Graefes Arch Clin Exp Ophthalmol (2011) 249:701–708
DOI 10.1007/s00417-011-1616-x

CORNEA

Assessment of limbus and central cornea in patients with keratolimbal allograft transplantation using in vivo laser scanning confocal microscopy: an observational study

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Received: 8 November 2010 / Revised: 30 December 2010 / Accepted: 31 December 2010 / Published online: 26 January 2011

Abstract
Background Keratolimbal allograft (KLAL) transplantation has been proved to be a useful surgical procedure for limbal stem cell deficiency patients. However, information about in vivo ocular surface changes in those patients is limited, due to the lack of a reliable and non-invasive technique for closely monitoring the changes of KLAL grafts. The aim of this study is to characterize the cellular changes in the limbus and central cornea after KLAL in patients with severe ocular chemical injury, using in vivo laser scanning confocal microscopy (LSCM).
Methods This is a prospective, noncomparative, observational case series. Twenty-three patients (23 eyes) with total limbal stem cell deficiency due to ocular chemical injury were recruited. KLAL with or without other concurrent surgery were performed. LSCM and slit-lamp examination were performed on the limbus and the central cornea before surgery and at 6 and 12 months postoperatively. Presence of palisades of Vogt, limbal basal epithelial cell density within the palisades of Vogt (LEC), limbal dendritic cells (DC) density, and central corneal basal epithelial cell (CEC) density were assessed by LSCM.
Results All patients completed 12 months of follow-up. Twenty-one patients were male and two were female, with a mean age of 39.5±12.5 years. Six cases were due to acid burns, and the others were alkali burns. Palisades of Vogt were observed in all surviving grafts but were absent in graft failure. The epithelial cells in the central cornea of the failed graft had lost the classic polygonal morphology of the normal corneal basal epithelial cells. The cell density of LEC and CEC decreased significantly, whereas DC density increased in the failed grafts over time.
Conclusions In vivo LSCM is a useful tool for monitoring the cellular changes in KLAL grafts, and has the potential to diagnose the failure of KLAL grafts at the cellular level.

Keywords Keratolimbal allograft · Confocal microscopy · Limbus · Cornea

Jiaxu Hong and Tianyu Zheng are the co-first authors.

Financial Disclosure The authors declare that no financial relationship exists with the organization that sponsored the research.

Conflict of Interest The authors declare no conflict of interest.

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Introduction

Limbal stem cells (LSCs) play a crucial role in maintaining a stable and transparent ocular surface. LSCs and their

progenies, the transient amplifying cells, are believed to locate at the basal layer of the limbal epithelium. They play an indispensable role in the self-renewal and wound repair of corneal epithelium [1, 2]. Severe chemical injury, thermal burn and Stevens–Johnson syndrome can lead to limbal stem cell deficiency (LSCD). These patients initially develop irregularity of the corneal epithelium, leading to decrease of vision. When the disease becomes more severe, they suffer from recurrent corneal epithelial erosions, and subsequent invasion of conjunctival epithelial cells onto the cornea leads to conjunctivalization and neovascularization of the cornea. Eventually, these patients become functionally blind from an opaque cornea.

In the past, patients with total LSCD had poor prognosis because treatment options were limited to penetrating keratoplasty, tarsorrhaphy and use of artificial tears [3]. These treatments do not correct the etiology of the disease, hence the outcome is disappointing. Using keratolimbal allograft (KLAL) transplantation to restore the LSCs population for LSCD has become a standard treatment when ex vivo expansion of autologous LSCs is not achievable [4–6].

Despite the wide clinical application of KLAL, information about In vivo ocular surface changes in KLAL patients was still limited due to the lack of a reliable and non-invasive technique for closely monitoring KLAL grafts. Impression cytology is traditionally used to assess the presence of goblet cells on the cornea, which is a diagnostic tool for LSCD [7, 8]. However, this method requests harvesting cells from the ocular surface, which restricts its repetitive application because of the invasive nature of the test and the potential injury to the patients [9]. In vivo laser scanning confocal microscopy (LSCM), which is a noninvasive tool to provide a real-time and three-dimensional structure of the ocular surface, has recently become a new method for diagnosing and monitoring ocular surface diseases [10–12]. Investigation of human corneoscleral limbus using LSCM has successfully elucidated the microstructures and age-related changes [13].

We previously reported that in vivo LSCM could effectively detect the changes on the ocular surface of severe chemical burn patients by the quantitative analysis of the limbus–palisade basal epithelial cells (LEC), dendritic cells (DC), and central corneal epithelial cells (CEC) [14, 15]. We found that there is much difference in the cellular morphology of the ocular surface in patients with severe chemical burns among diverse courses of the disease. Based on these findings, we believe that LSCM is a valuable aid in studying the ocular surface of patients with KLAL transplantation in vivo. Thus, the purpose of this study was to investigate the alterations in the morphology and quantitative parameters of the limbus and the central cornea after KLAL transplantation with different prognosis at 6 and 12 months postoperatively.

Methods

Subjects

Patients were enrolled from the Shanghai Ocular Chemical Injury Study [16]. Informed consent, according to the tenets of the Declaration of Helsinki, was obtained from all adults and parents of minors after explanation of the nature, risk and benefit of the study. This study was approved by the Ethics Committee of Shanghai Eye, Ear, Nose and Throat Hospital. Twenty-three patients (23 eyes) with LSCD caused by chemical burns between October 2007 and February 2009 were included. All patients presented with severe bilateral ocular burns or unilateral disease (grade III or higher according to the Roper–Hall classification), but preferred not to undergo surgery on the fellow eye [17].

LSCD was defined where there was presence of diffuse late fluorescein staining, conjunctivalization and vascularization of the cornea. In addition to slit-lamp examination, LSCD was confirmed by impression cytology in all cases, to detect the presence of conjunctival goblet cells on the corneal surface [18].

The surviving graft was defined by the maintaining of a smooth and clear corneal epithelial surface without fluorescence staining, while failed KLAL graft was defined when the corneal epithelium exhibited the similar presentation as LSCD defined above. In addition, failure of KLAL was also diagnosed when irreversible rejection occurred.

Operative procedure

Different ocular surface reconstructive methods were used, depending on the severity of ocular surface injury. KLAL alone was performed in 17 patients with corneal epithelium neovascularization. KLAL combined with penetrating keratoplasty was performed in four patients who had significant corneal opacity, and KLAL with deep lamellar keratoplasty in two patients who had diffuse stromal opacity but had normal Descemet's membrane and endothelium.

Postoperative management

After surgery, topical corticosteroid (0.1% dexamethasone) was used six times a day, and oral dexamethasone was administrated at a dose of 8 mg/d for 2 weeks. Topical

cyclosporin A (CsA) (0.05%) was applied four times a day. Oral CsA was used at 3 mg/kg intravenously for 1 week, and maintained its blood trough level at 50–150 ng/ml for at least 4 months. Blood pressure and serum creatinine were also monitored for side-effects. Preservative-free hyaluronic acid eye drops and 0.3% tobramycin eye drops were administrated three times daily.

When immunologic rejection developed, intensive immunosuppressant consisting of topical and systemic corticosteroids and CsA was repeated, using the same protocol as described above.

Examinations

Slit-lamp examination and LSCM were performed on all patients preoperatively and postoperatively at months 6 and 12 using the Heidelberg Retina Tomograph III / Rostock Cornea Module (Heidelberg Engineering GmbH, Dossenheim, Germany). It uses a 60× water-immersion objective lens (Olympus Europa GmbH, Hamburg, Germany) and a 670-nm diode laser as a light source, allowing a scanning area of 384×384 μm^2 with lateral and vertical resolutions of 1 μm and a magnification up to 800 times. Before examination, one drop of 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical, Japan) and Vidisic gel (0.2% Carbomer 940; Bausch & Lomb, Germany) were applied to the lower conjunctival sac. The patient's head was positioned in the head rest, and a fixation tool was used to position the eye. Images of the central cornea and limbal graft were recorded at one point along the *z*-axis as single scan or in the volume mode [13].

For each patient, the basal layer of the corneal and limbal epithelial cells was identified from all images. Three clear images for each parameter without motion blur or compression lines from limbus and central cornea were analyzed by a masked observer (JH). The cell density was obtained by using Cell Count® Software (Heidelberg Engineering GmbH) in manual mode, as described in previous reports [11–13]. The average of cell densities of LEC, DC and CCE from all three images for each patient were compared between different time points, and between patients with different outcomes.

Statistical analysis

Statistical analysis was performed using statistical software, SPSS for Windows, version 16.0 (SPSS, Inc., Chicago, IL, USA). The chi-square test was performed to detect the difference of graft survival rate at 6 and 12 months after surgery. The Mann–Whitney test or Student's *t*-test was used to compare the mean densities of LEC, DC, and CEC. A P value<0.05 was considered statistically significant.

Results

Clinical outcome

A total of 23 patients were recruited. Patients' clinical data and graft outcome are summarized in Table 1. Twenty-one patients were male and two were female. The mean age was 39.5±12.5 years, ranging from 15 to 63 years. Six cases were due to acid burns, and the others were alkali burns. Of these 23 KLAL procedures, the survival rate of limbal graft was 90.0% (20/23) at 6 months and 60.9% (14/23) at 12 months (Table 2). A significant decline of the survival graft rate was noted (χ^2=4.059, P=0.044) between these two time points.

LSCM investigations on base line

Before the KLAL, palisades of Vogt were absent under LSCM in all subjects. The epithelial–stromal boundary was flattened and neovascularization was observed. As shown in Fig. 1, the epithelial cells at the central cornea displayed as conjunctival epithelial morphology. In the corneal stroma, irregular fibrous tissue was observed and a hyperreflective background was noted.

LSCM investigations in KLAL follow-up

As shown in Fig. 2, in all surviving grafts, the palisades of Vogt extended into the limbal epithelium in a regular ridge-like arrangement. The limbus–palisade epithelium had a normal morphology with distinct cellular borderline. Infiltration of dendritic cells was observed at a low level. As expected, the central corneal epithelium had a polygonal shape and was regularly arranged. In contrast, the palisades of Vogt were poorly defined or absent in the failed grafts. The limbal epithelial cells were enlarged, with irregular arrangement. Within the limbal epithelium, a large number of dendritic cells were detected. Poor epithelization of the central cornea was found in most patients with graft failure, which was consistent with slit-lamp investigation. Corneal stromal cells in failed graft were often difficult to identify due to the hyperreflective fibrotic background.

The average cellular densities of LEC, DC, and CCE in the surviving and failed KLAL grafts are summarized in Table 2. There was a significant difference in these parameters between survival and failed grafts, no matter whether at 6 or 12 months postoperatively.

According to different outcomes, patients were divided into three groups: group 1 (14 eyes) consisted of grafts that

Table 1 Patient history and graft outcome

Patient	Age (years)	Gender	Eye	Preoperative VA	Time from Injury to KLAL (years)	Indication	Prior surgery	Combined surgery with KLAL	KLAL Outcome	
									6 months (VA)	12 months (VA)
1	24	Male	Left	HM	1.5	PED, VI	AMT	–	Survived (6/15)	Survived (6/15)
2	36	Male	Left	HM	12	PED, VI	–	PKP	Survived (6/30)	Survived (6/60)
3	42	Male	Right	LP	2	PED, VI	AMT, ECCE, Ahmed Valve	–	Failed (HM)	Failed (LP)
4	17	Female	Left	HM	14	PED, VI	–	PKP	Survived (6/15)	Failed (HM)
5	20	Male	Right	6/60	2	VI	AMT	–	Survived (6/24)	Survived (6/24)
6	41	Female	Left	CF	2	VI	–	–	Survived (6/12)	Survived (6/15)
7	15	Male	Left	CF	6	VI	–	–	Survived (6/30)	Failed (CF)
8	33	Male	Right	CF	13	VI	–	PKP	Survived (6/15)	Survived (6/18)
9	38	Male	Left	HM	2	VI	AMT	–	Survived (6/30)	Survived (6/60)
10	40	Male	Left	LP	3	VI	–	–	Survived (HM)	Survived (HM)
11	45	Male	Right	CF	20	VI	KLAL×2	LKP	Failed (HM)	Failed (HM)
12	26	Male	Left	CF	1.5	PED, VI	AMT×4	–	Survived (6/60)	Failed (HM)
13	63	Male	Left	HM	0.4	PED, VI	AMT	–	Survived (6/60)	Survived (6/90)
14	58	Male	Right	HM	2	PED	KLAL×2	LKP	Failed (LP)	Failed (LP)
15	63	Male	Right	6/15	1	PED	AMT	–	Survived (6/9)	Survived (6/9)
16	37	Male	Left	CF	1	PED	LKP	PKP	Survived (6/30)	Failed (HM)
17	40	Male	Left	6/60	1	PED, VI	AMT×2	–	Survived (6/15)	Survived (6/15)
18	61	Male	Left	HM	0.6	PED, VI	AMT	–	Survived (6/60)	Failed (HM)
19	50	Male	Right	HM	2	PED, VI	AMT×2	–	Survived (6/30)	Survived (6/60)
20	43	Male	Left	HM	1	VI	AMT×3	–	Survived (6/15)	Survived (6/36)
21	22	Male	Right	HM	0.6	VI	AMT×2	–	Survived (6/60)	Survived (6/60)
22	34	Male	Left	CF	1	VI	AMT	–	Survived (6//15)	Failed (6/30)
23	39	Male	Right	HM	3	VI	AMT	–	Survived (6/30)	Survived (6/60)

Abbreviations: VA = best-corrected visual acuity; PED = persistent epithelial defect; VI = visual impairment; AMT = amniotic membrane transplant; ECCE = extracapsular cataract extraction; PKP = penetrating keratoplasty; LKP = lamellar keratoplasty; CF = counting fingers at 1 meter; HM = hand movement at 1 meter; LP = light perception

Table 2 Differences in the parameters of surviving vs failed keratolimbal allograft over time

Group	Parameter[a]	Surviving KLAL graft	Failed KLAL graft	*P* value**
Postoperative month 6	Cases*	20	3	
	LEC	5994±201 cells/mm^2	3611±728 cells/mm^2	0.006
	DC	123±28 cells/mm^2	701±157 cells/mm^2	0.005
	CEC	5900±272 cells/mm^2	3202±313 cells/mm^2	0.011
Postoperative month 12	Cases*	14	9	
	LEC	5915±965 cells/mm^2	3297±136 cells/mm^2	<0.001
	DC	120±114 cells/mm^2	750±45 cells/mm^2	<0.001
	CEC	6010±1239 cells/mm^2	1681±329 cells/mm^2	<0.001

*Survival graft rate was statistically significant between 6 months and 12 months postoperatively using two-tailed chi-square tests (χ^2 = 4.059, P=0.044).

**Student's *t*-test was used to evaluate the difference in cellular densities between surviving and failed grafts.

[a] LEC = limbal suprabasal epithelial cells; DC = dendritic cells; CEC = central corneal basal epithelial cells

survived at both postoperative month 6 and month 12, group 2 (six eyes) consisted of grafts that survived at postoperative month 6 but failed at month 12, and group 3 (three eyes) were grafts that failed at both time points. Interestingly, the average density of DC significantly increased with time only in group 2. In contrast, the average densities of LEC and CCE decreased with time in group 2 (Table 3). However, there was no significant difference between these cell densities with time in group 1 and group 3.

Discussion

To the best of our knowledge, the present study is the first to characterize the microstructural changes in the limbus and central cornea after KLAL in patients with severe ocular chemical injury using LSCM. Investigation of the limbal graft in LSCD patients after KLAL using magnified photographic prints in the past was limited by the poor image quality due to low resolution. Some researchers have attempted to circumvent this limitation by examining the histopathology of patients' keratolimbal tissue obtained at the time of repeat surgery [19]. However, this only provides information of the transplants that have failed. Histopathology study cannot be obtained without biopsy, which is rarely performed due to the invasive nature of the procedure. With the improvement of the instrumentation, the new generation of LSCM is proven to be a very useful tool to study the in vivo microstructure at an acceptable resolution.

It has been proposed that the LSCs reside at the limbus, which also provides the niche to maintain the survival of LSCs. Palisades of Vogt might be vital in maintaining the normal state and function of LSC [20–22]. The morphology of palisades of Vogt in survived grafts were similar to that in normal humans observed by a previous study [12], with a wavy epithelium–stromal boundary or alternating epithelium–stromal cords, hyperreflective basal epithelial cells, and

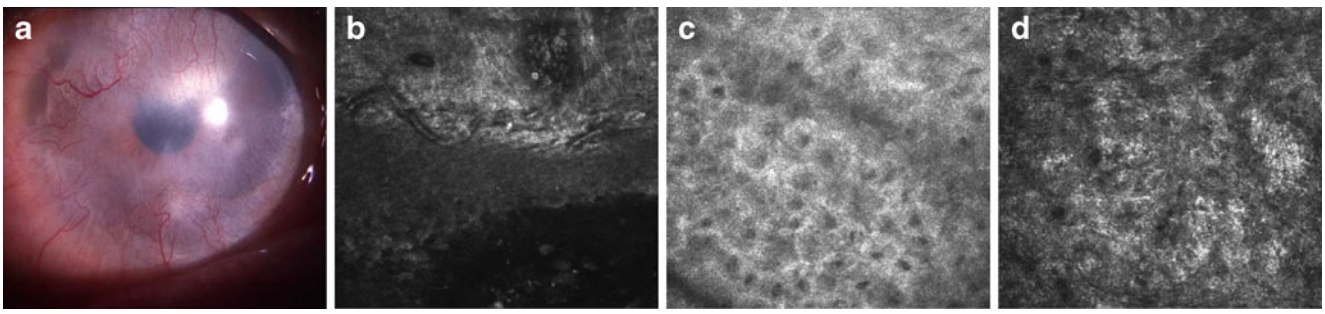

Fig. 1 Slit-lamp and confocal microscopic imaging of the limbus in patients with total limbal stem cell deficiency before keatolimbal allograft transplantation (Patient #8). **a** Slit-lamp examamination showed opacified corneal stroma, conjunctivalization and neovascularization of the corneal surface. **b** Confocal microscopic image of the limbus showed absence of palisades of Vogt, flat epithelium–stroma boundary, and presence of vessels in the stroma. **c** Confocal microscopic image of the central cornea showed polygonal cells with small nucleus that were consistent of conjunctival epithelium. **d** Confocal image of the central corneal stroma showed irregular collagen fibers and a relatively hyper-reflective background

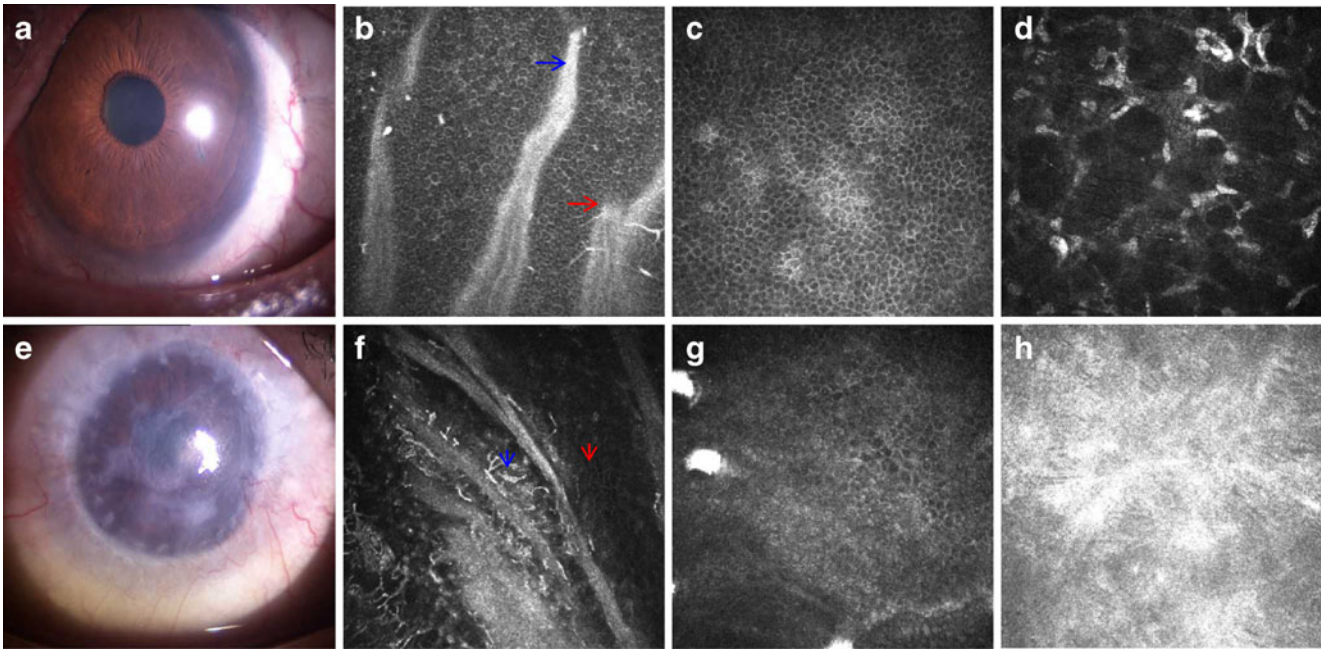

Fig. 2 Comparison of ocular surface in the surviving (Patient #10, **a**–**d**) and failed keratolimbal allograft (Patient # 4, **e**–**h**). **a** Slit-lamp photo showed a transparent cornea with a few vessels at the limbus. **b**–**d** Confocal images of the ocular surface in surviving KLAL graft. Palisades of Vogt (*blue arrow*) extend into the limbal epithelium in a regular arrangement, with slender vessels inside. A few dendritic cells (*red arrow*) were detected. The morphology of the limbalepithelial cells was normal (**b**). Central corneal epithelial cells displayed polygonal shape and are regular arranged (**c**). The morphology of central corneal stromal cells was normal (**d**). **e** Slit-lamp photo of failed KLAL graft showing an opacified cornea with neovascualrization. **f**–**h** Confocal images of ocular surface in a failed KLAL graft. **f** Palisades of Vogt were destroyed. The stromal process was thin and irregular. There was a large number of dendritic cells infiltration (*blue arrow*). The epithelial cells between stromal processes had relatively large cell bodies (*red arrow*) and irregular shape. **g** Central corneal epithelial cells were larger. **h** The central corneal stroma was hyperreflective

a slender blood vessel along each stromal papilla. These findings offer further evidence that the presence of palisades of Vogt in the limbus coincides with presence of epithelial stem/progenitor cells.

There were some conflicting results concerning the fate of transplanted limbal epithelial stem cells in patients receiving KLAL. Using primers that can detect variable nucleotide tandem repeats, Henderson et al. compared the genotype of surface epithelial cells from KLAL recipients with those of the donor eyes. Donor cells were not detected in any of the recipients 3 years after KLAL [23]. Others were able to detect donor cells in a majority of the recipients up to 3.5 years after transplant [24, 25]. Although LSCM could not be used to define the origin of epithelium, its ability to characterize the cellular structures and track their changes in vivo after transplantation would greatly enhance our understanding of the pathophysiology of these grafts.

Table 3 Changes in cell densities over time

Parameter[a]	Postoperative month 6	Postoperative month 12	*P* value[†]
Group 1[†] (survived at both postoperative month 6 and 12)			
LEC	5994±897 cells/mm^2	5915±965 cells/mm^2	0.809
DC	123±126 cells/mm^2	120±114 cells/mm^2	0.931
CEC	5900±1218 cells/mm^2	6010±1239 cells/mm^2	0.799
Group 2[†] (survived at postoperative month 6 but failed at month 12)			
LEC	6177±756 cells/mm^2	3383±399 cells/mm^2	<0.001
DC	139±118 cells/mm^2	774±129 cells/mm^2	<0.001
CEC	6312±1281cells/mm^2	3824±2191 cells/mm^2	0.037
Group 3[‡] (failed at both postoperative month 6 and month 12)			
LEC	3611±728 cells/mm^2	3297±407 cells/mm^2	0.358
DC	701±157 cells/mm^2	750±134 cells/mm^2	0.609
CEC	3202±313 cells/mm^2	2760±2417 cells/mm^2	0.766

[a] LEC = limbal basal epithelial cells; DC = dendritic cells; CEC = central corneal basal epithelial cells

Student's *t*-test (†) or Mann–Whitney test (‡) was used to evaluate the cellular densities

We found that the cell density of LEC and CCE was significantly decreased in failed grafts compared to surviving grafts. Moreover, for grafts that survived at postoperative month 6 but failed at month 12, the density of LEC and CCE decreased with time. And for grafts that survived or failed at both time points, the parameters showed no time-related difference. Thus, the density of LEC and CCE might be used as diagnostic and prognostic parameters after KLAL transplantation.

Furthermore, in vivo LSCM allowed identification and quantification of DC in KLAL patients. Dendritic cells are professional antigen-presenting cells (APC), [26] and they are found mostly in the normal peripheral corneal epithelium and anterior stroma [27]. They might play an important role in corneal graft rejection. Similarly, several studies have reported that persistent inflammation was a major risk factor for developing LSCD in humans [28] and failure of autologous limbal conjunctival transplantation in rabbits [29]. Rosenberg et al. found that dendritic cells, presumably Langerhans cells, were frequently seen at the level of the basal epithelium in corneas with a history of herpes simplex virus keratitis [30]. Recent evidence demonstrates that LC may be identical to immature DC in the cornea or limbus {Hamrah, 2003 #45}. Then, Zhivov et al. found that contact lens wearers revealed almost two-fold higher LC densities in the center and the periphery of the cornea, implying chronic mechanical irritation of the cornea in response to the contact lens as foreign body [31, 32]. In our study, dendritic cells were easily identified in patients (Fig. 2). The DC density remains low in the surviving grafts over time but increased significantly in the failed grafts. This observation suggests that DC density correlates with inflammation and could be used as a more sensitive measure to detect subclinical rejection.

There are several limitations in our study. Because of the high magnification of LSCM, the observed area in the LSCM images is smaller compared with that from histological analysis. To reduce this sampling error, we included three independent confocal scans in our analysis. The survival of the donor tissue was not confirmed by human leukocyte antigen, and the determination of KLAL graft survival was based on clinical grounds only. Nevertheless, the microstructural changes are consistent with the clinical presentation.

In conclusion, our study documents for the first time the cellular changes of the limbus and central cornea in KLAL grafts using in vivo confocal microscopy. The present study suggests that in vivo LSCM is a useful tool to detect pathological changes after keatolimbal transplantation. Furthermore, we proposed the cell density of LEC, CCE and DC as potential diagnostic parameters for KLAL transplantation in the future.

Acknowledgment This work was supported by grants from Scientific Research Program, Ministry of Health, China; National Natural Science Foundation of China (30872815); Excellent Leading Scholars Program, Science and Technology Commission of Shanghai Municipality (10XD1401100); Outstanding Doctoral Research Program of Fudan University.

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