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Research article

TNF-R1 and FADD mediate UVB-Induced activation of K^+ channels in corneal epithelial cells

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ABSTRACT

The goal of this study was to elucidate the role of Fas, TNF-R1, FADD and cytochrome *c* in UVB-induced K^+ channel activation, an early step in UVB-induced apoptosis, in human corneal limbal epithelial (HCLE) cells. HCLE cells were treated with Fas, TNF-R1 or FADD siRNA and exposed to 80 or 150 mJ/cm^2 UVB. K^+ channel activation and loss of intracellular K^+ were measured using whole-cell patch-clamp recording and ion chromatography, respectively. Cytochrome *c* was measured with an ELISA kit. Cells in which Fas was knocked down exhibited identical UVB-induced K^+ channel activation and loss of intracellular K^+ to control cells. Cells in which TNF-R1 or FADD were knocked down demonstrated reduced K^+ channel activation and decreased loss of intracellular K^+ following UVB, relative to control cells. Application of TNF-α, the natural ligand of TNF-R1, to HCLE cells induced K^+ channel activation and loss of intracellular K^+. Cytochrome *c* was translocated to the cytosol by 2 h after exposure to 150 mJ/cm^2 UVB. However, there was no release by 10 min post-UVB. The data suggest that UVB activates TNF-R1, which in turn may activate K^+ channels via FADD. This conclusion is supported by the observation that TNF-α also causes loss of intracellular K^+. This signaling pathway appears to be integral to UVB-induced K^+ efflux, since knockdown of TNF-R1 or FADD inhibits the UVB-induced K^+ efflux. The lack of rapid cytochrome *c* translocation indicates cytochrome *c* does not play a role in UVB-induced K^+ channel activation.

1. Introduction

A function of apoptosis is to remove abnormal or damaged cells which pose a threat to the organism. This process can be induced by intrinsic and extrinsic factors. Ultraviolet B (UVB) (280–315 nm) is an environmental hazard with the potential to induce apoptosis in human keratinocytes and corneal epithelial cells. In keratinocytes, UVB radiation can cause "sunburn" cells (Danno and Horio, 1987) which are quickly removed via apoptosis, presumably to prevent the development of basal and squamous cell skin cancer (Kulms and Schwarz, 2000).

Corneal epithelial cells are routinely sloughed from the ocular surface and replaced by cell division in the basal layer, so that the corneal epithelium turns over every 1–2 weeks (Hanna et al., 1961; Sharma and Coles, 1989; Cenedella and Fleschner, 1990). If UVB exposure from ambient sunlight triggered apoptosis, this would upset the innate balance of proliferation and sloughing (Ren and Wilson, 1994) and leave the cornea susceptible to erosion (Thoft and Friend, 1983; Ren and Wilson, 1994). We have previously proposed that a potential natural defensive mechanism against UVB-induced corneal epithelial apoptosis is the high concentration of K^+ in tear fluid (Botelho and Martinez, 1973; Rismondo et al., 1989; Singleton et al., 2009).

Loss of intracellular K^+ is a necessary early step in apoptosis, and inhibition of this efflux by application of K^+ channel blockers or an isosmotic increase in extracellular K^+ inhibits apoptosis (Hughes et al., 1997; Bortner et al., 1997). Lu et al. (2003) and Wang et al. (2003), studying rabbit and rat corneal epithelial cells, showed that a high dose of UVC activates K^+ channels, causing a K^+ efflux and subsequent apoptosis, which can be prevented by K^+ channel blockers.

The atmosphere filters out nearly all UVC, but UVB at doses equivalent to ambient outdoor levels can also trigger apoptosis. Within 1–2 min of exposure to UVB at 80–150 mJ/cm^2, K^+ channels are activated in human corneal limbal epithelial (HCLE) cells, as measured by patch-clamp recording (Singleton et al., 2009). In cell culture medium with 5.5 mM K^+, the same concentration as in

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interstitial fluids and plasma, this K^+ channel activation results in the loss of 50% of intracellular K^+ within 10 min, as determined by analyzing cell lysates using ion chromatography (Ubels et al., 2011). Exposure to 150 mJ/cm^2 UVB triggers activation of caspases −9, −8 and −3 and DNA fragmentation in HCLE cells (Singleton et al., 2009; Ubels et al., 2011, 2016). Ubels et al. (2011) demonstrated that culture medium with elevated extracellular K^+ (25 mM), similar to that in tear fluid, reduces K^+ loss following UVB-induced K^+ channel activation by diminishing the electrochemical gradient between the intracellular and extracellular fluids (Singleton et al., 2009), reducing UVB-induced apoptosis. Likewise, K^+ channel blockers Ba^{2+} and BDS-1 reduce UVB-induced K^+ currents and subsequent apoptosis (Singleton et al., 2009; Ubels et al., 2010, 2011; Glupker et al., 2016).

The observation that ambient levels of UVB cause loss of intracellular K^+ and apoptosis of HCLE cells led to the question of which signaling pathway triggered by UVB is responsible for activation of K^+ channels in these cells. The involvement of the apoptotic receptor Fas in UVB-induced apoptosis was suggested by reports from Aragane et al. (1998) and Rehemtulla et al. (1997), which implicated ligand-independent activation of Fas (CD95) as the instigator of UVB-induced apoptosis in keratinocytes, MCF-7, BJAB and Jurkat cells. Interest in tumor necrosis factor receptor 1 (TNF-R1) arose from a study by Sheikh et al. (1998) which provided evidence that UVB-induced apoptosis is mediated by ligand-independent activation of TNF-R1 in H1299 and MCF-7 cells. This was supported by Tong et al. (2006), who reported that UVB promotes TNF-R1 clustering. It is not known, however, if these receptors are involved in activation of K^+ channels in HCLE cells.

An alternative hypothesis is that UVB-induced K^+ channel activation occurs via the intrinsic apoptotic pathway involving cytochrome *c*, rather than the extrinsic pathway. An early step in the intrinsic pathway is the translocation of cytochrome *c* from the mitochondria to the cytosol, where it binds to apoptosis protease activating factor-1 (Apaf-1), forming an apoptosome, which in turn activates caspase-9. It is evident from our prior work that knockdown of Apaf-1 in HCLE cells results in diminished activation of caspases −9, −8 and −3 by UVB as well as decreased DNA fragmentation, whereas knockdown of Fas had little effect on UVB-induced caspase activation and DNA fragmentation in HCLE cells (Ubels et al., 2016), implying that UVB causes cytochrome *c* release from the mitochondria. A study by Platoshyn et al. (2002) showed that cytochrome *c* activates K^+ channels prior to inducing nuclear condensation in vascular smooth muscle cells. Motivated by these observations, we measured the time course of UVB-induced cytochrome *c* release in HCLE cells to determine whether cytochrome *c* release occurs prior to K^+ channel activation.

To test the involvement of Fas, TNF-R1 and FADD in the response to UVB in HCLE cells, siRNA was used to knock down Fas, TNF-R1 or FADD proteins. The treated cells were then exposed to 80 or 150 mJ/cm^2 UVB. K^+ channel activation and loss of intracellular K^+ were measured using whole cell patch-clamp recording and ion chromatography, respectively. To test the hypothesis that cytochrome *c* activates K^+ channels, translocation of mitochondrial cytochrome *c* to the cytosol was measured following exposure of cells to 150 mJ/cm^2 UVB.

2. Materials and methods

2.1. Cell culture

An immortalized human corneal limbal epithelial (HCLE) cell line was maintained in monolayer culture in Keratinocyte-Serum Free Medium (KSFM) (Life Technologies, Grand Island, NY), as previously described (Gipson et al., 2003; Singleton et al., 2009).

2.2. RNA interference

siRNAs for Fas, TNF-R1 or FADD were purchased from Qiagen (Valencia, CA). The siRNAs chosen had been functionally verified in human cells by the manufacturer. Their sequences are shown in Table 1. A negative control siRNA was not used in this study, because in a previous study we reported that Allstars negative control siRNA (Qiagen) had no effect on the response of K^+ channels and activation of apoptotic mechanisms in HCLE cells exposed to UVB (Ubels et al., 2016).

Prior to transfection, 2.5 μL/mL siLentFect (BioRad, Hercules, CA) and 25 nM siRNA were mixed with Opti-MEM (Life Technologies, Carlsbad, CA) and incubated together for 20 min at room temperature. HCLE cells, which had been grown to 30–50% confluence in six-well plates, were transfected using the Opti-MEM mixture according to the manufacturer's protocol. Knockdown of proteins was confirmed by SDS-PAGE and western blotting using rabbit anti-human monoclonal antibodies (Cell Signaling Technology, Danvers, Massachusetts) and Odyssey IRDye800 goat anti-rabbit secondary antibodies (Li-Cor, Lincoln, NE). Blots were imaged and scanned with a Li-Cor Odyssey Infrared Imaging System.

2.3. UVB exposure

The UVB dosages used are relevant to outdoor UVB-exposure in less than 1 h at noon at 40° north latitude, as measured at an angle of 45° above the southern horizon in the summer. The dosages were also chosen based on our previous studies (Singleton et al., 2009; Ubels et al., 2016; Glupker et al., 2016).

For ion chromatography and the cytochrome *c* ELISA, cells were grown to confluence in the four corner wells of six-well plates in Keratinocyte-Serum Free Medium. The cells were washed with HBSS and exposed to UVB (302 nm) using an Ultraviolet Products model UVM-57 lamp (UVP, Upland, CA) at a dose of 150 mJ/cm^2 while in Hanks Balanced Salt Solution (HBSS) without phenol red (Invitrogen, Carlsbad, CA). UVB intensity was measured using a Solarmeter Model 6.2 (Solartech, Inc., Harrison Twp., MI). Control cells underwent identical medium changes as exposed cells, but were not exposed to UVB radiation.

Cells used for patch-clamp recording were exposed to UVB radiation after access had been achieved and control currents were recorded, as described below. The UVB lamp was positioned 7.5 cm from the recording chamber and cells were exposed to UVB at a dose of 80 mJ/cm^2. We previously reported that doses of 80 or 150 mJ/cm^2 UVB have identical effects on activation of K^+ channels in HCLE cells and similar, dose-dependent effects on caspase activity and loss of K^+ from cells over a range of 50–200 mJ/cm^2 (Singleton et al., 2009; Ubels et al., 2010, 2011). However, we observed that the higher dose has an immediate effect on the cell

Table 1
Sequences of siRNAs (data provided by Qiagen). Sequences have been functionally verified in humans.

Hs FAS 7	
Target sequence	5′-AAGGAGTACACAGACAAAGCC-3′
Sense strand	5′-GGAGUACACAGACAAAGCCTT-3′
Antisense strand	5′-GGCUUUGUCUGUGUACUCCTT-3′
Hs FADD 5	
Target sequence	5′-AAGAAGACCTGTGTGCAGCAT-3′
Sense strand	5′-GAAGACCUGUGUGCAGCAUTT-3′
Antisense strand	5′-AUGCUGCACACAGGUCUUCTT-3′
HS TNFRSF1A 5	
Target sequence	5′-AAGTGCCACAAAGGAACCTAC-3′
Sense strand	5′-GUGCCACAAAGGAACCUACTT-3′
Antisense strand	5′-GUAGGUUCCUUUGUGGCACTT-3′