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Research article

Involvement of the extrinsic and intrinsic pathways in ultraviolet B-induced apoptosis of corneal epithelial cells

John L. Ubels [a, *], Courtney D. Glupker [a], Mark P. Schotanus [a], Loren D. Haarsma [b]

[a] Department of Biology, Calvin College, Grand Rapids, MI, USA
[b] Department of Physics and Astronomy, Calvin College, Grand Rapids, MI, USA

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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/cm^2 UVB and incubated in culture medium with 5.5 mM K^+. Knockdown 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.

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 and Martinez, 1973;

* Corresponding author. Department of Biology, Calvin College, 1726 Knollcrest Circle Dr. SE, Grand Rapids, MI 49546, USA.
E-mail address: jubels@calvin.edu (J.L. Ubels).

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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, 2003; Vu et al., 2001; 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/cm^2 (less than 2 h 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 min after exposure of HCLE cells to UVB, and this results in the loss of 50% of K^+_i within 10 min, 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 h 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.

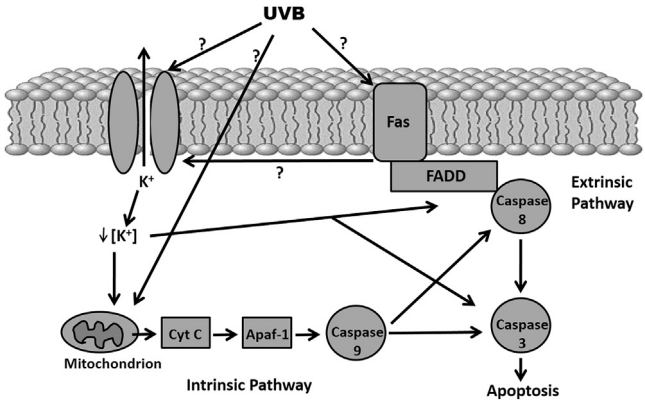

Fig. 1. This diagram illustrates relationships that are possibly involved in UVB-induced apoptosis of HCLE cells. UVB may directly affect K^+ channels, Fas or the mitochondrion. Fas activation by UVB may be required for activation of K^+ channels and caspases-8 and -3 via the extrinsic pathway. Loss of K^+_i upon activation of channels may directly activate the intrinsic pathway and/or may induce activation of caspase-8 and caspase-3.

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 osmolarity 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