American Academy of Ophthalmology
The Eye M.D. Association

In Vivo Confocal Microscopy 1 Year after Autologous Cultured Limbal Stem Cell Grafts

Emilio Pedrotti, MD,[1] Mattia Passilongo, MD,[1] Adriano Fasolo, MS,[1,2] Mario Nubile, MD,[3] Graziella Parisi, MD,[1] Rodolfo Mastropasqua, MD,[1,3] Sara Ficial, MD,[1] Marina Bertolin, BS,[2] Enzo Di Iorio, PhD,[4] Diego Ponzin, MD,[2] Giorgio Marchini, MD[1]

Purpose: To correlate clinical, impression cytologic, and in vivo confocal microscopy findings on the corneal surface after cultured limbal stem cell transplantation.

Design: Prospective, interventional, noncomparative, masked case series.

Participants: Thirteen patients with limbal stem cell deficiency after unilateral (9 eyes) or bilateral (2 eyes) chemical burn, liquid nitrogen injury (1 eye), or herpes simplex virus infection (1 eye).

Methods: Limbal cells were harvested from healthy or less affected eyes, cultured on 3T3 cells and fibrin glue, and transplanted to the patient's injured eye. Patients underwent clinical examination and impression cytologic examination of the central cornea before and 1 year after intervention. In vivo confocal microscopy scans were obtained in all corneal quadrants after 1 year. The interexamination agreement was established by calculation of the Cohen's κ coefficient.

Main Outcome Measures: Results of surgery were assessed considering clinical signs (successful: restoration of transparent, avascular, and stable corneal epithelium without neovascularization in central corneal surface; partially successful: recurrence of superficial neovascularization; failed: recurrent epithelial defects, pannus, and inflammation), phenotype of cells covering the corneal surface (conjunctivalized corneal surface: cytokeratin 12 [cK12]−negative and mucin 1 [MUC1]−positive cells; mixed epithelium: cK12-positive and MUC1-positive cells; corneal epithelium: cK12-positive and MUC1-negative cells), and cell morphologic features (corneal epithelium: multilayered polygonal and flat cells with hyperreflective nuclei; conjunctival epithelium: stratified cuboidal or polygonal cells, hyperreflective cytoplasm, and barely defined borders; epithelial transition: transition of epithelial cells from the cornea to the conjunctiva over the corneal surface).

Results: We found a moderate to substantial degree of concordance between confocal microscopy and clinical evaluation ($\kappa = 0.768$) and between confocal microscopy and impression cytologic analysis ($\kappa = 0.629$). Confocal microscopy showed that 46.2% of patients exhibited corneal epithelium in the central and peripheral cornea, 30.8% showed an irregular mixed corneal and conjunctival epithelium, and 23.0% showed conjunctival epithelium. Palisades of Vogt were absent in all (100.0%) patients, and the cornea−conjunctiva epithelial transition localized approximately 1 mm internally on the cornea.

Conclusions: Confocal microscopy provides objective measures of the corneal epithelium and may significantly improve the evaluation of outcomes after cultured limbal stem cell graft. *Ophthalmology 2015;■:1−9 © 2015 by the American Academy of Ophthalmology*

The diagnosis of limbal stem cell deficiency (LSCD) is essentially clinical, with intrinsic limitations associated with the interpretation of clinical signs, particularly in partial LSCD, because superficial corneal neovascularization, conjunctivalization, and ocular surface inflammation often are subtle and nonspecific.[1,2] Moreover, alterations of limbal anatomic features are not detectable simply with slit-lamp biomicroscopy, and the application of impression cytologic (IC) analysis can provide objective evidence of LSCD, although with significant limitations. In fact, IC analysis does not offer information on the deeper layers of the corneal epithelium, and some ambiguity on the specific markers of corneal and conjunctival epithelial cells is still a matter of concern.[3,4] Likewise, outcomes of limbal stem cell transplantation (LSCT) surgery usually are assessed based on subjective grading, and this raises the issue of how efficacy of treatment should be defined.[5]

Recently, a more reliable diagnostic procedure in patients with a suspected clinical diagnosis of LSCD was obtained using laser scanning in vivo confocal microscopy (IVCM).[6,7] Compared with clinical slit-lamp examination, IVCM resulted in less ambiguous microscopic morphologic-based differentiation of conjunctival and corneal-type epithelium[8,9]; in identification and quantification of goblet cells, dendritic cells and leucocytes; and in better recognition of alterations in the limbal anatomic features in eyes with LSCD.[10−12] In vivo confocal microscopy also showed a high degree of concordance with IC examination and offered the advantages of scanning a much wider area and the ability to observe deeper epithelial layers compared with

http://dx.doi.org/10.1016/j.ophtha.2015.04.004
ISSN 0161-6420/15

IC analysis. Moreover, IVCM allows a quick, less invasive, and repeatable real-time diagnostic examination. In this study, we used the laser scanning IVCM technique 1 year after LSCT and correlated the IVCM findings with the clinical signs and the results of IC immunofluorescence staining.

Methods

Patient Selection

A prospective, interventional, masked series of patients was evaluated using 3 different techniques. The study was carried out in agreement with the tenets of the Declaration of Helsinki. Institutional review board or ethics committee approval to carry out this study was obtained. Written informed consent for both treatment and participation in the study was obtained from all patients.

Patients with a history of unilateral or bilateral chemical burns or eye diseases leading to a diagnosis of LSCD were considered for LSCT. All patients were evaluated by slit-lamp biomicroscopy at baseline and at 3, 15, and 30 days and 3, 6, and 12 months after surgery. Before surgery, IC samples were obtained from the corneal surface of the injured eye in patients with unilateral injury and from both eyes in patients with bilateral injury. One year after LSCT, only operated eyes were sampled.

We used IVCM before surgery in patients with bilateral injury to evaluate limbal sectors that showed cytokeratin 12 (cK12)–positive cells at IC analysis and to target the biopsy site in eyes used as donors of limbal tissue. In the remaining patients, IVCM scans were performed only in operated eyes after 1 year. All clinical evaluations were performed by a single investigator (E.P.) masked to IC analysis and IVCM findings. All IC samples were analyzed by a single investigator (E.D.I.) masked to patients' clinical history and slit-lamp and IVCM results. In vivo confocal microscopy scans in all patients were performed by a single investigator (M.P.) and were reviewed independently by a second investigator (M.N.), both masked to slit-lamp and IC results.

Clinical Examination

Before surgery, every 7 days during the first 2 months after surgery, and then at 3, 6, and 12 months after surgery, the following clinical features were evaluated by slit-lamp biomicroscopy: (1) limbal anatomic features, (2) corneal neovascularization (total was defined as vessels covering the corneal surface and the limbus, and partial was defined as vessels only in the peripheral cornea or in some sectors of the central corneal surface), (3) irregularity and permeability of corneal epithelium by fluorescein staining (before surgery and after 3, 6, and 12 months), and (4) epithelial integrity and transparency.

To undergo LSCT, patients were required to have complete eyelid occlusion, Shirmer-I test results of more than 5 mm/5 minutes, and no ongoing anti-inflammatory treatments. The efficacy of treatment was assessed after 1 year and was classified as suggested by Rama et al[14] as (1) successful if a transparent, avascular, and intact corneal epithelium without neovascularization in the central corneal surface had been restored; (2) partially successful when superficial neovascularization had recurred, even if it was not as extensive as at the time of admission; (3) or failed if recurrent epithelial defects, pannus, and inflammation occurred at 1 year.[13]

Impression Cytologic Analysis

Cytologic samples of the corneal surface (1 sample/eye) were collected using Biopore membranes (Millicell-CM 0.4 μm; diameter, 12 and 30 mm; Millipore Corp, Bedford, MA). Each membrane was double stained with antibodies against cK12 (corneal specific, sc-17099, goat polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and mucin 1 (MUC1; conjunctival specific, H-295, sc-15333, rabbit polyclonal, 1:200; Santa Cruz Biotechnology) and was analyzed with laser scanning confocal microscopy (A1Rsi+ Laser Scanning Confocal Microscopy; Nikon Instruments, Inc, Melville, NY). Image analysis was performed using the NIS-Elements Advanced Research (Nikon Instruments, Inc.).[3] Impression cytologic samples were considered suitable for diagnostic purposes whenever more than 80% of the 10-mm central area of the membrane was covered by confluent (not scattered) cells.

The cK12 and MUC1 markers were selected because they can be used as individual markers of corneal and conjunctival epithelia, respectively; because overlapping is minimal; and because their expression is specific.[15] Single positivity for MUC1 or cK12 reflected completely conjunctivalized corneal surface or corneal epithelium, respectively. Copositivity for cK12 and MUC1 was interpreted as mixed epithelium.

In Vivo Confocal Microscopy

The HRT3 Rostock Cornea Module diode-laser 670-nm scanning microscope (Heidelberg Engineering GmbH, Heidelberg, Germany) was used under topical anesthesia with 0.4% oxybuprocaine and 1 drop of 0.2% polyacrylic gel between the contact cap of the objective lens and the contact lens applied to protect the limbus–corneal surface. Scans were obtained by moving the objective lens throughout the central and paracentral cornea and in 8 clock-hour positions of the limbal–corneal area (12, 6, 3, and 9—corresponding to superior, inferior, nasal, or temporal limbus—and intermediate positions between the above points of the limbal circumference) following methods previously described.[16]

At least 40 images from each area were obtained for each eye, with a total acquisition time of approximately 5 minutes. Morphologic features of epithelia on the corneal surface were defined as follows: (1) corneal epithelium, a multilayered epithelium with polygonal and flat cells with hyperreflective nuclei in the superficial layer, progressively decreasing in size in the intermediate layers, and small cells without detectable nuclei with reflective borders in the basal layer[17]; and (2) conjunctival epithelium, a stratified epithelium of cuboidal or polygonal cells, hyperreflective cytoplasm (with or without detectable nuclei), and barely defined borders. Goblet cells were highlighted by detection of round or oval cells with a highly reflective homogeneous cytoplasm scattered between epithelial cells. The detection of transition of epithelial cells from the corneal to the conjunctival morphologic features over the corneal surface (epithelial transition) was considered suggestive of the restoration of limbal function and was graded as reported by Nubile et al as (1) present when seen in all 4 quadrants from the central to the paracentral cornea, (2) partially present when seen in 1 to 3 quadrants, and (3) absent if not detected at all.[9] Stromal innervation and inflammatory cells in the 4 mm of the central cornea also were investigated and quantified following reported methods.[11,16]

Culture and Transplantation of Autologous Limbal Cells

Limbal epithelial cells were isolated from a superficial lamellar biopsy of the limbus (approximately 3 mm in tangential diameter