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

Massimo Busin, MD,1,2 Claudia Breda,3 Marina Bertolin, BSc,3 Cristina Bovone, MD,1,2 Diego Ponzin, MD,3 Stefano Ferrari, PhD,3 Vanessa Barbaro, PhD,3 Hossein Mostafa Elbadawy, PhD3,4

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.

Limbal autograft transplantation (LAT) is a well-known procedure used by a number of surgeons to treat unilateral limbal epithelial stem cell (LESC) deficiency.1e8 Unlike ex vivo LESC expansion,9,10 this method is ideal for treating severe chemical burns requiring a combined conjunctivalelimbal 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 (0.5e1 mm3) 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 mm2 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, 1e7 years). One site was located at the center of the 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 conjunctivalelimbal 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 # log10 (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 4,0 6-diamidino-2-phenylindole. To detect fluorescence, specimens were evaluated by means of an LSM-510 meta confocal laser microscope (Zeiss, Oberkochen, Germany).

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 ¼ 8; penetrating type, n ¼ 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 ¼ 0.74) were observed in the number of passages in culture between cells extracted from harvested and control sites (5.5!1.22 vs. 5.33!1.21). Also, the cumulative population doubling of the cells did not differ significantly (P ¼ 0.46) between the harvested and control sites (Fig 4C). Cells extracted from harvested or control sites were both capable of proliferating with comparable clonogenic ability (Fig 4D). The total value of colony-forming efficiency (n ¼ 6) at passage 1 was 38.61!8.01 for cells from harvested sites and 37.09!21.79 for cells from control sites (P ¼ 0.86), decreasing gradually to less than 1 on passage 5 in specimens from both harvested and control sites (Fig 4E). Again, no significant differences were found between the harvested and control sites at all passages. The capacity of cells from harvested and control sites to form colonies was performed during serial cultivation and showed similar percentages of clonogenic and aborted cells and total colony number (Fig 4F). These values did not differ from those obtained from normal, nonoperated, healthy eyes (>3%).12,13,21

Immunostaining

The marker of conjunctival cells mucin 1 showed a minimal positivity in the outermost layer of the epithelium (Fig 5A), but there were no significant differences (P ¼ 0.93) between harvested and control sites in the percentage of cells expressing mucin 1 (Fig 5B, C). The expression of the corneal epithelial cell marker K12 was detected in the epithelium of all collected samples (Fig 5A), and more than 80% of the cells extracted from the biopsies expressed K12 (Fig 5B). No significant differences (P ¼ 0.45) in K12- positive cell population were seen between harvested and control sites (Fig 5D). The expression of the limbal stem-cell marker p63 was detected in the basal layer of all sections from both harvested and control sites (Fig 6A). Limbal epithelial stem cells also could be extracted from both harvested and control sites (Fig 6B), and the LESC populations that were isolated did not differ significantly (P ¼ 0.40) between harvested and control sites (Fig 6C). The expression of the cell cycle marker Ki67 occasionally was detected in the bottom layers of the limbal epithelium of harvested and control sites (Fig 6A) and in most of the cells extracted from the biopsies (Fig 6B). The quantification of Ki67 in cells obtained from biopsies of harvested and control sites indicated no significant differences (P ¼ 0.89) in the proliferative capacity of the 2 types of site (Fig 6D).

Discussion

Recognition of the need to replenish the stem-cell population in LESC deficiency has led to a number of therapeutic approaches. In 1977, Thoft6 described for the first time the restoration of corneal epithelium by transplanting 4 small conjunctival autografts retrieved from the fellow eye. Subsequently, after identification of the basal epithelial cells of the limbus as presumptive stem cells of the corneal epithelium, Kenyon and Tseng,11 as well as other authors, modified the autograft technique specifically to include limbal tissue.2,4,6,7 Despite its effectiveness, a limitation of LAT has been considered to be the size of the limbal donation, because most surgeons fear that grafts that are too large would harm the functional integrity of the limbus in the donor eye. Advances in stem-cell cultures for the treatment of epidermal burns and the identification of the niche of corneal epithelial stem cells in the limbus suggest that clinical application of ex vivo cultured corneal epithelial stem cells could become a suitable alternative therapy to

limbal grafting. In 1997, Pellegrini et al10 described the successful reconstruction of the corneal epithelium in 2 patients with LESC deficiency using autologous cultured LESCs. Many reports since have described the clinical application of LESCs for the treatment of LESC deficiency, and recently the first stem cell-based medicinal product has been granted conditional marketing authorization by the European Medicines Agency. However, in eyes with severe chemical burns causing symblepharon and destruction of normal conjunctival anatomic features, LESC transplantation cannot be sufficient, and LAT still represents the only surgical option. To date, the safety of LAT with regard to the possible iatrogenic damage caused to the healthy donor eye has not been investigated, specifically when a large amount of tissue needs be transplanted. The findings of our study contrast with the current and common opinion that LAT affects limbus integrity in the donor eye and that sizing may be crucial to avoid iatrogenic damage. One year or more after LAT, it was possible to isolate LESCs from both harvested and control sites of the donor eye. In addition, in both harvested and control sites, LESCs exhibited comparable biological characteristics. Cell doubling, the number of cell passages in vitro, clonogenicity, and expression of K12 and mucin 1 markers all were substantially identical in the cells obtained from harvested and control sites. In particular, clonogenicity of more than 3% as obtained from both harvested and control sites represents a value considered normal for nonoperated, healthy eyes. This indicates that the presence of LESCs in the harvested sites may result from true restoration of the entire LESC population, rather than from simple spreading of the residual LESCs from the surrounding untouched areas, which would reduce the density of the LESCs and therefore depress this parameter to less than 3%.

The recovery of normal LESC parameters in the harvested sites and in the donor eye as a whole as early as 1 year after LAT indicates the possibility of harvesting LESCs from the same site for a second time, thus sparing the untouched part of the donor eye from surgical trauma and eventual related consequences. However, this does not solve the problem of sizing the graft used for LAT. Grafts that are too large could reduce the number of residual LESCs in the donor eye to a number that is less than compatible with the recovery of normal parameters seen after LAT with the graft size used in this series. Therefore, rather than using large limbal grafts, it may be advisable to perform a second LAT several months after the initial one, harvesting twice from the same site to obtain a final graft double in size. However, because abnormal corneal epithelial healing was observed as late as 4 to 8 months after repeat insult to the donor eye of animal models,22e24 a second harvest of LESCs from the same site should be considered with caution and only in the absence of other alternatives. In the past, minor abnormalities after LAT (i.e., partial limbal conjunctivalization, subconjunctival hemorrhages, filamentary keratitis) were described in the donor eye by several authors,1,25e30 although true limbal stem-cell deficiency was never proven. In our series, the presence of LESCs in the harvested sites was accompanied by palisades of Vogt in only 2 of 6 specimens; these eyes had the second and third longest follow-up (3 and 4 years, respectively) after LAT. We observed in the past the possibility of a missing correlation between LESC function and normal morphologic features, with positivity to corneal markers (K12) in the absence of the palisades of Vogt.31 Extensive damage to the ocular surface may be responsible for such a severe destruction of the corneal integrity that the time required for restoration of normal anatomic features is beyond the timeframe of our study, although reestablishment of normal function can be obtained as early as when donor LESCs are placed in the recipient eye. In conclusion, our study confirms previous observations of the effectiveness of LAT on the recipient eye, but demonstrates for the first time the harmlessness of the procedure on the donor eye. This implies the possibility of repeat LAT using grafts from the same previously harvested eye and broadens substantially the field of indications for the procedure.

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