ORIGINAL ARTICLE

UVB-Induced Activation of NF-κB is Regulated by the IGF-1R and Dependent on p38 MAPK

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To manage the frequent exposure to carcinogenic UVB wavelengths found in sunlight, keratinocytes have extensive protective measures to handle UVB-induced DNA damage. Recent *in vitro* evidence and epidemiological data suggest that one possible protective mechanism is dependent on the functional status of the IGF-1R signaling network. A second important signaling pathway regulating the response of keratinocytes to UVB involves the activation of the NF-κB transcription factor. Although it is clear that proper functioning of both the IGF-1R and NF-κB signaling networks are critical for the appropriate response of keratinocytes to UVB irradiation, it is currently uncertain if these two pathways interact. We now demonstrate that the activation of the NF-κB transcription factor by UVB is altered by the functional status of the IGF-1R. In the absence of ligand-activated IGF-1R, UVB-induced NF-κB consisted primarily of p50:p50 homodimers. Furthermore, the p38 kinase MAPK directs the subunit composition of NF-κB following UVB irradiation, most likely in an IGF-1R-dependent manner. We hypothesize that UVB irradiation leads to an activated p38 MAPK that is regulated in an IGF-1R-dependent manner, leading to NF-κB p50:RelA/p65 activation and a survival phenotype. In the absence of ligand-activated IGF-1R, UVB irradiation leads to the induction of NF-κB p50:p50 homodimers and a p38-dependent increased susceptibility to apoptosis.

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INTRODUCTION

The primary environmental factor that influences the development of skin cancer is exposure to sunlight, particularly wavelengths in the UVB spectrum (Kripke, 1999). When the epidermis is exposed to UVB, the genomes of cutaneous keratinocytes become damaged (Mullenders *et al.*, 1997). If UVB-induced DNA damage is allowed to persist in keratinocytes, alterations (or mutations) in genes will accumulate and cause these keratinocytes to deviate from the normal process of differentiation. This deviation from normal differentiation could eventually lead to a number of proliferative diseases, including basal cell carcinoma and squamous cell carcinoma (Yuspa and Dlugosz, 1991). Fortunately, keratinocytes have extensive protective measures to handle UVB-induced DNA damage (Hainaut, 1995). Recent *in vitro* evidence (Kuhn *et al.*, 1999) and epidemiological data (Chuang *et al.*, 2005) suggest that one possible protective mechanism involves the IGF-1 receptor (IGF-1R) signaling network. When normal human keratinocytes are grown *in vitro*, activated IGF-1Rs protect keratinocytes from UVB-induced apoptosis; however, although UVB-irradiated keratinocytes with activated IGF-1Rs survive, they are incapable of further cellular replication (Kuhn *et al.*, 1999). In the absence of IGF-1R activation, keratinocytes are more sensitive to UVB-induced apoptosis, but the keratinocytes that do survive retain the capacity to proliferate (Kuhn *et al.*, 1999). We hypothesize that the first two observations are part of the normal protective response of human skin to UVB exposure, that is maintaining the integrity of the protective barrier function of the epidermis while ensuring that UVB-damaged keratinocytes are not permitted to replicate DNA mutations. The third observation represents flawed protection from UVB damage, and the consequences of failed UVB protection may include malignant transformation of keratinocytes.

Activation of the NF-κB transcription factor prior to UVB irradiation will protect normal human keratinocytes from UVB-induced apoptosis, suggesting that the NF-κB signaling pathway is another important survival protein regulating the response of keratinocytes to UVB (Chaturvedi *et al.*, 2001; Adhami *et al.*, 2003; Lewis *et al.*, 2006; Lewis and Spandau, 2007). NF-κB consists of a family of proteins (including RelA/p65, c-Rel, RelB, p50 (NF-κB1), and p52 (NF-κB2)), which can homo- and heterodimerize (Chen and Greene, 2004; Schmitz *et al.*, 2004). The specific subunit composition of NF-κB determines its transcriptional activity. In unstressed

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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; DEVD-AMC, aspartic acid-glutamic acid-valine-aspartic acid-aminomethylcoumarin; EMSA, electrophoretic mobility shift assay; ERK, extracellular-regulated kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGF-1R, IGF-1 receptor; JNK, jun N-terminal kinase; MAPK, mitogen-activated protein kinase; OCT-1, octomer-binding protein-1; PIPES, piperazine-1, 4-bis(2-ethanesulfonic acid); TNFα, tumor necrosis factor-α

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epidermis, NF-κB plays a role in regulating appropriate keratinocyte differentiation (Seitz *et al.*, 1998, 2000). In response to a variety of endogenous and exogenous stresses, NF-κB is activated increasing the transcription of inflammatory cytokines. UVB irradiation of human keratinocytes leads to a dose- and time-dependent activation of NF-κB (Lewis *et al.*, 2006; Lewis and Spandau, 2007). However, the mechanism by which NF-κB becomes activated following UVB exposure in keratinocytes is very different from cytokine activation of NF-κB. NF-κB activation by UVB in keratinocytes is independent of IκBα degradation and independent of casein kinase 2, but is regulated by the p38 mitogen-activated protein kinase (MAPK) (Lewis and Spandau, 2007). Furthermore, UVB activation of NF-κB in keratinocytes does not result in phosphorylation of serine 536 on the RelA/p65 subunit that is commonly seen on transcriptionally active NF-κB (Lewis and Spandau, 2007). Accordingly, UVB-induced NF-κB-dependent gene activation was quite different from cytokine-induced NF-κB-dependent gene expression (Lewis and Spandau, 2007).

Although it is clear that proper functioning of both the IGF-1R and NF-κB signaling networks is critical for the appropriate response of keratinocytes to UVB irradiation, it is currently uncertain if these two pathways interact with one another. Therefore, the studies described here were designed to determine if the functional status of the IGF-1R in normal human keratinocytes influenced UVB-induced NF-κB activation.

RESULTS

Role of the IGF-1R in the UVB response of keratinocytes

Previously published reports from our laboratory (Kuhn *et al.*, 1999) as well as others (Kulik *et al.*, 1997; Heron-Milhavet *et al.*, 2001; Decraene *et al.*, 2002; Rochester *et al.*, 2005; Thumiger *et al.*, 2005) have demonstrated that the activation status of the IGF-1R is critical for the appropriate response of human keratinocytes to UVB irradiation. Based on these reports, we defined a model system to examine the response of keratinocytes to UVB irradiation in the presence or absence of activated IGF-1 receptors. Normal human keratinocytes were cultured in EpiLife-HKGS (human keratinocyte growth supplement) media (here called "Complete") that contain 5 μg ml^{-1} insulin. This concentration of insulin will activate both the insulin receptor and the IGF-1R. Keratinocytes were also grown in identical EpiLife-HKGS media in which the supplemental insulin had not been added (called "NoIn"). The activation status of the IGF-1R was then assayed by measuring IGF-1R-specific tyrosine phosphorylation. As seen in Figure 1a, the IGF-1R was only activated in Complete media but not in NoIn media. The overall expression of IGF-1R was not affected by growth in either EpiLife Complete or EpiLife NoIn media. The rapid activation of the IGF-1R by exogenous IGF-1 in both growth conditions demonstrated that the receptor-dependent signaling pathway was intact in both Complete and NoIn media. Activation of the IGF-1R could also be suppressed in Complete media by a small molecule inhibitor of the IGF-1R tyrosine kinase (I-OMe-AG 538). These results demonstrated that the functional

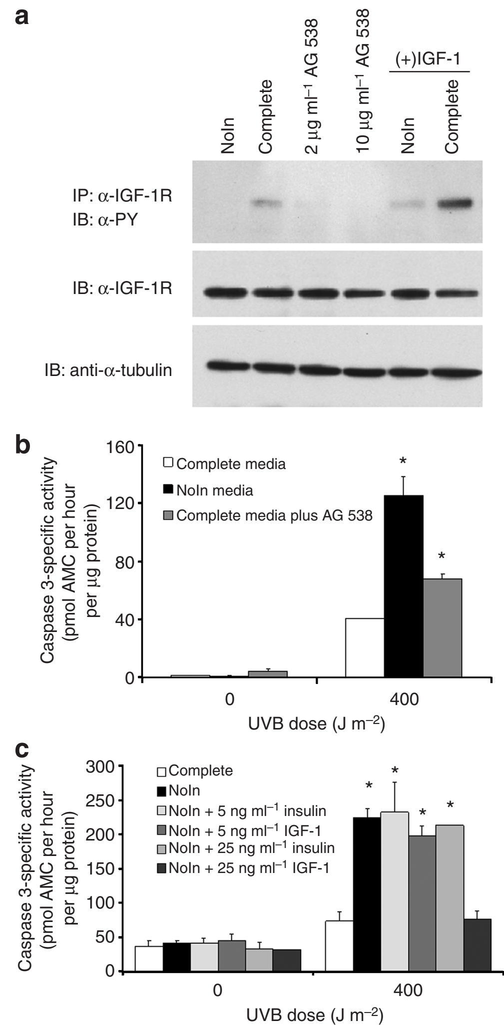

Figure 1. Activation status of the IGF-1R influences the response of human keratinocytes to UVB irradiation. (**a**) Normal human keratinocytes were grown in Complete EpiLife media or NoIn EpiLife media for 48 hours. Some keratinocytes grown in Complete media were treated with a small molecule inhibitor of IGF-1R tyrosine kinase (I-OMe-AG 538; 2 or 10 μg ml^{-1}) for 1 hour. As indicated, selected plates of keratinocytes grown in Complete or NoIn media were treated with 20 ng ml^{-1} IGF-1 for 15 minutes. Keratinocytes were harvested and equal quantities of cell lysate protein were immunoprecipitated with α-IGF-1R antibodies and Protein A/Protein G Sepharose beads. Immunoprecipitates were immunoblotted and probed with α-phosphotyrosine antibodies. Equal amounts of cell lysates were also immunoblotted and probed with either α-IGF-1R or α-alpha-tubulin antibodies as controls. (**b**) Normal human keratinocytes were grown in Complete or NoIn media for 48 hours. Portions of the cells grown in Complete media were treated with 10 μg ml^{-1} I-OMe-AG 538 for 1 hour. The keratinocytes were then irradiated with a dose of 0 or 400 J m^{-2} UVB. Keratinocytes were harvested at 6 hours post-irradiation and assayed for caspase 3 enzyme activity. Error bars indicate the standard deviation of the mean; asterisks indicate significant difference from control values ($P<0.01$, *t*-test). The data presented represent three independent assays. (**c**) Normal human keratinocytes were grown in Complete or NoIn media for 48 hours. Some of the keratinocytes grown in NoIn media were treated with 5 ng ml^{-1} insulin, 25 ng ml^{-1} insulin, 5 ng ml^{-1} IGF-1, or 25 ng ml^{-1} IGF-1 for 1 hour. The keratinocytes were then irradiated with doses of 0 or 400 J m^{-2} UVB. Six hours post-UVB, the cells were harvested and assayed for caspase 3-specific activity. Error bars indicate the standard deviation of the mean; asterisks indicate significant difference from control values ($P<0.005$, *t*-test). The data presented represent three independent assays.

status of the IGF-1R could be inhibited by ligand withdrawal. The cellular consequences of IGF-1R inactivation and the UVB response of keratinocytes grown in either Complete or NoIn media were examined by assaying for the induction of apoptosis (Kuhn *et al.*, 1999; Hurwitz and Spandau, 2000; Southall *et al.*, 2001; Lewis *et al.*, 2003; Li *et al.*, 2003). Keratinocytes grown in NoIn media were significantly more sensitive to UVB-induced apoptosis than keratinocytes grown in Complete media (Figure 1b). Specific inhibition of the IGF-1R by I-OMe-AG-538 in Complete media also resulted in increased sensitivity to UVB-induced apoptosis. As insulin has a high affinity for the insulin receptor and a low affinity for the IGF-1R, and the very high concentration of insulin contained in Complete media can activate both the insulin receptor and the IGF-1R, it is important to distinguish which receptor inactivation directly leads to the observed change in the keratinocyte UVB response. Therefore, insulin or IGF-1 was added back to NoIn media at concentrations that only activate their corresponding receptors. The addition of insulin to NoIn media did not change the sensitivity of keratinocytes to UVB-induced apoptosis (Figure 1c). However, the addition of IGF-1 to NoIn media restored the UVB response of keratinocytes to that seen in cells grown in Complete media (Figure 1c). As these data demonstrate, the altered UVB response observed in keratinocytes grown in NoIn media is due to a lack of IGF-1R activation. Therefore, to simplify the discussion of subsequent experiments, keratinocytes grown in NoIn media will be described as (−)IGF-1R keratinocytes and those grown in Complete media will be referred to as (+)IGF-1R keratinocytes (referring to the functional status of the IGF-1R in those keratinocytes).

Activation status of the IGF-1R alters the NF-κB DNA-binding pattern

The UVB response of keratinocytes consists of many different components, including activation of the transcription factor NF-κB. As the activation status of the IGF-1R can alter the sensitivity of keratinocytes to UVB-induced apoptosis, we questioned whether the IGF-1R would influence UVB-induced NF-κB activity. We have previously described that UVB irradiation of (+)IGF-1R keratinocytes leads to increased NF-κB DNA-binding activity, although less intensely and at a slower kinetic rate than keratinocytes stimulated with the cytokine tumor necrosis factor-α (TNFα) (Lewis and Spandau, 2007). Similarly, UVB-irradiated (−)IGF-1R keratinocytes demonstrated increased NF-κB DNA-binding activity; however, the DNA-binding pattern in (−)IGF-1R keratinocytes was quite different from that seen in (+)IGF-1R keratinocytes (Figure 2). Although UVB irradiation of both (+)IGF-1R and (−)IGF-1R cells resulted in two distinct NF-κB-specific shifted bands (A and B in Figure 2), the predominant band seen in (+)IGF-1R keratinocytes was Band A, whereas the predominant band observed in (−)IGF-1R keratinocytes was Band B. This observed shift in DNA-binding patterns was also seen in TNFα-induced NF-κB patterns, indicating that the shifted DNA-binding pattern was the result of the functional status of the IGF-1R and not unique to UVB exposure.

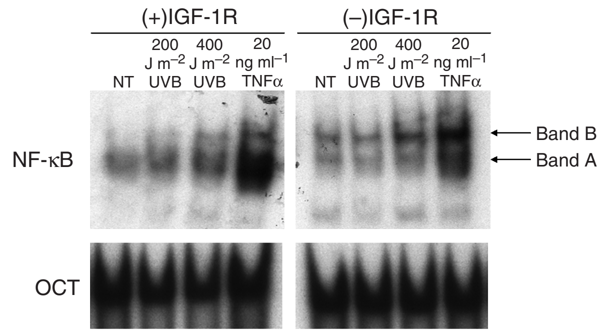

Figure 2. The activation status of the IGF-1R alters the UVB-induced NF-κB DNA-binding pattern. (+)IGF-1R keratinocytes (grown in EpiLife Complete media) or (−)IGF-1R keratinocytes (grown in EpiLife NoIn media) were irradiated with doses of 0, 200, or 400 J m^{-2} of UVB. Additional sets of keratinocytes were treated with 20 ng ml^{-1} TNFα. Keratinocytes were harvested for EMSA assays at 1 hour (TNFα-treated) or 4 hours (UVB-treated) post-treatment. Cell lysates were used in EMSA assays with OCT- or NF-κB-specific oligonucleotides. Two bands (A and B) specific for NF-κB DNA binding are indicated by arrows. Experiments using OCT oligonucleotides were controls to confirm the equivalent integrity of the cell lysates.

UVB-irradiation of (−)IGF-1R keratinocytes induces NF-κB p50/p50 homodimers

The classical cytokine-induced NF-κB signaling pathway leads to the activation of subunits comprised predominantly of p50:RelA/p65 heterodimers (Perkins, 2004). Previously we have reported that electrophoretic mobility shift assay (EMSA) analysis of TNFα-treated keratinocyte cell lysates revealed two NF-κB-specific bands, an intense faster-migrating band (corresponding to Band A) and a less predominant slower-migrating band (corresponding to Band B) (Lewis and Spandau, 2007). Supershift EMSA analysis identified Band A to consist of p50:RelA/p65 heterodimers and Band B to contain primarily p50:p50 homodimers. To confirm the identity of the NF-κB subunits induced to bind NF-κB oligonucleotides from (−)IGF-1R keratinocytes following UVB irradiation, cell lysates were analyzed by identical supershift EMSA assays utilizing antibodies with specificities for p50, p52, or RelA/p65 (Figure 3). Similar to the results previously observed in TNFα-induced NF-κB activation (Lewis and Spandau, 2007), the addition of α-p50 antibodies to UVB-treated keratinocyte cell lysates eliminated most of Band B, demonstrating that Band B consisted predominately of p50:p50 homodimers. As p50-specific antibodies did not reduce the intensity of Band A, this band in UVB-irradiated keratinocytes must contain very little if any p50 subunits. In contrast, the addition of α-RelA/p65 antibodies completely eliminated Band A, demonstrating the presence of the RelA/p65 subunit as a major component of this complex. Antibodies specific for p52 also eliminated Band A, suggesting that Band A in UVB-irradiated normal human keratinocyte lysates was primarily composed of p52:RelA/p65 heterodimers. The combined results from Figures 2 and 3 indicate that the activation status of the IGF-1R influences the subunit composition of UVB-induced NF-κB. UVB irradiation of (+)IGF-1R keratinocytes induced NF-κB consisting of primarily p50:RelA/p65 heterodimers, whereas UVB irradiation of (−)IGF-1R keratinocytes induced NF-κB p50:p50 homodimers.

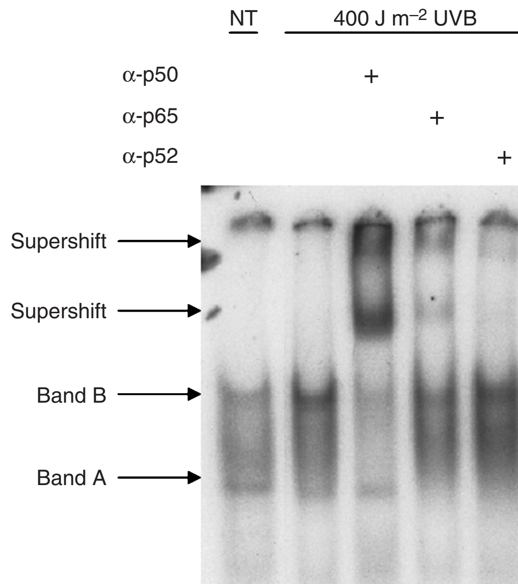

Figure 3. UVB irradiation of human keratinocytes with inactive IGF-1Rs induces NF-κB containing p50:p50 homodimers. (−)IGF-1R keratinocytes were irradiated with 0 (NT) or 400 J m^{-2} of UVB. Four hours following UVB irradiation, keratinocytes were harvested and cell lysates assayed for NF-κB-specific DNA-binding activity. Specific components of the NF-κB DNA-binding complex were identified by the addition of antibodies specific to p50, p52, or RelA/p65 for 30 minutes prior to EMSA analysis. The positions of Band A, Band B, and antibody-dependent supershifted bands are indicated by arrows. DNA-binding patterns and intensities using OCT oligonucleotides were equivalent (data not shown).

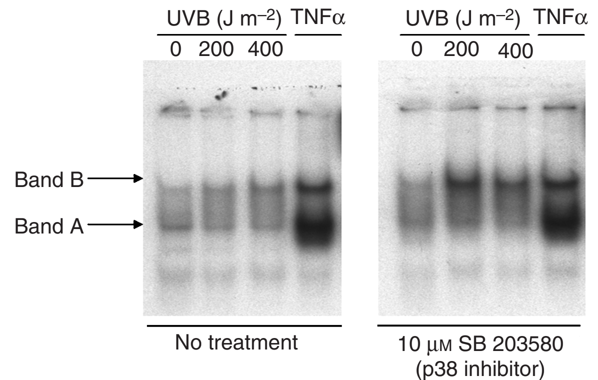

Figure 4. p38 MAPK inhibits the formation of UVB-induced NF-κB p50:p50 homodimers. Normal human keratinocytes ((+)IGF-1R) were untreated or treated with an inhibitor of p38 MAPK (SB 203580) for 1 hour. The keratinocytes were then irradiated with 0, 200, or 400 J m^{-2} of UVB or treated with 20 ng ml^{-1} TNFα. Keratinocytes were harvested at 4 hours post-UVB treatment or 1 hour following TNFα treatment. Specific NF-κB DNA-binding activity was determined by EMSA analysis of cell lysates. Bands A and B are indicated by arrows. DNA-binding patterns and intensities using OCT oligonucleotides were equivalent (data not shown).

IGF-1R-dependent regulation of UVB-induced NF-κB requires p38

UVB irradiation of normal human keratinocytes initiates a rapid activation of the p38 MAPK (Lewis and Spandau, 2007). Previous reports from our lab have implicated p38 in UVB-induced NF-κB activation. To further investigate the role of p38, (+)IGF-1R keratinocytes were treated with SB 203580, an inhibitor of p38 activity, or vehicle. NF-κB DNA binding was then induced by UVB irradiation or treatment with TNFα. EMSA analyses of cell lysates derived from these keratinocytes demonstrated that when p38 activity was inhibited, Band B formation was increased in (+)IGF-1R keratinocytes (Figure 4). In contrast, no effect of SB 203580 treatment on TNFα-induced NF-κB DNA binding was observed. These data suggest that UVB-induced NF-κB activation resulting in p50:RelA/p65 heterodimers is dependent on the activation status of the IGF-1R and functional p38.

Activation status of the IGF-1R does not influence UVB-induced MAPK activation

As the DNA-binding pattern and composition of NF-κB subunits induced by UVB irradiation in (−)IGF-1R keratinocytes was the same as in (+)IGF-1R keratinocytes in the presence of p38 inhibitors, the role of the activation status of the IGF-1R on UVB-induced p38 activation was examined. (−)IGF-1R and (+)IGF-1R keratinocytes were irradiated with UVB and cell lysates harvested at various time points post-irradiation. The activation status of p38, jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK) was then assessed by immunoblot analysis using ERK, p38, and JNK phosphothreonine/phosphotyrosine-specific antibodies. Both p38 and JNK were activated within 15 minutes following UVB irradiation (Figure 5) and remained activated for at least 8 hours. As previously described, ERK was rapidly inactivated following exposure to UVB. UVB did not affect the total amount of ERK, p38, or JNK protein during the 8 hours post-irradiation period. However, the patterns of UVB response for ERK, p38, and JNK were not significantly different in (+)IGF-1R keratinocytes or (−)IGF-1R keratinocytes, indicating that the IGF-1R does not affect UVB-induced MAPK activation.

The IGF-1R modulates the function of p38 following UVB-induced activation

To determine if the activation status of the IGF-1R influenced the function of UVB-induced ERK, JNK, or p38, (+)IGF-1R or (−)IGF-1R keratinocytes were treated with specific small molecule inhibitors of ERK (PD 98059), JNK (SP 600125), or p38 (SB 203580 or PD 169316) and irradiated with 0 or 400 J m^{-2} of UVB. The influence of the inhibitors was then evaluated by assaying for changes in UVB-induced apoptosis. Inhibition of ERK activity prior to UVB irradiation led to increased susceptibility to UVB-induced apoptosis regardless of the activation status of the IGF-1R (Figure 6a). In contrast, inhibition of JNK activity protected keratinocytes from UVB-induced apoptosis; however, SP 600125 was equally protective in both (+)IGF-1R and (−)IGF-1R cells (Figure 6b). Pretreatment of keratinocytes with either of two distinct p38 inhibitors had no effect on UVB-induced apoptosis of (+)IGF-1R cells (Figure 6c). Conversely, inhibition of p38 activity in (−)IGF-1R keratinocytes protected keratinocytes from an increase in UVB sensitivity due to IGF-1R functional

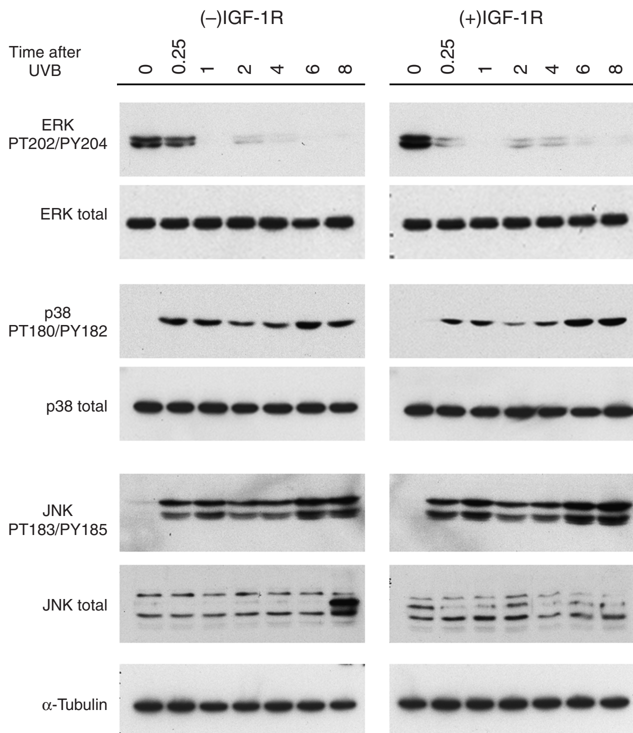

Figure 5. Functional status of the IGF-1R does not affect UVB-induced MAPK activation. (+)IGF-1R keratinocytes (grown in EpiLife Complete media) or (−)IGF-1R keratinocytes (grown in EpiLife NoIn media) were irradiated with doses of 0 or 400 J m^{-2} of UVB and harvested at the indicated time following irradiation. Immunoblots were probed with antibodies that specifically recognize the designated proteins.

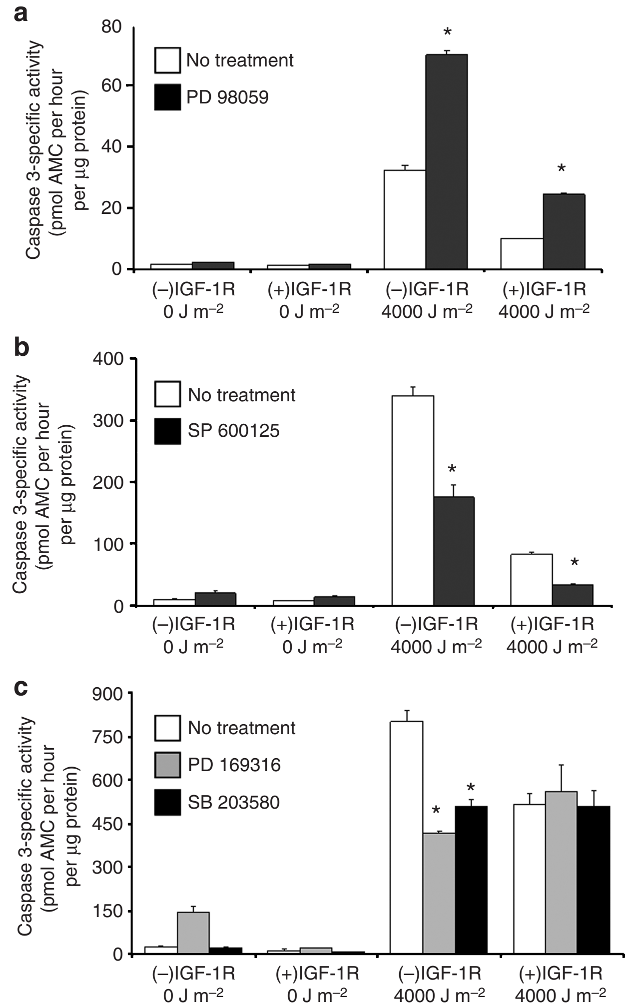

Figure 6. UVB-induced p38 MAPK activity is dependent on the functional status of the IGF-1R. (+)IGF-1R keratinocytes (grown in EpiLife Complete media) or (−)IGF-1R keratinocytes (grown in EpiLife NoIn media) were treated with (**a**) a small molecule inhibitor of ERK activity, 10 μM PD 98059, (**b**) a small molecule inhibitor of JNK activity, 10 μM SP 600125, or (**c**) a small molecule inhibitor of p38 activity, 10 μM PD 169316 or 10 μM SB 203580 for 1 hour. Keratinocytes were then irradiated with 0 or 400 J m^{-2} of UVB and harvested 6 hours post-irradiation. Cell lysates were assayed for caspase 3-specific activity. Error bars indicate the standard deviation of the mean; asterisks indicate significant difference from control values ($P<0.005$, *t*-test). The data presented represent three independent assays.

inactivation (Figure 6c). These data suggest that signaling through the IGF-1R inhibits UVB-induced p38 functions, which results in a muting of the apoptotic signal.

DISCUSSION

The ability of keratinocytes to respond appropriately to the UV components of sunlight is essential for the inherent resistance of the skin to non-melanoma skin cancer. The development of non-melanoma skin cancer occurs when there is a breach in these normal protective mechanisms, particularly in aged epidermis. Previous reports from our laboratory, derived from both *in vitro* and epidemiological data, have demonstrated the importance of the IGF-1R signal transduction pathway to the appropriate response of keratinocytes to UVB irradiation. In our *in vitro* keratinocyte model system, activation of the IGF-1R directs the UVB response of keratinocytes toward two phenotypic outcomes, survival and stress-induced premature senescence. In the absence of a functionally active IGF-1R, keratinocytes have an increased susceptibility to UVB-induced apoptosis; however, the surviving keratinocytes also fail to prevent the replication of UVB-damaged DNA (Kuhn *et al.*, 1999). We have now demonstrated that the activation of the NF-κB transcription factor by UVB is altered by the functional status of the IGF-1R. It is intriguing to speculate that the IGF-1R-dependent shift in UVB-induced NF-κB subunit composition we described in these studies is involved in the IGF-1R-dependent survival of keratinocytes to UVB irradiation. The NF-κB transcriptional subunits we observed that were activated by UVB in the absence of a ligand-activated IGF-1R consisted primarily of p50:p50 homodimers. In general, these p50:p50 homodimers have been associated with changes in the specific genes that are activated by NF-κB; in particular, the p50:p50 homodimer has been linked to the repression of traditional NF-κB-dependent genes (Grundstrom *et al.*, 2004; Williams *et al.*, 2005). Recently, we described differences in the transcriptional profile of NF-κB target genes following either UVB-induced activation or cytokine-induced activation of NF-κB (Lewis and Spandau,

2007). We believe that in the absence of appropriate IGF-1R signaling, UVB-induced NF-κB gene expression is further skewed toward the repression of these target genes.

Previously, we have reported that UVB induction of NF-κB activity occurs via a mechanism distinct from the canonical cytokine-induced pathway (Lewis and Spandau, 2007). During those studies, we found that the MAPK p38 was involved in UVB-induced NF-κB activation. These current experiments now demonstrate that p38 directs the subunit composition of NF-κB following UVB irradiation, most likely in an IGF-1R-dependent manner. These results prompted us to examine the role of the functional status of the IGF-1R on UVB-dependent MAPK activation. Therefore, we examined the activation of ERK, JNK, and p38 following UVB irradiation in both (+)IGF-1R and (−)IGF-1R keratinocytes. The examination of MAPK activation by UVB has been exhaustively described in cultured keratinocytes; however, to maximize the detection of activated MAPK proteins, many of the previous reports have grown the keratinocytes to confluence and then removed all growth factors prior to UVB irradiation (Assefa *et al.*, 1997; Chen and Bowden, 1999; Peus *et al.*, 1999). In contrast, the experiments described in our studies used actively proliferating, subconfluent populations of keratinocytes growing in Complete media. Because of the proliferative nature of these keratinocytes, abundant phosphorylated ERK protein is evident in unirradiated keratinocytes. In these growth conditions, UVB irradiation leads to the rapid inactivation of ERK (Nakamura *et al.*, 2001; Iordanov *et al.*, 2002). However, this rapid dephosphorylation of ERK occurred in both (+)IGF-1R and (−)IGF-1R keratinocytes. In contrast, both p38 and JNK were rapidly phosphorylated following UVB irradiation, and the proteins remained activated for the entire 8-hour period of the experiment. Once again, the activation of both p38 and JNK was independent of the functional status of the IGF-1R. As the IGF-1R did not affect the UVB-induced activation of ERK, p38, or JNK, we examined whether the IGF-1R altered the function of any of the activated MAPK proteins. This was accomplished using specific small molecule inhibitors of the MAPK proteins in both (+)IGF-1R and (−)IGF-1R keratinocytes and assaying for changes in UVB-induced apoptosis. Inhibition of ERK activity resulted in an increased sensitivity to UVB-induced apoptosis, suggesting that ERK functioned as a survival factor. Inhibition of JNK activity protected keratinocytes from UVB-induced apoptosis, implicating JNK in apoptotic signaling pathways. However, the effect of the ERK and JNK inhibitors was identical in both (+)IGF-1R and (−)IGF-1R keratinocytes, demonstrating the activity of these two MAPK proteins was independent of the functional status of the IGF-1R. In contrast, inhibition of p38 activity in (+)IGF-1R keratinocytes did not affect UVB-induced apoptosis, but in (−)IGF-1R keratinocytes blocking p38 activity inhibited the increased UVB sensitivity found in (−)IGF-1R keratinocytes. Therefore, although the IGF-1R does not directly influence the UVB-dependent activation of p38, collectively these data implicate p38 as a downstream modulator of the IGF-1R-dependent UVB response in keratinocytes.

By integrating the results of the data presented here with previously reported data, we have suggested a working model to describe the relationship between the IGF-1R, p38, and NF-κB (Figure 7). We hypothesize that in cells containing ligand-activated IGF-1Rs, UVB irradiation leads to an activated p38 MAPK whose apoptosis-inducing function is suppressed in an IGF-1R-dependent manner, and to NF-κB p50:RelA/p65 activation with an accompanying survival phenotype. If the IGF-1R is inactivated, UVB irradiation leads to the induction of NF-κB p50:p50 homodimers and, in the absence of IGF-1R signaling, UVB-induced p38 activation leads to an increased susceptibility to apoptosis. These data fit well with recent reports in other cell types that have attributed the effects of IGF-1R signaling to be dependent on p38 (Heron-Milhavet *et al.*, 2001). Although Figure 7 is drawn with arrows connecting the IGF-1R and p38, we do not have evidence that there is a direct interaction between these proteins. It is possible that there are intermediate proteins downstream of the IGF-1R that will ultimately interact with p38. A promising candidate for one of these intermediary proteins is the serine/threonine protein kinase apoptosis signal-regulating kinase 1 (ASK1) (Nagai *et al.*, 2007). ASK1 binds to the IGF-1R regardless of the functional status of the IGF-1R. Activation of the IGF-1R leads to the tyrosine phosphorylation of ASK1 that suppresses ASK1 kinase activity (Galvan *et al.*, 2003). Inactive IGF-1R does not phosphorylate ASK1 and subsequently does not inhibit ASK1 kinase activity. One of the substrates of ASK1 is p38 and the activation of p38 by ASK1 leads to apoptosis in certain cell types (Sayama *et al.*, 2001; Matsukawa *et al.*, 2004; Hsieh and Papaconstantinou, 2006). Furthermore, the ASK1–p38 pathway was recently implicated in UVB-induced apoptosis in human keratinocytes (Van Laethem *et al.*, 2006).

In summary, we have defined a model system *in vitro* to examine the role of the functional status of the IGF-1R in the UVB response of human keratinocytes. The subunit composition of UVB-induced NF-κB was dependent on the activation status of the IGF-1R and the p38 MAPK.

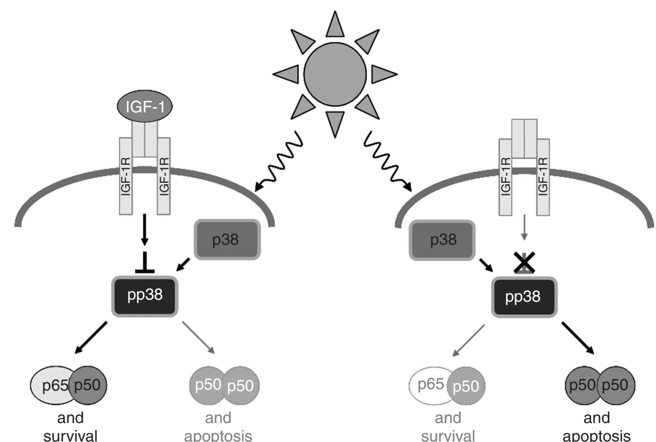

Figure 7. Model for the proposed interactions between the IGF-1R, p38, and NF-κB in normal human keratinocytes following UVB irradiation.

MATERIALS AND METHODS

Normal human keratinocyte cell culture

Normal human keratinocytes were isolated from neonatal foreskin tissue (Kuhn *et al.*, 1999) that was discarded following elective circumcision. Isolated keratinocytes were grown in EpiLife media (Cascade Biologics, Portland, OR) supplemented with HKGS (Cascade Biologics) and 1,000 U penicillin–streptomycin (Roche Molecular Biochemicals, Indianapolis, IN). All experiments were conducted using subconfluent, low-passage primary normal human keratinocytes. The Indiana University School of Medicine Institutional Review Board approved experiments using human keratinocytes via expedited review.

UVB irradiation

UVB irradiation of normal human keratinocytes was accomplished using two Philips FS20T12 UVB broadband light sources. The intensity of the UVB source was measured prior to each experiment using an IL1700 radiometer and an SED240 UVB detector (International Light, Newburyport, MA) at a distance of 8 cm from the UVB source to monolayer of cells. Normal human keratinocytes were irradiated in EpiLife media (Cascade Biologics) and returned to standard incubation conditions (37°C and 5% CO_2). EpiLife medium absorbs all of the UVC wavelengths emanating from the light source without absorbing significant amounts of UVB wavelengths.

Caspase 3 assay

Caspase 3 proteolytic activity in cell lysates was measured using a synthetic fluorogenic substrate (DEVD-AMC; Alexis Biochemicals, San Diego, CA) as previously described by Nicholson *et al*. (1995) and Kuhn *et al*. (1999). Briefly, keratinocyte cell pellets were suspended in lysis buffer (50 mM PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol), subjected to one freeze–thaw cycle, and placed on ice. Any cellular debris was removed by centrifugation. An aliquot of the cell lysate was added to caspase 3 reaction buffer (100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 10% sucrose, 0.1% Chaps, 10 mM dithiothreitol, 0.1 mg ml^{-1} bovine albumin, and 50 μM DEVD-AMC (aspartic acid-glutamic acid-valine-aspartic acid-aminomethylcoumarin) substrate) and incubated at 37°C for 1 hour. Release of the fluorescent AMC moiety was measured using a Hitachi F2000 spectrophotofluorimeter (excitation, 380 nm; emission, 460 nm). The fluorescent intensity was converted to picomoles 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, Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assay

Washed keratinocyte 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 dithiothreitol, 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 (octomer-binding protein-1) (control) consensus binding sites (Promega Corporation). Labeled ^{32}P samples were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel. Gels were dried onto Whatman filter paper and shift visualized by autoradiography. Specific components of the DNA-binding complex were identified by the addition of antibodies to p50, p52, or relA/p65 (Upstate Biotech Inc., Charlottesville, VA) for 30 minutes at room temperature prior to polyacrylamide gel electrophoresis.

Immunoprecipitation

Keratinocyte cultures were washed twice with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) containing complete protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 10 mM sodium orthovanadate, and 1 mM sodium fluoride. Keratinocytes were further disrupted by a sonic dismembranator, seven 3-second bursts on ice. The total protein concentration of the clarified cell lysate was then determined (Bio-Rad Protein Assay Reagent, Bio-Rad, Hercules, CA) and IGF-1R was immunoprecipitated from 500 μg of total cell lysate by incubation with 10 μg of α-IGF-1R antibody (C-20; Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C followed by 2 hours with 30 μl of Protein G Plus/Protein A agarose (Calbiochem, La Jolla, CA). The agarose–protein complex was collected by centrifugation, washed, and separated on an appropriate concentration of polyacrylamide gel.

Immunoblots

Cells were lysed in radioimmunoprecipitation buffer (150 mM NaCl 50 mM Tris-HCl, 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 (Protein Assay Reagent; Bio-Rad). Equivalent amounts of cellular protein were separated on 7.5% (IGF-1R) or 12% (MAPK and α-tubulin) polyacrylamide gels and then transferred to Immobilon polyvinylidine difluoride membranes (Millipore Corporation, Bellerica, MA). Specific proteins were identified using antibodies to α-phosphotyrosine-horseradish peroxidase-conjugated antibody 4G10 (Upstate USA, Charlottesville, VA), α-phosphothreonine^{202}-phosphotyrosine^{204} ERK, α-phosphothreonine^{183}-phosphotyrosine^{185} JNK, α-phosphothreonine^{180}-phosphotyrosine^{182} p38, α-ERK, α-JNK, α-p38 (Cell Signaling Technologies, Danvers, MA), α-IGF-1R (Santa Cruz Biotech, Santa Cruz, CA), 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, UK) and MR-Biomax film (Kodak, Rochester, NY).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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