

UVB-induced Senescence in Human Keratinocytes Requires a Functional Insulin-like Growth Factor-1 Receptor and p53

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To cope with the frequent exposure to carcinogenic UV B (UVB) wavelengths found in sunlight, keratinocytes have acquired extensive protective measures to handle UVB-induced DNA damage. Recent *in vitro* and epidemiological data suggest one these protective mechanisms is dependent on the functional status of the insulin-like growth factor-1 receptor (IGF-1R) signaling network in keratinocytes. During the normal UVB response, ligand-activated IGF-1Rs protect keratinocytes from UVB-induced apoptosis; however, as a consequence, these keratinocytes fail to proliferate. This adaptive response of keratinocytes to UVB exposure maintains the protective barrier function of the epidermis while ensuring that UVB-damaged keratinocytes do not replicate DNA mutations. In contrast, when keratinocytes are exposed to UVB in the absence of IGF-1R activation, the keratinocytes are more sensitive to UVB-induced apoptosis, but the surviving keratinocytes retain the capacity to proliferate. This aberrant UVB response represents flawed protection from UVB damage potentially resulting in the malignant transformation of keratinocytes. Using normal human keratinocytes grown *in vitro*, we have demonstrated that activation of the IGF-1R promotes the premature senescence of UVB-irradiated keratinocytes through increased generation of reactive oxygen species (ROS) and by maintaining the expression of the cyclin-dependent kinase inhibitor p21^{CDKN1A}. Furthermore, IGF-1R-dependent UVB-induced premature senescence required the phosphorylation of p53 serine 46. These data suggest one mechanism of keratinocyte resistance to UVB-induced carcinogenesis involves the induction of IGF-1R-dependent premature senescence.

INTRODUCTION

Cellular senescence is defined as an irreversible arrest in cellular replication in otherwise metabolically active cells. First identified as a phenomenon controlling the longevity of cells cultured *in vitro* (Hayflick and Moorhead, 1961), senescent cells have now been identified *in vivo* to accumulate in aging tissues (Dimri *et al.*, 1995; Pendergrass *et al.*, 1999; Toussaint *et al.*, 2002; Herbig *et al.*, 2006). Replicatively senescent cells result from the critical erosion of telomeres after repeated cellular divisions (Harley *et al.*, 1990; Kim *et al.*, 2002; Blackburn, 2005). Although single specific cellular markers for senescent cells *in vitro* can be inconclusive, senescent cells can be identified by their syndromic phenotype: replication failure; a flattened, spreading cellular morphology; sustained expression of cyclin-dependent kinase inhibitors; and the expression of senescence-associated β -galactosidase (Kim *et al.*, 2002; Blackburn, 2005). Senescence can also be induced before replication exhaustion by factors that induce cellular stress, *i.e.*, oxidative stress, DNA damage, inappropriate oncogene activation (Behrend *et al.*, 2005;

Ben-Porath and Weinberg, 2005; Dasari *et al.*, 2006; Mallette *et al.*, 2007). The induction of senescence after stress-related injury is a defense mechanism to avert carcinogenesis (Campisi, 2005; Dimri, 2005).

In the skin, keratinocytes are repeatedly exposed to UV B radiation (UVB) in sunlight that if left unchecked can potentially cause widespread skin cancer (Melnikova and Ananthaswamy, 2005). Exposure to UVB causes both DNA damage and oxidative stress in irradiated keratinocytes (Mullenders *et al.*, 1997). Fortunately, keratinocytes have developed many specific mechanisms to handle the unrelenting bombardment of carcinogenic insults. The *in vitro*-cultured keratinocyte model system has provided us with a wealth of information regarding how keratinocytes respond to UVB irradiation (Cotton and Spandau, 1997; Kumar *et al.*, 1999; Kuhn *et al.*, 1999; Qin *et al.*, 2002; Zhang *et al.*, 2002; Lewis *et al.*, 2003, 2006; Chaturvedi *et al.*, 2004; Lewis and Spandau, 2007a,b). In addition to the severity of UVB-induced DNA damage, events at the cell membrane of the keratinocyte, and their subsequent signal transduction cascades, critically control the fate of UVB-irradiated keratinocytes. We have examined one of these cell surface events, the activation of the insulin-like growth factor-1 receptor (IGF-1R; Kuhn *et al.*, 1999; Lewis and Spandau, 2007b), and we described three key features affecting the cellular outcome of UVB-irradiated human keratinocytes: 1) ligand-activation of the IGF-1R promotes protection from UVB-induced apoptosis; 2) although UVB-irradiated keratinocytes with activated IGF-1Rs survive, they are incapable of further cellular replication; and

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Abbreviations used: IGF-1R, insulin-like growth factor-1 receptor; PBS, phosphate-buffered saline; UVB, ultraviolet B.

3) 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 were part of the normal protective response of human skin to UVB exposure, i.e., 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. Previously, we have reported that the survival pathway controlling the IGF-1R-dependent UVB response in human keratinocytes involved the nuclear factor- κ B and p38 signaling pathways (Lewis and Spandau, 2007b). We now report that UVB irradiation induces premature senescence in human keratinocytes that is dependent on a functional IGF-1R and subsequent signaling through p53.

MATERIALS AND METHODS

Cell Culture

Normal human keratinocytes were isolated from neonatal foreskin tissue (Kuhn *et al.*, 1999). Isolated keratinocytes were grown in EpiLife Complete media (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement (Cascade Biologics) and 1000 U of penicillin-streptomycin (Roche Molecular Biochemicals, Indianapolis, IN). EpiLife NoIn media is identical to EpiLife Complete *except* it contains no insulin. Subsequently, keratinocytes grown in EpiLife NoIn media are described as (-)IGF-1R keratinocytes, and cells grown in EpiLife Complete media are referred to as (+)IGF-1R keratinocytes (referring to the functional status of the IGF-1R in those keratinocytes; Lewis and Spandau, 2007b). All experiments were conducted using subconfluent, low-passage primary normal human keratinocytes. The n-tert and lif-tert keratinocytes (Dickson *et al.*, 2000) were treated identically to normal keratinocytes. The n-tert cell lines expressing p53 mutants were clonally selected after the transduction of the parental n-tert keratinocytes. Retroviruses were constructed by inserting the relevant p53 gene from pCMV-p53WT, p53S46A, or p53S46D (Ichwan *et al.*, 2006) into the pBABE-puro vector.

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 before each experiment by using an IL1700 radiometer and a 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 then they were returned to standard incubation conditions (37°C and 5% CO₂). EpiLife medium absorbs all of the UVC wavelengths emanating from the light source without absorbing significant amounts of UVB wavelengths.

Senescence-associated β -Galactosidase Assays

Keratinocytes were washed twice with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde/0.2% glutaraldehyde at room temperature for 10 min. After two additional washes with PBS, 2 ml of staining solution (150 mM sodium chloride, 25.2 mM sodium phosphate dibasic, 7.36 mM citric acid, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 ng/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside, pH 6.0; Dimri *et al.*, 1995) were added to the cells, and they were incubated at 37°C overnight. The cells were again washed with PBS and photographed by bright field microscopy to count blue cells and phase contrast microscopy to count total cells. At least four fields (100 \times magnification; ~200–600 cells/field) were counted for each plate of cells; at least two plates of cells for each condition (or cell type) were assayed in each experiment.

Immunoblotting

Cell cultures were washed twice with ice-cold PBS and lysed with radioimmunoprecipitation assay buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40) containing complete protease inhibitor (Roche Molecular Biochemicals), 10 mM sodium orthovanadate, and 1 mM sodium fluoride for 20 min on ice. Keratinocytes were further disrupted by a sonic dismembrator, seven 3-s bursts on ice. Cellular debris was eliminated by two rounds of centrifugation. The total protein concentration of the clarified cell lysate was then determined (Bio-Rad Protein Assay Reagent; Bio-Rad, Hercules, CA). Forty micrograms of cell

lysate was separated on an SDS-polyacrylamide gel, and the proteins were transferred to Immobilon P (Millipore, Billerica, MA) membranes by semidry electrophoresis (Bio-Rad, Melville, NY). After transfer, the membranes were incubated in TSB buffer (150 mM sodium chloride and 100 mM Tris-base, pH 7.5) and 5% nonfat dry milk for 2 h at room temperature. Antibodies were diluted in TSB plus milk (mouse antibodies) or TSB and 5% bovine serum albumin (rabbit antibodies), and they were added to the membrane overnight at 4°C. The membranes were washed three times with TSB containing 0.1% Tween 20. Secondary antibody conjugated to horseradish peroxidase was diluted in TSB plus milk and incubated on the membrane for 30 min. The membrane was washed as described above, and then specific protein bands were identified by chemiluminescence (ECL Plus; GE Healthcare, Piscataway, NJ).

Caspase-3 Assay

Caspase-3 proteolytic activity was measured in keratinocyte cell lysates by using a synthetic fluorogenic substrate (DEVD-AMC; Alexis Laboratories, San Diego, CA) as described previously (Kuhn *et al.*, 1999). Briefly, keratinocyte cell pellets were suspended in lysis buffer (50 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, and 1 mM dithiothreitol [DTT]) for 30 min on ice. Epidermal and cellular debris were removed by centrifugation. An aliquot of the cell lysate was added to a caspase-3 reaction buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 10 mM DTT, 0.1 mg/ml bovine albumin, and 50 μ M DEVD-AMC substrate) and incubated at 37°C for 1 h. Release of the fluorescent 7-amino-4-methylcoumarin (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 with the fluorescent intensity of standards of AMC (Invitrogen, Carlsbad, CA). The specific activity of caspase-3 in cell lysates was then determined after the total protein concentration of the cell lysates was measured (Bio-Rad Protein Assay Reagent).

Reactive Oxygen Species (ROS) Assays

Keratinocytes were washed with PBS, and the cells were loaded with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA; Invitrogen; 10 μ M dye solution in HEPES-balanced saline) for 15 min. Keratinocytes were irradiated with desired dose of UVB, incubated an additional 15 min, and then washed and harvested in fresh HEPES-balanced saline. Fluorescence of the H₂DCFDA was read at 480-nm excitation/520-nm emission in the Hitachi F2000 spectrophotofluorimeter. The approximate cell number in each sample was determined by subsequent staining with 4,6-diamidino-2-phenylindole (DAPI) (fluorescence read at 358-nm excitation/461-nm emission).

RESULTS

Low Doses of UVB Induce Cellular Senescence Only in Keratinocytes with a Functional IGF-1R

To determine whether the IGF-1R is involved in UVB-induced premature senescence in keratinocytes, normal human keratinocytes were grown in EpiLife Complete [(+)IGF-1R] or EpiLife NoIn [(-)IGF-1R] media for 24 h. The keratinocytes were then irradiated with a dose of 0 or 100 J/m² UVB. Fifteen hours after irradiation, the media on both (+)IGF-1R and (-)IGF-1R keratinocytes were replaced with EpiLife Complete media. At 72 h after irradiation, the keratinocytes were assayed for senescent-associated β -galactosidase activity, indicated by the development of blue pigment in senescent cells (Dimri *et al.*, 1995). Figure 1, A–D, depicts representative photomicrographs of the stained keratinocyte cultures from these assays. UVB irradiation of (+)IGF-1R keratinocytes resulted in a high frequency of β -galactosidase-positive cells (Figure 1B), and the flattened morphology of these keratinocytes was also indicative of senescent cells. In contrast, UVB irradiation of (-)IGF-1R keratinocytes failed to induce a demonstrable increase in blue-stained cells (Figure 1D) compared with unirradiated keratinocytes (Figure 1, A and C). When the percentages of senescent keratinocytes were tabulated for each experimental group, only UVB-irradiated (+)IGF-1R keratinocytes had a significant increase in senescent keratinocytes. These results correlate with our previous data demonstrating the failure of UVB-irradiated (+)IGF-1R keratinocytes to proliferate.

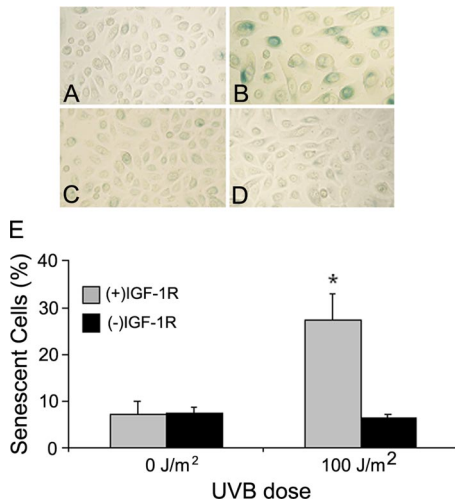


Figure 1. Low doses of UVB irradiation induce cellular senescence only in keratinocytes containing functional IGF-1 receptors. Normal human keratinocytes were grown in EpiLife Complete media [(+)IGF-1R, A and B] or EpiLife NoIn media [(-)IGF-1R, C and D] for 24 h. At that time, keratinocytes were irradiated with 0 (A and C) or 100 (B and D) J/m² UVB. Eighteen hours after irradiation, the media on all of the dishes was replaced with EpiLife Complete media. Seventy-two hours after irradiation, the keratinocytes were assayed for the expression of senescence-associated β -galactosidase. (A–D) Photomicrographs of keratinocyte cultures stained for the presence of senescence-associated β -galactosidase activity (positive cells are blue). (E) Compiled percentage of senescent cells from each culture condition. Error bars indicate the SEM; the asterisk indicates significant difference between irradiated (+)IGF-1R and (-)IGF-1R keratinocyte percentages ($p < 0.01$; t test). The data represent three independent assays.

erate, whereas UVB-irradiated (-)IGF-1R keratinocyte retained the ability to proliferate (Kuhn *et al.*, 1999).

The induction of a stress-induced senescent phenotype can be caused by oxidative stress (Behrend *et al.*, 2005; Ben-Porath and Weinberg, 2005; Dasari *et al.*, 2006). We hypothesized that the failure of (-)IGF-1R keratinocytes to undergo stress-induced senescence after UVB irradiation was due to either the inability of these cells to respond to UVB-induced oxidative stress or a deficiency in the generation of ROS after UVB irradiation. The first possibility was examined by stressing (+)IGF-1R and (-)IGF-1R keratinocytes with hydrogen peroxide and monitoring the induction of senescence. Both (+)IGF-1R and (-)IGF-1R keratinocytes responded to exogenous oxidative stress by undergoing stress-induced senescence (Figure 2, B and D) compared with untreated keratinocytes (Figure 2, A and C), indicating that both types of keratinocytes were capable of undergoing senescence. The induction of ROS by UVB irradiation was then examined by loading (+)IGF-1R and (-)IGF-1R keratinocytes with an indicator dye (CM-H₂DCFDA) to measure the generation of ROS after UVB irradiation. As seen in Figure 2E, keratinocytes with functionally active IGF-1Rs generated higher levels of ROS after UVB irradiation than keratinocytes with inactive IGF-1Rs. These data were consistent with the induction of a senescent phenotype in UVB-irradiated (+)IGF-1R keratinocytes. In contrast, (-)IGF-1R keratinocytes accumulate lower levels of ROS after UVB irradiation, and subsequently, they do not undergo premature senescence.

Sustained Induction of p21^{CDKN1A} after UVB Irradiation Occurs Only in Keratinocytes Containing a Functional IGF-1R

The induction of a senescent phenotype is accompanied by the sustained expression of at least one member of the

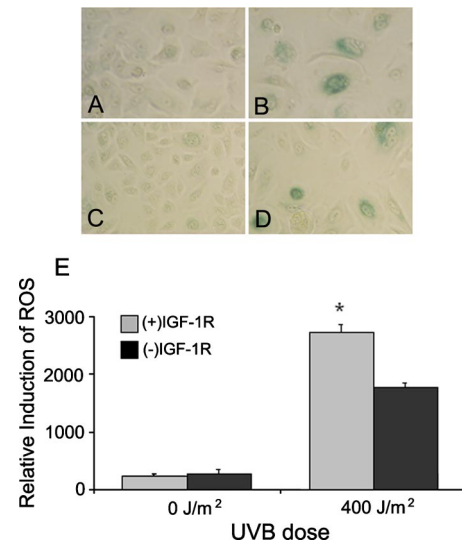


Figure 2. The IGF-1R regulates the production of ROS following UVB irradiation. Keratinocytes were grown in EpiLife Complete [(+)IGF-1R] or EpiLife NoIn [(-)IGF-1R] media for 24 h. (A–D) Both (+)IGF-1R keratinocytes (A and B) and (-)IGF-1R keratinocytes (C and D) were treated with 0 (A and C) or 600 μ M H₂O₂ (B and D) for an additional 24 h. At that time, keratinocyte were examined for the induction of senescence by assaying for the presence of senescence-associated β -galactosidase activity. (E) To measure the production of ROS, both (+)IGF-1R and (-)IGF-1R keratinocytes were loaded with CM-H₂DCFDA dye for 15 min before UVB irradiation. Cells were harvested at 15 min after irradiation, and the fluorescence was measured. The relative induction of ROS was standardized for total cell number by the subsequent staining and measurement of DAPI fluorescence. Error bars indicate the SE of the mean; the asterisk indicates significant difference between irradiated (+)IGF-1R and (-)IGF-1R keratinocyte percentages ($p < 0.01$, t test). The data represent three independent assays.

cyclin-dependent kinase inhibitor family of proteins, namely, p16^{CDKN2A}, p21^{CDKN1A}, or p27^{CDKN1B} (Pajalunga *et al.*, 2007). Therefore, we examined the expression of these proteins after UVB irradiation in both (+)IGF-1R and (-)IGF-1R keratinocytes. In our cultured keratinocyte model, we could not reproducibly detect p16^{CDKN2A} or p27^{CDKN1B} protein, either before or subsequent to UVB irradiation (data not shown). However, consistent with the experiments assaying UVB-induced senescence, the activation status of the IGF-1R influenced the stability of the CDKI p21^{CDKN1A} after UVB irradiation (Figure 3). The p21^{CDKN1A} protein was rapidly degraded within 1 h after UVB irradiation of (-)IGF-1R keratinocytes. In contrast,

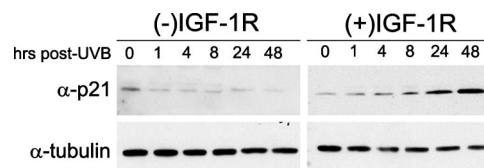
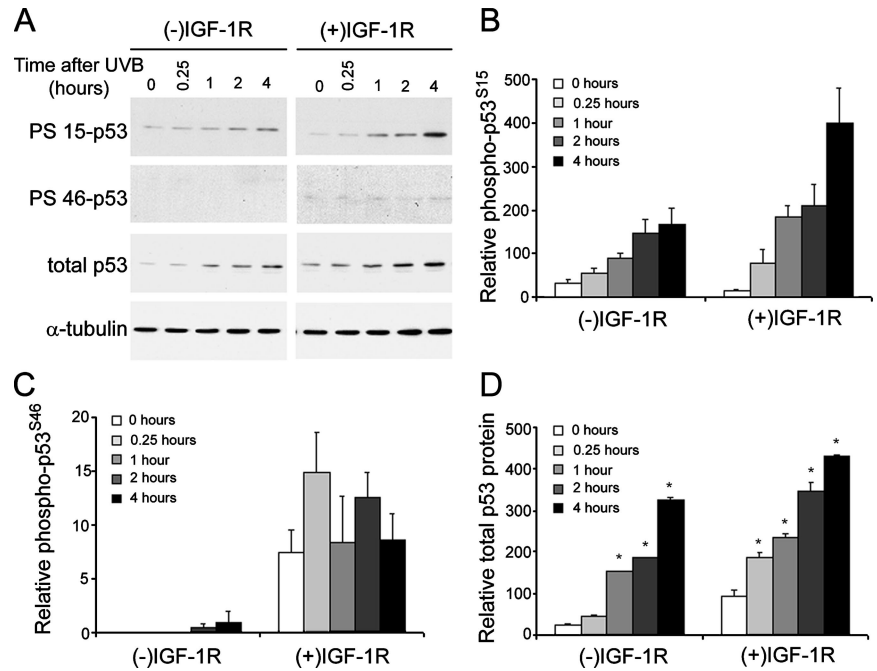


Figure 3. Keratinocytes containing a functionally inactive IGF-1R fail to accumulate p21^{CDKN2A} after UVB irradiation. Normal human keratinocytes were grown in EpiLife Complete [(+)IGF-1R] or EpiLife NoIn [(-)IGF-1R] media for 24 h. At that time, the keratinocytes were irradiated with a dose of 400 J/m² UVB. At the indicated times after irradiation, keratinocytes were harvested and assayed for the expression of p21^{CDKN2A} and α -tubulin via immunoblot analysis.

Figure 4. A functional IGF-1R is required for phosphorylation of serine 46 on p53. (A) Keratinocytes were treated as described in Figure 3 and harvested at the indicated times after irradiation (UVB, 400 J/m²). Keratinocyte cell lysates were analyzed for the presence of phosphorylated serines at amino acids 15 (PS 15-p53) and 46 (PS 46-p53) on p53 by using phospho-specific antibodies. The expression of p53 protein (independent of phosphorylation status) and α -tubulin were also assayed. Densitometry (ImageJ, National Institutes of Health; <http://rsb.info.nih.gov/ij/>) of the expression of phospho-p53^{S15} (B), phospho-p53^{S46} (C), or total p53 protein (D) relative to α -tubulin expression in either (-)IGF-1R keratinocytes or (+)IGF-1R keratinocytes harvested at the indicated time after a UVB dose of 400 J/m². The data represent three independent assays. Error bars indicate the SEM; the asterisks indicate significant difference between unirradiated keratinocytes ($p < 0.05$, t test).



the level of p21^{CDKN1A} in (+)IGF-1R keratinocytes after UVB irradiation steadily increased over the entire 48-h assay. Again, these data are consistent with our model for the normal UVB response in keratinocytes; the induction of stress-induced premature senescence, which is perhaps maintained by persistent levels of the growth inhibitory p21^{CDKN1A} protein. When the IGF-1R is inactive in keratinocytes, an inappropriate response to UVB irradiation occurs. These cells do not become senescent and they can continue to proliferate, which is consistent with the rapid degradation of the growth inhibitory protein p21^{CDKN1A}.

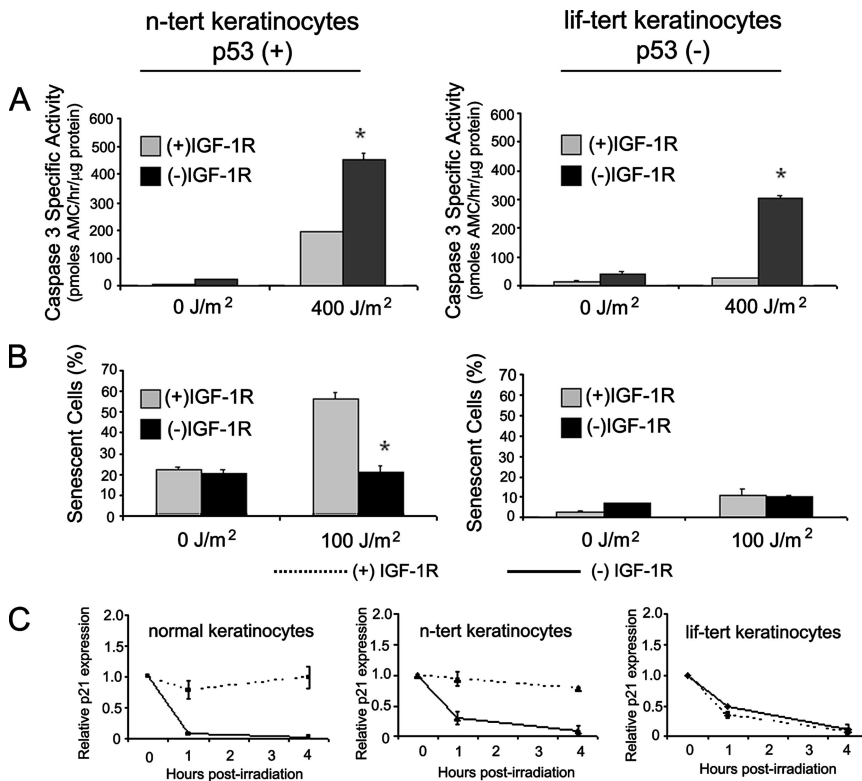
Keratinocytes without a Functional IGF-1R Fail to Phosphorylate Serine 46 on p53

The IGF-1R-dependent protein kinase signaling cascade was exhaustively examined after UVB irradiation. Surprisingly, no differences in the activation of AKT, BCL-X_L, BAX, inhibitor of nuclear factor- κ B, RELA/p65 (data not shown), extracellular signal-regulated kinase, p38, or c-Jun NH₂-terminal kinase (Lewis and Spandau, 2007b) were observed between (-)IGF-1R and (+)IGF-1R keratinocytes. However, the activation of p53 after UVB irradiation was influenced by the activation status of the IGF-1R (Figure 4A). Activation of the IGF-1R resulted in higher levels of UVB-induced total p53 protein, increased phosphorylation of serine 15 on p53 (p53^{S15}), and increased phosphorylation of serine 46 on p53 (p53^{S46}). Interestingly, the phosphorylation of p53^{S15} (Figure 4B) and the induction of total p53 protein (Figure 4D) were only marginally higher in (+)IGF-1R keratinocytes compared with (-)IGF-1R keratinocytes. However, detectable phosphorylation of p53^{S46} only occurred in (+)IGF-1R keratinocytes, and it was generally not observed in (-)IGF-1R keratinocytes (Figure 4C). Unlike p53^{S15} phosphorylation, the phosphorylation of p53^{S46} was not influenced by UVB irradiation.

IGF-1R-dependent UVB Response of Human Keratinocytes Requires Phosphorylation of p53^{S46}

The limited replicative life span of normal human keratinocytes complicates the introduction and analysis of mutant

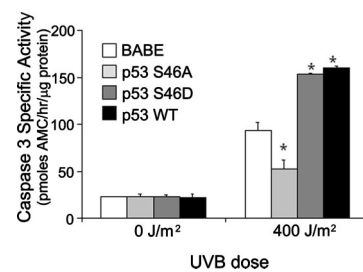
proteins via retroviral transduction. Therefore, it would be advantageous to find an immortal human keratinocyte cell line that could serve as a model for the IGF-1R-dependent UVB response of normal human keratinocytes. Previously, we have demonstrated that the HaCaT immortal keratinocyte cell line was not an appropriate model for the response of normal human keratinocytes to UVB irradiation (Lewis *et al.*, 2006). Subsequently, we have compared normal human keratinocytes with n-tert keratinocytes (Dickson *et al.*, 2000) that were immortalized by ectopic expression of the catalytic subunit of telomerase hTERT. Similar to normal keratinocytes, (-)IGF-1R n-tert cells were more sensitive to UVB-induced apoptosis (Figure 5A; Kuhn *et al.*, 1999; Lewis and Spandau, 2007b). Furthermore, (-)IGF-1R n-tert cells do not undergo UVB-induced premature senescence (Figure 5B, compare with Figure 1E). In addition, the changes in protein expression in n-tert keratinocytes after UVB irradiation (p53, p53^{S15}, p53^{S46}, and p21) were very similar to those changes observed in normal keratinocytes (data not shown). Our previous data had suggested that p53 might be a component of the IGF-1R-dependent UVB response. We tested the role of p53 in this process by comparing the UVB response of n-tert keratinocytes with a similarly hTERT-immortalized keratinocyte cell line derived from an individual with Li-Fraumeni syndrome (lif-tert). In the lif-tert keratinocyte cell line, both p53 alleles are disrupted, resulting in no functional p53 activity (Dickson *et al.*, 2000). Comparison of the response of n-tert keratinocytes and lif-tert keratinocytes (Figure 5, A and B) validate the importance of p53 in the normal (+)IGF-1R response of keratinocytes to UVB irradiation. In contrast to normal human keratinocytes and n-tert keratinocytes, (+)IGF-1R lif-tert keratinocytes do not undergo measurable UVB-induced apoptosis (Figure 5A) and (+)IGF-1R lif-tert keratinocytes also do not become senescent after low dose UVB irradiation (Figure 5B). Similarly, p21 expression is lost in lif-tert keratinocytes after UVB irradiation regardless of the functional status of the IGF-1R (Figure 5C). These data validated the use of n-tert keratinocytes as a surrogate for normal human keratinocytes and suggested the importance of p53 in the IGF-1R-dependent UVB response.



time, the keratinocytes were irradiated with a dose of 400 J/m² UVB. At the indicated times after irradiation, keratinocytes were harvested and assayed for the expression of p21^{CDKN2A} and α -tubulin via immunoblot analysis. Relative expression of p21^{CDKN2A} was determined from autoradiographs by using ImageJ software (<http://rsb.info.nih.gov/ij/>) and normalization with α -tubulin expression. The graphs represent data compiled from at least three independent assays.

Based on the differential phosphorylation of p53^{S46} in (+)IGF-1R keratinocytes in contrast to (-)IGF-1R keratinocytes (Figure 4), we created clones of n-tert keratinocytes expressing p53 proteins altered at amino acid 46 (Ichwan *et al.*, 2006). These mutants include p53^{S46A}, in which the p53 gene was altered so that the wild-type serine found at amino acid 46 is replaced by an alanine. Due to this mutation, the p53^{S46A} mutant protein cannot be phosphorylated at amino acid 46 (an inactivating mutation). The mutant p53^{S46D} contains a p53 gene in which the serine at amino acid 46 was replaced by an aspartic acid residue. This substitution results in an amino acid side chain that resembles a phosphorylated serine residue; thus, this mutant will function in a constitutively activated manner (an activating mutation). The n-tert cells transduced with only the retrovirus vector (BABE) or a retrovirus containing the wild-type p53 gene (p53^{WT}) served as negative and positive controls. As seen in Figure 6, in keratinocytes containing a functional IGF-1R, the p53^{S46A} mutation rendered keratinocytes resistant to UVB-induced apoptosis. This result is consistent with experiment shown in Figure 5A, which demonstrates that inactivation of p53 disrupts the UVB-induced apoptotic signal in (+)IGF-1R keratinocytes. If p53 expression was removed from normal control (p53^{S46D} and p53^{WT} clones), n-tert keratinocytes became more sensitive to UVB-induced apoptosis (Figure 6). As seen in Figure 7A, phosphorylation of p53^{S46} was also essential for UVB-induced senescence in (+)IGF-1R keratinocytes. Low-dose UVB irradiation of n-tert (+)IGF-1R p53^{S46A} keratinocytes failed to become senescent. The activating mutation (p53^{S46D}) or overexpression of p53 (p53^{WT}) did not significantly alter the percentage of senescent cells

after UVB irradiation compared with the vector-only (BABE) (+)IGF-1R keratinocytes (Figure 7A). No phosphorylation of p53^{S46} was observed in n-tert (-)IGF-1R keratinocytes (similar to results in Figure 4), so it was not surprising that n-tert p53^{S46A} (-)IGF-1R keratinocytes were identical to control



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Figure 6. Phosphorylation of serine 46 on p53 is necessary for UVB-induced apoptosis. Independent clones of n-tert keratinocytes transduced with retroviruses expressing the native p53 gene (p53 WT), a p53 gene containing an inactivating mutation resulting from a substitution of an alanine for the serine at amino acid 46 (p53 S46A), a p53 gene containing an activating mutation resulting from a substitution of an aspartic acid for the serine at amino acid 46 (p53 S46D), or containing only the vector sequences (BABE) were irradiated with the indicated dose of UVB. Six hours after irradiation, the keratinocytes were harvested and assayed for caspase-3-specific activity. Error bars indicate the SEM; the asterisks indicate significant differences between irradiated BABE clones and p53 clones keratinocyte specific activities ($p < 0.01$, t test). The graph represents data compiled from at least three independent clones of each cell type.

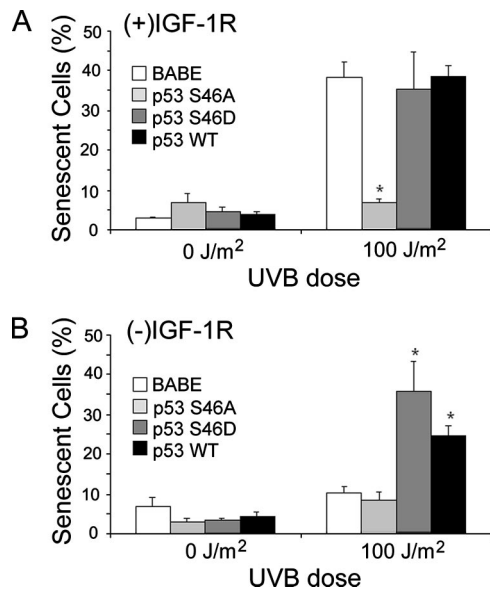


Figure 7. Phosphorylation of serine 46 on p53 is critical for IGF-1R-dependent UVB-induced senescence. The n-tert keratinocyte clones described in Figure 6 were grown in EpiLife Complete [(+)IGF-1R; A] or EpiLife Nolin [(-)IGF-1R; B] media for 24 h. The keratinocytes were then irradiated with the indicated dose of UVB. Fifteen hours after irradiation, the media on all of the keratinocytes were replaced with EpiLife Complete media. Seventy-two hours after irradiation, the keratinocytes were assayed for senescence-associated β -galactosidase activity. The graphs represent the compiled percentage of senescent cells from each culture condition by using at least two independent clones of each cell type. Error bars indicate the SEM; the asterisks indicate significant differences between irradiated BABE clones and p53 clones percentages ($p < 0.01$, t test).

(BABE) n-tert (-)IGF-1R keratinocytes in failing to become senescent after low-dose UVB irradiation. However, either activating mutations at serine 46 (p53^{S46D}) or ectopic expression of p53 (p53^{WT}) in (-)IGF-1R keratinocytes restored low-dose UVB-induced senescence (Figure 7B). These data indicate that UVB-induced senescence in human keratinocytes requires signaling through a functional IGF-1R and subsequent phosphorylation of p53^{S46}.

DISCUSSION

To better understand the biological consequences of UVB-irradiated skin, we have used an in vitro model system to probe the response of normal human keratinocytes to UVB exposure. Using this model system, we have reported previously that the response of human keratinocytes to UVB irradiation was dependent on the activation status of the IGF-1R (Kuhn *et al.*, 1999; Lewis and Spandau, 2007b). In the presence of a functionally active IGF-1R, the response of keratinocytes to UVB irradiation was dependent on the dose of UVB delivered. At low doses of UVB, keratinocytes are able to completely repair all of the UVB-damaged DNA and continue normal cellular functions. At high doses of UVB, the UVB-induced DNA damage is extensive and it cannot be completely repaired by the keratinocytes. At these high doses of UVB, the keratinocytes respond to the severely damaged DNA by undergoing apoptosis. In between these extreme doses of UVB, if the DNA damage is significant enough that the keratinocyte cannot completely repair the

damaged DNA, the keratinocyte undergoes mitotic arrest. We have now demonstrated that this mitotic arrest is in fact premature senescence. Furthermore, we have shown that the induction of senescence by UVB requires a functionally active IGF-1R at the time of UVB irradiation.

If we can extrapolate the findings we observed with cultured keratinocytes in vitro to the response of keratinocytes in the skin, they would correlate with these expected outcomes. To prevent UVB-induced carcinogenesis, replicating keratinocytes cannot be permitted to pass UVB-induced mutations to their progeny. Therefore, if possible, UVB-damaged DNA must be repaired. If the damage to the genome is too great to completely repair, keratinocytes must prevent the passage of damaged DNA via alternative mechanisms. One mechanism would be by inducing UVB-induced apoptosis. However, if this was the default mechanism in the absence of complete DNA repair, too many keratinocytes would be lost from the epidermis, and the barrier function of the epidermis would be severely compromised. Therefore, the induction of premature senescence would be a preferred response to intermediate exposure of UVB, allowing for the continued presence of UVB-damaged keratinocytes and disallowing propagation of UVB-damaged DNA.

In the skin, keratinocytes express the IGF-1R, but they do not synthesize IGF-1 (Tavakkol *et al.*, 1992). Dermal fibroblasts support the proliferation of keratinocytes in the epidermis by secreting IGF-1. Interestingly, as dermal fibroblasts become senescent in vitro, their capacity to produce IGF-1 is severely diminished (Ferber *et al.*, 1993); therefore, in aged skin keratinocytes may be provided with a reduced supply of IGF-1. We think this decrease in IGF-1 expression with advancing age is a major component of the increase in nonmelanoma skin cancer seen in geriatric patients. A corollary of this hypothesis would be that individuals with an increased activation of the IGF-1R might have some protection from UVB-induced skin cancer that could be detected by a decrease in skin cancer incidence. IGF-1 and insulin have very similar molecular structures, and high concentrations of insulin will activate the IGF-1R. Patients with type II diabetes frequently have to take exogenous systemic insulin to overcome their insulin resistance. We hypothesized that the high systemic insulin levels in these patients would compensate for an aging-dependent decline in IGF-1 in the skin by inadvertently maintaining IGF-1R activation despite declining levels of IGF-1. If this were true, our control population would have an increasing incidence of skin cancer with advancing age, whereas the type 2 insulin-using diabetic patient cohort would not see a change in skin cancer incidence with age. In fact, the use of insulin was protective of the age-dependent increase in skin cancer incidence (Chuang *et al.*, 2005).

The role of p53 in regulating apoptosis has been well documented in a variety of tissues in response to a plethora of cellular insults (Meek, 2004; Yee and Vousden, 2005). More recently, p53 has been reported to be essential for the control of cellular senescence, both replicative- and stress-induced (Itahana *et al.*, 2001). The outcome of p53 activation, be it apoptosis or senescence, may be determined by the specific type of activation via posttranslational modification (Brooks and Gu, 2003; Xu, 2003; Moll *et al.*, 2005). One of the most important posttranslational modifications of p53 responsible for inducing apoptosis is the phosphorylation of serine 46 on p53. Phosphorylation of p53^{S46} has been described to alter specific p53-dependent gene expression (Mayo *et al.*, 2005); disrupt interactions with HMD2, leading to p53 stabilization (Xu, 2003; Sullivan *et al.*, 2004); and be essential for p53-dependent apoptosis (Sun *et al.*, 2007; We-

sierska-Gadek *et al.*, 2007). The data we have presented concur with a role for p53^{S46} phosphorylation inducing apoptosis after high doses of UVB. Additionally, we have now demonstrated that phosphorylation of p53^{S46} in keratinocytes was dependent on the functional activation of the IGF-1R. Failure to phosphorylate p53^{S46} in (+)IGF-1R keratinocytes resulted in an inhibition of UVB-induced apoptosis. However, UVB-induced apoptosis in (–)IGF-1R keratinocytes was independent of p53^{S46} phosphorylation. In contrast to previous reports (Sun *et al.*, 2007; Wesierska-Gadek *et al.*, 2007), our data also demonstrated that UVB-induced premature senescence after low-dose UVB exposure required p53^{S46} phosphorylation.

At least two kinases have been reported to phosphorylate p53^{S46}, the p38 MAPK (Xu, 2003; Sun *et al.*, 2007) and HIPK2 (DiStefano *et al.*, 2005; Wesierska-Gadek *et al.*, 2007). We have previously demonstrated that the activation status of the IGF-1R does not influence UVB-induced activation of p38 MAPK (Lewis and Spandau, 2007b). However, the functional status of the IGF-1R does regulate the activity of UVB-activated p38 MAPK (Lewis and Spandau, 2007b). It is provocative to think that one of the downstream effectors regulating p38 MAPK-dependent signaling would be p53^{S46} phosphorylation. However, as of yet we have no information linking IGF-1R function and the substrate specificity of the p38 MAPK.

In summary, we have shown that low doses of UVB can induce premature senescence in normal human keratinocytes *in vitro*. The induction of senescence by UVB was only observed in keratinocytes with functionally active IGF-1 receptors. Ligand-activated IGF-1R resulted in p54^{S46} phosphorylation, which was required for UVB-induced senescence. We hypothesize that to evade the carcinogenic potential of UVB exposure to the skin, the normal UVB response of human keratinocytes is to undergo premature senescence. Furthermore, this protective response to UVB irradiation requires a functionally active IGF-1R and the subsequent downstream phosphorylation of p53^{S46}.

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