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Involvement of the Extrinsic and Intrinsic Pathways in Ultraviolet B-Induced Apoptosis of Corneal Epithelial Cells

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Abstract

The goal of this study was to elucidate the pathway by which UVB initiates efflux of K⁺ and subsequently apoptosis in human corneal limbal epithelial (HCLE) cells. The initial focus of the study was on the extrinsic pathway involving Fas. HCLE cells transfected with Fas siRNA were exposed to 80–150 mJ/cm² UVB and incubated in culture medium with 5.5 mM K⁺. Knock down of Fas resulted in limited reduction in UVB-induced caspase-8 and -3 activity. Patch-clamp recordings showed no difference in UVB-induced normalized K⁺ currents between Fas transfected and control cells. Knockdown of caspase-8 had no effect on the activation of caspase-3 following UVB exposure, while a caspase-8 inhibitor completely eliminated UVB activation of caspase-3. This suggests that caspase-8 is a robust enzyme, able to activate caspase-3 via residual caspase-8 present after knockdown, and that caspase-8 is directly involved in the UVB activation of caspase-3. Inhibition of caspase-9 significantly decreased the activation of caspases-8 and -3 in response to UVB. Knockdown of Apaf-1, required for activation of caspase-9, resulted in a significant reduction in UVB-induced activation of caspases-9, -8, and -3. Knockdown of Apaf-1 also inhibited intrinsic and UVB-induced levels of apoptosis, as determined by DNA fragmentation measured by TUNEL assay. In UVB exposed cultures treated with caspase-3 inhibitor, the percentage of apoptotic cells was reduced to control levels, confirming the necessity of caspase-3 activation in DNA fragmentation. The lack of effect of Fas knockdown on K⁺ channel activation, as well as the limited effect on activation of caspases-8 and -3, strongly suggest that Fas and the extrinsic pathway is not of primary importance in the initiation of apoptosis in response to UVB in HCLE cells. Inhibition of caspase-8 and -3 activation following inhibition of caspase-9, as well as reduction in activation of caspases-9, -8, and -3 and DNA fragmentation in response to Apaf-1 knockdown support the conclusion that the intrinsic pathway is more important in UVB-induced apoptosis in HCLE cells.

Keywords

apoptosis; cornea; corneal epithelium; potassium channels; caspase; Apaf-1; tear film

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1. Introduction

Both the cornea and skin are exposed to levels of ambient ultraviolet B (UVB) radiation (280–315 nm) that can damage these tissues. In the epidermis “sunburn cells” that have been overexposed to UVB go into apoptosis and are eliminated to prevent these damaged cells from becoming cancerous (Kulms and Schwarz, 2000). In the corneal epithelium a delicate balance is maintained among migration of cells from the stem cell niche into the basal layer, mitosis in the basal layer, cell differentiation in the wing cell layers and sloughing from the superficial layer (Thoft and Friend, 1983). This balance is required to maintain a smooth optical surface on the cornea, provide a barrier from the environment and prevent painful exposure of corneal nerves. Damage by UVB has the potential to upset this balance by increasing the shedding rate of superficial cells above the normal level (Ren and Wilson, 1994), and recent papers have drawn attention to public health concerns about ocular UVB exposure (Lucas, 2011; Coroneo, 2011; Lin et al. 2013). The cornea protects the lens and retina from damage by absorbing the majority of ambient UVB radiation (Ringvold, 1998; Kolozvari et al., 2002; Podskochy, 2004). In spite of this exposure, the corneal epithelium does not appear to be highly susceptible to damage by ambient UVB. As discussed in more detail below, our overall hypothesis (Singleton et al., 2009) is that the high concentration of K^+ in tear fluid, 25 mM (Botelho et al., 1973; Rismondo et al., 1989), may help to protect the corneal epithelium from adverse effects of ambient UVB by preventing loss of intracellular K^+ when cells are exposed to UVB.

Exposure to UVB radiation causes cells to activate numerous intersecting signaling pathways that lead to apoptosis. A common factor in activation of apoptotic pathways appears to be activation of K^+ channels in the cell membrane, loss of intracellular K^+ (K^+_i) down its concentration gradient and activation of caspases (Bortner et al., 1997; Hughes et al., 1997; Yu et al., 1997; Wang et al., 1999; Vu et al., 2001; Wang et al., 2003; Shimmura et al., 2004; Arrebola et al. 2006; Singleton et al., 2009) however, the mechanisms by which UVB activates K^+ channels and how K^+ loss leads to apoptosis needs further investigation.

We have previously reported that exposure of human corneal limbal epithelial (HCLE) cells in culture to UVB at 80–200 mJ/cm² (less than two hours of solar radiation at most latitudes) activates K^+ channels, as determined by patch-clamp recording and measurement of K^+_i loss. K^+ channels are activated within 1–2 minutes after exposure of HCLE cells to UVB, and this results in the loss of 50% of K^+_i within 10 minutes, as determined by measurement of $[K^+]$ in cell lysates by ion chromatography. The UVB-induced K^+ current can be partially blocked by the $K_v3.4$ channel blocker BDS-1, by Ba^{2+} or by exposure of the cells to elevated extracellular K^+ (K^+_o), confirming that UVB is activating K^+ channels (Singleton et al., 2009; Ubels et al., 2010, 2011).

Exposure of HCLE cells to UVB activates the initiator caspase-8 and the effector caspase-3, with maximal activity occurring by 6 hr after exposure. This leads to apoptosis, as confirmed by the TUNEL assay. We have shown that this UVB-induced apoptosis can be partially inhibited by incubation of the cells in medium with 25–100 mM K^+ (as compared to the normal culture medium concentration of 5.5 mM) (Singleton et al., 2009; Schotanus et al.,

2011). This effect of elevated $[K^+]_o$ shows that loss of K^+_i is involved in that activation of caspase-3, caspase-8 and DNA fragmentation and also suggests that high $[K^+]_o$ can protect HCLE cells from UVB.

The purpose of the present study was, in the context of numerous previous studies of apoptosis, to investigate in HCLE cells pathways by which UVB activates K^+ channels, caspase activity, and the ways in which these mechanisms interact (Fig. 1). We initially focused on the extrinsic, Fas-activated pathway. Subsequently we investigated the effects of UVB and elevated K^+_o on the intrinsic apoptotic pathway.

In the extrinsic death pathway Fas ligand binds to the receptor protein, Fas, on the cell membrane which in turn, via the adaptor protein FADD, activates the initiator caspase, caspase-8. This group of proteins is known as the death-inducing signaling complex, or DISC (Ashkenazi and Dixit, 1998; Kulms and Schwarz, 2000). It is evident from our research on HCLE cells (Singleton et al., 2009) and the work of others on skin cells and macrophages that an early event in UVB-induced apoptosis is activation of caspase-8 (Kulms et al., 1999; Sodhi and Sethi, 2004). It has been proposed that UVB and UVA cause activation of Fas independently of Fas ligand (Aragane et al., 1998; Zhuang and Kochevar, 2003), although the mechanism by which UV interacts with Fas is unknown. It has also been shown that the loss of function mutation of Fas in MRL/lpr mice causes decreased apoptosis of keratinocytes in response to UVB (Takahashi et al., 2001). These events cause caspase-8 activation leading to apoptosis. It has also been reported that in Jurkat cells, Fas activation by Fas ligand causes activation of $K_v1.3$ channels via FADD recruitment (Storey et al., 2003). It is not known, however, if activation of Fas by UVB requires loss of K^+ , or whether activation of K^+ channels in HCLE cells is a downstream event from activation of Fas. We investigated the role of Fas in UVB-induced apoptosis of HCLE cells by knocking down Fas using siRNA, followed by measurement of UVB-induced caspase-8 and caspase-3 activity and by patch-clamp recording of whole cell K^+ currents.

An alternative hypothesis is that UVB may activate the intrinsic apoptotic pathway in HCLE cells via loss of K^+_i or by a direct effect on the mitochondria. This is suggested by a report that, in immortalized human keratinocytes, UVB-induced apoptosis requires caspase-9, but is death receptor-independent (Daher et al., 2006). This hypothesis was tested in the present study by measurement of UVB-induced activation of caspase-9 in the presence of elevated $[K^+]_o$ and measurement of UVB-induced activation of caspase-8 and caspase-3 after caspase-9 inhibition. The effect of knockdown of apoptosis protease activating factor-1 (Apaf-1) (Acehan et al., 2002; Kugler et al., 2005) on UVB-induced caspase activation and DNA fragmentation was also investigated.

2. Materials and Methods

Methods for cell preparation, transfection, biochemical analyses and electrophysiology are described in this section. The experimental design is described in more detail in the context of the presentation of the results.

2.1 Cell Culture

Human corneal limbal epithelial (HCLE) cells were grown to confluence and maintained as monolayers in 6-well plates in Keratinocyte-SFM (KSFM, Life Technologies, Grand Island, NY), as previously described (Singleton et al., 2009). It should be noted that this cell line is often grown under conditions that lead to the formation of stratified constructs that are several cell layers thick and express mucins typical of the in vivo corneal epithelium (Gipson et al., 2003). We have previously shown that stratified and monolayer HCLE cells respond similarly to UVB and incubation in elevated $[K^+]_o$ (Schotanus et al., 2011). Therefore, for convenience in timing of experiments, transfection with siRNA and collection of cells for caspase assays and flow cytometry, monolayers were used in the present study.

For exposure of cells to elevated concentrations of K^+_o , custom made KSFM with 100 mM K^+ and reduced $[Na^+]$, to maintain osmolality at 290 mOsm/l (Life Technologies), was mixed with standard medium to achieve the desired $[K^+]$ of 25–100 mM. Following exposure to UVB, cells were incubated in KSFM with 5 mM K^+ or in medium with elevated K^+ for 4–6 hours before measurement of caspase activity or TUNEL staining. Control cells, not exposed to UVB, were incubated in KSFM with 5.5 mM K^+ . It should be noted that in our initial study of effects of UVB on HCLE cells we reported that incubation of cells not exposed to UVB in medium with K^+ concentrations up to 100 mM has no adverse effect on cell viability and does not activate apoptosis (Singleton, et al., 2009). Therefore controls incubated with elevated $[K^+]$ were not included in the present study.

2.2 RNA interference

Small interfering RNAs (siRNA) were purchased from Qiagen (Valencia, CA). HCLE cells were grown to 30–50% confluence and transfected with siRNAs to Fas (25 nM), caspase-8 (25 nM), Apaf-1 (50 nM), or All-Stars negative control siRNA (50 nM) using siLentFect (BioRad, Hercules, CA), according to manufacturers' protocols. Optimal transfection conditions and siRNA concentrations were determined in preliminary experiments (data not shown). After transfection, cells were incubated for 72 hours. Knock-down of proteins was confirmed by SDS-PAGE and western blotting using appropriate rabbit anti-human monoclonal antibodies (Cell Signaling Technology, Danvers, MA) and Odyssey IRDye800 goat anti-rabbit secondary antibody (Li-Cor, Lincoln, NE). All control data in knockdown experiments are from cells transfected with the negative control siRNA. Blots were imaged and scanned with a Li-Cor Odyssey Infrared Imaging System.

2.3 Exposure to UVB

Cells were exposed to UVB radiation (302 nm) at 80 or 150 mJ/cm² using a UVM-57 lamp (Ultraviolet Products, Upland, CA) under conditions that have been previously described (Singleton et al., 2009). The doses of UVB were chosen based on previous studies and are relevant to ambient outdoor exposure in less than 2 hr at midday in the summer at 42° north latitude.

2.4 Caspase Activity Assays

Activity of caspases-3, -8 and -9 was measured using fluorometric caspase assay kits (BioVision, Milpitas, CA) according to the manufacturer's instructions, as previously

described. Protein was measured using the Bio-Rad assay. The caspase-8 inhibitor, Z-IEDT-FMK, caspase-3 inhibitor, Z-DEVD-FMK, and caspase-9 inhibitor, Z-LEHD-FMK, were purchased from R&D Systems (Minneapolis, MN). The optimal concentration (10 μ M) of these inhibitors for caspase inhibition was determined in preliminary experiments based on the manufacturer's recommendation.

For measurement of UVB-induced caspase activity, following exposure to UVB and incubation for 4–6 hr, the culture medium was collected from the wells so that apoptotic cells that had been released from the cell layer could be included in the analysis. Adherent cells were then removed from the plate using TrypLE-Express (Life Technologies) and combined with the cells in the retained medium (Singleton et al., 2009).

2.5 TUNEL Assay

DNA fragmentation was measured using an APO-BrdU TUNEL assay kit (Invitrogen Molecular Probes, Carlsbad, CA). After exposure to UVB and incubation, floating and adherent cells were combined, fixed in 1% paraformaldehyde/PBS, washed in PBS, and suspended in 70% EtOH. The cells were labeled according to manufacturer's instructions and analyzed on a BD FACSCaliber flow cytometer (BD Biosciences, San Jose, CA), as previously described (Singleton et al., 2009).

2.6 Patch-clamp Recording

Recording of UVB-induced K^+ channel activation was done as previously described (Singleton et al., 2009; Ubels et al., 2010, 2011). Briefly, whole-cell voltage-clamp current recordings were made using standard amphotericin-B perforated patch techniques. Pipette solution was (in mM) 145 K-methanesulfonate, 2.5 $MgCl_2$, 2.5 $CaCl_2$, 5 HEPES (pH 7.3). Bath solution was (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose (pH 7.4). The holding potential was -80 mV and the recording protocol consisted of 250 ms duration voltage steps from -80 mV to +120 mV in 10 mV increments. Current was recorded by an Axon Instruments Axopatch 200B (Molecular Devices, Sunnyvale, CA) and analyzed by accompanying software (Clampex/Clampfit 9.2). Access resistance, capacitance, and most leak resistance were compensated by amplifier circuitry, with remaining leakage currents subtracted offline. An AutoMate Scientific (Berkeley, CA) perfusion pencil was used to apply the $K_v3.4$ channel blocker, BDS-1 (Alamone Labs, Jerusalem), to cells at a concentration of 1 μ M. To expose cells to UVB, after control K^+ currents were recorded, the UVB lamp was placed 7.5 cm from the recording chamber and the cell was exposed to an 80 mJ/cm² dose of UVB (6 min 40 sec duration). The lamp was then removed and recording was continued within 2 minutes of UVB exposure. The dose of UVB was chosen based on our previous studies. Doses above this level have an immediate effect on the cell that make it difficult to maintain a high resistance seal of the electrode to the membrane or a low resistance access to the cell interior.

2.7 Data analysis

Most statistical comparisons were made using one-way analysis of variance and the Student-Newman-Keuls or Dunnet's test, as appropriate, or the t-test. All data were tested for normal

distribution by the Shapiro-Wilk test and non-parametric tests were used when appropriate. Statistical analysis was conducted using SigmaPlot 12 (Systat Software, Inc., San Jose, CA).

3. Results

3.1 The extrinsic pathway – Fas and caspase signaling

To determine whether Fas signaling is required for initiation of apoptosis in response to UVB, HCLE cells were treated with Fas siRNA and incubated for 72 hr. Figure 2A illustrates a representative experiment with knockdown of Fas as compared to expression in cells treated with negative control siRNA. In all transfection experiments that were conducted (including preliminary experiments to establish optimal conditions, data not shown), Fas expression was knocked down to 4 – 15% of control levels.

Following knockdown of Fas, HCLE cells were exposed to UVB at 150 mJ/cm² and incubated in KSFM with 5.5 mM K⁺ for 6 hr followed by measurement of caspase-8 and caspase-3 activity. In agreement with our previous reports, these caspases were strongly activated by UVB. Knockdown of Fas caused a statistically significant, but limited, reduction in UVB-induced caspase-8 and caspase-3 activation (Fig. 2B).

We also tested whether Fas is involved in a signaling pathway that leads to UVB-induced activation of K⁺ channels. It was confirmed that exposure to UVB at 80 mJ/cm² causes an immediate increase in K⁺ channel activation in cells treated with negative control siRNA (Fig 3A, Control) that is identical to the effect of UVB previously reported in untreated cells, and that this current is blocked by BDS-1 (Singleton et al., 2009; Ubels et al., 2010). Similarly, UVB activates K⁺ currents in HCLE cells after Fas knockdown (Fig. 3A). There was no significant difference in the normalized K⁺ current between control cells and the Fas knockdown cells (Fig. 3B).

In the DISC complex Fas, via FADD, activates the initiator caspase-8 which in turn activates the effector caspase-3. The small effect of knockdown of Fas on UVB-induced activation of these caspases suggests that UVB is acting on caspase-8 via an additional mechanism or that caspase-8 is not required for UVB-induced activation of caspase-3. To investigate these possibilities we first transfected HCLE cells with caspase-8 siRNA followed by incubation for 72 hr. This resulted in knockdown of caspase-8 protein expression to 17% of control levels (Fig. 4A). These cells were then used to determine the effect of caspase-8 knockdown on UVB-induced activation of caspase-3.

Control cells and caspase-8 knockdown cells were exposed to 150 mJ/cm² UVB and incubated for 6 hr in KSFM with either the normal concentration of 5.5 mM K⁺ or 50 mM K⁺. As expected, UVB strongly activated caspase-3 in control cells and this activation was inhibited by 50 mM K⁺ (Fig. 4B). In caspase-8 knockdown cells UVB exposure and incubation in elevated [K⁺_o] had the identical effect.

The lack of an effect of caspase-8 knockdown on activation of caspase-3 suggested that caspase-8 is a highly robust enzyme, so that the residual caspase that was expressed after transfection with siRNA is adequate to activate caspase-3. Alternatively, although caspase-8

is activated by UVB, it is possible that this activation is not required for UVB induced activation of caspase-3. To test this possibility, untransfected HCLE cells were exposed to 150 mJ/cm² UVB and incubated for 6 hr in the presence of 10 μ M caspase-8 inhibitor. Inhibition of caspase-8 eliminated the activation of caspase-3 by UVB (Fig. 5). Based on this observation, an additional experiment was conducted in which cells were transfected with caspase-8 siRNA followed by exposure of the cells to UVB and incubation for 6 hours in the absence and presence of caspase-8 inhibitor, the purpose being to determine whether UVB-induced caspase-3 activation after caspase-8 knockdown was, in fact, due to residual caspase-8 activity. In agreement with the data in Figure 4, caspase-3 was activated 33.4 fold by UVB in the transfected cells. Caspase-8 inhibitor reduced this UVB-induced caspase-3 activity to a 5-fold activation.

3.2 The intrinsic pathway – Caspase-9 and Apaf-1

The apparent lack of involvement of Fas knockdown in UVB-induced caspase-8, caspase-3 and K⁺ channel activation, and the demonstration that caspase-8 activity is required for activation of caspase-3 in response to UVB exposure, raises the question of how these caspases are activated. In our initial study of the role of K⁺ in UVB-induced apoptosis of HCLE cells we investigated the mitochondrial potential and caspase-9 activation; however, we did not emphasize these observations because of the relatively weak effect of elevated K⁺_o on this pathway after exposure of cells to UVB (Singleton et al., 2009). In the present study we returned to the intrinsic pathway based on the possibility that UVB-induced caspase-9 activation is required for activation of caspases-8 and -3.

HCLE cells were exposed to UVB at 150 mJ/cm² and in preliminary studies it was determined that maximal activation of caspase-9 occurs by 4 hr after treatment (data not shown). In agreement with our previous report, when HCLE cells were incubated in 25 -100 mM K⁺_o for 4 hr after exposure to UVB there was a significant, dose-dependent inhibition of caspase-9 activation (Fig. 6.) The magnitude of this inhibition was not, however, as great as the effect of elevated [K⁺_o] on UVB-induced caspase-3 activity (Fig. 4.)

To determine if caspase-9 activation is required for UVB-induced activation of caspase-8 and caspase-3, HCLE cells were exposed to UVB and incubated for 6 hr in the presence of 10 μ M caspase-9 inhibitor. Inhibition of caspase-9 resulted in complete inhibition of UVB-induced activation of caspase-8 and also resulted in significant suppression of activation of caspase-3 by UVB to nearly control levels (Fig. 7).

During apoptosis initiated by the intrinsic pathway cytochrome c is released by the mitochondria and causes heptamerization of Apaf-1 (Acehan et al., 2002). Apaf-1 in turn recruits and activates caspase-9. To provide further evidence that the effect of UVB in apoptosis of HCLE cells is via the intrinsic pathway, the effect of knockdown of Apaf-1 on caspase activation was investigated. The use of siRNA was chosen because inhibitors of Apaf-1 are not readily available. The results of a representative knockdown of Apaf-1 are shown in Figure 8A. For all experiments the protein expression was reduced to about 35% of control, however, this reduction in Apaf-1 expression was adequate to cause a significant and marked inhibition of the UVB-induced activation of caspases-9, -8 and -3 (Fig. 8B).

If knockdown of Apaf-1 is inhibiting UVB-induced activation of the caspase cascade in HCLE cells, then ultimately apoptosis, as indicated by DNA fragmentation, should be inhibited. Control and Apaf-1 knockdown cells were exposed to 150 mJ/cm² UVB, and after 6 hours apoptosis was measured by the TUNEL assay. UVB exposure caused an increase in the percentage of stained control cells, while no difference was detected between exposed and untreated cells after knockdown of Apaf-1 (Fig. 9). It should be noted that knockdown of Apaf-1 also reduced the baseline number of apoptotic cells in cultures not exposed to UVB, as compared to control.

Finally, since Apaf-1 appears to be important for UVB-induced activation of caspase-3 experiments were conducted to confirm the requirement for activation of caspase-3 in DNA fragmentation. HCLE cells were exposed to 150 mJ/cm² UVB and incubated for 6 hr in the absence or presence of caspase-3 inhibitor. In UVB-treated cultures with the inhibitor, the percentage of apoptotic cells remained at control levels (Fig. 10).

4. Discussion

4.1 The extrinsic pathway

In this study of the mechanisms of apoptosis as related to activation of K⁺ channels in HCLE cells exposed to UVB, we initially focused on the extrinsic pathway. This was based on the abundant literature showing that UVB activates Fas independently of Fas ligand in HeLa cells, keratinocytes and lymphocytes (Aragane et al., 1998; Takahashi et al., 2001; Kulms et al., 2002). Our results, however, suggest that in HCLE cells the intrinsic pathway may have a greater role in the response to UVB exposure.

Knockdown of Fas had no effect on activation of K⁺ channels in HCLE cells by UVB, and the effect on UVB-induced activation of caspase-8 and caspase-3, although significant, was limited. In keratinocytes and melanoma cells UV activation of apoptosis appears to be strongly related to activation of Fas by a Fas ligand-independent mechanism. This is reported to occur via clustering of Fas in the cell membrane and by increased expression of Fas. The clustering of Fas is detectable by 30 min after UVB exposure and is maximal by 6 hr (Aragane et al., 1998; Bang et al., 2003), which is in agreement with the time course of activation of apoptosis that we observe post-UVB in HCLE cells. In preliminary studies to our original work on this problem we found that activation of caspase-8 and caspase-3 is at least half maximal by 3 hr after UVB exposure and approaches a maximum by 6 hr. The strong activation of caspases and detection of apoptotic cells by the TUNEL assay in HCLE cells at 6 hr post-UVB is clearly evident in the present study and in our previous reports. This is in contrast to reports on keratinocyte and melanoma cell lines which suggest that a similar level of apoptosis does not occur until 16–24 hours post-UVB (Aragane et al., 1998; Wang and Li, 2006). It has also been reported that UVB increases Fas mRNA and Fas protein expression in keratinocytes, macrophages and melanoma cells (Bang et al., 2002; Sodhi and Sethi, 2004, Wang and Li, 2006). This expression peaks in 6–24 hours, depending on the cell type. Although we have not measured Fas expression in HCLE cells after UVB exposure, the relatively rapid response of caspases to UVB exposure and the limited effect of Fas knockdown suggest that a change in Fas expression is unlikely to be involved in the effect of UVB on HCLE cells.

We proposed that if Fas is activated by UVB in HCLE cells and in turn activates K^+ channels then knockdown of Fas would reduce the activation of $K_v3.4$ channels by UVB. This hypothesis was based on the report by Storey et al. (2003) that $K_v1.3$ channels are activated when Fas ligand is applied to Jurkat cells and a report by Bortner et al. (2001) that exposure of Jurkat cells to an agonistic anti-Fas antibody causes changes in membrane potential of Jurkat cells. In contrast to these observations, knockdown of Fas had no effect on UVB activation of K^+ currents in HCLE cells. An important difference exists between the activation of K^+ currents in HCLE and Jurkat cells. In Jurkat cells the increase in channel activation is detectable 30 minutes after application of Fas ligand, but exposure to the ligand for 20 minutes or less had no effect. In the Bortner et al. study, membrane potential changes in response to anti-Fas are detectable 4 hr after exposure. The present study demonstrates a lack of effect of Fas knockdown on K^+ channel activation and very rapid activation of these channels in response to UVB (less than 2 min). This strongly suggests that Fas is not involved in the response of HCLE cells to UVB and that another, as yet unidentified, signaling mechanism is involved in the increase in K^+ current and loss of K^+_i in response to UVB.

The minimal effect of Fas knockdown on apoptosis in HCLE cells was unexpected since knockdown of Fas in melanoma cells lines completely inhibits UVB-induced activation of caspase-8 and apoptosis (Wang and Li, 2006). A more recent report is, however, in agreement with our findings. It was reported by Hedrych-Ozimina et al. (2011) that keratinocytes cultured from the skin of Fas knockout mice go into UVB-induced apoptosis at the same rate as cells from control mice, showing that while Fas may be involved in the response to UVB it is not necessarily required. In the cornea the decreased importance of Fas may serve as a protective mechanism, decreasing the vulnerability of the corneal epithelium to damage by ambient, outdoor UVB, to which the epidermis is highly susceptible.

We have previously reported that caspase-8 is activated by UVB in HCLE cells, but that the magnitude of the response is not as great as the activation of caspase-3, nor is the inhibition of caspase-8 activity by elevated K^+_o as great as the inhibition of caspase-3 activity (Singleton et al., 2009; Schotanus et al., 2011). Since caspase-8 is part of DISC, and Fas does not appear to be important for UVB-induced apoptosis, this led us to question whether caspase-8 is required for UVB-induced apoptosis of HCLE cells. We tested this hypothesis by measurement of UVB-induced caspase-3 activation in normal HCLE cells and after knockdown of caspase-8, in the absence or presence of caspase-8 inhibitor. Residual caspase-8 in knockdown cells was adequate to activate caspase-3 upon UVB exposure, but the loss of UVB-induced caspase-3 activation in the presence of caspase-8 inhibitor shows that caspase-8 is essential. Based on the Fas knockdown data, however, caspase-8 possibly is activated via a different mechanism, perhaps the intrinsic pathway.

4.2 The intrinsic pathway

In our original study of effects of UVB on HCLE cells and inhibition of apoptosis by elevated $[K^+_o]$, we tested the effect of UVB and elevated $[K^+_o]$ on caspase-9 (Singleton et al., 2009). Since the effect of high $[K^+_o]$ in inhibiting this caspase was much less than the

effect on caspases -8 and -3, we turned our attention to the extrinsic pathway, as described above. Because of the findings concerning Fas in this study, we returned to caspase-9, confirming that this caspase is activated by UVB and that elevated K^+_o at a concentration as low as 25 mM has significant inhibitory effect on UVB-induced caspase-9 activity. This suggests that caspase-9 may be responsible for activation of caspase-8 and subsequent steps in UVB-induced apoptosis, which was confirmed by measurement of caspase-8 and -3 activity in the presence of caspase-9 inhibitor. The importance of the intrinsic pathway in the response of HCLE cells to UVB was confirmed by the lack of activation of caspases-9, -8 and -3 and the lack of TUNEL staining after knockdown of Apaf-1. The importance of Apaf-1 in this pathway is also shown by the decrease in the baseline level of apoptosis in Apaf-1 knockdown cultures not exposed to UVB. Our data on Apaf-1 are supported by the work of Feng et al. (2012) who showed that Apaf-1 deficiency inhibits UVC-induced apoptosis of mouse fibroblasts. We therefore conclude that the intrinsic pathway is more important than the extrinsic pathway in UVB-induced apoptosis of HCLE cells. It must be noted that p53 is inactivated in the HCLE cell line, so that we cannot eliminate a possible involvement of p53 in corneal epithelial apoptosis in intact corneas.

Having provided evidence that Fas is apparently not required for UVB-induced activation of K^+ channels and having shown the importance of the intrinsic pathway in HCLE cell apoptosis in response to UVB, it remains to further elucidate the pathway by which K^+ channels are activated and to determine if K^+ loss from the cytoplasm is a primary or secondary event in apoptosis. Preliminary data from our laboratory suggest that knockdown of tumor necrosis factor receptor 1 (TNF-R1) or FADD inhibits UVB-induced loss of K^+ from HCLE cells, and it has been reported that UVB can cause apoptosis of lung and breast carcinoma cells due to ligand independent activation of TNF-R1 (Sheikh et al., 1998), which is known to activate FADD (Ashkenazi and Dixit, 1998). Based on this information we are pursuing the hypothesis that TNF-R1 initiates a signaling pathway that activates K^+ channels. It will also be important to investigate further the earliest step in the intrinsic pathway, release of cytochrome c from the mitochondria, since it has been reported that normal intracellular levels of K^+ can inhibit formation of the apoptosome in response to cytochrome c release (Cain et al., 2001). Cytochrome c has been reported to activate K^+ channels (Platoshyn et al., 2002), so a next step in our work will be to investigate the interrelationships among cell surface receptors, K^+ channels and the intrinsic apoptotic pathways in HCLE cells.

5. Conclusion

Several mechanisms exist that can protect the ocular surface from UVB, including physical shade by the eyebrows and chemical protection by lactoferrin in tears and intracellular ascorbate and tryptophan, all of which absorb UV radiation (Ringvold, 1998; Fujihara et al., 2000; Kolozvari et al., 2002; Choy et al., 2011). Prevention of activation of the intracellular signaling pathways discussed in this report would also help to prevent damage by ambient UVB-induced apoptosis in the corneal epithelium. As we have previously proposed (Singleton et al., 2009; Ubels et al., 2006; 2012), the lacrimal functional unit appears to be adapted to participate in protection of the corneal epithelium from UVB by secreting tears with a relatively high concentration of K^+ . Bathing of the ocular surface in tear fluid with

this high $[K^+]$ may attenuate the loss of intracellular K^+ and subsequent apoptosis when channels in corneal epithelial cells are activated by UVB.

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References

- Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*. 2002; 9:423–432. [PubMed: 11864614]
- Aragane Y, Kulms D, Metze D, Wilkes G, Pöppelmann B, Luger TA, Schwarz T. Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J Cell Biol*. 1998; 140:171–182. [PubMed: 9425165]
- Arrebola F, Fernández-Segura E, Campos A, Crespo PV, Skepper JN, Warley A. Changes in intracellular electrolyte concentrations during apoptosis induced by UV irradiation of human myeloblastic cells. *Am J Physiol Cell Physiol*. 2006; 290:C638–649. [PubMed: 16162654]
- Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science*. 1998; 28:1305–1308. [PubMed: 9721089]
- Bang B, Gniadecki R, Larsen JK, Baadsgaard O, Skov L. In vivo UVB irradiation induces clustering of Fas (CD95) on human epidermal cells. *Exp Dermatol*. 2003; 12:791–798. [PubMed: 14714559]
- Bang B, Rygaard J, Baadsgaard O, Skov L. Increased expression of Fas on human epidermal cells after in vivo exposure to single-dose ultraviolet (UV) B or long-wave UVA radiation. *Br J Dermatol*. 2002; 147:1199–1206. [PubMed: 12452871]
- Bortner CD, Gomez-Angelats M, Cidlowski JA. Plasma membrane depolarization without repolarization is an early molecular event in anti-Fas-induced apoptosis. *J Biol Chem*. 2001; 276:4304–4314. [PubMed: 11050080]
- Bortner CD, Hughes FM, Cidlowski JA. A primary role for K^+ and Na^+ efflux in the activation of apoptosis. *J Biol Chem*. 1997; 272:32436–32542. [PubMed: 9405453]
- Botelho SY, Martinez EV. Electrolytes in lacrimal gland fluid and in tears at various flow rates in the rabbit. *Am J Physiol*. 1973; 225:606–609. [PubMed: 4726493]
- Cain K, Langlais C, Sun XM, Brown DG, Cohen GM. Physiological concentrations of K^+ inhibit cytochrome c-dependent formation of the apoptosome. *J Biol Chem*. 2001; 276:41985–41990. [PubMed: 11553634]
- Choy CK, Cho P, Benzie IF. Antioxidant content and ultraviolet absorption characteristics of human tears. *Optom Vis Sci*. 2011; 88:507–511.10.1097/OPX.0b013e31820e9fe2 [PubMed: 21336228]
- Coroneo M. Ultraviolet radiation and the anterior eye. *Eye Contact Lens*. 2011; 37:214–224.10.1097/ICL.0b013e318223394e [PubMed: 21670690]
- Daher A, Simbulan-Rosenthal CM, Rosenthal DS. Apoptosis induced by ultraviolet B in HPV-immortalized human keratinocytes requires caspase-9 and is death receptor independent. *Exp Dermatol*. 2006; 15:23–34. [PubMed: 16364028]
- Feng R, Han J, Ziegler J, Yang M, Castranova V. Apaf-1 deficiency confers resistance to ultraviolet-induced apoptosis in mouse embryonic fibroblasts by disrupting reactive oxygen species amplification production and mitochondrial pathway. *Free Radic Biol Med*. 2012; 52:889–897.10.1016/j.freeradbiomed.2011.11.028 [PubMed: 22226830]
- Fujihara T, Nagano T, Endo K, Nakamura M, Nakata K. Lactoferrin protects against UV-B irradiation-induced corneal epithelial damage in rats. *Cornea*. 2000; 19:207–211. [PubMed: 10746454]

- Gipson IK, Spurr-Michaud S, Argüeso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci.* 2003; 44:2496–2506. [PubMed: 12766048]
- Hedrych-Ozimina A, Behrendt K, Hao Z, Pofahl R, Ussath D, Knaup R, Krieg T, Haase I. Enhanced contact allergen- and UVB-induced keratinocyte apoptosis in the absence of CD95/Fas/Apo-1. *Cell Death Differ.* 2011; 18:155–163.10.1038/cdd.2010.83 [PubMed: 20689557]
- Hughes FM, Bortner CD, Purdy GD, Cidlowski JA. Intracellular K⁺ suppresses the activation of apoptosis in lymphocytes. *J Biol Chem.* 1997; 272:30567–30576. [PubMed: 9374553]
- Kolozvari L, Nogradi A, Hopp B, Bor Z. UV absorbance of the human cornea in the 240- to 400-nm range. *Invest Ophthalmol Vis Sci.* 2002; 43:2165–2168. [PubMed: 12091412]
- Kugler W, Buchholz F, Köhler F, Eibl H, Lakomek M, Erdlenbruch B. Downregulation of Apaf-1 and caspase-3 by RNA interference in human glioma cells: consequences for erucylphosphocholine-induced apoptosis. *Apoptosis.* 2005; 10:1163–1174. [PubMed: 16151649]
- Kulms D, Düsselmann H, Pöppelmann B, Ständer S, Schwarz A, Schwarz T. Apoptosis induced by disruption of the actin cytoskeleton is mediated via activation of CD95 (Fas/APO-1). *Cell Death Differ.* 2002; 9:598–608. [PubMed: 12032668]
- Kulms D, Pöppelmann B, Yarosh D, Luger TA, Krutmann J, Schwarz T. Nuclear and cell membrane effects contribute independently to the induction of apoptosis in human cells exposed to UVB radiation. *Proc Natl Acad Sci U S A.* 1999; 96:7974–7979. [PubMed: 10393932]
- Kulms D, Schwarz T. Molecular mechanisms of UV-induced apoptosis. *Photodermatol Photoimmunol Photomed.* 2000; 16:195–201. [PubMed: 11068857]
- Lin DP, Chang HH, Yang LC, Huang TP, Liu HJ, Chang LS, Lin CH, Chen BY. Assessment of ultraviolet B-blocking effects of weekly disposable contact lenses on corneal surface in a mouse model. *Mol Vis.* 2013; 19:1158–1168. [PubMed: 23734085]
- Lucas RM. An epidemiological perspective of ultraviolet exposure--public health concerns. *Eye Contact Lens.* 2011; 37:168–175.10.1097/ICL.0b013e31821cb0cf [PubMed: 21670693]
- Platoshyn O, Zhang S, McDaniel SS, Yuan JX. Cytochrome c activates K⁺ channels before inducing apoptosis. *Am J Physiol Cell Physiol.* 2002; 283:C1298–1305. [PubMed: 12225992]
- Podskochy A. Protective role of corneal epithelium against ultraviolet radiation damage. *Acta Ophthalmol Scand.* 2004; 82:714–717. [PubMed: 15606469]
- Ren H, Wilson G. The effect of ultraviolet-B irradiation on the cell shedding rate of the corneal epithelium. *Acta Ophthalmol (Copenh).* 1994; 72:447–452. [PubMed: 7825410]
- Ringvold A. Corneal epithelium and UV-protection of the eye. *Acta Ophthalmol Scand.* 1998; 76:149–153. [PubMed: 9591943]
- Rismondo V, Osgood TB, Leering P, Hattenhauer MG, Ubels JL, Edelhauser HF. Electrolyte composition of lacrimal gland fluid and tears of normal and vitamin A-deficient rabbits. *CLAO J.* 1989; 15:222–229. [PubMed: 2776293]
- Schotanus MP, Koetje LR, Van Dyken RE, Ubels JL. Stratified corneal limbal epithelial cells are protected from UVB-induced apoptosis by elevated extracellular K⁺ *Exp Eye Res.* 2011; 93:735–740.10.1016/j.exer.2011.09.005 [PubMed: 22019354]
- Sheikh MS, Antinore MJ, Huang Y, Fornace AJ. Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1. *Oncogene.* 1998; 17:2555–2563. [PubMed: 9840918]
- Shimmura S, Tadano K, Tsubota K. UV dose-dependent caspase activation in a corneal epithelial cell line. *Curr Eye Res.* 2004; 28:85–92. [PubMed: 14972714]
- Singleton KR, Will DS, Schotanus MP, Haarsma LD, Koetje LR, Bardolph SL, Ubels JL. Elevated extracellular potassium inhibits apoptosis of corneal epithelial cells exposed to UV-B radiation. *Exp Eye Res.* 2009; 89:140–151.10.1016/j.exer.2009.02.023 [PubMed: 19289117]
- Sodhi A, Sethi G. Involvement of fas/fas ligand in ultraviolet B-induced apoptosis of murine peritoneal macrophages. *Toxicol Mech Methods.* 2004; 14:215–222.10.1080/15376520490434449 [PubMed: 20021133]
- Storey NM, Gómez-Angelats M, Bortner CD, Armstrong DL, Cidlowski JA. Stimulation of K_v1.3 potassium channels by death receptors during apoptosis in Jurkat T lymphocytes. *J Biol Chem.* 2003; 278:33319–33326. [PubMed: 12807917]

- Takahashi H, Ishida-Yamamoto A, Iizuka H. Ultraviolet B irradiation induces apoptosis of keratinocytes by direct activation of Fas antigen. *J Invest Dermatol Symp Proc.* 2001; 6:64–68.
- Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci.* 1983; 24:1442–1443. [PubMed: 6618809]
- Ubels JL, Hoffman HM, Srikanth S, Resau JH, Webb CP. Gene expression in rat lacrimal gland duct cells collected using laser capture microdissection: evidence for K⁺ secretion by duct cells. *Invest Ophthalmol Vis Sci.* 2006; 47:1876–1885. [PubMed: 16638994]
- Ubels JL, Schotanus MP, Bardolph SL, Haarsma LD, Koetje LR, Louters JR. Inhibition of UV-B induced apoptosis in corneal epithelial cells by potassium channel modulators. *Exp Eye Res.* 2010; 90:216–222.10.1016/j.exer.2009.10.005 [PubMed: 19874821]
- Ubels JL, Van Dyken RE, Louters JR, Schotanus MP, Haarsma LD. Potassium ion fluxes in corneal epithelial cells exposed to UVB. *Exp Eye Res.* 2011; 92:425–431.10.1016/j.exer.2011.02.019 [PubMed: 21377460]
- Ubels JL, Gipson IK, Spurr-Micheud SJ, Tisdale AS, Van Dyken RE, Hatton MP. Gene expression in human accessory lacrimal glands of Wolfring. *Invest Ophthalmol Vis Sci.* 2012; 53:6738–6747.10.1167/iops.12-10750 [PubMed: 22956620]
- Vu CC, Bortner CD, Cidlowski JA. Differential involvement of initiator caspases in apoptotic volume decrease and potassium efflux during Fas- and UV-induced cell death. *J Biol Chem.* 2001; 276:37602–37611. [PubMed: 11431480]
- Wang L, Li T, Lu L. UV-induced corneal epithelial cell death by activation of potassium channels. *Invest Ophthalmol Vis Sci.* 2003; 44:5095–5101. [PubMed: 14638703]
- Wang L, Xu D, Dai W, Lu L. An ultraviolet-activated K⁺ channel mediates apoptosis of myeloblastic leukemia cells. *J Biol Chem.* 1999; 274:3678–3685. [PubMed: 9920918]
- Wang Y, Li G. ING3 promotes UV-induced apoptosis via Fas/caspase-8 pathway in melanoma cells. *J Biol Chem.* 2006; 281:11887–11893. [PubMed: 16520380]
- Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW. Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science.* 1997; 278:114–117. [PubMed: 9311914]
- Zhuang S, Kochevar IE. Ultraviolet A radiation induces rapid apoptosis of human leukemia cells by fas ligand-independent activation of the Fas death pathway. *Photochem Photobiol.* 2003; 78:61–67. [PubMed: 12929750]

Highlights

UVB activates K^+ channels in corneal epithelial cells leading to caspase-8 and -3 activation and apoptosis.

Elevated extracellular K^+ inhibits UVB induced apoptosis.

Knockdown of Fas does not inhibit K^+ channel and caspase activation.

Apaf-1 is essential for UVB-induced activation of caspases-9, -8 and -3 and apoptosis of corneal epithelial cells.

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

Fig. 1.

This diagram illustrates relationships that are possibly involved in UVB-induced apoptosis of HCLE cells. UVB may directly affect K^+ channels, Fas or the mitochondrion. Fas activation by UVB may be required for activation of K^+ channels and caspases-8 and -3 via the extrinsic pathway. Loss of K^+_i upon activation of channels may directly activate the intrinsic pathway and/or may induce activation of caspase-8 and caspase-3.

Fig. 2.
A. Knockdown of Fas in HCLE cells. Lanes 1–3: control, lanes 3–6: 72 hr after transfection with 25 nM Fas siRNA. Arrow indicates position of Fas protein. B. Effect of Fas knockdown to less than 15% of control levels on activation of caspase-8 and caspase-3, 6 hr after exposure to 150 mJ/cm² UVB. Fas knockdown had a limited, but statistically significant effect on UVB-induced caspase-8 and caspase-3 activity as compared to control cells. * Values are not significantly different from one another. UVB-treated values (150 mJ/cm²) differ from each other and from untreated (No UVB) values. (ANOVA and Student-Newman-Keuls test, mean $\pm$ SD, n=17 – caspase-8 and n=6 – caspase-3, p = 0.05).

Fig. 3.

A. Current-voltage relationships in response to 10 mV voltage steps in control HCLE cells and after knockdown of Fas. K^+ channels are activated within 2 minutes after exposure cells to 80 mJ/cm² UVB. The UVB-activated current is blocked by BDS-1. B. Normalized UVB-activated currents at +60 mV do not differ between control cells (n=11) and cells treated with Fas siRNA (t-test, n=12, p = 0.05).

Fig. 4.

A. Knockdown of caspase-8 in HCLE cells. Lanes 1–3: control; lanes 3–6: 72 hr after transfection with 25 nM caspase-8 siRNA. Arrow indicates position of caspase-8 protein. B. Effect of caspase-8 knockdown to less than 17% of control levels on activation of caspase-3, 6 hr after exposure to 150 mJ/cm² UVB. Caspase-8 knockdown does not reduce UVB-induced caspase-3 activity. Note inhibition of UVB-induced caspase-3 activation in the presence of 50 mM K⁺_o. Values with the same symbols are not significantly different from one another but differ from all other values. (ANOVA and Student-Newman-Keuls test, mean ± SD, n=9, p = 0.05).

Fig. 5. UVB-induced caspase-3 activity in the presence of the caspase-8 inhibitor, Z-IEDT-FMK. Inhibition of caspase-8 prevents activation of caspase-3 by 150 mJ/cm² UVB, showing that caspase-8 is required for caspase-3 activation in HCLE cells. *significantly different than all other values (Boxes indicate median, 25th and 75th percentiles, whiskers indicate 10th and 90th percentiles, Kruskal-Wallis ANOVA on Ranks and Dunn's test, n=12, p = 0.05)

Fig. 6.

Effect of elevated $[K^+]_o$ on activation of caspase-9 in HCLE cells exposed to UVB at 150 mJ/cm². Cells were incubated in KSFM with isosmotic 5.5 – 100 mM K⁺ for 4 hr after exposure to UVB. * significantly different than cells exposed to UVB and incubated in 5.5 mM K⁺. Marked values also differ from one another. (ANOVA and Student-Newman-Keuls test, mean $\pm$ SD, n=12, p = 0.05,)

Fig. 7. UVB-induced caspase-8 and caspase-3 activity in the presence of the caspase-9 inhibitor Z-LEHD-FMK 6h after exposure of HCLE cells to 150 mJ/cm² UVB. Caspase-9 is required for activation of caspase-8 and caspase-3. *significantly different than all other values. Unmarked values do not differ. (ANOVA and Student-Newman-Keuls test, mean $\pm$ SD, n=12, p = 0.05)

Fig. 8. A. Knockdown of Apaf-1. Lanes 1 and 2: control; lanes 3 and 4: 72 hr after transfection with 50 nM Apaf-1 siRNA. Arrow indicates position of Apaf-1 protein. B. Effect of Apaf-1 knockdown to less than 35% of control levels on activation of caspases-9, -8 and -3, respectively, 6 hr after exposure to 150 mJ/cm² UVB. *significantly different than all other values. Unmarked values do not differ. (ANOVA and Student-Newman-Keuls test, mean $\pm$ SD, n=12, p = 0.05)

Fig. 9. Effect of knockdown of Apaf-1 on UVB-induced apoptosis of HCLE cells. Cells were exposed to UVB at 150 mJ/cm² and apoptotic cells were detected by the TUNEL assay and flow cytometry at 6 hr. Values marked * are not significantly different from one another. # significantly different from all other values. (ANOVA and Student-Newman-Keuls test, n=12 samples of 10,000 cells, p = 0.05)

Fig. 10.

Effect of inhibition of caspase-3 on UVB-induced apoptosis of HCLE cells. Cells were exposed to UVB at 150 mJ/cm² and apoptotic cells were detected by the TUNEL assay and flow cytometry at 6 hr. * significantly different than other values. (ANOVA and Student-Newman-Keuls test, n=12 samples of 10,000 cells each, p = 0.05)