Published in final edited form as:

Ocul Surf. 2017 October ; 15(4): 749–758. doi:10.1016/j.jtos.2017.05.010.

Proteasomes in corneal epithelial cells and cultured autologous oral mucosal epithelial cell sheet (CAOMECS) graft used for the ocular surface regeneration

Fawzia Bardag-Gorce, PhD*, Richard Hoft, MD, Imara Meepe, Julio Garcia, Kumar Tiger, Andrew Wood, Amanda Laporte, Derek Pan, Andrew Makalinao, Robert Niihara, Joan Oliva, PhD, Arjie Florentino, Amber M. Gorce, Jeremy Stark, Daileen Cortez, Samuel W. French, MD, and Yutaka Niihara, MD¹

Los Angeles Biomedical Research Institute (LA BioMed) at Harbor-UCLA Medical Center, Torrance, CA 90502, USA

Abstract

Purpose: This study focuses on characterizing proteasomes in corneal epithelial cells (CEC) and in cultured autologous oral mucosal epithelial cell sheets (CAOMECS) used to regenerate the ocular surface.

Methods: Limbal stem cell deficiency (LSCD) was surgically induced in rabbit corneas. CAOMECS was engineered and grafted onto corneas with LSCD to regenerate the ocular surface.

Results: LSCD caused an increase in inflammatory cells in the ocular surface, an increase in the formation of immunoproteasomes (IPR), and a decrease in the formation of constitutive proteasome (CPR). Specifically, LSCD-diseased CEC (D-CEC) showed a decrease in the CPR chymotrypsin-like, trypsin-like and caspase-like activities, while healthy CEC (H-CEC) and CAOMECS showed higher activities. Quantitative analysis of IPR inducible subunit (B5i, B2i, and B1i) were performed and compared to CPR subunit (B5, B2, and B1) levels. Results showed that ratios B5i/B5, B2i/B2 and B1i/B1 were higher in D-CEC, indicating that D-CEC had approximately a two-fold increase in the amount of IPR compared to CAOMECS and HCEC. Histological analysis demonstrated that CAOMECS-grafted corneas had a re-epithelialized surface, positive staining for CPR subunits, and weak staining for IPR subunits. In addition, digital quantitative measurement of fluorescent intensity showed that the CPR B5 subunit was significantly more expressed in CAOMECS-grafted corneas compared to non-grafted corneas with LSCD.

Conclusion: CAOMECS grafting successfully replaced the D-CEC with oral mucosal epithelial cells with higher levels of CPR. The increase in constitutive proteasome expression is possibly responsible for the recovery and improvement in CAOMECS-grafted corneas.

*Corresponding author. Department of Medicine, LA BioMed at Harbor UCLA Medical Center, 1124 W. Carson Street, Torrance, CA 90502, USA, fgorce@labiomed.org (F. Bardag-Gorce).

¹Dr. Yutaka Niihara is the CEO and President of Emmaus Life Science Inc., the sponsor of this research. The other authors have no commercial or proprietary interest in any concept or product described in this article.

Keywords

Corneal epithelial cells (CEC); Cultured autologous oral mucosal epithelial; cell sheets (CAOMECS); Limbal stem cell deficiency; Proteasome

1. Introduction

Corneal epithelium is an important component of the ocular surface. The epithelial cell layer has barrier and other functions that protect the eye from external threats, such as trauma, thermal and chemical burns, and infectious agents. These threats, and other systemic, autoimmune inflammatory diseases (e.g., Stevens-Johnson syndrome and ocular pemphigoid), sometimes cause severe damage to the limbal stem cells and to the corneal epithelium. When limbal stem cell deficiency (LSCD) occurs, the corneal epithelium can no longer maintain a healthy and transparent corneal surface. LSCD is frequently associated with neovascularization, conjunctivalization, and opacification of the corneal surface. The underlying molecular mechanism of corneal opacification is complex. Signaling pathways for angiogenesis and inflammation are the two most important and intricate pathways involved when the corneal surface is undergoing conjunctivalization and opacification caused by LSCD. The ubiquitin proteasome pathway (UPP) regulates the key transcriptional factors of these two, as well as many other, signaling pathways [1–4].

Proteasome-mediated protein degradation is essential for the regulation of many vital cellular mechanisms, such as cell cycle progression (cyclins), apoptosis, transcriptional activation (p53, I κ B- α , HIF- α), immune response, and signal transduction [5]. Proteasome is now considered a cellular defense mechanism, as it also removes abnormal and other damaged proteins generated by mutations, translational errors, or oxidative stress [6,7]. The proteasome is a dynamic structure that exists in different forms: the 20S, the 26S (20S + two regulatory complexes 19S), the immunoproteasome (20S + two 11S regulating complexes), the proteasome hybrid (20S + 11S + 19S), and some other variant forms [2]. Each of these proteasomes has several specific functions in various important cellular mechanisms, such as cell cycle, NF κ B activation and angiogenesis [3,4].

The 26S proteasome is a complex macromolecule with multiple subunits that perform proteolytic activities in the ATP-ubiquitin-mediated proteolytic pathway. The 19S regulatory complexes of the 26S proteasome are formed by a basal ring of 9 ATPase and non-ATPase subunits and a lid of 10 non-ATPase subunits that recognize, unfold, and translocate ubiquitinated substrates designated for proteolysis by the proteasome [2]. The 20S proteasome catalytic core is formed by 14 α and 14 β subunits (7 α 7 β 7 β 7 α) and is in a latent state. Several chemical compounds, proteins, and regulatory complexes function as activators of this latent 20S form [1,2]. They are termed gate-openers, as they provoke the opening of the gate at the α -type subunits of the 20S proteasome [1], and facilitate the access of designated proteins to the catalytic chamber formed by the β -type subunits to be degraded [1,2].

Angiogenesis is associated with hypoxia-dependent molecular events caused by activating the transcriptional factor HIF-1 α , and consequently a downstream upregulation of VEGF

[8] and other angiogenic factors, such as matrix metalloproteases (MMP-2, MMP-3), transforming growth factors (TGF) alpha and beta, and bFGF. Under normoxic conditions, clearance of HIF-1alpha requires the proteasome. However, under hypoxic conditions, HIF-1 alpha becomes stabilized, thus encouraging development of various vision-threatening pathologies [4]. The newly formed blood vessels allow neutrophil and macrophage infiltration [9], which provide key cytokines and growth factors to the angiogenic bed [10].

Neutrophils and macrophages are predominantly present in injured cornea, along with alpha smooth muscle actin and new blood vessels [11]. Both types of inflammatory infiltrating cells are intricately involved in corneal angiogenesis [12]. Moreover, in response to infectious pathogens [13] and various stimuli, the host's inflammatory response promotes the activation of the transcriptional factor or nuclear factor kB (NF-kB) [14], and the induction of cytokine production, thus leading to corneal opacification and loss of vision [15]; to induce NFkB activation, the ubiquitin proteasome pathway is required for the degradation of NF-kB inhibitory protein-IkB [16]. In the last decade, diseased corneal epithelium has been successfully regenerated in experimental animals and in humans, using cultured autologous oral mucosal epithelial cell sheet (CAOMECS) grafting [11,17,18]. However, the molecular mechanism by which CAOMECS grafting suppresses corneal vascularization is not fully understood.

In the present study, we hypothesize that CAOMECS grafting improves the activity of the constitutive proteasome in the injured ocular surface with LSCD, which in turn may contribute to improvement in the health of the ocular surface observed after treatment [11]. Surgically-induced LSCD was created in a rabbit model (as previously reported [11]). The injured and diseased ocular surface was treated with CAOMECS grafts applied to the corneal surface 3 months after the experimental induction of LSCD. After 6 months of follow-up, (which included periodic slit lamp examinations and photographs), the rabbits were sacrificed and ocular surface tissue from the cornea was histologically examined. The distribution of proteasome subunits in corneas with experimental LSCD that received CAOMECS grafts was compared to corneas with experimental LSCD that did not receive the grafts (sham treatment) and to healthy normal corneas. To our knowledge, our results document for the first time the characterization of proteasome distribution in corneal epithelium and the dysfunction of CPR in diseased corneal epithelium with LSCD. In addition, we report the recovery and improvement of CPR activity in LSCD corneas grafted with CAOMECS.

2. Material and methods

2.1. Animal experiment

New Zealand white rabbits weighing 2.5–3 kg were used. They were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council. The experimental protocol was performed as previously reported [11]. Briefly, rabbits were sedated, subjected to lamellar limbectomy and followed for 3 months until LSCD was confirmed stable by an ophthalmologist.

2.2. Corneal epithelial cells (CEC) harvest

Healthy (control) and LSCD-diseased rabbit corneal epithelial cells were harvested by exposing the corneas to 20% isopropyl alcohol. The corneas were then washed three times with saline, and epithelial cells were scraped and removed from the corneal surface, using a small scalpel blade. Healthy rabbit corneal epithelial cells (H-CEC) were collected the day of the lamellar limbectomy that induced LSCD. LSCD-diseased epithelial cells (D-CEC) were collected approximately 3 months after limbectomy on the day of CAOMECS grafting. Both epithelial cell samples (from the same rabbits) were then used for proteasome biochemical analysis. The cells were suspended in lysis buffer containing 20 mM Tris-HCl pH = 7.5; glycerol 10% EGTA 1 mM; DTT 1 mM; protease and phosphatases inhibitor cocktail as instructed by the supplier (Sigma, St Louis, MO). Cell membranes were disrupted by pipetting 25 times the cells through a hypodermic 18G needle.

2.3. Impression cytology

Millicell® culture inserts 12 mm in diameter, 0.4 mm pore size, with mixed cellulose membrane (Millipore, Billerica, MA) were used to sample corneal surface cells. The inserts were placed on the cornea for a few seconds, then slowly removed. The inserts were allowed to dry for 1 h at room temperature. The inserts were then fixed in 10% neutral buffered formalin overnight and conserved in 70% ethanol prior to immunohistochemical staining analysis.

2.4. CAOMECS engineering

After LSCD was confirmed to be stable, the rabbit's oral mucosa was biopsied, and the tissue was cut in pieces and placed in Dispase solution to dissociate the tissue and isolate the epithelial cells (as described in Ref. [11]). Epithelial cells were then cultured as previously reported [11] to engineer a CAOMECS graft. After 2 weeks of culture, two cell sheets were cultured and harvested as a multilayered epithelium-like tissue. One cell sheet was grafted back onto the rabbit cornea and the second cell sheet was used for proteasome biochemical and histological analysis. CAOMECS-grafted rabbits were then followed for 6 months to examine the therapeutic effects of the CAOMECS graft in reducing vascularization and cornea opacification. The detailed protocol was reported in a previous study [11].

2.5. Proteasome enzyme assay

One µg of total protein from sample homogenates was used. The reaction mixture contained 50 mM Tris-HCl pH = 8, 1 mM DTT, and 40 µM Suc-Leu-Leu-Val-Tyr-AMC (Sigma Aldrich) substrate for chymotrypsin-like activity or 40 µM of Boc-Leu-Ser-Thr-Arg-AMC for trypsin-like activity or 100 µM of Ac-Gly-Pro-Leu-Asp-AMC (Sigma Aldrich) for Caspase-like activity. To distinguish the 26S proteasome activity from the 20S proteasome activity, 5 mM ATP was added to the reaction mixture. The mixture was then incubated for 30 min at 37 °C. The reaction was stopped by adding 100 mM monochloroacetate and 30 mM sodium acetate pH = 4.3. Fluorescence was determined by measuring the release of AMC (λ excitation: 370 nm, λ emission: 410 nm), using a Perkin Elmer LS 30 spectrofluorometer.

2.6. Western blot analysis

Two µg of total protein from sample homogenates were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris-HCl (pH = 8.3), 192 mM Glycine and 20% methanol. The membranes were probed with primary antibodies against proteasome subunits (CPR beta 5, beta, beta2, beta5i, beta2i, beta1i (Enzo Life Sciences, Inc.). IκB (Santa Cruz Inc., Santa Cruz CA Appropriate species polyclonal and monoclonal HRP-conjugated antibodies were used as the secondary antibodies. The membranes were subjected to chemiluminescence detection using Luminal according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

2.7. Immunohistochemistry

Immunohistochemical analysis was performed on three types of harvested corneal epithelial specimens: 1) normal, healthy corneas that did not have induced LSCD and did not undergo CAOMECS grafting, 2) corneal specimens with experimentally-induced LSCD that did not undergo CAOMECS grafting, but instead underwent a sham surgical procedure consisting of scraping and removing the superficial tissue from the LSCD cornea surface, and 3) corneal specimens with CAOMECS grafts. Each sample was fixed in 10% neutral buffered formalin. The processed tissues were sectioned, and the slides were stained with H&E and in immunofluorescent staining using deltaNp63 (Biocare Medical, Concord, CA), Ki-67, (Santa Cruz Inc., Santa Cruz CA), proteasome CPR beta5, beta2, beta1 (Enzo Life Sciences, Inc.), proteasome IPR beta5i, beta1i (Enzo Life Sciences, Inc.) and beta2i (Abcam, Cambridge, MA) antibodies.

Alexa Fluor® 488 and Alexa Fluor® 568 donkey anti-rabbit/mouse/goat fluorophore-conjugated secondary antibodies were used. DAPI or propidium iodide (Invitrogen, Eugene, OR) for staining cell nuclei was also used. A Nikon 400 fluorescent microscope was used to analyze the slides. Picture processing and analysis were performed using Adobe Photoshop CS5.

2.8. Morphometric analysis

Healthy corneas, CAOMECS-grafted corneas, and non-grafted corneas that were subjected to sham surgery (surface scraping) were fixed in formalin, embedded in paraffin and sectioned. These specimens were used for immunofluorescent staining to quantify proteasome subunits expression. The intensity of fluorescence was quantified using Nikon morphometric software. The intensity profile appears as a graph of sequential intensity changes. The software provides numeric data by recording numeric intensity of the peaks on the graph. For each sample examined, one random snip was taken, and 4 to 7 measurements were recorded from the intensity profile graph (Fig. 8).

2.9. Statistics

Data were obtained from at least three separate experiments. Bars represent mean values ± SEM. P values are determined by oneway ANOVA and Student-Newman-Keuls for multiple group comparisons (Sigma-Stat softdisk, San Francisco, CA). Statistical significance is set at $p = \text{or} < 0.05$. Bar graphs were shown as Mean ± SEM, $n = 3-5$.

3. Results

To create LSCD, a lamellar limbectomy was performed following the experimental protocol previously reported [11]. Slit lamp examinations and photographic analysis were performed every 3–4 weeks after limbectomy to assess the development and progression of corneal vascularization and fibrovascular pannus tissue (PT) formation. The amount of corneal vascularization and PT formation in each rabbit eye undergoing limbectomy varied and was stabilized 2–3 months after limbectomy (Fig. 1A–C). When PT grew on the ocular surface, cornea opacification was greater, and details of the iris surface were more difficult to visualize [11]. Under mild sedation, corneal epithelial cells were then harvested from healthy-control (H-CEC) and LSCD-diseased (D-CEC) rabbits for subsequent proteasome enzyme assay and quantitative analysis of proteasome subunits. PT was dissected separately after CEC sampling. Fig. 1D shows that proteasome chymotrypsin-like activity was significantly reduced in PT compared to H-CEC and D-CEC, indicating CPR dysfunction in severely diseased corneal epithelial cells.

Rabbits were then subjected to oral mucosal biopsy. Epithelial cells were isolated, seeded, and cultured for 2 weeks in order to produce CAOMECS graft [19]. Two cell sheets were engineered from one 6 mm biopsy. One cell sheet was grafted back onto the rabbit cornea. One half of the 2nd cell sheet (Fig. 2A) was processed for immunofluorescent staining. The other half of the 2nd graft was used for proteasome enzyme assay and quantitative analysis of proteasome subunits. Fig. 2B showed that basal cells of CAOMECS expressed progenitor stem cell deltaN-p63 and showed a healthy proliferative capacity as indicated by Ki-67 staining (Fig. 2C). Apical squamous cells were negative for these two biomarkers, reflecting CAOMECS epithelial polarity. All CAOMECS epithelial layers stained positive for CPR beta subunits (Fig. 2D and E).

Six months after CAOMECS grafting, rabbits were sacrificed for histological analysis [11]. Healthy and LSCD-diseased corneas were stained with CPR and IPR catalytic subunits. The histological analysis showed that LSCD-diseased corneas stained negative for CPR beta catalytic subunits, and stained positive for IPR beta catalytic subunits (Fig. 3A–L).

Proteasome chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-T) were measured, as previously reported [20], in healthy and diseased corneal epithelial cell lysates (H-CEC and DCEC) for comparison to CAOMECS. Chymotrypsin-like is the major proteasome activity that cleaves amino acid chains after hydro-phobic amino acids. Trypsin-like activity is the 2nd major proteasome activity that cleaves after the basic amino acids Lys and Arg. Caspase-like activity is similar to that of caspases Cys proteases that have essential roles in apoptosis. Fig. 4A shows that the three proteasome activities were decreased in D-CEC while CAOMECS showed a significantly higher chymotrypsin-like activity than DCEC and an almost similar activity than H-CEC.

The three catalytic subunits of CPR, beta5 (B5), beta2 (B2), and beta1 (B1), are respectively responsible for chymotrypsin-like, trypsin-like and caspase-like activities.

In inflamed milieu and in the presence of high levels of cytokines, these catalytic subunits are replaced by homologous subunits: B5i/LMP7, B2i/LMP10 (MECL1), and B1i/LMP2.

These new inducible subunits form the immunoproteasome (IPR). Quantitative analysis of immunoproteasome subunit levels showed that DCEC presented high levels of the three IPR subunits and low levels of CPR subunits (Fig. 4C).

IPR and CPR subunits expression was analyzed in healthy oral mucosal epithelial cells and the immunofluorescent staining showed no difference between IPR and CPR subunits, reflecting a homeostatic distribution (Fig. 5A) possibly specific of buccal tissue. However, when healthy conjunctiva was compared to diseased conjunctiva, results showed that CPR B5 subunit expression was weakly detected in D-Cj, while IPR B5i expression was similarly detected (Fig. 5B).

Chronic inflammation as a result of CPR dysfunction was detected by an elevation of neutrophil infiltration (Fig. 6). Cell samples were collected by impression cytology from the surface of healthy and diseased central cornea and processed for H&E staining (Fig. 6B, C and D). As determined by the pathologist, 1 month after limbectomy, LSCD-diseased corneas showed marked presence of macrophages (Fig. 6B, arrow), which dissipated 3 months later to let neutrophils populate the corneal surface (Fig. 6C).

NFkB signaling plays a pivotal role in inflammatory responses, immune responses, cell growth, tissue differentiation, and apoptosis [21]. Fig. 6E shows that activated NFkB in D-CEC was detected by the level of phosphorylated form of NFkB p65 protein. This result was confirmed by the low levels of total Ikb protein levels, Ikb being an inhibitory protein to NFkB activation and translocation to nucleus for pro-inflammatory signaling.

Corneal tissue sections from CAOMECS-grafted rabbits were then analyzed and compared to healthy and diseased corneas (ungrafted or sham cornea). Immunofluorescent staining for CPR subunits was performed to investigate if grafted cornea recovered a functional proteasome pathway 6 months after CAOMECS grafting (Fig. 7).

CAOMECS-grafted corneas showed a positive staining of CPR subunits B5, B1 and B2, corroborating the results shown in Fig. 4, where a higher expression of these subunits can be seen in CAOMECS compared to IPR subunits. The expression of CPR subunits in CAOMECS-grafted cornea (Fig. 7, GE) was higher compared to CPR subunits expression in LSCD sham corneas. The three CPR subunits stained weakly in LSCD sham corneas that were not grafted (Fig. 7, SE B5, SE B1 and SE B2).

Morphometric analysis was performed to quantify fluorescent intensity in corneal tissue sections stained with B5 subunits of CRP. Fig. 8 shows that fluorescent intensity was significantly higher in CAOMECS-grafted cornea than in sham corneas (GE vs SE $p < 0.001$), supporting the observation that CAOMECS grafting regenerated corneal epithelium with epithelial cells that presented higher levels of constitutive proteasome.

4. Discussion

The ubiquitin proteasome pathway has been a very active area of research for at least 10–15 years. Proteasomes have been studied by many investigators in many different organ systems. Even though progress in understanding the proteasome system in other major

organs has been rapid, there have been very few published studies related to proteasome function in the anatomical structures of the eye.

The ubiquitin proteasome pathway is known to be the major proteolytic pathway in the cell and is responsible for the degradation and removal of damaged unwanted proteins. Our present study investigates changes in the proteasome population in healthy, normal corneal epithelial cells compared to corneal epithelial cells present in an experimental model of LSCD. To our knowledge, the only reports investigating proteasome in CEC have focused on the immunoproteasome (IPR) as a key player in ocular surface diseases [22,23], even though IPR represents only a small percentage of the proteasome population in the absence of inflammation. CPR, the most abundant proteasome complex, is the key organelle responsible for degradation and clearance of abnormal or damaged proteins tagged with ubiquitin or with other ubiquitin-like modifier. CPR is also the least stable proteasome in conditions of stress [24–26], so the CPR is the first proteasome to be impaired after cell injury. Immunoproteasomes (IPR's) are highly expressed after exposure of most cells to high levels of cytokines. The three catalytic beta subunits of CPR, B5, B1 and B2 are replaced by homologous IPR subunits, B1i (or LMP2), B2i (or LMP10, MECL1, and B5i (or LMP7) [25]. Due to their distinct peptidase sites, the IPR catalytic subunits cleave proteins in a manner different than CPR subunits. IPR subunits generate peptides capable of binding to MHC class I molecules, thereby serving an important role in antigen presentation [26–28].

Using our rabbit model of LSCD, CEC were collected, lysed and assayed for characterization of the proteasome population 3 months after limbectomy [11]. All three activities of CPR (Chymotrypsin-like, Trypsin-like and Caspase-like activities) were significantly decreased in LSCD-diseased CEC compared to healthy CEC, indicating a decrease in the function of the constitutive ubiquitin proteasome pathway when LSCD is present. It is well known that failure of CPR to clear the ubiquitinated proteins impairs the process of protein quality control. This results in the accumulation of abnormal proteins within the cell, which causes abnormal cell homeostasis and other impairments in cell function. In our study, rabbits with LSCD developed variable amounts of fibrovascular tissue or pannus tissue (PT) that significantly contributed to corneal opacification [11]. PT was dissected and proteasome chymotrypsin-like activity was assayed. Our results showed a significant decrease of CPR chymotrypsin-like activity in pannus tissue compared to normal CEC. PT also had lower levels of CPR activity compared to LSCD-diseased corneal epithelial cells (D-CEC) with no observable pannus tissue. These results indicate that CPR activity is most severely impaired when PT growth occurs.

A quantitative analysis of CPR subunits was performed to assess the expression of CPR catalytic beta subunits B5, B2 and B1. Results showed that D-CEC expressed low levels of these catalytic beta subunits, and higher levels of IPR subunits B5i, B2i and B1i. In addition, the ratio of densitometric measurements for each IPR subunit compared to its constitutive CPR subunits (IPR/CPR) showed higher levels of IPR subunits in diseased CEC; this indicated that D-CEC were characterized by a preferential expression of the IPR and that, following limbectomy for experimental creation of LSCD, there was a shift in the proteasome population toward IPR formation. The histological analysis also confirmed that

CPR subunit expression was positive in H-CEC and negative in D-CEC. Conversely, IPR subunit expression was negative in H-CEC and positive in D-CEC. CPR failure has been extensively reported to cause significant accumulation of altered and aggregated proteins, thus leading to severe cellular disorders, e.g., in the brain (neuro-degenerative diseases [29]), the liver (Mallory Denk bodies [30]) and cornea (cataract [31]). Bardag-Gorce et al. have previously demonstrated the shift from CPR to IPR in proteasome population and the consequent MDB formation in the liver cells [30]. In addition, Bardag-Gorce et al. have demonstrated that decreased CPR activity in alcoholic liver disease was not necessarily caused by proteasome inhibition, but rather a post-translational modification of proteasome subunits that obstructs CPR assembly [1]. Therefore, whether it is hepatic or corneal tissue, proteasome activity shifting from CPR to IPR one is similar in these two tissues, and can occur as a result of either alcoholic or LSCD insults.

In a previous study [11], CAOMECS was shown to successfully regenerate the ocular surface of rabbit with LSCD and to significantly reduce neovascularization. However, the mechanism of action by which CAOMECS regenerates cornea is not yet fully defined. We proposed that CAOMECS regenerates cornea by populating the ocular surface with healthy epithelial cells containing functional CPR. Autologous oral mucosa was biopsied, and epithelial cells were isolated and cultured to engineer CAOMECS grafts. The present study indicates that CAOMECS proteasomal Chymotrypsin-like activity—the major activity in CPR—was significantly higher compared to healthy and LSCD-diseased CEC. We did not find any significant difference between CAOMECS and H-CEC in Trypsin-like and Caspase-like activities, which confirmed that CAOMECS contains active CPR. In addition, the quantitative analysis showed that CAOMECS contained low levels of IPR subunits when compared to the LSCD-diseased CEC.

We also used immunofluorescent staining analysis of corneal tissue sections to assess the distribution of proteasomes in CAOMECS-grafted corneas in comparison to healthy and LSCD-diseased non-grafted corneas. Healthy corneas showed CPR subunit expression in all corneal epithelial cells, including the basal and supra-basal cells. LSCD sham corneas that were not grafted with CAOMECS showed very weak expression of CPR subunits. CAOMECS-grafted cornea epithelial cells stained positive for CPR subunits, corroborating the results from CAOMECS quantitative analysis prior to grafting. However, the three CPR subunits tend to be differently distributed in CAOMECS-grafted corneas as compared to healthy corneas because they were mainly positive in apical cells. It is possible that, 6 months after CAOMECS grafting, a wound healing process is still occurring, which causes reduced staining for CPR subunits. Other mechanisms such as inflammation and angiogenesis are involved in the wound healing process. Both mechanisms are respectively controlled by the master transcriptional factors NFkB and Hif-1 alpha/VEGF, and both transcriptional factors are specifically regulated by the proteasome CPR. These results suggest that, to a certain extent, CAOMECS grafts provided healthy epithelial cells with functional CPR for the ocular surface, reduced IPR formation, and possibly more rapid and improved recovery of the ocular surface.

Many cells and tissues express low amounts of IPR subunits in the absence of cytokine stimulation — or inflammation. High expression of IPR is usually found in macrophages, T

cells and B cells, and requires induction by IFNs or TNF alpha [32–34]. However, it is not certain if IPR over-expression is required to counteract the pathology, or is a symptom of the disease. IPR has been associated with inflammatory diseases [35], cancer and tumor growth regulation [36], and neurodegenerative diseases [37]. The literature reflects the general idea that IPR is involved in inflammatory conditions associated with oxidative stress. IPR is induced to not only activate cytokine productions, but also to increase oxidized and altered protein clearance [38–40]. It is possible that the higher proteolytic capacity of IPR is required to maintain cell viability in physiologically adverse situations. The studies of IPR activity highlight other functions of the IPR in addition to generating peptides for antigen presentation. However, it is still not known how the IPR specifically processes proteins designated by the ubiquitination system for proteolysis and protein quality control. Does IPR process oxidized proteins blindly as does the 20S proteasome without the need for the ubiquitination system? Or is there a different ubiquitin-like system that assists the IPR with other functions? Many questions are still unanswered.

The literature reports that the mechanism of action of oral mucosal epithelial cells resides in the renewal capacity of its basal cells positive for the progenitor stem cell marker deltaNp63 [11,17]. It is not known whether the grafted cells replace or revive progenitor stem cells of the host by secreting growth factors by chemotactic stimuli or by other mechanisms. We propose that the grafted oral mucosa epithelial cells seed a functional ubiquitin proteasome pathway that enhances unwanted protein clearance and transcriptional factors process. A functional CPR will not only reduce NFkB activation and reduce inflammation, but also improve VEGF clearance and thus block angiogenesis and neovascularization of central cornea [41]. Developing proteasome CPR activation and/or IPR inhibition in eye research for LSCD treatment may reduce excessive inflammation and reduce or resolve the negative consequences of LSCD.

5. Conclusion

CAOMECS grafting successfully reconstructed the ocular surface and seeded healthy epithelial cells with low levels of IPR and a functional CPR. The results of our study suggest that a functional ubiquitin proteasome pathway could be the primary therapeutic mechanism of action that CAOMECS grafts provide to an ocular surface with LSCD.

Acknowledgements

This research was supported by Emmaus Life Sciences Inc., Torrance CA 90503 USA.

References

- [1]. Bardag-Gorce F, Venkatesh R, Li J, French BA, French SW. Hyper-phosphorylation of rat liver proteasome subunits: the effects of ethanol and okadaic acid are compared. *Life Sci* 2004;75(5): 585–97. [PubMed: 15158368]
- [2]. Gomes AV, Zong C, Edmondson RD, Li X, Stefani E, Zhang J, et al. Mapping the murine cardiac 26S proteasome complexes. *Circulat Res* 2006;99(4):362–71. [PubMed: 16857966]
- [3]. Brown M, Cohen J, Arun P, Chen Z, Van Waes C. NF-kappaB in carcinoma therapy and prevention. *Expert Opin Ther Targets* 2008;12(9):1109–22. [PubMed: 18694378]

- [4]. Vadlapatla RK, Vadlapudi AD, Mitra AK. Hypoxia-inducible factor-1 (HIF-1): a potential target for intervention in ocular neovascular diseases. *Curr Drug Targets* 2013;14(8):919–35. [PubMed: 23701276]
- [5]. Pickart CM, Cohen RE. Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol* 2004;5(3):177–87. [PubMed: 14990998]
- [6]. Yang Y, Yu X. Regulation of apoptosis: the ubiquitous way. *FASEB J Off. Publ Fed Am Soc Exp Biol* 2003;17(8):790–9.
- [7]. Rechsteiner M, Hill CP. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. *Trends Cell Biol* 2005;15(1): 27–33. [PubMed: 15653075]
- [8]. Chen P, Yin H, Wang Y, Wang Y, Xie L. Inhibition of VEGF expression and corneal neovascularization by shRNA targeting HIF-1 α in a mouse model of closed eye contact lens wear. *Mol Vis* 2012;18:864e73. [PubMed: 22511848]
- [9]. Shaw JP, Chuang N, Yee H, Shamamian P. Polymorphonuclear neutrophils promote rFGF-2-induced angiogenesis in vivo. *J Surg Res* 2003;109(1):37–42. [PubMed: 12591233]
- [10]. Berger EA, McClellan SA, Vistisen KS, Hazlett LD. HIF-1 α is Essential for effective PMN bacterial killing, antimicrobial peptide production and apoptosis in *Pseudomonas aeruginosa* keratitis. *PLoS Pathog* 2013;9(7): e1003457. [PubMed: 23874197]
- [11]. Bardag-Gorce F, Oliva J, Wood A, Hoft R, Pan D, Thropay J, et al. Carrier-free cultured autologous oral mucosa epithelial cell sheet (CAOMECS) for corneal epithelium reconstruction: a histological study. *Ocular Surf* 2015;13(2): 150–63.
- [12]. Sonoda KH, Nakao S, Nakamura T, Oshima T, Qiao H, Hisatomi T, et al. Cellular events in the normal and inflamed cornea. *Cornea* 2005;24(8 Suppl):S50–4. [PubMed: 16227824]
- [13]. Gao N, Kumar A, Yu FS. Matrix Metalloproteinase-13 as a target for suppressing corneal ulceration caused by *Pseudomonas aeruginosa* infection. *J Infect Dis* 2015;212(1):116–27. [PubMed: 25589337]
- [14]. Ma P, Wang Z, Pflugfelder SC, Li DQ. Toll-like receptors mediate induction of peptidoglycan recognition proteins in human corneal epithelial cells. *Exp Eye Res* 2010;90(1):130–6. [PubMed: 19799901]
- [15]. Alves M, Calegari VC, Cunha DA, Saad MJ, Velloso LA, Rocha EM. Increased expression of advanced glycation end-products and their receptor, and activation of nuclear factor kappa-B in lacrimal glands of diabetic rats. *Diabetologia* 2005;48(12):2675–81. [PubMed: 16283249]
- [16]. El-Hashim AZ, Renno WM, Abduo HT, Jaffal SM, Akhtar S, Benter IF. Effect of inhibition of the ubiquitin-proteasome-system and I κ B kinase on airway inflammation and hyperresponsiveness in a murine model of asthma. *Int J Immunopathol Pharmacol* 2011;24(1): 33–42.
- [17]. Burillon C, Huot L, Justin V, Nataf S, Chapuis F, Decullier E, et al. Cultured autologous oral mucosal epithelial cell sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. *Investig. Ophthalmol Vis Sci* 2012;53(3):1325–31. [PubMed: 22064987]
- [18]. Utheim TP, Utheim ØA Khan QE, Sehic A. Culture of oral mucosal epithelial cells for the purpose of treating limbal stem cell deficiency. *J Funct Biomater* 2016;7(1). pii: E5. [PubMed: 26938569]
- [19]. Bardag-Gorce F, Oliva J, Wood A, Niihara H, Makalinao A, Sabino S, et al. Microarray analysis of oral mucosal epithelial cell sheet. *Tissue Eng Regen Med* 2013;10(6):362–70.
- [20]. Bousquet-Dubouch MP, Nguen S, Bouyssié D, Burlet-Schiltz O, French SW, Monsarrat B, et al. Chronic ethanol feeding affects proteasome-interacting proteins. *Proteomics* 2009;9(13): 3609e22. [PubMed: 19609968]
- [21]. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008;454(7203):436–44. [PubMed: 18650914]
- [22]. Ferrington DA, Roehrich H, Chang AA, Huang CW, Maldonado M, Bratten W, et al. Corneal wound healing is compromised by immunoproteasome deficiency. *PLoS One* 2013;8(1):e54347. [PubMed: 23365662]

- [23]. Lomako J, Lomako WM, Carothers Carraway CA, Carraway KL. Regulation of the membrane mucin Muc4 in corneal epithelial cells by proteosomal degradation and TGF-beta. *J Cell Physiol* 2010;223(1):209–14. [PubMed: 20054827]
- [24]. Bardag-Gorce F, Oliva J, Lin A, Li J, French BA, French SW. Proteasome inhibitor up regulates liver antioxidative enzymes in rat model of alcoholic liver disease. *Exp Mol Pathol* 2011;90(1):123–30. [PubMed: 21036165]
- [25]. Klotzel PM. Antigen processing by the proteasome. *Nat Rev Mol Cell Biol* 2002;3:179–87.
- [26]. Gaczynska M, Rock KL, Goldberg AL. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* 1993;365(6443):264–7. [PubMed: 8396732]
- [27]. Kincaid EZ, Che JW, York I, Escobar H, Reyes-Vargas E, Delgado JC, et al. Mice completely lacking immunoproteasomes show major changes in antigen presentation. *Nat Immunol* 2011;13(2):129–35. [PubMed: 22197977]
- [28]. Rock KL, Goldberg AL. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu Rev Immunol* 1999;17:739–79. [PubMed: 10358773]
- [29]. Le W Role of iron in UPS impairment model of Parkinson's disease. *Park Relat Disord* 2014;1(20 Suppl):S158–61.
- [30]. Bardag-Gorce F, Oliva J, Li J, French BA, French SW. SAME prevents the induction of the immunoproteasome and preserves the 26S proteasome in the DDC-induced MDB mouse model. *Exp Mol Pathol* 2010;88(3):353–62. [PubMed: 20223233]
- [31]. Wride MA, Geatrell J, Guggenheim JA. Proteases in eye development and disease. *Birth Defects Res Part C, Embryo Today Rev* 2006;78(1):90–105.
- [32]. Seifert U, Bialy LP, Ebstein F, Bech-Otschir D, Voigt A, Schröter F, et al. Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell* 2010;142(4):613–24. [PubMed: 20723761]
- [33]. Shin EC, Seifert U, Kato T, Rice CM, Feinstone SM, Klotzel PM, et al. Virus-induced type I IFN stimulates generation of immunoproteasomes at the site of infection. *J Clin Investigat* 2006;116(11):3006–14.
- [34]. Barton LF, Cruz M, Rangwala R, Deepe GS, Jr, Monaco JJ. Regulation of immunoproteasome subunit expression in vivo following pathogenic fungal infection. *J Immunol Baltim Md* 1950) 2002;169(6):3046–52.
- [35]. Groettrup M, Kraft R, Kostka S, Standera S, Stohwasser R, Klotzel PM. A third interferon-gamma-induced subunit exchange in the 20S proteasome. *Eur J Immunol* 1996;26(4):863–9. [PubMed: 8625980]
- [36]. Ferrington DA, Gregerson DS. Immunoproteasomes: structure, function, and antigen presentation. *Prog Mol Biol Transl Sci* 2012;109:75–112. [PubMed: 22727420]
- [37]. Díaz-Hernández M, Hernández F, Martín-Aparicio E, Gómez-Ramos P, Morán MA, Castaño JG, et al. Neuronal induction of the immunoproteasome in Huntington's disease. *J Neurosci Off. J Soc Neurosci* 2003;23(37):11653–61.
- [38]. Krüger E, Klotzel PM. Immunoproteasomes at the interface of innate and adaptive immune responses: two faces of one enzyme. *Curr Opin Immunol* 2012;24(1):77–83. [PubMed: 22296715]
- [39]. Pickering Andrew M, Davies Kelvin JA. Differential roles of proteasome and immunoproteasome regulators Pa28ab, Pa28g and Pa200 in the degradation of oxidized proteins. *Archives Biochem Biophys* 2012;523(2):181–90.
- [40]. Johnston-Carey HK, Pomatto LC, Davies KJ. The immunoproteasome in oxidative stress, aging, and disease. *Crit Rev Biochem Mol Biol* 2016;20:1–14.
- [41]. Liu S, Lai L, Zuo Q, Dai F, Wu L, Wang Y, et al. PKA turnover by the REGγ-proteasome modulates FoxO1 cellular activity and VEGF-induced angiogenesis. *J Mol Cell Cardiol* 2014;72:28–38. [PubMed: 24560667]

Fig. 1.

A, B and C show respectively healthy rabbit cornea, 3 months after lamellar limbectomy without (B) and with (C) fluorescein staining. LSCD was stable and the cornea looked opaque with blood vessel all over the central cornea. D shows proteasome chymotrypsin-like activity in healthy control (H-CEC), LSCD-diseased (D-CEC) and in fibrovascular pannus tissue (PT). Note that proteasome activity was significantly reduced in D-CEC and pannus tissue (PT) showed the lowest level of proteasome activity.

Fig. 2.

A is rabbit harvested CAOMECS. B, C, D, and E are immunofluorescent staining, respectively with antibodies against DeltaN-p63, Ki67, CPR beta5 subunits, and CPR beta1. Note that basal cells were positive for DeltaN-p63, a marker for progenitor stem cell, and for Ki-67, a proliferation marker. CPR subunits stained both the nuclei and cytoplasm of basal and suprabasal cells.

Fig. 3.

Proteasomes CPR and IPR catalytic beta subunits immunofluorescent staining is in green. Red is nuclear staining. A, B, and C are rabbit healthy corneas stained with CPR subunits B5, B1 and B2, respectively. D, E, and F are rabbit LSCD-diseased corneas stained with CPR subunits B5, B1 and B2, respectively. G, H, and I are rabbit healthy corneas stained with IPR subunits B5i, B1i and B2i, respectively. J, K, and L are rabbit LSCD-diseased corneas stained with IPR subunits B5i, B1i and B2i, respectively. Note that the three CPR subunits expression was decreased, while the inducible IPR subunits expression was increased in LSCD diseased corneas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Proteasome activities were found to be decreased in LSCD-induced diseased corneal epithelial cells (D-CEC) compared to healthy corneal epithelial cells H-CEC and to engineered CAOMECS (A) (mean $\pm$ SEM $n = 3$). B is a schematic illustration of immunoproteasome (IPR) assembly after exposure of most cells to high levels of cytokines. C is western blot analysis comparing IPR to CPR catalytic subunits. D-CEC showed significantly high levels of IPR subunits compared to H-CEC and to CAOMECS. The results also showed that CAOMECS CPR subunit levels were significantly higher than D-CEC and were almost similar to H-CEC (Mean $\pm$ SEM $n = 3$).

D-CEC vs. H-CEC	D-CEC vs. CAOMECS	H-CEC vs. CAOMECS
Beta5i/Beta5: t -test, $p < 0.001$	Beta5i/Beta5: t -test, $p < 0.001$	Beta5i/Beta5: t -test, $p = 0.835$
Beta2i/Beta2: t -test, $p < 0.001$	Beta2i/Beta2: t -test, $p < 0.001$	Beta2i/Beta2: t -test, $p = 0.071$
Beta1i/Beta1: t -test, $p = 0.17$	Beta1i/Beta1: t -test, $p = 0.05$	Beta1i/Beta1: t -test, $p = 0.079$

Fig. 5.

A: Proteasome subunits analysis of normal oral mucosa tissue showed localization in basal and suprabasal epithelial cells (white arrow). There was no noticeable difference between IPR and CPR staining in normal oral mucosa tissue. B is healthy conjunctival (H-Cj) and diseases (D-Cj) stained with IPR subunit B5i and CPR subunit B5. Note that conjunctiva weakly stained for B5, especially in D-Cj.

Fig. 6.

A is rabbit LSCD-diseased cornea showing high vascularization and opacification. B and C are central cornea cells collected by IC and H&E stained, respectively, 1 month and 3 months after limbectomy. D is healthy corneal cells. Note that LSCD cornea cells contained small nuclei (C), indicating the presence of neutrophil cells (C), when compared to normal epithelial cells with bigger nuclei and a rectangular cellular shape (D) (Magnification 40 $\times$). E is a semi-quantitative analysis of phosphorylated NFkB showing NFkB activation and IκB level as an inhibitory protein of NFkB. Note that NFkB activation was high in D-CEC while total IκB was significantly low as compared to healthy H-CEC.

Fig. 7.

Proteasome subunits immunofluorescent staining (green) analysis of cornea tissue sections (red is nuclear staining with propidium iodide, 40 $\times$): NE: Normal Eye; GE: Grafted Eye; SE: Sham Cornea. Note that CAOMECS grafted cornea tissue section showed a mostly apical expression of CPR subunits (B5, B1 and B2). The sham cornea tissue section weakly expressed CPR subunit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Digital fluorescent intensity quantification of CPR B5 stained NE: Normal Eye; GE: CAOMECS grafted Eye; and SE: Sham cornea. Note that fluorescent intensity was significantly higher in CAOMECS grafted cornea (GE) compared to sham cornea (SE) ($p < 0.001$).