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The safety of photochemical tissue bonding for treating damaged corneal epithelium using limbal stem cells pre-cultured on human amniotic membrane

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ABSTRACT

We previously demonstrated the feasibility of treating limbal stem cell deficiency (LSCD) with limbal stem cells (LSCs) pre-cultured on human amniotic membrane (HAM), using a suture-free technique called photochemical tissue bonding (PTB). However, important issues regarding the safety and the influence of PTB on LSCs have not been elucidated. In this study, LSCs, isolated from rabbit eyes and identified by cell markers, were labeled with BrdU prior to cultivation on de-epithelialized HAM to fabricate grafts. Rabbit LSCD models were created and randomly divided into groups for transplantation of fabricated grafts using sutures or PTB ($n = 10$). Possible phototoxicity of PTB to LSCs was analyzed in vitro and in vivo. Restoration of corneal epithelium was evaluated at 28 days after grafting. Our results showed that phototoxicity did not occur in the LSCs cultured on HAM after PTB in vitro. Transplantation of grafts with PTB restored the damaged cornea epithelium effectively and no significant influences on LSC characteristics were found in both sutured and PTB groups. BrdU positive cells were tracked at 28 days post grafting suggesting that the restored epithelium was derived from the in vitro fabricated HAM/LSC graft. These data suggest that PTB is a safe and potential strategy for securing LSC/HAM grafts that produces with better outcomes than sutured attachment.

1. Introduction

The corneal epithelium is a rapidly regenerating stratified epithelium, which plays a critical role in maintaining corneal transparency and integrity of the ocular surface. The maintenance of the corneal epithelium is achieved by limbal stem cells (LSCs), which are located at the border of the cornea and conjunctiva [1]. Several factors, including chemical or thermal injuries or severe diseases, such as Stevens-Johnson syndrome, can cause the dysfunction or absence of LSCs population. Therefore, the epithelial integrity and stability is highly affected by corneal chronic inflammation and vascularization.

Surgical transplantation of limbal epithelial cells is capable of restoring a normal corneal phenotype with regression of vascularization and inflammation and improvement in optical clarity [2]. However, limbal autograft from a normal contralateral eye has the potential risk of inducing the limbal stem cell deficiency of the donor eye. Limbus taken from a living related or cadaveric eye as an allograft is limited by the donor resource and life long immunosuppression [3]. Expanded limbal stem cells ex vivo from a small limbal biopsy can minimize the damage and reduce the morbidity of donor eyes [4]. Cultivating the LSCs on human amniotic membrane (HAM) as a graft (LSC/HAM) for treating LSCD seems to be a potential method and has been intensively investigated recently [4–6].

Kochevar and Redmond recently developed a new suture-free technique called photochemical tissue bonding (PTB), which has been successfully applied for repair of variety of tissues including skin, peripheral nerve, blood vessels, and tendon [7–12]. Also, our previous studies demonstrated that PTB secured a fabricated LSC/HAM graft in a rabbit LSCD model with relatively better outcomes compared to sutured attachment in terms of corneal transparency, neovascularization, inflammation, and collagen organization [13]. These results suggested that PTB is an effective and promising technique for transplantation procedure. However, several issues remain unclear regarding the safety of PTB and the differentiation of LSCs after transplantation with PTB. The PTB procedure involves

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applying Rose Bengal dye (RB) to the graft, placing the graft on the cornea, then exposing the area to green light (532 nm), One important concern is that activation of RB with green light releases reactive oxygen species (ROS), which have a potential toxicity to cells in vitro [10,14,15]. Thus, we asked: can this toxic feature of PTB affect the viability and survival rate of LSCs cultivated on HAM graft? A second concern is whether the differentiation of transplanted LSCs on the graft would be altered by PTB. It has been reported that ex vivo cultured LSCs on HAM that restored the corneal epithelium also maintained the characteristics of stemness [16]. Thus, we asked: does PTB alter the phenotype of LSCs and influence the efficacy of epithelium restoration after transplantation?

In this study, we used our previously established rabbit model of LSCD, in which the corneal epithelium is removed surgically and the total limbus and conjunctival epithelial cells are depleted by sodium hydroxide. The lesion was then repaired by BrdU-labeled fabricated grafts, which were secured either with PTB or with sutures. After transplantion, the tracking of BrdU labeling was performed. In addition, the phenotype of transplanted LSCs and the restoration of corneal epithelium were evaluated.

2. Materials and methods

2.1. Fabrication of HAM/LSC grafts

The grafts were constructed according to our previous method with minor modifications [13]. In brief, limbal rings obtained from sacrificed rabbits were cut into small pieces and then placed into culture plates with one drop of fetal bovine serum (FBS) (Invitrogen-Gibco BRL, Grand Island, NY, USA) for overnight incubation. The next day, full culture medium was added and changed every other day. The tissue should be removed within 3 days to avoid the outgrowth of other type of cells including fibroblasts. The cultivated LSCs were examined by phase contrast microscopy every other day and images were taken. LIVE/DEAD reagents (Sigma–Aldrich, St. Louis, MO, USA) were employed to further assess the morphology and viability of cultured LSCs. The live cells showed green fluorescence (calcein) and the dead cells showed red fluorescence (ethidium) under fluorescent microscopy [17].

Human amniotic membrane was obtained from discarded placenta after scheduled Caesarean section deliveries under a protocol approved by the Partners Healthcare System Institutional Review Board. Under sterile conditions, the epithelial layer was removed after incubation in 0.25% trypsin containing 0.02% EDTA (Sigma–Aldrich]. The absence of epithelium was confirmed on H&E-stained sections. The de-epithelialized HAM was placed, basement membrane side up, on the porous membrane support of a 6 well transwell plate (Millipore Corporation, Boston, MA). The bottom of the well contained pre-cultured mitomycin-treated 3T3 fibroblast (NIH 3T3; American Type Culture Collection, VA) as feeder cells. Single LSCs suspension was added onto the HAM at a density of $2 \times 10^5/cm^2$. Upon confluence of the LSCs (1 week), air-lifting was performed by lowering the level of the medium to the bottom of the membrane to allow stratification of the LSCs.

2.2. Immunofluorescent staining of LSCs markers

Similar to many other stem cells, LSCs lack a specific molecular marker and a combination of different cell markers is usually employed to identify these cells. Following the methods in our previous study [13], immunohistochemical staining was performed for P63, K3 and integrin β1, which are supposed to be expressed by LSCs (p63, integrin β1) or mature epithelial cells (K3). In brief, the primary cultured LSCs were fixed with 4% paraformaldehyde for 10 min. Permeabilization was performed with 0.1% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) in PBS at room temperature for 10 min when P63 (nucleus marker) was stained. Goat serum (10%) was applied to block non-specific bonding sites. Monoclonal primary antibodies against P63 (1:100), K3 (1:200) and integrin β1 (1:50) (Santa Cruz, Biotechnology Inc. Santa Cruz, CA, USA) were applied and the cells or samples incubated at 4 °C overnight. A secondary antibody conjugated with FITC (Santa Cruz, Biotechnology Inc. Santa Cruz, CA, USA) was applied at room temperature for 45 min. Finally, the samples were mounted with mounting solution (Dako Cytomation Kyoto, Japan), containing DAPI to stain nuclei. An Olympus Confocal FV1000 image system (Olympus Co., Ltd, Tokyo, Japan) was used to record images.

2.3. BrdU labeling and tracking

To label the cultured LSCs for tracking post surgery, Full medium containing 10 μM BrdU was added for 24 h when the cells had grown to 60% confluency in 35 mm dishes. All samples were fixed in cold methnol at 4 °C for 10 min and processed for BrdU immunofluorescent staining as previously described [18]. In brief, after rehydration in PBS for 5 min, samples were incubated with 2 N HCl at 37 °C for 60 min to denature DNA and neutralized in boric acid (pH 8.5) for 20 min. Incorporated BrdU was detected by immunofluorescent staining with a rabbit anti-BrdU polyclonal antibody (1:100), followed by incubation with goat anti-rabbit IgG (1:300) and counterstaining with DAPI for nuclei.

2.4. Phototoxicity to LSCs cultured in monolayers or in fabricated grafts

To determine the phototoxicity of PTB to LSCs before transplantation, monolayer LSCs on plastic dishes or on HAM were treated with and followed by viability assay with DEAD/LIVE reagents (Sigma–Aldrich). The monolayer cultured LSCs was used as control. The PTB procedure is the same as previously described [8,9,19]. Briefly, the cultured monolayer LSCs or HAM/LSC was washed with PBS twice, then 0.1% (w/v) RB (Sigma–Aldrich) in PBS was applied to the cells or HAM/LSC for approximate 5 min. The LSC or HAM/LSC were then exposed to green light (532 nm) at an irradiance of $0.4\ W/cm^2$ to produce a fluence of $80\ J/cm^2$ using a CW Nd:YAG 532 nm laser (Iridex OcuLight TX, Mountain View, CA). The LSCs and HAM/LSC were cultivated with media for overnight and assayed by DEAD/LIVE reagents according to the instruction. Under fluorescent microscopy, the live and dead cells showed green and red fluorescence, respectively. By counting the number of green and red cells in randomly selected fields, the percentage of live cells was calculated.

2.5. Rabbit model of LSCD and treatments

New Zealand white rabbits (2–2.5 kg; Charles River Laboratory, Wilmington MA USA) were anesthetized using 3.5–4.5% isoflurane for induction and 2% for maintenance. Under sterile conditions, 0.5 M NaOH was applied to the limbal area of the left eye for 45 s to destroy the limbus and the eye was washed with PBS 3 times to wash away the excess alkali and remove the limbal cells [13,20,21]. A biopsy punch with 10 mm diameter was used to cut the anterior stroma. The entire corneal epithelium and anterior part of stroma was then surgically removed. The creation of corneal defects and depletion of limbus was confirmed by histological examination.

The rabbits were then randomly divided into PTB and control sutured group ($n = 10$ each), in which the HAM/LSC grafts were secured by PTB or sutures respectively. 0.1% RB was applied to the HAM/LSC graft stroma surface as well as the created cornea wound bed for approximate 5 min, and then the graft was placed on the corneal defect followed by exposure to a round green light