 (N-16)	Polyclonal	Goat	Human K12	Santa Cruz Biotechnology, Santa Cruz, CA	1/100
K13	ks13.1	Monoclonal	Mouse	Human K13	Chemicon International, Inc., Temecula, CA	1/100
K19	NCL-CK19/b170	Monoclonal	Mouse	Human K19	Vision Biosystems, Novocastra, Newcastle upon Tyne, UK	1/100

present biomicroscopic examination compatible with LSCD, consisting in persistent epithelial defect, chronic inflammation, corneal neovascularization, conjunctivalization, and stromal scarring with corneal opacity and loss of vision. Each patient should be older than 18 years of age. For both healthy and pathologic patients, exclusion criteria were sensitivity to drugs that provide local anesthesia or active infection of the external eye.

We scored LSCD as mild when the visual axis was covered with normal corneal epithelium and peripheral neovascularization and conjunctivalization and when the patient was relatively asymptomatic. LSCD was severe when there was a central corneal involvement with complete conjunctivalization and 360° neovascularization with significant irritation.

Tissue samples were collected from cadaveric tissues of deceased donors: corneoscleral buttons comprising corneal, limbal, and conjunctival epithelia, not suitable for therapeutic use, were obtained from the tissue and cell bank (Lyon, France). Permission to use the donated tissues for research was obtained from all donor families.

Cells were obtained by cytologic impression of cornea and bulbar conjunctiva of 10 healthy volunteers (3 males and 7 females; mean age, 61.0 ± 25.0 years), and of patients with a clinical diagnosis of LSCD [*n* = 10 eyes of 9 patients; 4 males and 5 females; mean age, 50.3 ± 13.2 years with etiologies of aniridia (*n* = 1), chemical burn (*n* = 2), Lyell syndrome (*n* = 2), infections (*n* = 2), postradiotherapy (*n* = 1), rosacea (*n* = 1), and exposure keratitis with facial palsy (*n* = 1)] (see Table 3 in the following text). All patients were recruited consecutively at the eye clinic at Edouard Herriot Hospital (Lyon, France), between December 2009 and March 2010. Prior informed consent was obtained from the subjects after the nature and possible consequences of the study were explained.

Impression Cytology

The cells were collected by IC on nitrocellulose filters (Millipore, Cork, Ireland) after topical anesthesia with drops of 0.04% oxybuprocaine. The sampling sites chosen were the central cornea and the remote temporal bulbar conjunctiva (10 mm from the limbus) for healthy patients, and areas of maximum corneal conjunctivalization for those clinically suspected of LSCD. In healthy patients, two ICs were performed at each corneal and bulbar conjunctiva site, ensuring that the same site was not impressed twice, at the risk of collecting basal cells whose immunocytochemical characteristics differ from those of suprabasal cells. In diseased patients, only one sample per site was taken. Cellular contamination of the filter by the sample taker was avoided by using nonsterile, powder-free gloves. The cells thus collected were immediately transferred to glass slides (Menzel-Gläser, Superfrost Plus;

Thermo Scientific,) using light finger pressure. The same filter was thus impressed on a maximum of three different slides.

The cells were fixed in acetone at −20°C and kept frozen.

Immunostaining

Primary antibodies were against K3/K76 and K12, both specifics of corneal EC, and K13 and K19, specifics of conjunctival EC (Table 1). The secondary antibody was a commercial IgG (H+L) (AlexaFluor 488; Invitrogen, diluted 100-fold) for K3/K76, K13, and K12 immunostaining. For K19 immunostaining, the secondary antibody was either of two commercial IgGs (H+L) in validation steps (AlexaFluor 488; Invitrogen, diluted 100-fold) or IgG (H+L) in diagnostic steps (AlexaFluor 568; Invitrogen, diluted 100-fold) (Table 2). For the corneal markers K3/K12, the positive control was the cornea with its collar and the negative control was the bulbar conjunctival tissue of a deceased patient. For the conjunctival markers K13/K19, the positive control was the bulbar conjunctival tissue and cornea was the negative control. In all immunostainings, anisotype negative control (IgG) was carried out.

Design of the Study

The single labeling techniques for K3, K12, K13, and K19 were validated using both healthy cells from ICs and tissues from deceased donors; the double-labeling techniques for K12/K13 and K12/K19 were carried out on corneoscleral buttons unsuitable for transplantation from healthy deceased donors (Step 1).

The simple labeling of K13 and double labeling of K12/K19 were carried out on patients with a clinical diagnosis of LSCD (Step 2).

RESULTS

Validation of the Labeling Method

The demographic and clinical data of patients clinically diagnosed with LSCD are summarized in Table 3.

The IC was simple, fast, and easy to carry out for the operator, painless and atraumatic but uncomfortable for the 10 healthy patients: 3 males and 7 females, aged 61 ± 25 years. The results obtained in terms of cell density were very reproducible for the same corneal or conjunctival tissue studied. Indeed, 100% of conjunctival ICs provided a high rate of return, giving carpets of many joint cells containing at least 200 cells per field, at a magnification ×200. Conversely, 100% of corneal ICs yielded only a handful of isolated cells, a maximum 17 cells/field at a magnification ×200 (Fig. 1).

TABLE 2. Secondary Antibodies Used during the Qualification of Markers (I) and Qualification of the Diagnostic Test (II)

Step	Antigen	Secondary Antibodies	Host	Immunogen	Manufacturer	Dilution
1	K3/K76	Alexa Fluor 488 IgG FITC	Goat	Mouse	Invitrogen Corporation, Carlsbad, CA	1/100
	K13					
	K19					
2	K13	Alexa Fluor 488 IgG FITC	Goat	Mouse	Invitrogen Corporation, Carlsbad, CA	1/100
	K19	Alexa Fluor 568 IgG TRITC	Rabbit	Mouse	Invitrogen Corporation, Carlsbad, CA	1/100
1,2	K12	Alexa Fluor 488 IgG FITC	Donkey	Goat	Invitrogen Corporation, Carlsbad, CA	1/100

TABLE 3. Clinical and Immunocytochemical Characteristics of Patients with Clinically Suspected LSCD

Eye	Sex	Age (y)	Laterality	Score	Visual Acuity	Pathology	K13	K19	K12
1	Female	71	Left	Severe	LP+	Ocular rosacea	+	+	–
2	Male	41	Right	Severe	LP+	Chemical burn (acid)	+	+	–
3	Female	53	Left	Severe	LP+	Corneal herpetic infection	+	+	+
4	Male	71	Right	Mild	CF at 50 cm	Trachoma	+	+	–
5	Female	51	Right	Severe	CF at 50 cm	Facial palsy	+	NA	NA
6	Female	33	Left	Severe	LP+	Aniridia	+	+	–
7	Female	43	Right	Severe	LP+	Lyell syndrome	+	+	–
8	Female	43	Left	Severe	CF at 10 cm	Lyell syndrome	+	+	–
9	Male	41	Right	Severe	LP+	Postradiation burn	+	+	–
10	Male	49	Right	Severe	CF at 50 cm	Chemical burn (acid)	+	+	–

LP, light perception; CF, counts fingers; NA, not analyzable.

The cell density decreased significantly as the filters were impressed, this operation not being renewable more than three times.

As shown in Figure 1, strong K3 expression was observed in the suprabasal layers of the conjunctival EC (Fig. 1A), and all the layers of the whole corneal EC (Fig. 1C,D), but absent of the limbus (Fig. 1B), suggesting a lack of sensitivity of K3. On the contrary, K12 expression was restricted to the whole corneal epithelium (all layers) (Figs. 1E, 1H, 1I) and the suprabasal layers of the limbal EC (Figs. 1E, 1G) only. As opposed to K3, K12 was never observed in the conjunctival EC (Figs. 1E, 1F), confirming that K12 is a specific marker of the cornea.

Strong K13 expression was restricted to the conjunctival epithelium (all layers) (Figs. 1E, 1F) and the suprabasal layers of the limbal EC (Figs. 1E, 1G) only, but was never observed in the whole corneal EC (Figs. 1E, 1H, 1I), confirming that K13 is a specific marker of the conjunctiva. As the same, K19 expression was restricted to the conjunctival epithelium (all layers) (Fig. 1L) and the suprabasal layers of the limbal EC (Fig. 1M) only and was never observed in the central corneal EC (Fig. 1O). Nevertheless, as opposed to K13, some weak staining of K19 was observed in the basal cells of the peripheral corneal EC (Fig. 1N). Thus, K19 was considered as a specific marker of the conjunctiva compared with the central cornea. All the IgG isotype negative controls were negative.

The K12 and K13, thus qualified, were retained for the second stage. Because ICs were performed on the central

cornea, we selected K19 too as marker of conjunctival differentiation, considering the higher sensitivity of a double-staining K13/K19 in detecting conjunctival EC.

On the contrary, the lack of corneal specificity of K3 led to its abandonment.

The K12/K19 double labeling was successfully qualified on bulbar conjunctival and corneal tissue.

Labeling of Cells of Patients with a Clinical Diagnosis of LSCD

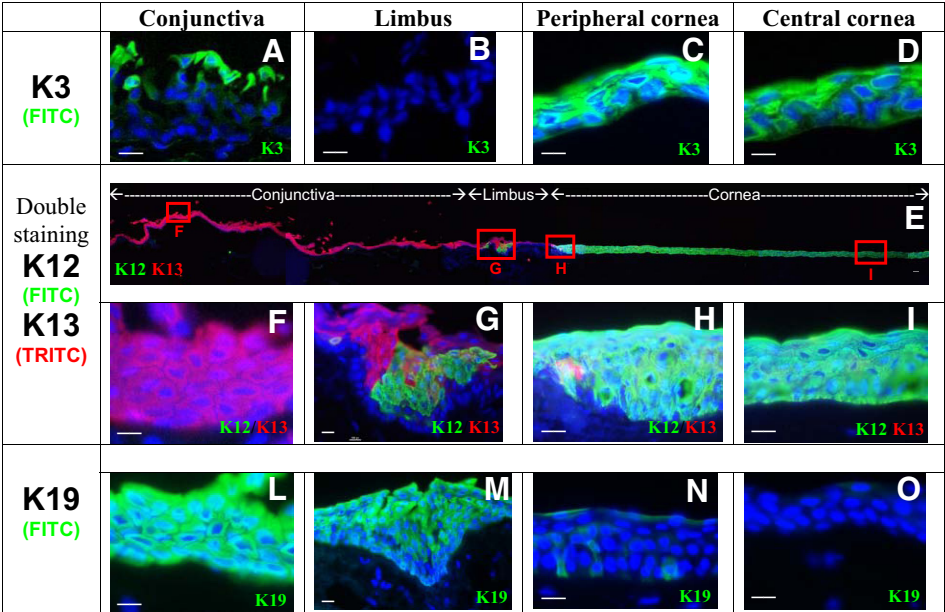
During the second stage, carrying out IC proved painless but uncomfortable for patients with LSCD. No side effects were found linked to sampling. It was easy to identify the areas of maximum conjunctivalization by the naked eye.

In patient 5, the sampling had been made difficult by the presence of a symblepharon, limiting the palpebral fissure (Fig. 2). This sample was not analyzable because of a lack of cellularity on the second printed blade (Fig. 2).

With the exception of patient 5, all samples included a large number of cells, respecting the architecture of the epithelial tissue and intercellular junctions. The second slide impressed often contained fewer cells than the first but still in sufficient numbers to allow its interpretation.

Finally, on slides impressed too strongly, the cell cytoplasm appeared disorganized and the cell nuclei were poorly individualized.

FIGURE 1. Expression of epithelial cell markers in corneoscleral buttons of healthy deceased donors comprising conjunctiva, limbus, and cornea. The expression pattern of keratin 3 (K3) (green, A–D), keratin 12 (K12) (green, E–I), keratin 13 (K13) (red, E–I), and keratin 19 (K19) (green, L–O) were analyzed. K13 is specific of conjunctival differentiation because it is specifically located in the limbus (E, G) and conjunctival epithelia (E, F). The same pattern is found for K19 (L, M, O), although some weak staining is observed in the basal layers of the peripheral cornea (N). K12 is specific of corneal differentiation because it is specifically localized in the limbus (E, G) and cornea (E, H, I). K3, present in both corneal (C, D) and conjunctival (A) epithelia, is not a specific marker. Scale bar: 100 μ m (A–D, F–O) and 50 μ m (E).



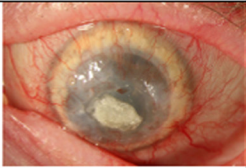
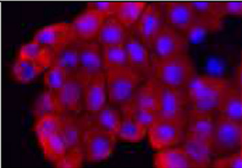
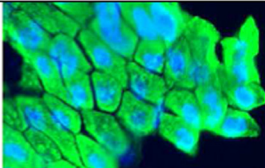
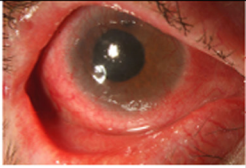
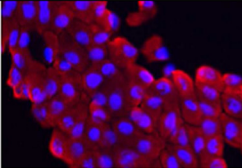
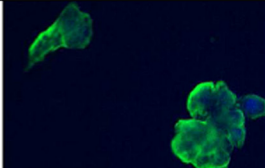
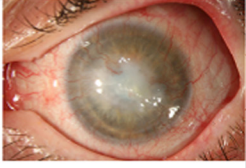
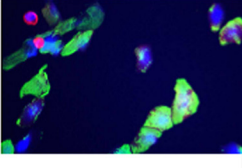
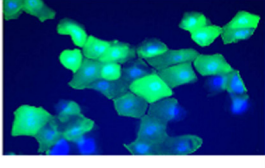
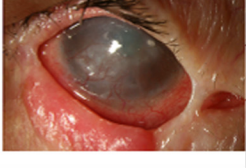

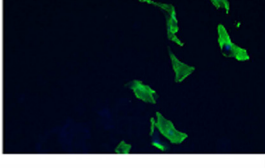
Patient	Biomicroscopic characteristics	Corneal IC Double labeling technique for K12 (green) / K19 (red)	Corneal IC Simple labeling technique For K13 (green)
Nº1			
Nº4			
Nº3			
Nº5			

FIGURE 2. Biomicroscopic characteristics and corneal impression cytology of four of the stage 2 patients. Patient 1: there is a strong expression of conjunctival differentiation markers (K13, K19), whereas K12, a marker of corneal differentiation, is absent. Patient 4: These same characteristics are found at a clinically mild LSCD, demonstrating the sensitivity of the diagnostic test. Patient 3: the coexistence of corneal and conjunctival markers indicates a weak form of the disease. Patient 5: the existence of a symblepharon limiting the opening of the palpebral fissure made cell collection impossible.

For 8 of 10 patients (patients 1, 2, 4, 6, 7, 8, 9, and 10), collected cells strongly expressed K13 and K19 but were negative for K12, confirming the presence of a conjunctival epithelium (Fig. 2). It should be noted that this conjunctival phenotype was found in patient 4, who had a mild form of the disease (Fig. 2). Patient 3 presented a mixed phenotype, with K13, K19, and K12 expression on almost 100%, 20%, and 80% of cells, respectively (Fig. 2).

DISCUSSION

The first step in this study showed that the cytological impression technique is very suitable for collecting conjunctival epithelial cells. It can collect many cells while preserving intercellular junctions. In contrast, in healthy subjects, it retrieves few corneal epithelium cells, the pauci-desquamative character of which partly explains the difficulty of collecting them. A rich cytologic impression is therefore a characteristic specific to conjunctival epithelium and the high retrieval rate of cells by corneal IC represents in itself a criterion of conjunctival differentiation and thus a diagnosis of LSCD.

Among the markers that we selected in accordance with current data in the literature, K12 has been shown to be an excellent marker for corneal differentiation, and K13 an excellent new marker for conjunctival differentiation, as recently confirmed by the work of Ramirez-Miranda et al.²⁶ Moreover, K13 is a more reliable marker for distinguishing between the corneal and conjunctival epithelia compared with K19. Indeed, we should carefully consider the conjunctival specificity of K19 due to its staining in the basal cells of the peripheral corneal EC, as reported too in several recent studies.^{14,17,26–28}

Moreover, K3, the most widely used marker, has not been shown to be specific to the corneal epithelium.

One hypothesis for this K3 nonspecificity is that, in this study, we sampled from the bulbar conjunctiva, far from the cornea to avoid contamination of our conjunctival impressions by corneal cells, whereas the bibliographic data indicating the specificity of K3 tend to focus on the study of the near-limbus conjunctiva.²⁹ It has, in fact, been reported that K3 was expressed by noncorneal tissues, including bulbar conjunctiva,^{11,14,30} nose, and oral mucosa.³¹ As shown in Figure 1, strong K3 expression was observed in the suprabasal layers of the conjunctival EC (Fig. 1A) but absent of the limbus (Fig. 1B).

A second hypothesis on the lack of K3 specificity is that the expression of our antibody AE5 in the conjunctiva is due to the detection of K76. Indeed, today, the specificity of the AE5 clone that we used is in question, because the latter would recognize the K3 and K76 isoforms. K76 is a keratin that is specific to suprabasal cells of the masticatory epithelium (gum and palate),³¹ and its presence in conjunctival cells still remains to be proven.

The second step in this study showed that IC produces strong returns for LSCD with 90% of samples suitable for analysis. Unfavorable anatomic conditions, however, are a contraindication to sampling.

This diagnostic test has proved very sensitive, with 8 positive samples of 9 tested here, and capable of diagnosing LSCD at a mild form of the disease (Fig. 2, patient 4). The negative predictive value of the test was confirmed on healthy histologic tissue.

In the case of patient 3, we identified a mixed corneal and conjunctival epithelium (K12, K13, and K19+). This patient had a herpetic keratitis with major corneal opacification and advanced corneal neovascularization, which are not specific of LSCD but can result from the viral infection. After reading the diagnostic test, we questioned our clinical diagnosis of severe

LSCD and accepted, despite biomicroscopic findings, the possibility of a mild form of the disease. Thus, K12 could be used to quantify the severity of the disease, its detection defining mild LSCD. However, we must accept that significant sampling error does exist in the frequency of K12+ cells sampled, as shown in healthy subjects, where few corneal epithelial cells were retrieved by IC. Perhaps the quantification of K13 and/or K19+ cells could be useful as a measure to determine the degree of LSCD. More mild LSCD should be sampled to answer this question.

Finally, K7 has shown to be another new specific marker of conjunctival differentiation.¹⁷ The immunocytochemical search for both K7 and K13 by corneal IC could be more efficient than the K13/K19 couple in diagnosing LSCD.

CONCLUSION

We have described the use of impression cytology of the corneal surface, coupled to the detection of K12-K13-K19 keratins, for diagnosing limbal stem cell deficiency.

This study revealed that IC is a cell collection technique that is simple, rapid, reproducible, painless, and atraumatic. K13 is a new marker of conjunctival differentiation. The detection of both keratins K19 and K13 by corneal impression cytology gives a highly sensitive diagnosis for LSCD. The search for keratin K12 could quantify the severity of the disease, distinguishing between mild (positive K12) and severe (negative K12) forms. The choice of these three markers overcomes the variability in the density of goblet cells on the one hand, and the lack of specificity of corneal K3 on the other, while maintaining a high positive predictive value of the test result through the use of two conjunctival differentiation markers.

The restoration of the corneal epithelium is a prerequisite for a successful corneal transplant in LSCD patients, and only this will allow the return of transparency to the eye surface.

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