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Corneal Epithelial Stem Cells Repopulate the Donor Area within 1 Year from Limbus Removal for Limbal Autograft

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Purpose: To determine whether limbal epithelial stem cells (LESCs) repopulate the site harvested for limbal autograft transplantation (LAT), the expression of LESCs markers was evaluated in bioptic specimens obtained from the donor area 12 months or more after surgery.

Design: Interventional case series.

Participants: Patients who underwent LAT for unilateral acquired limbal stem cell deficiency after chemical burn.

Methods: Corneal limbal explants were obtained from 2 sites, the harvested area and the untouched control area, in the donor eyes of 6 patients who previously underwent LAT for unilateral acquired limbal stem cell deficiency after chemical burn. Limbal epithelial stem cells were isolated, and cellular, immunohistochemistry, and histologic parameters were assessed to compare differences between LESCs isolated from harvested or control sites.

Main Outcome Measures: Presence of LESCs 1 year or more after LAT.

Results: Specific markers (p63, Ki67, K12), percentage of LESCs, cell doubling, and number of passages in culture did not differ significantly between harvested and control sites. However, the distinctive structure of the palisades of Vogt was found only in 2 of 6 harvested sites.

Conclusions: Limbal epithelial stem cells repopulate the donor site as early as 1 year after limbus removal for LAT. Autologous transplantation of conjunctiva and limbus are safe procedures and can be performed in cases that cannot be treated by simple grafting of LESCs cultured ex vivo. *Ophthalmology 2016;■:1−8 © 2016 by the American Academy of Ophthalmology*

Limbal autograft transplantation (LAT) is a well-known procedure used by a number of surgeons to treat unilateral limbal epithelial stem cell (LESC) deficiency.[1−8] Unlike ex vivo LESC expansion,[9,10] this method is ideal for treating severe chemical burns requiring a combined conjunctival−limbal transplantation and for restoring the normal anatomic features of the ocular surface. Comparisons have been made between the ex vivo expansion of cells on special scaffolds or amniotic membrane later transplanted onto the ocular surface and the transplantation of the entire tissue, namely limbal or limboconjunctival explants.[2,11] Because only a small biopsy specimen (0.5−1 mm^3) is harvested for cell isolation, transplantation of ex vivo expanded LESCs is considered a minimally invasive technique, and thus unlikely to cause any significant harm to the healthy fellow eye. Instead, despite the effectiveness of the procedure, concerns have been raised about the possible iatrogenic damage caused by LAT to the healthy donor eye, especially when a large portion of tissue has to be transplanted. However, to date, the effect of LAT on the donor eye has not been investigated. In this study, quantification of LESCs, according to predefined standards,[9,12] was used as an indicator of the regenerative ability of the cornea to evaluate the limbal stem cell existence and functionality at the donor sites of patients who underwent LAT for unilateral secondary LESC deficiency.

Methods

We reviewed the charts of all patients with acquired unilateral stem-cell deficiency who had undergone LAT at our institution between January 2005 and December 2014. All eyes had been operated on by the same surgeon (M.B.) at "Villa Igea-Villa Serena" Private Hospitals in Forlì, Italy, using the technique previously published.[4] In December 2014, all patients were asked to enter a study aimed at assessing the presence of LESCs in the harvested site and in a control site of the donor eye. The study followed the tenets of the 1964 Declaration of Helsinki and was approved by the local ethics committee (Comitato Etico Ospedali Privati Forlì); a detailed informed consent form was signed by all patients who agreed to enter the study.

Two specimens, 1 mm^2 each in surface area, were obtained from 2 sites at the limboscleral junction of 6 eyes that had served as donor sites for LAT in 6 patients with unilateral acquired stem-cell deficiency after chemical burn (average time after LAT, 3.5±2.07 years; range, 1−7 years). One site was located at the center of the

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harvested area (harvested site), whereas the other site was chosen 180° apart (the control site; Fig 1). A 1-mm conjunctival incision was performed 1 mm posterior to the limbus. The conjunctiva then was separated from Tenon's capsule and the dissection was extended centrally with a crescent knife until clear cornea was encountered. Thus, the excised tissue specimen included the limbus. At the end of surgery, each specimen was split into 2 parts. Cells were extracted from the first part and serially propagated until they exhausted their capacity to proliferate (life span assay). Cryosections were obtained from the second half of each specimen and submitted for histologic and immunohistochemistry analysis. The proliferation capacity of cells extracted from the harvested sites was compared with that of cells obtained from the control sites. In addition, the molecular characteristics of the explants were assessed to rule out possible changes resulting from the collection of the conjunctival–limbal graft.

Cell Culture, Clonogenicity, and Cell Doubling

Limbal epithelial stem cells were isolated from fresh limbal biopsy specimens, cultivated as previously described,[10,13] and identified using 3 different parameters: cell size,[12] p63 positivity,[14] and high nucleus-to-cytoplasm size ratios.[15,16] Cell quantification was performed as described previously.[12] The percentage of stem cells was calculated as the number of stem cells divided by the total number of cells. For the tissue sections, fluorescence positivity was used to determine the distribution of fluorescence in the different layers of the biopsy.

The colony-forming efficiency assay was used to evaluate in vitro cell survival based on the ability of a single cell to grow into a colony. For the life span assay, cells were plated and propagated serially until they reached senescence. At each passage, the colony-forming efficiency assay was performed to obtain information about cell doublings and clonogenicity, as described previously.[17,18] Colonies were stained with crystal violet (1:100) and counted to distinguish between clonogenic or aborted ones, thus allowing us to assess the quality of the culture.[13]

Cell doublings and cumulative population doublings of each culture were calculated using the following equation: $3.322 \times \log^{10}(UCY/I)$, where UCY is the cell yield at that passage and I is the number of clonogenic cells found.[13,14]

Immunostaining

To characterize the cellular phenotype (conjunctival or corneal epithelium) present in each biopsy, staining of tissue sections and isolated cells was performed.[19] In addition, the expression of the transcription factor p63 was tested in the basal cells of the limbal epithelium and isolated cells of harvested and control sites.[14,18,20] To do so, as previously described,[9] sections and cells cytospun onto glass slides were analyzed using primary antibodies against the following antigens: actin (Santa Cruz, Milan, Italy), p63 (Dako, Milan, Italy), keratin 12 (Santa Cruz), mucin 1 (Santa Cruz), and Ki67 (Dako). Fluorescein isothiocyanate secondary antibodies (Santa Cruz) were used. Nuclei were stained with 4,′6-diamidino-2-phenylindole. To detect fluorescence, specimens were evaluated by means of an LSM-510 meta confocal laser microscope (Zeiss, Oberkochen, Germany).

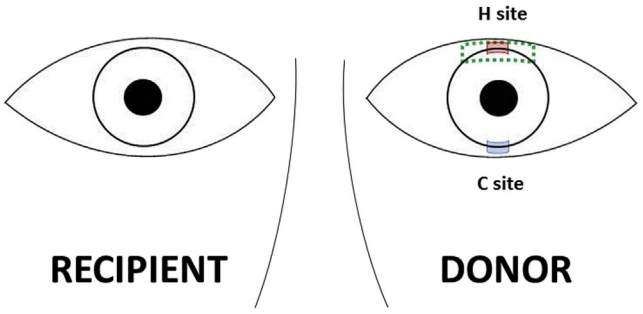

Figure 1. Schematic representation of the harvest (H) and control (C) sites evaluated in our study.

Data Analysis

Statistical analysis was performed using 2-tailed paired-sample t tests. The level of significance was set at $P < 0.05$ for all experiments.

Results

Clinical Outcomes

Eighteen patients underwent LAT at our institution during the study period. All donor eyes recovered uneventfully with no postoperative clinical signs of limbal stem-cell deficiency, and epithelial growth was completed within 4 weeks in all cases. All but 1 graft succeeded, with the only exception demonstrating total necrosis within 4 days from transplantation and subsequent secondary healing of the bare sclera.

In 8 patients, removal of the corneal pannus improved vision in the recipient eye to a level considered satisfactory by the patient (20/50 or better); in the other 10 patients, LAT was followed by keratoplasty (lamellar type, n = 8; penetrating type, n = 2). To prove the effectiveness of LAT, 2 excised donor buttons were submitted for immunohistochemistry: the specimens stained positive for human epithelial cytokeratin and negative for conjunctival cells marker mucin 1 in both cases. At the time of writing this article, the lamellar grafts were clear with vision of 20/40 or better in all patients but one. One of the full-thickness grafts had failed because of irreversible immunologic rejection occurring after bacterial keratitis, whereas in the other, vision of 20/50 was achieved.

Of the 6 eyes evaluated in the present study, 2 did not undergo any corneal procedure, whereas in 4, a lamellar keratoplasty was performed 6 to 18 months after LAT. Clinical recovery of the recipient eyes and of the donor sites was monitored with no major adverse reactions and events to be mentioned (Fig 2).

Histologic Assessment of Morphologic Features

Phenotypic characterization of the putative niche of stem cells showed that all epithelial layers as well as distinctive palisades of Vogt were represented normally in all biopsy samples from the control sites. No stromal or epithelial irregularities were noted. Limbal sections from patients 1 and 2 showed a similar normal structure also in the harvested sites. However, in the other 4 patients, the typical anatomic features of the palisades of Vogt were lost. In these specimens, the epithelium of the limbal area was twice as thick as that of the control site, with elongated epithelial cells. Irregularities in the stromal fibers also were observed, with larger gaps and detachment of the epithelium in few areas (Fig 3).

Quantification of Cells, Clonogenicity, and Cell Doubling

As shown in Figure 4A, B, no significant differences ($P = 0.74$) were observed in the number of passages in culture between cells extracted from harvested and control sites (5.5±1.22 vs.