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Short communication

Effects of activated omental cells on rat limbal corneal alkali injury

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ABSTRACT

Omental cells (OCs) are shown to help wound healing. The purpose of this study is to investigate if OCs improve cornea repair after alkali injury by subconjunctival injection of activated OCs in rats. Forty eight hours after limbal corneal alkali injury, fresh isolated OCs were injected subconjunctivally into the recipient rat's eye. Prior to the injury and at 0, 4 and 8 days after injury, the eyes were examined using slit lamp biomicroscopy. Corneal opacification and corneal neovascularization were graded in a masked fashion. The inflammatory response to the injury was evaluated by counting neutrophil cell numbers in the cornea under microscope. There was no significant difference in corneal opacification between the control and OCs treatment groups; however, the corneal neovascularization was significantly less in the eyes treated with OCs as compared to the controls. Also OCs treatment markedly decreased neutrophil infiltration after corneal-limbal alkali injury. Our results suggest that OCs may have a beneficial role in corneal healing after limbal corneal alkali injury by suppressing inflammatory cell infiltrates and corneal neovascularization.

Corneal epithelial renewal and repair is dependent upon stem cells located in the limbus, the narrow zone between the cornea and bulbar conjunctiva (Rama et al., 2010). These cells are able to produce transit amplifying cells that migrate centripetally toward the central cornea to populate the basal layer of the corneal epithelium, further dividing and differentiating (Kinoshita et al., 1981; Tseng, 1989). Limbal stem cell deficiency (LSCD) can develop in traumatic, immunologic, or genetic diseases that affect the ocular surface. LSCD leads to conjunctivalization, with corneal vascularization and opacification and subsequent loss of vision (Bakhtiari and Djalilian, 2010). Restoration of ocular surface integrity and the limbal barrier by limbal stem cell transplantation is essential in patients with limbal stem cell deficiency (Fernandes et al., 2004). Current management of LSCD depends on the laterality and the severity of the disease. In unilateral corneal stem cell deficiency, treatment options include autologous conjunctivolimbal autografts (CLAU), ex vivo stem cell transplantation, or an allograft if the patient refuses surgery on the normal eye. For severe bilateral disease, options include keratolimbal allografts (KLAL) with immunosuppression or living related allografts (lr-CLAU), or some combination of the two. Autologous limbal stem cell transplantation is not applicable in cases of total bilateral limbal stem cell deficiency.

The search for an alternative source of stem cells for the limbus and for corneal stromal repair has led researchers to examine other non corneal stem cell sources including adipose tissue (Arnalich-Montiel et al., 2008), where mesenchymal stem cells have been found to be present (Ye et al., 2006). Adipose tissue itself is relatively expendable and lipoaspirate can easily be obtained and processed in large quantities; additionally, these cells can be harvested from the patient, thereby avoiding the need for immunosuppressive therapy. The omentum, a pad of abdominal fat present in all human beings, has long been known to have the power to heal injured organs once it has adhered to the damaged site, either naturally or deliberately by surgery.

Some of omental cells (OCs) have the potential to express important pluripotent stem cell markers. Some of OCs can be activated by foreign bodies *in vivo* and become a rich source of growth factors. They may engraft into injured tissue and serve as stem cells themselves (Litbarg et al., 2007; Shah et al., 2012; Singh et al., 2008). They have been shown to produce new insulin producing cells when transplanted into the pancreas of diabetic rats and have also been shown to assist in liver regeneration (Singh et al., 2007, 2009).

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The activated omentum has also been shown to contain immunomodulatory cells that assist in regeneration of injured tissue: $CD45^+$, Gr^+ and myeloid derived suppressor cells. $CD45^-$ cells have also been shown to be present in this tissue, suppressing Th17 cells, which play an important role in inflammation (Shah et al., 2012).

The clinical application of these activated OCs has not yet been explored for limbal stem cell deficiency. Our study investigates the effects of subconjunctival injection of activated OCs on rat limbal corneal alkali injury. Alkali chemical injuries to the eye cause many complications by the nature of their injury. By increasing the pH of tissues and causing saponification of fatty acids, alkalis are able to penetrate the epithelium and reach underlying stromal tissue. Alkali injury of the cornea has been shown to be detrimental to the limbal stem cell population. Damage to limbus is seen clinically as limbal "blanching", a sign of vascular death. Without the stem cells for regeneration, the healing corneal epithelium often undergoes "conjunctivalization," and is subsequently vascularized. Chronic inflammation and breakdown subsequently incur as well (Wagoner et al 2000). Therefore, our study uses a rat limbal corneal alkali injury model to study the effects of activated OCs on limbal stem cell deficiency.

Adult male Fisher rats (250–300 g) were used in this study. Animal care and experimental procedures were performed in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee, Loyola University Chicago. All procedures were performed on animals anesthetized with ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). Topical 0.5% proparacaine hydrochloride (Alcaine, Alcon) was applied to the eye prior to limbal corneal alkali injury. OCs were isolated from the donor Fisher rats (Shah et al., 2012). The donor Fisher rats (200–250 g) were injected intraperitoneally with 5 ml of polydextran particle slurry (Bio-gel P-60, Biorad Laboratories, Richmond, Calif.; 1:1 in PBS). The rats were sacrificed on day 7 and the expanded omentum was harvested aseptically and gently chopped into small pieces. These pieces were then digested in 1 mg/ml of collagenase type I (Sigma, St. Louis, MO) for 30 min at 37 °C. The OCs were washed and separated over a ficoll gradient. Cells from the interface were passed through a cell strainer. After washing and centrifugation, cells were finally suspended in sterile saline. Limbal corneal alkali injuries to the eye of the deeply anesthetized recipient rats were induced via 20 s of exposure to the limbal and peripheral corneal area with a donut-shaped filter paper ring (8 mm outside and 4 mm inside diameter) soaked in 0.5 N NaOH. The eyes were immediately rinsed with sterile saline (10 ml). This paper ring was used to destroy limbal cells while preserving central cornea. The animals were then randomly allocated into a control group or treatment group. The control group ($n = 6$ eyes) was treated with a subconjunctival injection of sterile saline 48 h after limbal corneal alkali injury. The treatment group ($n = 6$ eyes) received subconjunctival injections of activated OCs (0.5×10^6 cells/eye in 100 μl sterile saline), freshly isolated from the donor rats. The total volume was divided among 3 different areas of the conjunctiva. To verify that the volume had been delivered, slit lamp exam confirmed a localized elevation of the conjunctiva without leakage of the medication. Prior to the injury and at 4 and 8 days after injury, the eyes were examined using slit lamp biomicroscopy. Corneal opacification and corneal neovascularization were graded in a masked fashion. Using a laboratory slit lamp, a previously described scoring system (Sonoda and Streilein, 1992) was employed to measure the relative degree of corneal opacification with grading between 0 and 5+: 0 = clear and compact cornea; 1+ = minimal superficial opacity; 2+ = mild deep (stromal) opacity with pupil margin and iris vessels visible; 3+ = moderate stromal opacity with only pupil margin visible; 4+ = intense stromal opacity with anterior chamber visible; 5+ = maximal corneal opacity with total obscuration of the anterior chamber. Corneal neovascularization (NV) was graded between 0 and 3 per corneal quadrant, with increments of 0.5, using a grid system based on the centripetal extent of neovascular branch outgrowth from the corneoscleral limbus (Choi et al., 2009; Dana and Streilein, 1996). Dilated limbal vessels not penetrating the corneal stroma were not considered representative of corneal NV. The scores for each quadrant were then summed to derive the corneal NV indices (range, 0–12) for each eye at an each given time point. The rats were euthanized immediately after examination on day 4 and 8 post injury by CO_2 inhalation. Enucleated eyes were fixed in 10% formaldehyde and embedded in paraffin; sections (5 μm) were stained with hematoxylin and eosin. Slides were analyzed with an optic microscope at 400× magnification (Nikon ECLIPSE E600 Japan) and pictures were taken with a digital camera (Q IMAGING). Three fields in each sample were used for the neutrophil cell counting.

The mean neovascularization (NV) scores were 4.00 ± 1.10 in the control group and 2.50 ± 1.22 in the OC treated groups at 4 days after limbal corneal alkali injury; at 8 day post-injury, the mean NV scores were 5.33 ± 1.03 in the control group and 2.17 ± 0.75 in the OC treated group. The data demonstrated that corneal neovascularization was significantly less in eyes injected with OCs compared to controls. The mean corneal opacity score was 4.33 ± 0.81 in the control group and 3.50 ± 1.22 in the group treated with OCs at 4 days post-limbal corneal alkali injury; at 8 days, the mean opacity score was 4.17 ± 0.98 in the control group and 4.00 ± 1.09 in the OC treated group (Fig. 1). Our data showed that there was no significant difference in the corneal opacification between the eyes injected with OCs versus the control although the corneal opacity was less in the OC treated group compared to the control treated group at 4 days post injury. Corneal histology demonstrated a greater density of neutrophils that had infiltrated into the corneal stroma in the control group as compared to the OC treated group (Fig. 1). Furthermore, the histology also showed that there was more stromal neovascularization within the control group as compared to the OC treated group (Fig. 2).

The wound healing properties of the human omentum have been well-described (Liebermann-Meffert, 2000; Litbarg et al., 2007), but the underlying cellular and molecular mechanisms involved in the omentum-mediated prevention of inflammation and healing of injured organs remains poorly understood. The omentum produces high levels of vascular endothelial growth factor (VEGF) when "activated" by injury (Singh et al., 2008). Human CD34+ omental cells produced angiogenic growth factors bFGF and VEGF. When implanted in rats, the omental cells promoted neovascularization (Garcia-Gomez et al., 2005). It is thought that the corneal limbal stem cells and the corneal epithelium help create an avascular environment (Ellenberg et al., 2010). The corneal epithelial cells produced a variety of anti-angiogenic factor including angiostatin, endostatin, restin, neostain and thrombospondins in maintaining the angiogenic balance of avascularity (Lim et al., 2009). Ma et al. found that human limbo-corneal epithelial cells, cultivated on amniotic membrane, expressed enhanced secretion of endostatin and restin, matrix metalloproteases involved in anti-angiogenic activity (Ma et al., 1999). Furthermore, transplanted limbal SCs reduced neovascularization by increasing the secretion of antiangiogenic factors like neostatin and restin, causing regression of corneal blood vessels (Ellenberg et al., 2010; Ma et al., 2006). Our finding showed that activated OCs after corneal alkali injury inhibited angiogenesis. The difference between our finding and the results from Garcia-Gomez et al. may be due to different cell populations used as they used relatively