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Stratified corneal limbal epithelial cells are protected from UVB-induced apoptosis by elevated extracellular K⁺

Mark P. Schotanus¹, Leah R. Koetje¹, Rachel E. Van Dyken¹, and John L. Ubels^{1,2,*}

¹Department of Biology, Calvin College, 3201 Burton St. SE, Grand Rapids, Michigan 49546 USA

²Department of Ophthalmology, Wayne State University School of Medicine, Detroit, Michigan, USA

Abstract

The goal of this study was to determine whether elevated [K⁺] protects stratified corneal epithelial cells from entering apoptosis following exposure to ambient levels of UVB radiation. Human corneal-limbal epithelial (HCLE) cells were stratified to form multilayered constructs in culture. The cells were exposed to UVB doses of 100 – 250 mJ/cm² followed by incubation in medium with 5.5 – 100 mM K⁺. The protective effect of K⁺ was determined by measuring the caspase-3 and -8 activity and TUNEL staining of the stratified HCLE constructs. In response to UVB exposure, activation of apoptotic pathways peaked at 24 hours. Caspase-8 in stratified cells was activated by exposure to UVB at 100 – 250 mJ/cm², and activity was significantly reduced in response to 50 or 100 mM K⁺. Caspase-3 was activated in the stratified cells in response to 100 – 250 mJ/cm² UVB and showed a significant reduction in activity in response to 25, 50 or 100 mM K⁺. DNA fragmentation, as indicated by TUNEL staining, was elevated after exposure to 200 mJ/cm² UVB, and decreased following incubation with 25 – 100 mM K⁺. These results show that in a culture system that models the intact corneal epithelium elevated extracellular K⁺ can reduce UVB-induced apoptosis which is believed to be initiated by loss of K⁺ from cells. This is the basis of damage to the corneal epithelium caused by UVB exposure. Based on these observations it is suggested that the relatively high K⁺ concentration in tears (20–25 mM) may play a role in protecting the corneal epithelium from ambient UVB radiation.

Keywords

apoptosis; caspase-3; caspase-8; cornea; corneal epithelium; tear film; potassium; ultraviolet

1. Introduction

In vitro apoptosis caused by chemical agents, serum withdrawal and UV radiation in several cell types, including lymphocytes, neurons, smooth muscle and corneal epithelial cells, involves activation of K⁺ channels and loss of intracellular K⁺ (Bortner et al., 1997; Bortner et al., 2007; Hughes et al., 1997; Krick et al., 2001; Lu et al., 2003; Redman et al., 2007; Wang et al., 2003; Yu et al., 1997). It is believed that this loss of K⁺ from the cells is an

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*Corresponding author: John L. Ubels, Department of Biology, Calvin College, 3201, Burton St. SE, Grand Rapids, MI, 49546 USA, Telephone: 616-526-6219, Fax: 616-526-6501, jubels@calvin.edu.

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important factor in the activation of apoptotic pathways, since incubation of lymphocytes and neuronal cells in extracellular K^+ concentrations ($[K^+]_o$) at 100 mM or 25 mM, respectively, inhibits apoptosis (Hughes et al., 1997; Yu et al., 1997). These observations are intriguing because the ocular surface epithelium, which is exposed to ambient outdoor UVB radiation, is continually bathed in tear fluid with a K^+ concentration ($[K^+]$) of 20–25 mM (Botelho et al., 1973; Rismondo et al., 1989).

The studies cited above led to our overall hypothesis that the relatively high $[K^+]$ in tears reduces the loss of intracellular K^+ and inhibits the activation of apoptotic mechanisms when ocular surface cells are exposed to ambient UVB radiation. In support of this hypothesis, we have recently reported that exposure of human corneal limbal epithelial (HCLE) cells (Gipson et al., 2003) to UVB at levels relevant to ambient UVB exposure activates K^+ channels (Singleton et al., 2009; Ubels et al., 2010) which leads to loss of intracellular K^+ (Ubels et al., 2011). The loss of $[K^+]_i$ causes apoptosis by activating the initiator caspase-8 and the effector caspase-3, leading to DNA fragmentation (Singleton et al., 2009; Ubels et al., 2010). Incubation of the cells in elevated extracellular K^+ (K^+_o) at concentrations similar to those found in tears, or blocking K^+ channels, reduces UVB-activated K^+ currents, prevents loss of K^+ from HCLE cells by reducing the K^+ concentration gradient across the cell membrane and ultimately inhibits UVB induced apoptosis (Singleton et al., 2009; Ubels et al., 2010, 2011).

The studies cited above were conducted using HCLE cells in monolayer culture which was necessary for technical reasons, including analyses by flow cytometry and patch-clamp recording. Under appropriate conditions, this cell line can be induced to differentiate and stratify without exposure to an air interface. The stratified cells express mucins, galectin-3, and cell junction proteins (Argüeso et al., 2006, 2009; Gipson et al., 2003), and they establish an epithelial barrier closely modeling an in vivo corneal epithelium (Argüeso et al., 2009; Lim et al., 2009). The purpose of the present study was to perform experiments on UVB-induced apoptosis in stratified HCLE cells to determine whether increased $[K^+]_o$ would also have a protective effect under these conditions.

2. Materials and methods

2.1. Cell Culture

HCLE cells were plated in 6-well plates, trans-well inserts or chamber slides in Keratinocyte-Serum Free Medium (K-SFM, Invitrogen, Carlsbad, CA) and grown to confluence in a 1:1 mixture of K-SFM and low calcium DMEM/F12 with 0.3 mM Ca^{2+} , as described by Gipson et al. (2003). The cells were then placed in DMEM/F12 medium (Mediatech, Manassas, VA) with 1 mM Ca^{2+} , 10% FCS (Hyclone Laboratories, Logan, UT) and 10 ng/ml EGF for 4–7 days until stratified.

2.2. Exposure to UVB at 302 nm

The medium was changed to Hank's Balanced Salt Solution (HBSS) without phenol red (Invitrogen) and a UVM-57 lamp (Ultraviolet Products, Upland, CA) was placed 16.5 cm above the cells. UVB intensity was measured with a Solarmeter Model 6.2 (Solartech, Inc., Harrison Twp., MI) and the time of exposure was controlled to achieve doses ranging from 100–250 mJ/cm². These levels of UVB are relevant to levels of exposure in less than 1 hour at mid day at 40° north latitude (Singleton et al., 2009).

2.3. Exposure to elevated concentrations of extracellular K^+

A custom-made K-SFM with 100 mM K^+ and reduced $[Na^+]$ to maintain osmolarity at 290 mOsm/l (Invitrogen) was mixed with standard K-SFM medium (5.5 mM K^+) to achieve the

desired $[K^+]$ of 25–100 mM. After exposure to UVB, cells were incubated for 24–30 hours in medium containing 5.5, 25, 50 or 100 mM K^+ . In the caspase and barrier function experiments control cells, not exposed to UVB, were incubated in K-SFM with the standard 5.5 mM K^+ or with 100 mM K^+ to insure that any changes observed were not due simply to elevated $[K^+]_o$.

2.4. Caspase activation

Stratified HCLE cells in 6-well plates were exposed to UVB and incubated for 24 hours with 5.5 – 100 mM $[K^+]_o$. The cells were removed from the wells by incubation in TrypLE Express for 10–12 minutes (Invitrogen) followed by scraping. Caspase-8 activity was measured using a fluorometric protease assay kit, and caspase-3 activity with a colorimetric protease assay kit (Invitrogen), as previously described (Singleton et al., 2009). Protein was measured using the Bio-Rad assay and enzyme activity was expressed as relative fluorescence units or optical density units/mg protein.

2.5. TUNEL assay

Stratified cells on 8-chamber Lab-Tek slides (NalgeNunc International, Rochester, NY) were exposed to 200 mJ/cm² UVB, and incubated for 24 hr in medium with 5.5 – 100 mM K^+ . Control cells were not exposed to UVB and were incubated in medium with 5.5 mM K^+ . In each experiment cells in 4 chambers were subjected to each condition, and the experiment was repeated 3 times. DNA damage was visualized using an *In Situ* Cell Death Detection Kit - TMR Red (Roche Applied Science, Indianapolis, IN), according to manufacturer's instructions, and the nuclei were stained with DAPI using Prolong Gold Antifade Reagent (Invitrogen). Cells were imaged using an Axiovert 200 imaging system with AxioVision software (Carl Zeiss MicroImaging LLC, Thornwood, NY). The total number of apoptotic cells in the central 0.4 mm² of each chamber was counted using ImageJ software (National Institutes of Health, Bethesda, MD).

3. Results

3.1. Caspase-8 activation

In initial experiments caspase-8 activity was measured 6 hours after exposure to UVB at 100–250 mJ/cm². In contrast to monolayer cells (Singleton et al., 2009) caspase-8 was activated only 1.67-fold compared to control at this time point. Caspase-8 was activated 3-fold 24 hours after exposure to UVB. Therefore all subsequent experiments, including studies of caspase-3 activation and TUNEL staining, were conducted 24 hours after UVB exposure. The level of activation was not dose-related over a range of 100–250 mJ/cm² (Fig. 1). When cells exposed to 100 or 150 mJ/cm² UVB were incubated in medium with 25–100 mM K^+ during this 24 hour period, caspase-8 activation was attenuated in the presence of 50 or 100 mM K^+ . After exposure to 250 mJ/cm² UVB, 100 mM K^+ was effective in reducing caspase-8 activation.

3.2. Caspase-3 activation

Exposure to 100 – 250 mJ/cm² UVB also activated caspase-3 in stratified HCLE cells without a clear effect of dose (Fig. 2). Incubation of cells exposed to 100 mJ/cm² UVB in medium with 25 – 100mM K^+ resulted in a significant reduction of UVB-induced caspase-3 activity. After exposure to 150 mJ/cm² UVB, a trend toward reduction of caspase-3 activation was observed in medium with 25 or 50 mM K^+ , while 100 mM K^+ was effective after treatment with all three doses of UVB.

3.3. TUNEL assay

Because a UVB dose-response was not observed in the caspase experiments, the TUNEL assay for DNA damage was conducted only at a UVB dose of 200 mJ/cm², which is intermediate to the doses of 150 and 250 mJ/cm² used in the caspase assays. A low number of apoptotic cells was observed in control cultures. DNA damage increased markedly 24 hours after UVB treatment and incubation in medium with 5.5 mM K⁺. Incubation with 25 or 50 mM K⁺ following UVB exposure significantly reduced the number of TUNEL-stained cells, while staining was not different than control levels in UVB-treated cultures incubated in medium with 100 mM K⁺ (Figs. 3 and 4).

4. Discussion

Exposure to UVB had the expected effects on apoptosis in stratified HCLE cells, based on results of previous monolayer culture studies (Singleton et al., 2009; Ubels et al., 2010), activating caspases -8 and -3 and leading to DNA degradation. The evidence for apoptosis in the present study is consistent with, and extends the observations of others who have studied UV-induced apoptosis in the cornea (Ren and Wilson, 1994; Podskochy et al., 2000; Lu et al., 2003; Shimmura et al., 2004).

Among the many pathways involved in apoptosis, there is a direct link in the pathway from caspase-8 activation to activation of caspase-3 which then activates mechanisms in the nucleus that damage DNA. In our previous work on monolayer HCLE cells we showed that this pathway was more strongly activated by UVB, and better protected by high [K⁺]_o, than apoptotic pathways mediated by the mitochondria and caspase-9 (Singleton et al., 2009). The pathway investigated in the present study has high relevance for UVB-induced apoptosis since there is evidence that caspase-8 can be activated in the death inducing signaling complex via Fas ligand-independent activation of Fas by UV (Aragane et al., 1998; Kulms et al., 1999).

The effects of UVB on stratified cells differed quantitatively from its effects on monolayer HCLE cells. Activation of caspases by UVB peaked at 24 hours in stratified cultures compared to 6 hours in monolayer cultures (Singleton et al., 2009). This observation is in agreement with Clarke et al. (1990) who exposed rabbit eyes in vivo to 50 mJ/cm² UVB. They showed by scanning electron microscopy that UVB-induced exfoliation of superficial corneal epithelial cells increases markedly by 24 hours and peaks at 48 hours post-exposure. The dependence of the degree of activation of caspases-8 and -3 on UVB dose was not as strong in stratified cells as in monolayer cells. The reason for this lack of a detectable dose response is unknown at this time; however, several reasonable hypotheses may be proposed. First, a difference between monolayer and stratified cells was not unexpected since we have previously reported that more highly differentiated, stratified corneal epithelial cells in culture are less susceptible to damage in toxicologic studies than monolayer cultures (Hoffman et al., 2007; Lim et al., 2009).

Second, the effects of UVB on superficial and deeper cell layers may not be uniform. Corneal epithelial cells are reported to contain several components that protect the cornea. The epithelial cells contain tryptophan, which absorbs UVB, and ascorbate, which also absorbs UVB and can protect cells from UV-generated reactive oxygen species (Kolozvari et al., 2002; Ringvold, 1998). In a stratified model these mechanisms may protect deeper cells due to absorption of UVB by more superficial cells which are directly exposed to UV. When total caspase activity is measured in all of the cells of a stratified construct, this degree of protection may mask the enzyme activation in superficial layers making it more difficult to detect a dose-response. In contrast, monolayer cells are exposed uniformly to

UVB so that caspases are activated equally in all cells, allowing greater sensitivity and precision in the caspase assay.

Third, we have recently reported that after activation of K^+ channels in HCLE cells by UVB, intracellular K^+ levels are rapidly restored by Na^+/K^+ ATPase activity which may be an intrinsic mechanism for protection from UVB (Ubels et al., 2011). Using the same argument proposed above, if deeper cells are partially protected from UVB, this ion pump mechanism may be more effective, again leading to reduced apoptosis in deeper cell layers and therefore less sensitivity in detection of caspase activation in the superficial cells.

In our previous studies of HCLE cells grown as undifferentiated monolayers, 25 mM K^+_o , which is near the concentration found in tears, prevented UVB-induced loss of K^+ from the cells and consistently offered at least partial inhibition of apoptosis (Singleton et al., 2009; Ubels et al., 2010, 2011). In the present study the protective effect of increasing K^+_o concentrations on any parameter tended to be related to the dose of UVB but 25 mM K^+_o was not consistently effective. Specifically, 25 mM K^+_o did not inhibit caspase-8 activation at all, while 50 mM K^+_o was effective in cells exposed to 100 or 150 mJ/cm² UVB. In contrast, 25 mM K^+_o reduced caspase-3 activation in cells exposed to 100 mJ/cm² UVB but was not effective at higher UVB doses. Elevated $[K^+_o]$ was effective in reducing UVB-induced DNA damage since TUNEL staining of cells exposed 200 mJ/cm² UVB was significantly reduced in the presence of 25 and 50 mM K^+_o and remained at control levels in 100 mM K^+_o . It should be noted that the possible protection of deeper cells from UVB as discussed above, would not interfere with detection by microscopy of TUNEL staining in superficial cells.

If 25 mM K^+_o was effective in inhibiting DNA damage in stratified HCLE cells, why was inhibition of caspases-8 and -3 by elevated $[K^+_o]$ less effective than expected? This may be related to the well-known fact that amplification occurs along signaling cascades. Amplification in the caspase-8, caspase-3 pathway includes a positive feedback loop via caspase-6 (Slee et al., 1999; Legewie et al., 2006; Wurstle et al., 2010). This suggests that effects of UVB and elevated $[K^+_o]$ that might not be detectable at the initiation point of the pathway, caspase-8, may become detectable at the level of DNA degradation due to amplification. It is also possible that reduction of UVB-induced loss of K^+ from the cells by elevated $[K^+_o]$ affects one or more of the other apoptotic mechanisms that we have not yet investigated. Examples could include inhibition of caspase-6 which is involved in the amplification loop mentioned above or promotion of activity of the apoptosis inhibitor, XAIP (Choi et al., 2007). The complexity of apoptotic pathways and the large number of signaling molecules involved provide ample opportunity for further studies of this problem.

The cornea and tears have several components that can protect the cornea itself, as well as the lens and retina, from UVB. In addition to the presence of tryptophan and ascorbate in the epithelial cells cited above, lactoferrin, which can absorb UV radiation, is present in tears, (Fujihara et al., 2000), and it has recently been reported that tear fluid strongly absorbs UVB (Choy et al., 2011). We also recently showed that after activation of K^+ channels by UVB and loss of over 50% of K^+ from corneal epithelial cells, which occurs within 10 minutes, active transport rapidly restores intracellular K^+ levels by 60 minutes after UVB exposure (Ubels et al., 2011). This recovery does not occur in other cell types, such as lymphocytes, when they are exposed to UV (Arrebola et al., 2006; Bortner et al., 2008), suggesting that this is a protective mechanism in corneal epithelial cells which, unlike most other cells, are normally exposed to UVB.

The role of the long-recognized high K^+ concentration in tears has not been fully elucidated, although Fullard and Wilson (1986) reported that bathing the surface of normal corneas with medium containing 19 mM K^+ reduces sloughing of superficial epithelial cells. The results

of the present study using a model of the intact corneal epithelium are in essential agreement with our previous observations using monolayer HCLE cells. Taken together the data suggest that by reducing loss of K^+ from cells when K^+ channels are activated by UVB, the relatively high K^+ concentration in tears may work together with the other mechanisms cited to protect the cornea from ambient UVB. This may contribute to the control of the normal process of shedding and renewal of superficial epithelial cells (Thoft and Friend, 19), thereby preventing chronic damage to the eye by ambient UVB.

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Highlights

Apoptosis occurs in stratified human corneal epithelial cells exposed to UVB.

UVB-induced caspase-8 activation is inhibited by ≥ 50 mM extracellular K^+ .

Extracellular $K^+ \geq 25$ mM inhibits caspase-3 activation and DNA damage.

The high K^+ in tears (20–25 mM) may help to protect cornea from ambient UVB.

Fig. 1.

UVB activates caspase-8 in stratified HCLE cells. Elevated $[K^+]_o$ reduces caspase-8 activity during a 24 hour incubation following UVB exposure. Within UVB-exposed groups unmarked values differ from marked values and from each other. Marked values do not differ. Controls were not exposed to UVB. 100 mM K^+ has no effect on caspase-8 activity in control cells. (ANOVA and Student-Newman-Keuls test, $p \leq 0.05$, $n=20$)

Fig. 2.

Caspase-3 in stratified HCLE cells is activated 24 hours after exposure to UVB. At the lowest dose, 100 mJ/cm², activation is reduced by incubation with [K⁺]_o as low as 25 mM. *significantly different than all other values. #significantly different than all other values but not from each other. Unmarked values within groups do not differ. 100 mM K⁺ has no effect on caspase-3 activity in control cells. (ANOVA and Student-Newman-Keuls test, $p \leq 0.05$, $n=16$)

Fig. 3. DNA damage in HCLE cells, as detected by TUNEL staining, 24 hours after exposure to UVB. Incubation in elevated $[K^+]_o$ reduces UVB-induced apoptosis. Each image is a mosaic of the central 0.4 cm^2 of a well on an 8-chamber slide. The DAPI stained image shows cell density in a representative chamber. Bar = 1 mm

Fig. 4. UVB-induced TUNEL staining of HCLE cells is significantly reduced by incubation in elevated $[K^+]_o$. Values marked with the same symbol do not differ. (ANOVA and Student-Newman-Keuls test, $p \leq 0.05$, $n=12$)