Aberrant NF-κB Activity in HaCaT Cells Alters their Response to UVB Signaling

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The immortalized keratinocyte cell line called HaCaT has been used in experiments as a convenient substitute for cultured normal human keratinocytes. However, some molecular differences have been identified that distinguish HaCaT cells from normal human keratinocytes, including differences in the NF-κB signaling pathway and in their response to UVB irradiation. NF-κB is a widely expressed transcription factor that is activated by a cacophony of stimuli, including inflammatory mediators such as TNFα and oxidative stressors such as UVB exposure. This report delineates and further elucidates the aberrant NF-κB signaling pathway and its effect in HaCaT cells exposed to UVB radiation or inflammatory mediators. We demonstrate that NF-κB DNA binding is activated by both UVB and TNFα, but discrepancies in the activation of key upstream signaling pathway components such as AKT phosphorylation and IκBα degradation exist. Disruption of the constitutive NF-κB activity in HaCaT cells resulted in alterations in NF-κB signaling that were more consistent with the NF-κB signaling pathway in normal human keratinocytes. These studies suggest that caution should be used in extrapolating the biological responses of HaCaT cells to those of normal human keratinocytes in the absence of confirmatory experiments.

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INTRODUCTION

Proper functioning of the epidermis is of paramount importance to human survival. The integrity of the epidermis is maintained in part by the ability to continually renew the cells comprising the tissue. This function is dependent on mechanisms that control the correct balance of proliferating and differentiating cells in the stratified structure and any disturbance of this balance can lead to skin diseases. Various signaling pathways controlling epidermal homeostasis have been identified, including signaling cascades that result in the activation of the transcription factor NF-κB (Seitz *et al.*, 1998; Kaufman and Fuchs, 2000; Seitz *et al.*, 2000a; Hu *et al.*, 2001; Nickoloff *et al.*, 2002; Takao *et al.*, 2003). Within the stratified epidermis, NF-κB is located in the cytoplasm in the proliferative basal layer cells and shifts to the nucleus in the nonproliferating suprabasal layers cells. While the molecular mechanisms controlling this process have not been completely identified, the change in cellular localization suggests a role for NF-κB in progression of keratinocytes from proliferation to growth arrest in the epidermis. In part, the inhibition of keratinocyte proliferation by NF-κB can be attributed to the induction of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ (Seitz *et al.*, 2000b). Additional studies have suggested that NF-κB suppresses keratinocyte proliferation by inhibiting TNFR1- and JNK-dependent activation of CDK4 expression (Zhang *et al.*, 2005). NF-κB is comprised of the *rel* family of transcription factors and consists of a heterodimer composed of subunits, p50 (NF-κB1), p65 (*rel*A), p52 (NF-κB2), c-*re*l, or *rel*B (Kaufman and Fuchs 2000; Karin *et al.*, 2002). In unstimulated cells, NF-κB dimers are maintained in the cytoplasm by association with the inhibitory IκBα protein, which blocks the nuclear localization signal of NF-κB. The activation of NF-κB can induce a variety of cellular responses, including the induction of inflammation, cell proliferation, differentiation, or apoptosis. These divergent cell responses to NF-κB activation are defined by cell-specific and stimuli-specific mechanisms (Hinata *et al.*, 2003). A multitude of stimuli activate the NF-κB signaling pathway, including TNFα, lipopolysaccharides, the interleukin family (e.g. IL-1 and IL-8), lipid mediators such as platelet-activating factor (PAF), (Southall *et al.*, 2001), and external stress inducers such as ultraviolet light (Chaturvedi *et al.*, 2001; Adhami *et al.*, 2003).

Reports from several laboratories have shown that disturbances of the NF-κB signaling pathway lead to changes in keratinocyte cell growth and epidermal thickness (Seitz *et al.*, 1998), causing a breakdown in homeostasis, resulting in a loss of function. Defects in NF-κB and NF-κB signaling pathway components are observed in a number of skin abnormalities. For example, patients with incontinentia pigmenti have mutations in IKKγ (NEMO) (Schmidt-Supprian

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Abbreviations: IGF-1R, insulin-like growth factor-1 receptor; IκBα, Inhibitor of NF-κB alpha; IKK, IκB kinase; IκBsr, IκBα super-repressor; NF-κB, nuclear factor kappa B; TNFα, tumor necrosis factor alpha

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et al., 2000; Smahi *et al.*, 2002). Mice lacking IKK subunits IKKγ or IKKβ, die during embryogenesis due to extensive liver apoptosis (Li *et al.*, 1999) and mice expressing an IκBα super repression display impaired hair follicle and exocrine glands due to apoptosis, analogous to that seen in the human disorder hypohydrotic ectodermal dysplasia (Smahi *et al.*, 2002; Courtois, 2005). While the exact nature of the function of NF-κB in normal epidermal homeostasis is unclear, data derived from experimental model systems suggest that NF-κB plays a role in the suppression of carcinogenesis (Hoger linden *et al.*, 1999; Bell *et al.*, 2002; Karin *et al.*, 2002). In rodent models, targeted inhibition of NF-κB function specifically in mouse keratinocytes renders the animals susceptible to the development of squamous cell carcinoma (Hogerlinden *et al.*, 1999; Fujioka *et al.*, 2003). Furthermore, in genetically modified human keratinocytes, NF-κB is required to suppress the induction of carcinogenesis due to transforming mutations in the RAS oncogene (Dajee *et al.*, 2003).

The immortalized human keratinocyte cell line HaCaT has been used as a substitute model for primary human keratinocyte cells. Indeed many features of HaCaT cells and primary human keratinocytes are similar, for example, they both display distinct keratinocyte morphology and both undergo UVB-induced apoptosis (Qin *et al.*, 1999; Chaturvedi *et al.*, 2001; Nickoloff *et al.*, 2002; Lewis *et al.*, 2003b). However, distinct differences in the biology of HaCaT cells and primary human keratinocytes have been described. Alterations in the molecular events necessary for NF-κB activation have been reported in HaCaT cells, which include a diminished induction of Creb binding protein (CBP), p65/RelA or IKKβ after IFNγ and TPA treatment (Chaturvedi *et al.*, 2001). Additionally, compared to primary human keratinocytes, HaCaT cells display an enhanced sensitivity to UVB-induced apoptosis (Chaturvedi *et al.*, 1999; Lewis *et al.*, 2003a, b) and lack intact p53 alleles.

This study investigates the mechanisms of NF-κB activation in HaCaT cells exposed to UVB irradiation and inflammatory mediators. HaCaT cells were found to contain a constitutive aberrant NF-κB activity that is not found in primary human keratinocytes. Significant differences were also found between HaCaT cells and primary human keratinocytes in their response to UVB irradiation. When the constitutive NF-κB activity in HaCaT cells was disrupted, the response of HaCaT cells to UVB became closer to that of primary human keratinocytes.

RESULTS

Induction of NF-κB activity prior to irradiation protects HaCaT cells from UVB-induced apoptosis

In many cell types, the induction of the NF-κB signaling cascade serves to protect cells from a variety of cellular stresses. Previous reports from our lab have demonstrated the protective effect of NF-κB activity in other epithelial cells as well as primary human keratinocytes (Southall *et al.*, 2001). This function was examined in HaCaT cells by treating cells with either TNFα or CPAF (an analog of the lipid inflammatory mediator platelet-activating factor) to induce NF-κB activity, and subsequently irradiating the cells with a lethal dose of UVB. As shown in Figure 1, the pre-activation of NF-κB protected HaCaT cells from UVB-induced apoptosis, as demonstrated by the reduction in caspase 3 specific activity in TNFα- and CPAF-treated cells. This response in HaCaT cells is consistent with previously described survival functions of NF-κB in primary human keratinocytes.

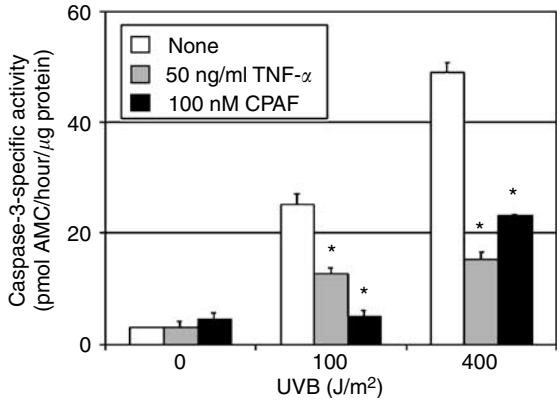

Figure 1. Activation of NF-κB prior to UVB irradiation protects HaCaT cells from UVB-induced apoptosis. HaCaT cells were untreated or treated with the indicated concentrations of TNFα or CPAF. At 30 minutes following treatment, the cells were irradiated with 0, 100, or 400 J/m^2 of UVB. At 9 hours after irradiation, the HaCaT cells were harvested and the induction of apoptosis was determined by measuring the level of caspase 3 specific activity in the cell lysates. Error bars indicate SD of the mean; asterisks indicate significant difference from untreated (*t*-test, $P<0.02$). The graph is representative of at least three independent experiments.

Aberrant constitutive NF-κB activity in unstimulated HaCaT cells

As described above, several differences in the NF-κB signaling pathway have been described in HaCaT cells that are distinct from that found in normal human keratinocytes (Chaturvedi *et al.*, 1999, 2001; Muller *et al.*, 2003). Therefore, the ability of UVB to induce NF-κB DNA-binding activity was compared in HaCaT cells and normal human keratinocytes. HaCaT cells and primary human keratinocytes were exposed to a single high dose of UVB and the cells were harvested at specific times following irradiation. The DNA-binding activity of NF-κB was then determined in cell lysates by electrophoretic mobility shift assays using specific NF-κB oligonucleotides. Oligonucleotides specific for the OCT transcription factor were used as controls because OCT binding is unaffected by UVB irradiation. As described previously, unstimulated normal human keratinocytes have very little active NF-κB DNA-binding activity (Figure 2). However, following UVB irradiation, NF-κB DNA-binding activity increases in a time-dependent manner, with significant NF-κB DNA-binding activity apparent between 2 and 4 hours post-irradiation. In contrast to normal human keratinocytes, unirradiated HaCaT cell lysates contained substantially higher levels of NF-κB DNA-binding activity (Figure 2). Similar to normal human keratinocytes, UVB irradiation of HaCaT cells led to a time-dependent increase in NF-κB DNA-binding activity (Figure 2). OCT DNA-binding activity was unchanged following UVB irradiation in either

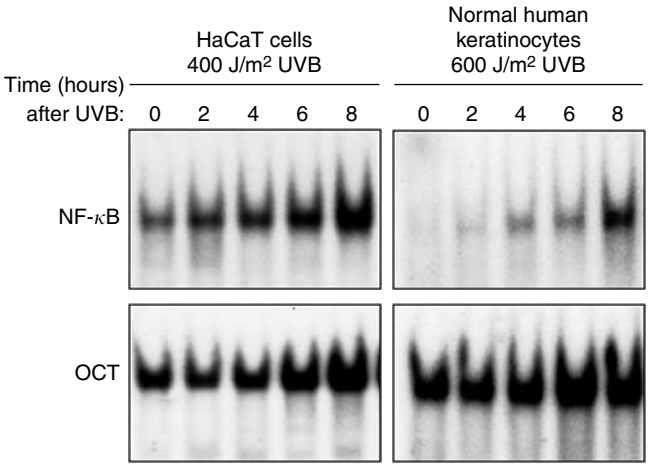

Figure 2. HaCaT cells have a constitutive uninduced level of NF-κB activity that is not found in primary human keratinocytes. HaCaT cells and primary human keratinocytes were unirradiated or irradiated with the indicated doses of UVB. At the specific times indicated following irradiation, the cells were harvested and cell lysates assayed for NF-κB or OCT (control) DNA-binding activity. The results presented in this figure are representative of at least three independent experiments.

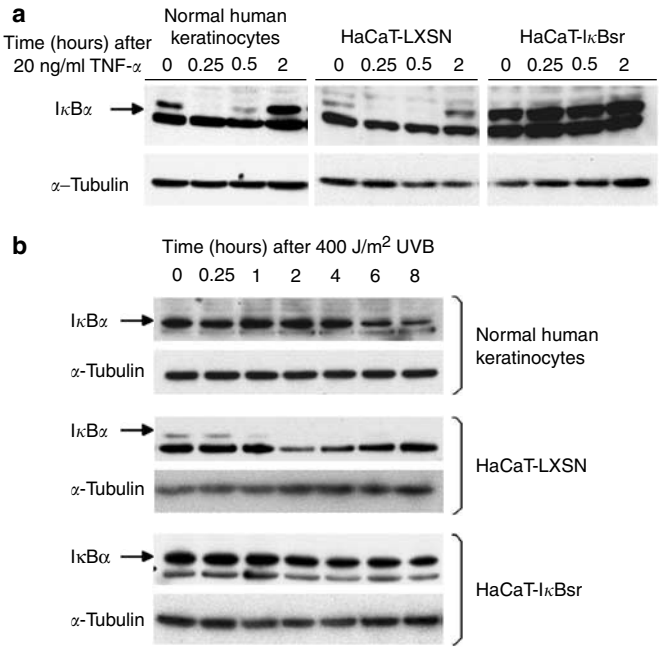

Figure 3. UVB-dependent activation of NF-κB in HaCaT cells utilizes a different signal transduction pathway than primary normal human keratinocytes. (**a**) Primary normal human keratinocytes, HaCaT-LXSN cells, and HaCaT-IκBsr cells were treated with 20 ng/ml TNFα. Cells were harvested at the indicated times following treatment. The presence of IκBα was determined on immunoblots probed with anti-IκBα antibodies (indicated by the arrow, just above the nonspecific crossreactive band). To verify that cell lysates contained equal concentrations of protein, the lysates were also probed with antibodies to α-tubulin. (**b**) Primary human keratinocytes, HaCaT-LXSN cells, and HaCaT-IκBsr cells were irradiated with 400 J/m^2 of UVB and the cells harvested at the indicated times postirradiation. Cell lysates were analyzed as described in (**a**). The data in this figure are representative of two (HaCaT-LXSN and HaCaT-IkBsr cells) or three (NHK) independent experiments.

cell type. Therefore, UVB stimulated NF-κB activity in both HaCaT cells and primary human keratinocytes. However, unstimulated HaCaT cells contained an unusually high degree of NF-κB DNA-binding activity that was not observed in normal human keratinocytes.

UVB-induced NF-κB activation in HaCaT cells is dependent on IκB degradation

To further examine the differences in UVB activation of NF-κB in HaCaT cells and normal human keratinocytes, we utilized a HaCaT cell line that was engineered to be unresponsive to NF-κB activation. This cell line (HaCaT-IκBsr) contains a gene encoding IκBα in which activating serines at amino acid positions 32 and 36 were changed to alanines, rendering the protein resistant to phosphorylation by IKK (Southall *et al.*, 2001). These cells were compared with HaCaT-LXSN cells containing only the vector sequences. IκBα is a member of a family of proteins which inhibits NF-κB nuclear translocation by binding to it. Stimulation of the NF-κB signaling cascade involves IKK, which phosphorylates IκBα. Phosphorylated IκBα is targeted for ubiquitin-dependent degradation, which releases the NF-κB proteins to translocate to the nucleus and become functionally active. The induction of NF-κB activity by TNFα in both normal human keratinocytes and HaCaT cells is accompanied by a rapid degradation of IκBα (Figure 3a, arrow). As IκBα gene expression is activated by NF-κB, baseline levels of IκBα are restored within 2 hours following TNFα treatment. As HaCaT-IκBsr cells express an IκBα protein that is resistant to phosphorylation and degradation, TNFα treatment does not affect the IκBα protein in HaCaT-IκBsr cells. In contrast to TNFα-induced NF-κB activation, UVB irradiation does not alter IκBα protein expression in normal human keratinocytes until at least 6 hours postirradiation (Figure 3b, arrow). At 8 hours post-irradiation, the expression of IκBα was only reduced by 50%, which occurs well after the detection of UVB-induced NF-κB DNA-binding activity. This result suggests that UVB activation of NF-κB in primary human keratinocytes is independent of IκBα degradation. However, UVB activation of NF-κB in HaCaT cells is accompanied by a corresponding decrease in IκBα protein (Figure 3b). These results imply that UVB activates NF-κB in normal human keratinocytes and HaCaT cells by distinct mechanisms. As expected, expression of the IκBsr protein is unaffected by UVB irradiation in HaCaT-IκBsr cells (Figure 3b).

Inhibition of constitutive NF-κB activity in HaCaT cells reduces UVB-induced apoptosis

As described above, UVB activates NF-κB in HaCaT cells through a mechanism that is different from primary human keratinocytes. The molecular inactivation of the NF-κB signaling cascade in normal human keratinocytes has been previously reported to not influence UVB-induced apoptosis (Qin *et al.*, 1999). Therefore, we were interested to see if the inhibition of the aberrant constitutive NF-κB activity in HaCaT cells altered the sensitivity of these cells to

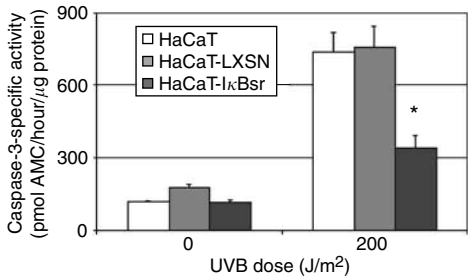

Figure 4. Molecular inhibition of aberrant NF-κB activity in HaCaT cells results in decreased sensitivity to UVB-induced apoptosis. HaCaT, HaCaT-LXSN, and HaCaT-IκBsr cells were irradiated with 0 or 200 J/m^2 of UVB and the cells were harvested 9 hours postirradiation. The induction of apoptosis in these cells was determined by measuring the caspase 3 specific activity in the cell lysates. Error bars indicate SD of the mean; asterisks indicate significant difference from untreated cells (*t*-test, $P<0.005$). The graph presented in this figure is representative of three independent experiments.

UVB-induced apoptosis. As shown in Figure 4, the mutant IκBsr in HaCaT cells led to a reduction of UVB-induced apoptosis. These data provide evidence that the constitutive NF-κB activity in HaCaT cells contributes to their increased sensitivity to UVB-induced apoptosis.

An increased basal level of activated AKT accompanies the aberrant NF-κB activity in HaCaT cells

To explore how the elevated basal level of NF-κB influences the behavior of HaCaT cells in response to UVB irradiation, we examined the activation status of the AKT protein, a component of the NF-κB survival pathway, in unirradiated cells. Cell lysates from HaCaT, HaCaT-LXSN, HaCaT-IκBsr, or normal human keratinocytes were assayed for the presence of AKT or AKT phosphorylated at serine 473. The levels of total AKT protein were determined by densitometry of the immunoblots adjusted for total protein by comparison to levels of α-tubulin. The percentage of phosphorylated AKT at serine 473 was determined by comparing the relative densitometry of immunoblots using antibodies that recognize activated AKT to adjusted values of total AKT protein. Similar levels of phosphorylated AKT were found in unstimulated HaCaT and HaCaT-LXSN cells. This level was significantly higher than the basal levels of phosphorylated AKT in normal human keratinocytes (Figure 5a). Reducing the unstimulated level of NF-κB in HaCaT cells through the expression of the IκBsr mutant led to a corresponding reduction in the basal level of AKT activation similar to that seen in normal human keratinocytes. Therefore, cells containing a high background level of NF-κB activity also contained a high basal level of phosphorylated AKT. One of the functions previously reported for the AKT protein in keratinocytes is to promote cell survival following UVB irradiation (Kuhn *et al.*, 1999; Madrid *et al.*, 2000). We examined the role of AKT in HaCaT-LXSN cells versus HaCaT-IκBsr cells in response to UVB irradiation using a pharmaceutical inhibitor of AKT. Pretreatment of normal keratinocytes with the AKT inhibitor led to an increase in UVB-induced apoptosis (Figure 5b). In contrast, pretreatment of HaCaT-LXSN cells with the AKT inhibitor did not influence the induction of apoptosis by UVB. Inhibition of

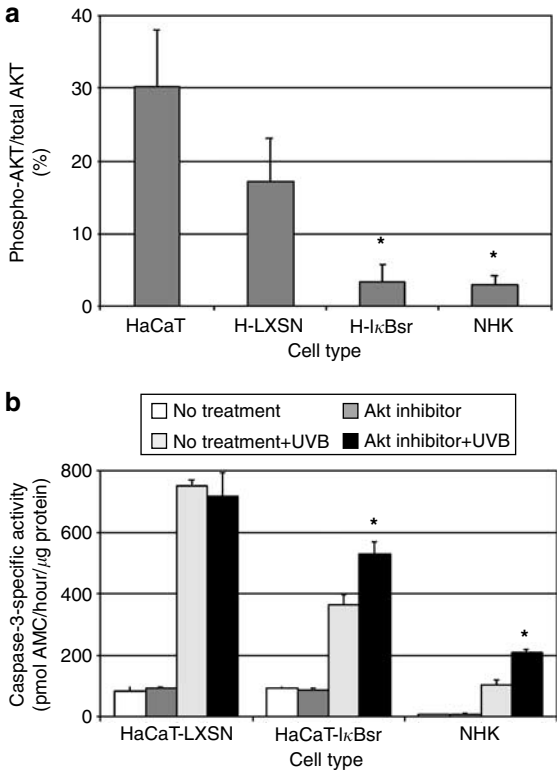

Figure 5. HaCaT cells with aberrant NF-κB activity contain high levels of activated AKT. (**a**) Immunoblots containing HaCaT, HaCaT-LXSN, HaCaT-IκBsr, and primary normal human keratinocyte cell lysates were analyzed for total AKT protein and activated AKT protein that was specifically phosphorylated at serine 473. Equal loading of cell lysates was confirmed using immunoblots probed with antibodies to α-tubulin. The percentage of phosphorylated AKT was determined following densitometric measurement of radiographs and analysis by ImageJ densitometry software (NIH). Data for primary human keratinocytes and HaCaT cells were collected from four separate experiments and data for HaCaT-LXSN and HaCaT-IκBsr cells were derived from two independent experiments. Error bars indicate SD of the mean; asterisks indicate significant difference from HaCaT cells (*t*-test, $P<0.01$). The data are derived from at least three independent experiments. **(b)** HaCaT-LXSN cells, HaCaT-IκBsr cells, and primary normal human keratinocytes were untreated or treated with 10 μM of AKT inhibitor III (Calbiochem) for 1 hour and then the cells were irradiated with either 0 or 200 J/m^2 of UVB. Cells were harvested 6 hours postirradiation and the induction of apoptosis was measured by assaying the caspase 3 specific activity in cell lysates. Error bars indicate SD of the mean; asterisks indicate significant difference from untreated UVB-irradiated cells (*t*-test, $P<0.05$). The data are representative of at least three independent experiments.

AKT activity in HaCaT-IκBsr cells resulted in an increased sensitivity to UVB-induced apoptosis similar to that seen in normal human keratinocytes. These results provide more evidence that the inhibition of the basal NF-κB activity in HaCaT-IκBsr cells restored the UVB response of these cells to an activity similar to that of normal human keratinocytes.

Ablation of the constitutive NF-κB activity in HaCaT cells restores the role of the IGF-1R in the response of the cells to UVB-induced apoptosis

The importance of the IGF-1R in effecting the response of primary human keratinocytes to UVB irradiation has been

well documented (Kuhn *et al.*, 1999). In contrast to normal human keratinocytes, HaCaT cells are less dependent on the activity of the IGF-1R in response to UVB exposure. To determine if the high basal levels of active NF-κB in HaCaT cells affected the influence of the IGF-1R, HaCaT-LXSN and HaCaT-I*k*Bsr cells were grown in culture medium that allowed fully functional IGF-1Rs (EpiLife complete medium) or in media in which the activation of the IGF-1R was suppressed (EpiLife no insulin medium or EpiLife complete medium containing the IGF-1R-specific inhibitor AG 538). Inhibition of IGF-1R in HaCaT-LXSN cells, either by withdrawal of ligand from the media or by treatment with a small-molecule inhibitor, did not significantly influence the induction of apoptosis following UVB irradiation (Figure 6a). However, inhibition of IGF-1R function in HaCaT-IκBsr cells prior to UVB irradiation led to a dramatic increase in apoptosis (Figure 6b) similar to that seen in normal human keratinocytes (Kuhn *et al.*, 1999).

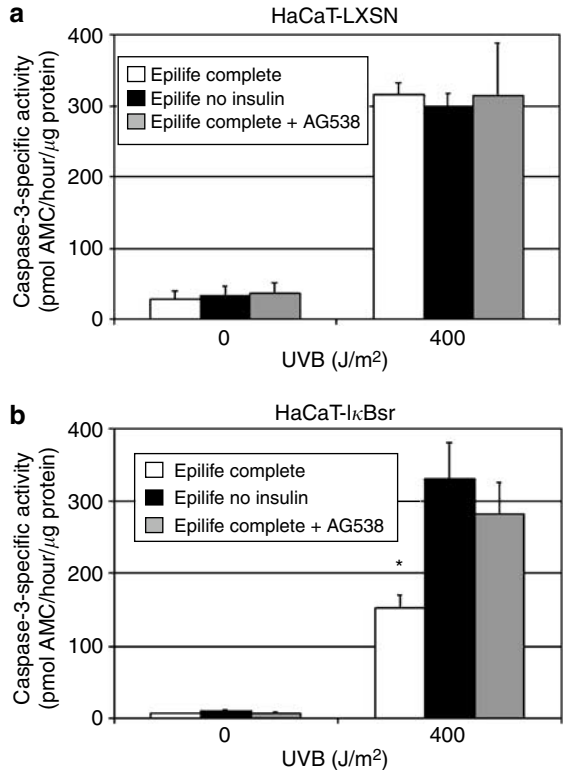

Figure 6. Inhibition of the aberrant NF-κB activity renders HaCaT cells sensitive to IGF-1R-dependent UVB-induced apoptosis. (**a**) HaCaT-LXSN and (**b**) HaCaT-IκBsr cells were grown in EpiLife keratinocyte media (EpiLife complete) or EpiLife keratinocyte media deficient in IGF-1R ligands (EpiLife no insulin) for 24 hours. Some of the cells grown in EpiLife complete were treated with 10 μM I-Ome-AG 538 (a specific small molecule inhibitor of the IGF-1R) for 30 minutes. Following AG 538 treatment, the cells were irradiated with 0 or 400 J/m^2 of UVB. 9 hours postirradiation, the cells were harvested and the induction of apoptosis determined by measuring the caspase-3 specific activity in cell lysates. Error bars indicate SD of the mean; asterisks indicate significant difference from no insulin- or AG 538-treated UVB-irradiated cells (*t*-test, $P<0.02$). The data presented in this figure are representative of at least three independent experiments.

DISCUSSION

Previous reports from many laboratories as well as our own laboratory's studies have indicated the importance of the NF-κB signaling pathway in the response of primary human keratinocytes to UVB irradiation. In addition, many studies have used the immortalized human keratinocyte cell line HaCaT as a surrogate for normal human keratinocytes in similar studies. However, a number of reports have indicated that there are many differences in how normal human keratinocytes and HaCaT cells respond to UVB irradiation. HaCaT cells have been shown to be more sensitive to UVB-induced apoptosis, responding to lower doses of UVB and undergoing UVB-induced apoptosis in a significantly shorter timeframe. The experiments presented in our studies demonstrate that there are some similarities in the response of these two cell populations to UVB; however, there are also many significant differences and these differences should preclude the use of HaCaT cells as a surrogate for UVB studies of normal human keratinocytes.

In both normal human keratinocytes and HaCaT cells, the induction of NF-κB activity by inflammatory mediators prior to UVB irradiation results in enhanced survival of the cells. Additionally, in both cell types, exposure to UVB irradiation evokes an activation of NF-κB as measured by an increase in specific DNA binding. In both cell types, this response increases with the dose of UVB delivered and the time following irradiation. However, the unstimulated level of NF-κB DNA-binding activity in HaCaT cells is much higher than that observed in normal human keratinocytes. In our studies, unstimulated normal human keratinocytes contained very little NF-κB DNA-binding activity, while unstimulated HaCaT cells possessed very significant levels of unprovoked, constitutive NF-κB DNA-binding activity. This aberrant constitutive NF-κB activity in HaCaT cells is a harbinger of differences in the UVB response of normal human keratinocytes and HaCaT cells.

The mechanism of NF-κB activation by TNFα in both normal human keratinocytes and HaCaT cells appears to follow the classical pathway involving degradation of IκBα. However, UVB-induced activation of NF-κB in normal human keratinocytes occurs in the absence of IκBα degradation. In contrast, activation of NF-κB in HaCaT cells following UVB irradiation does require IκBα degradation. These data suggest that the signaling pathway following UVB irradiation in HaCaT cells has been altered from the original keratinocyte response. Furthermore, the constitutive NF-κB activity observed in HaCaT cells was shown to lead to a high constitutive level of activated AKT protein. In most signal transduction schemes, the activation of NF-κB is believed to be a downstream effect of the serine/threonine kinase AKT, as IKK has been demonstrated to be one of the many substrates of activated AKT (Karin, 1999; Ozes *et al.*, 1999). Unstimulated normal human keratinocytes contain low levels of activated AKT protein. In contrast, HaCaT cells have a high constitutive level of activated AKT protein. The high level of phosphorylated AKT was dependent on the constitutive NF-κB activity found in HaCaT cells. If the aberrant NF-κB activity was inactivated by expression of the IκBα

super-repressor, the level of activated AKT fell to similar levels observed in normal human keratinocytes. NF-κB activation of AKT protein expression and phosphorylation has been previously described (Meng *et al.*, 2002; Meng and D'Mello, 2003) and elevated levels of activated NF-κB and AKT are frequently observed in cells derived from epithelial tumors (Dhawan *et al.*, 2002; Saleem *et al.*, 2004; Sweeney *et al.*, 2004). The connection between the increased NF-κB and AKT activities in HaCaT cells can be further validated by experiments examining the response of the cells to UVB irradiation following inactivation of AKT activity. In normal human keratinocytes, suppression of AKT activity leads to an increase in the sensitivity of the cells to UVB-induced apoptosis, consistent with the survival function attributed to AKT. However, in HaCaT cells, inhibition of AKT activity had no effect on the induction of UVB-induced apoptosis. Instead, the inactivation of the constitutive NF-κB activity reduced the background level of AKT phosphorylation and restored the AKT-dependent survival pathway following UVB irradiation.

The aberrant NF-κB activity found in HaCaT cells contributes to the increased sensitivity of HaCaT cells to UVB-induced apoptosis. When the constitutive NF-κB activity was suppressed, HaCaT cells were found to be more resistant to UVB-induced apoptosis. These data are counter-intuitive to the expected role of NF-κB following cell stress because, in most cases, NF-κB functions as a survival factor. However, the altered NF-κB-dependent signal transduction pathways observed in HaCaT cells actually serve to enhance UVB-induced apoptosis. We have previously described the role of the IGF-1R during the response of normal human keratinocytes to UVB irradiation. Activation of the IGF-1R prior to UVB irradiation results in enhanced survival through an AKT-dependent pathway. In contrast, the UVB response in HaCaT cells was shown to be independent of IGF-1R activation. When the aberrant NF-κB activity in HaCaT cells was inhibited, the role of the IGF-1R on UVB-induced apoptosis was restored.

In summary, while HaCaT cells have been conveniently used as an immortalized substitute for primary human keratinocytes, there are many differences between the cell types, especially in the response of the cells to UVB irradiation. Exposure of HaCaT cells to UVB induces a significantly higher level of apoptosis compared to that seen in primary human keratinocytes. HaCaT cells also possess an aberrant elevated NF-κB activity compared to primary human keratinocytes. We propose that this high constitutive level of NF-κB found in HaCaT cells leads to abnormal UVB responses. Therefore, the use of HaCaT cells as a surrogate for normal human keratinocytes in studies designed to investigate UVB-dependent responses should be discouraged.

MATERIALS AND METHODS

Reagents

Human recombinant TNF-α was obtained from PeproTech, Rocky Hill, NJ. NF-κB and OCT-1 oligonucleotide for probes were from Promega Corporation, Madison, WI and γ-^{32}P-ATP was purchased from GE Healthcare, Princeton, NJ. Caspase-3 fluorogenic substrate (aspartic acid-glutamic acid-valine-aspartic acid (DEVD)-AMC) was from Alexis Biochemicals, San Diego, CA. AKT inhibitor III was from Calbiochem, La Jolla, CA. All other chemicals were obtained from Sigma (St Louis, MO), Invitrogen (Carlsbad, CA), BioRad (Hercules, CA), or Roche Molecular Biochemicals (Indianapolis, IN).

Cell culture

HaCaT keratinocyte cells were grown in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Nova-Tech, Inc, Grand Island, NY) and 1000 U penicillin–streptomycin (Roche) unless otherwise stated. Cells were subconfluent at the time of experiments. Primary human keratinocytes were isolated from discarded neonatal foreskin tissue as described previously. Primary human keratinocytes were grown in EpiLife keratinocyte media (Cascade Biologics, Portland, OR) containing 1000 U penicillin–streptomycin, and human keratinocyte growth supplement (Cascade Biologics). HaCaT-LXSN and HaCaT-IκBsr cells were generously provided by Dr Jeffrey Travers (Newton *et al.*, 1999; Southall *et al.*, 2001). In some experiments, HaCaT cells were grown in EpiLife keratinocyte media. We have observed similar results when HaCaT cells were grown in either Dulbecco's modified Eagle's or EpiLife media. All experiments were performed on cells at approximately 75% confluence. All experiments received Institutional Review Board approval in compliance with the declaration of Helsinki principles.

UVB irradiation

UVB irradiation of cultured cells was accomplished using a Philips F20T12/UV-B source (270–390 nm; containing 2.6%. UVC, 43.6% UVB, 53.8% UVA). The intensity of the UVB source was measured prior to each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA). HaCaT cells were irradiated in PBS, after which the original medium with or without supplements was returned to each culture dish. Primary human keratinocytes were irradiated in EpiLife medium, because serum-free EpiLife medium absorbs UVC wavelengths without absorbing significant amounts of UVB. At the doses of UVB used in these studies, we have observed similar results if HaCaT cells were irradiated in either PBS or EpiLife media.

Caspase-3 assay

Caspase-3 proteolytic activity in cell lysates was measured using a synthetic fluorogenic substrate DEVD-AMC (Alexis Biochemicals) as previously described by Hurwitz and Spandau, (2000) and Nicholson *et al*, (1995). Cell pellets were re-suspended in caspase lysis buffer (50 mM piperazine-1,4-bis[2-ethanesulfonic acid, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT) subjected to one freeze–thaw cycle and placed on ice. Cellular debris was removed by centrifugation. An aliquot of the cell lysate was added to caspase reaction buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-[(3-chamidopropyl)-dimethyl-ammino]-1-propanesulfonate, 10 mM DTT, 0.1 mg/ml bovine albumin, and 50 mM CPP32-AMC substrate) and incubated at 37°C for 1 hour. Release of the fluorescent 7-amino 4-methylcoumarin (AMC) moiety was measured using a Hitachi F2000 Spectrophotometer (excitation, 380 nm; emission, 460 nm). The fluorescent intensity was converted to pmoles of AMC by comparison to the fluorescent intensity of standards of AMC (7-amino 4-methylcoumarin; Molecular Probes, Eugene, OR). The

specific activity of caspase-3 in cell lysates was then determined after the total protein concentration of the cell lysates was measured (Protein Assay Reagent Dye, BioRad).

Electrophoretic mobility shift assay (EMSA)

Washed cell pellets were re-suspended in EMSA whole-cell extraction buffer (20 nM HEPES, pH 7.5,. 400 nM KCL, 0.5 nM EDTA, 0.1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM sodium fluoride, 10 mM sodium orthovanadate, and 0.5 mM Pefabloc-sc) and subjected to three freeze–thaw cycles. Cellular debris was removed by centrifugation and the protein concentrations of the whole-cell lysates were determined. Equal quantities of cell lysates (2.5 μg) were incubated with ^{32}P-labeled oligonucleotide probes for NF-κB or OCT-1 (control) consensus binding sites (Promega Corporation). Labeled ^{32}P samples were separated by electrophoresis on a 6% nondenaturing acrylamide gel. Gels were dried onto Whatman filter paper and shift visualized by autoradiography.

Immunoblots

Cells were lysed in RIPA buffer (150 mM NaCl 50 mM Tris, pH 8.0, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing complete protease C (Roche), 1 mM sodium fluoride, and 10 mM sodium orthovanadate. Cell lysates were disrupted by several short bursts of sonication on ice and protein concentrations were determined (BioRad protein assay dye reagent; BioRad). Equivalent amounts of cellular protein were separated on 12% polyacrylamide gels and then transferred to Immobilon PVDF membranes (Millipore Corporation, Bellerica, MA). Specific proteins were identified using antibodies to AKT and phospho-AKT serine 473 (Cell Signaling Technologies, Danvers, MA), IκBα (Santa Cruz Biotech, Santa Cruz, CA), or α-tubulin (Sigma-Aldrich), and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies). The proteins recognized by the specific antibodies were detected using ECL PLUS enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire) and MR-Biomax film (Kodak, Rochester, NY).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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