E6 proteins from low-risk human papillomavirus types 6 and 11 are able to protect keratinocytes from apoptosis via Bak degradation

Michael P. Underbrink,1,2 Crystal Dupuis,2 Jia Wang1 and Stephen K. Tyring3

1Department of Microbiology & Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA
2Department of Otolaryngology, University of Texas Medical Branch, Galveston, TX 77555, USA
3Department of Dermatology, University of Texas Health Science Center, Houston, TX 77030, USA

Correspondence
Michael P. Underbrink
mpunderb@utmb.edu

INTRODUCTION

Low-risk human papillomavirus (HPV) types 6 and 11 are responsible for the mucosal infections which cause recurrent respiratory papillomatosis (RRP) and genital warts (Cheah & Looi, 1998). It is estimated that the incidence of RRP is 1–4 per 100 000 people in the USA (Larson & Derkay, 2010), whilst genital warts affect 160–289 per 100 000 people (Chesson et al., 2012). These troublesome infections carry a significant burden to the healthcare system at an estimated cost of $0.5 billion per year in the USA alone (Chesson et al., 2012). RRP poses a unique and very challenging problem for patients affected by the disease. First, there are currently no treatments which cure this disease. Thus, the mainstay of treatment relies on surgical debridement of the areas within the larynx and upper aerodigestive tract that contain proliferating papillomas (Goon et al., 2008). Due to the infectious and proliferative nature of RRP, affected patients are prone to frequent recurrences and multiple surgical procedures (Derkay, 1995). Not only does this pose a significant risk of damage to delicate structures important for voice, but rapid and excessive proliferation of papillomas can lead to life-threatening airway obstruction if not addressed quickly. Second, there are no effective medical treatments to help control aggressive disease and reduce the surgical burden of the current treatment paradigm (Stamataki et al., 2007). Finally, and perhaps more importantly, there is not the wealth of epidemiological and experimental evidence for low-risk HPV types that exists for the high-risk HPV counterparts which cause cervical, genitourinary, and head and neck oropharyngeal cancers (zur Hausen, 1996).

In high-risk HPVs, the E6 protein has long been a topic of extensive investigation. The most widely known function of high-risk E6 is its ability to complex with E6-associated protein (E6AP) and target p53, a cellular tumour suppressor that regulates cell cycle arrest and apoptosis in damaged cells, for proteasomal degradation (Huibregtse et al., 1993, 1995; Scheffner et al., 1990). High-risk E6 also possesses redundant mechanisms for circumventing apoptosis, including the inactivation of p53 via p300 binding (Patel et al., 1999), inhibition of both the intrinsic and extrinsic...
pathways via targeting Bak and FADD (Fas-associating protein with death domain) for degradation (Garnett et al., 2006; Jackson et al., 2000), respectively, and attenuation of the UV-induced transactivation of several pro-apoptotic genes (Fas, Puma, Apaf-1 and PIG3) (Giampieri et al., 2004). In contrast, the low-risk E6 proteins either do not possess comparable transforming activities (Barbosa et al., 1991) or, more commonly, have not been investigated for similar molecular effects. The low-risk E6 proteins do, however, share significant homology with high-risk E6 oncoproteins (Howie et al., 2009), and retain some of the abilities that may provide mechanisms for promoting cellular proliferation, disrupting apoptosis and uncoupling cellular differentiation (Gupta et al., 2003; Jha et al., 2010; Park et al., 2001). One retained function between low- and high-risk E6 proteins is the ability of HPV 16E6, 18E6 and 11E6 to bind Bak in vitro and stimulate Bak degradation using an in vivo assay in human 293 cells (Thomas & Banks, 1999). Recently, Underbrink et al. (2008) showed that the E6 proteins from multiple betapapillomavirus types, in addition to high-risk E6 types, degrade Bak and reduce the induction of apoptosis in keratinocytes. Here, we show that the E6 proteins from low-risk RRP-related HPV6 and HPV11 also possess the ability to degrade Bak when expressed in keratinocytes and protect those cells from apoptosis, predominantly via disrupting the intrinsic pathway. These findings not only suggest a conserved function of E6 by both high- and low-risk alphapapillomaviruses, but more importantly add to the limited molecular studies on RRP-related HPV infections that will be important for targeting novel therapeutics for the treatment of these diseases.

RESULTS

E6 proteins of low-risk RRP-related HPVs degrade Bak following UV-B treatment of human keratinocytes

Previous studies have reported that the E6 proteins from high-risk alphapapillomavirus (16, 18), intermediate-risk cutaneous alphapapillomavirus (10, 77) and several betapapillomavirus HPV (5, 8, 20, 22, 38, 76, 92, 96) types prevent the normal accumulation of Bak seen in UV-B-treated keratinocytes (Jackson et al., 2000; Underbrink et al., 2008). To investigate whether the low-risk mucosal alphapapillomavirus HPV responsible for RRP share this ability, HPV 6E6 and 11E6 proteins were expressed in N-TERT-immortalized human keratinocytes (NHKs) and confirmed using reverse transcription (RT)-PCR (Fig. 1a). E6-expressing cell lines were also subjected to real-time PCR to further confirm relative expression levels compared with both vector LXSN and high-risk 16E6 control cells (Fig. 1b). Interestingly, in multiple generated E6-expressing cell lines, the high-risk E6 transcript was consistently expressed at higher levels (10-fold) than in the low-risk E6-expressing cell lines.

The ability of the low-risk E6 proteins to degrade Bak was then compared with high-risk 16E6 and vector LXSN control cells. The cells were treated with UV-B, harvested at 4, 8, 16 and 24 h post-treatment, and subjected to immunoblotting analysis for detection of p53 and Bak (Fig. 2a). UV-B irradiation of LXSN-expressing N-TERT-1 keratinocytes triggered the induction of p53 and the accumulation of Bak, confirming previously reported cellular responses to UV-B irradiation (Laitio & Laiho, 2005). As expected, 16E6-expressing cells targeted p53 for degradation, both constitutively and after UV-B irradiation. Also confirming previous studies, 16E6 reduced Bak levels maximally by 24 h following UV-B treatment. In the low-risk 6E6- and 11E6-expressing cells, p53 was induced similar to vector LXSN control cells. The levels of Bak were also reduced in the low-risk E6 cells with maximal effect at 24 h, very similar to high-risk 16E6. As noted in our previous studies with E6-expressing keratinocytes (Underbrink et al., 2008), the low-risk E6 proteins did not alter the constitutive levels of Bak, but rather reduced the levels beginning ~8 h after UV exposure with maximal effect at 24 h (Fig. 2b).

We also examined Bak mRNA levels following UV treatment to ensure that the reduced levels of Bak were not due to transcriptional changes. 6E6-, 11E6-, 16E- and
LXSN-expressing keratinocytes were exposed to UV-B irradiation, and total RNA harvested at 0, 8 and 24 h. Real-time RT-PCR was performed using SYBR green with Bak-specific primer sets and compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). When compared with LXSN control cells, there was no significant change in Bak mRNA levels at any time point following UV treatment in any of the E6-expressing cells (Fig. 2c). Whilst there appears to be a transcriptional effect on 16E6 at 8 h, this did not approach statistical significance ($P<0.065$). These data confirmed that the decreases in total Bak protein were not due to changes at the transcriptional level.

The ability of E6 to disrupt Bak activation, which is an indirect measure of general apoptotic response, was then examined in E6-expressing cells. 293T cells were transiently transfected with haemagglutinin (HA)-tagged 6E6, 11E6, 16E6 or GFP and immunostained with HA and Bak antibodies. HA-positive cells were then collected by flow cytometry, and activated Bak levels were determined and the mean fluorescent intensity of Bak was plotted. Data represent mean±SD for each sample ($n=3$). Statistical difference between the E6-expressing cells and the LXSN vector control at the indicated time point: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Fig. 2. Low-risk E6 proteins disrupt apoptosis. (a) Representative immunoblot showing the levels of Bak, p53 and nucleolin (Nuc) in E6-expressing and vector control N-TERT NHKs. Cells were mock treated (−) or treated with UV-B (15 mJ cm$^{-2}$) and harvested at the indicated time points (4, 8, 16 and 24 h after UV treatment). (b) Quantification of the levels of Bak protein following UV treatment. Values represent the mean±SD levels of total Bak protein normalized to the levels seen in non-UV treated cells from three independent experiments. (c) No change in Bak mRNA levels in LXSN- and E6-expressing cells following UV treatment. Cells were treated with UV-B (15 mJ cm$^{-2}$) and harvested at the indicated time points (0, 8 and 24 h after treatment). Relative levels of Bak mRNA were calculated using the $\Delta\Delta CT$ method with GAPDH to normalize mRNA levels within each sample. Values shown are the mean±SD changes within each sample compared with before treatment in each cell line ($n=4$). (d) Bak activation is reduced in E6-expressing cells. 293T cells were transfected with HA-tagged 6E6, 11E6, 16E6 or GFP and immunostained with HA and Bak antibodies. HA-positive cells were then collected by flow cytometry, and activated Bak levels were determined and the mean fluorescent intensity of Bak was plotted. Data represent mean±SD for each sample ($n=3$). Statistical difference between the E6-expressing cells and the LXSN vector control at the indicated time point: *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

http://jgv.microbiologyresearch.org
betapapillomavirus HPVs occurs through proteosomal degradation (Jackson et al., 2000; Simmonds & Storey, 2008; Underbrink et al., 2008). We also tested this phenomenon of proteosomal Bak degradation with low-risk 6E6 and 11E6 expression (Fig. 3). 6E6, 11E6, 16E6 and LXSN vector control N-TERT cells were irradiated with UV-B in the presence or absence of the proteasome inhibitor MG132 and then harvested at 24 h. In control cells, the usual responses of Bak and p53 were seen with or without MG132 exposure at 24 h. By comparison, the reduction in Bak levels seen 24 h after UV treatment of 6E6- and 16E6-expressing cells is reversed in the presence of MG132 (Fig. 3a). As the reversal effect on 11E6-expressing cells was not as robust using MG132 (Fig. 3c), we repeated these experiments using a more potent proteasome inhibitor, which allowed longer exposure of the keratinocytes to the inhibitor due to a greater dilutional effect on the DMSO carrier. In the presence of MG262, the accumulation of Bak at 24 h was more robust in all of the E6-expressing cells (6E6, 11E6 and 16E6), as shown in Fig. 3(b), which when quantified more closely mimicked the treatment of LXSN control cells at 24 h (Fig. 3d). Of note, p53 degradation in 16E6-expressing cells was also reversed in the presence of proteasome inhibition, as expected, although slightly less in the presence of MG262 versus MG132. Our findings supported that reduction of Bak in 6E6-, 11E6- and 16E6-expressing keratinocytes occurred via proteosomal degradation.

**Low-risk HPV E6 expression does not affect the overall kinetics of other Bcl-2 family members after UV-B treatment**

The activation of the intrinsic apoptosis pathway after UV exposure is controlled by many related proteins of the Bcl-2 family (Kulms & Schwarz, 2000). In keratinocytes, it has been shown that the crucial Bcl-2 family members for regulating apoptosis through the intrinsic pathway are Noxa, Bcl-xL, Mcl-1 and Bak (Fig. 4a) (Willis et al., 2005). Puma, Bcl-2 and Bax have a less important role in UV-induced keratinocytes, but are also important for this regulation in other cell types. Therefore, we wanted to compare the expression and induction of other relevant Bcl-2 family members in low-risk E6-expressing keratinocytes versus high-risk E6 and control cells (Fig. 4b). All cell lines were exposed to UV-B irradiation, and harvested at 0, 2, 4, 8 and 24 h after treatment. The UV treatment of LXSN control keratinocytes caused the induction of p53 and the p53-responsive pro-apoptotic BH3 protein Noxa, beginning at ~4 h and maximally at 8 h after UV exposure. The low-risk 6E6- and 11E6-expressing cells exhibited the identical response of p53 and Noxa induction and the induction of Bcl-xL, Bcl-2, Mcl-1 and Bak (Fig. 4b). The levels of these proteins were determined by immunoblot analysis (Fig. 4c) and quantified (Fig. 4d). Our findings supported that reduction of Bak in 6E6-, 11E6- and 16E6-expressing keratinocytes occurred via proteosomal degradation.
induction. Interestingly, the high-risk 16E6 cells, which did not contain identifiable levels of p53, also showed induction of Noxa, but at a slightly delayed time point (8–24 h) than controls. Not surprisingly, Noxa has also been reported to mediate programmed cell death in a p53-independent manner (Ploner et al., 2008), which our findings supported. Expression of the pro-apoptotic BH3 protein Puma was not induced after UV treatment in any of the cell lines, but rather was slightly reduced by 24 h, which was consistent with other reports that Noxa is predominantly responsible for UV-induced apoptosis in keratinocytes (Naik et al., 2007). The levels of the pro-apoptotic protein Bax were induced by 8 h in both control and E6-expressing cells.

The anti-apoptotic Bcl-2 family members Mcl-1, Bcl-xL, and Bcl-2 showed variable expression patterns. As expected, Mcl-1 levels were degraded maximally at 4 h in LXSN control keratinocytes. The low-risk 6E6- and 11E6-expressing cells showed a similar pattern of Mcl-1 degradation. In high-risk 16E6-expressing cells, the maximal degradation of Mcl-1 was slightly delayed to 8 h, which correlated with the delay in Noxa induction seen in a p53-independent manner. The levels of Bcl-xL
remained unchanged following UV-B treatment in both control and E6-expressing cells. Bcl-2 was minimally expressed and the levels were slightly reduced 24 h after UV-B exposure in both control and E6-expressing cells. Taken together, these results indicated that, excluding the degradation of Bak, the normal signalling events that initiate apoptosis through the intrinsic pathway were not disrupted by low-risk 6E6 and 11E6 expression.

Low-risk HPV E6 proteins protect keratinocytes from apoptosis

We then investigated whether the degradation of Bak seen in E6-expressing cells could protect keratinocytes from apoptosis caused by cytotoxic damage with UV treatment. UV-B irradiation is known to activate apoptosis through multiple pathways, involving both the extrinsic and intrinsic pathways (Assefa et al., 2005; Kulms & Schwarz, 2000).
Both pathways require the recruitment of initiator caspases: caspase-8 via the extrinsic and caspase-9 via the intrinsic pathway. Once the initiator caspases are activated they, in turn, cleave the effector caspases, such as caspase-3. Expression of E6, and thus Bak degradation, should abrogate this caspase signalling activation following UV-B treatment. Thus, we analysed levels of caspase-3 via immunostaining in LXSN control cells after UV-B exposure and compared those levels with those of E6-expressing cells. Following UV-B treatment, fewer vector control cells than non-treated cells were present and the remaining cells showed a large number of caspase-3-positive cells, which exhibited green fluorescence and condensed nuclei in the assay (Fig. 5a). In comparison, the 6E6-, 11E6- and 16E6-expressing cells showed only a very slight decrease in the number of cells following treatment, and a much lower number of caspase-3-positive cells. Relative to LXSN vector control cells, the E6-expressing cell lines showed a significant decrease in caspase-3-positive, or apoptotic cells, by over one half (Fig. 5d).

At physiologically relevant doses, caspase activation in response to UV radiation occurred mainly via the intrinsic, mitochondria-driven pathway, downstream of cytochrome c release (Assefa et al., 2005). As the release of cytochrome c depends on Bak pore formation, decreased levels of Bak protein should result in a defect of cytochrome c release following UV-B treatment. We therefore tested our cells for the release of cytochrome c following UV-B treatment. After UV treatment, again, fewer vector cells were present and a large number of those cells showed an appearance in cytochrome c staining or absent staining with nuclear fragmentation typical of apoptosis, as indicated by the white arrows in Fig. 5(b). By comparison, the E6-expressing cells all had fewer cells with cytochrome c dispersion. Relative to controls, all of the E6-expressing cell lines showed less than half of the levels of cytochrome c release following UV-B treatment, which was statistically significant (Fig. 5e).

As death receptors on the cell membrane are also involved in UV-induced apoptosis via FADD activation of caspase-8 signalling through the extrinsic pathway (Yuan et al., 2012), we also tested our cell lines for caspase-8 staining. After exposure to UV, the E6-expressing cell lines showed a slight decrease in caspase-8-positive or green fluorescent cells (Fig. 5c). However, this effect was variable in the low-risk 6E6- and 11E6-expressing cells, and did not reach statistical significance. By comparison, the high-risk 16E6-expressing cells showed a significant decrease in caspase-8-positive staining compared with controls (Fig. 5f).

**DISCUSSION**

Previous studies have determined that the E6 protein from high-risk alpha- and betapapillomavirus HPV types gives cells protection from apoptosis after genotoxic exposure (Jackson et al., 2000; Leverrier et al., 2007; Thomas & Banks, 1998; Underbrink et al., 2008). This protective effect provides HPV-infected cells with an advantage over normal tissue and promotes tumorigenesis. It is unknown whether low-risk RRP-related HPV types also possess similar abilities that confer a cytoprotective advantage to infected keratinocytes. This ability would offer affected cells not only a proliferative advantage in vivo, but may also explain the resistance to clinical treatments for RRP.

We investigated the ability of the RRP-related low-risk alphapapillomavirus HPV types to degrade Bak in keratinocyte cultures. This would reveal a conserved function of papillomaviruses regardless of transforming activity or the ability to contribute to carcinogenesis. Both of the low-risk 6E6 and 11E6 proteins were able to degrade Bak following UV-B treatment to the same extent as their high-risk 16E6 counterpart (Fig. 2), and this was not due to changes in transcriptional levels of Bak mRNA.

To confirm a cytoprotective effect of Bak degradation in E6-expressing keratinocytes, we tested the ability of our cells to blunt the apoptotic response. The activated caspase-3 and cytochrome c assays confirmed significant differences between the effects of UV-B treatment on vector control versus E6-expressing cells in both high- and low-risk HPV types. Interestingly, only the high-risk 16E6-expressing cells revealed a significant decrease in caspase-8 activation following UV-B treatment. This finding supports other studies showing that the high-risk E6 proteins are able to bind the death effector domain of FADD and accelerate its degradation, which prevents activation of pro-caspase-8 and the extrinsic apoptotic pathway (Garnett et al., 2006). In contrast, the cytoprotective effect conferred on cells by low-risk 6E6 and 11E6 expression is limited to the ability to degrade Bak and disrupt the intrinsic apoptotic pathway.

To ensure that the protective effect seen for E6-expressing cells is strictly due to Bak degradation, we also examined other Bcl-2 family members within the intrinsic pathway of apoptosis. The expected kinetics of these mediators after UV-B exposure was confirmed in control cells as well as the E6-expressing cells. There were no significant differences between controls versus low- and high-risk E6 keratinocytes, other than the ability to degrade Bak seen in the E6-expressing cells.

E6-mediated degradation of Bak has been shown repeatedly to occur via the proteasomal pathway (Jackson et al., 2000; Underbrink et al., 2008), as we have now shown with low-risk 6E6 and 11E6. This degradation does not occur constitutively with E6 expression, but relies on activation of Bak after UV-B treatment. Recently, the HECT domain E3 ubiquitin ligase HERC1 was identified to be required for E6-mediated Bak degradation (Holloway et al., 2014). The authors also found that the E6 protein is able to recruit HERC1, which contains a putative BH3 domain that can bind to phosphorylated Bak, when cells are damaged by UV. This ability seems conserved in multiple E6 proteins, including the low-risk RRP-related HPVs.
The ability of the low-risk RRP-related 6E6 and 11E6 proteins to repress the apoptotic response has interesting clinical implications. Photodynamic therapy (PDT) using a photosensitizer [m-tetra(hydroxyphenyl)chlorin] has been reported to be effective for the treatment of RRP in multiple clinical studies, but not curative (Shikowitz et al., 2005). As photosensitizers accumulate preferentially within the mitochondria and PDT can lead to an apoptotic response, the ability of this clinical treatment relies on a functional apoptotic response for maximum effect (Dougherty et al., 1998). However, because of the inherent resistance of E6-expressing cells to apoptosis through the mitochondrial pathway, some of the expected clinical response may be circumvented at the molecular level. This may explain why the treatment of respiratory papillomas is so difficult and predominantly quickly recurs after initial surgical treatments, including treatments that target apoptosis, such as PDT. Overcoming the cytoprotective response that E6 expression confers on these cells certainly has clinical implications and is an important area for future studies, currently being performed in our laboratory.

In summary, we have found that the low-risk alphapapillomavirus E6 proteins (HPV6 and HPV11) have a critical conserved function with the high-risk HPVs, i.e. the ability to cause the proteasomal degradation of activated Bak. This finding reveals a new function of the low-risk HPV types that gives them a cytoprotective advantage over normal, uninfected cells. Future studies, focused towards overcoming this response and identifying additional molecular effects of the low-risk E6 proteins, are being examined in order to discover novel targets for the effective clinical treatment of RRP and other diseases caused by HPV infection.

**METHODS**

**Tissue culture.** N-TERT-immortalized NHKs, and the generated N-TERT E6 cell lines (6, 11 and 16) and vector control (LXSN) cells were grown in EpiLife medium, human keratinocyte growth supplement (Life Technologies), and penicillin/streptomycin. 293T cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) containing 10 % FBS and penicillin/streptomycin.

**Plasmids.** Full-length HPV DNA from types 6, 11 (kindly provided by Dr. E.-M. de Villiers, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and 16 (Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) were used as the templates for subsequent PCRs in order to clone the E6 genes into the pLXSN vector. The PCR primers used for the untagged versions of E6 were: 6E6 forward 5'-AAAAAGACGGCTGACTTATGGAAGATGCGAATGGCC-3'/reverse 5'-AGAAAGCTGGTGTCTTCAATGGTAAATTC-3'; 11E6 forward 5'-AAAGACGGCTGACTTATGGAAGATGCGAATGGCC-3'/reverse 5'-AGAAAGCTGGTGTCTTCAATGGTAAATTC-3'; 11E6 forward 5'-ATGGAAAGCAGGCTGCATTATGGAAAGTGCAAATGCCTC-3'/reverse 5'-TACCTCAACACGCGTTTGTGT-3'; 11E6 forward 5'-ATGGAAAAGTAAAGATGCGTCCT-3'/reverse 5'-TATTTCACACAAACGCGCTTGTT-3'; 16E6 forward 5'-ATGCACCAAAAGAGAACTGC-3'/reverse 5'-AGAAGCTGGTGTCTTCAATGGTAAATTC-3'. The primers for actin were acquired from QuantuRNA® β-Actin Internal Standards kit (ThermoFisher Scientific).

**Real-time RT-PCR.** RNA was isolated with a RNeasy Mini kit (Qiagen). Briefly, 600 µl Buffer RLT was added to each 10 cm plate, and cells were harvested by scraping and immediately transferred to a QShredder tube. After centrifugation, the aqueous phase was transferred and mixed with an equal volume of 2-propanol, added to an RNeasy spin column, and centrifuged at 10 000 g for 15 s. The RNeasy spin column membrane was then washed with Buffer RW1 and centrifuged at 10 000 g for 15 s. The RNeasy spin column membrane was then washed with Buffer RPE twice at 10 000 g for 15 s and 2 min. The RNA was then eluted from the RNeasy spin column in RNase-free H₂O. A Qiagen One-Step RT-PCR kit was then used for PCR amplification according to the manufacturer’s protocol. PCR amplification was used to identify 300 bp amplicons with the designated E6 primers: 6E6 forward, 5'-ATGGAAGCTGCCAATTGCTC-3'/reverse 5'-TACCTCAACACGCGTTTGTGT-3'; 11E6 forward 5'-ATGGAAAAGTAAAGATGCGTCCT-3'/reverse 5'-TATTTCACACAAACGCGCTTGTT-3'; 16E6 forward 5'-ATGCACCAAAAGAGAACTGC-3'/reverse 5'-AGAAGCTGGTGTCTTCAATGGTAAATTC-3'. The primers for actin were acquired from QuantuRNA® β-Actin Internal Standards kit (ThermoFisher Scientific).

**RT-PCR.** RNA was isolated with a RNeasy Mini kit (Qiagen). Briefly, 600 µl Buffer RLT was added to each 10 cm plate, and cells were harvested by scraping and immediately transferred to a QShredder tube. After centrifugation, the aqueous phase was transferred and mixed with an equal volume of 2-propanol, added to an RNeasy spin column, and centrifuged at 10 000 g for 15 s. The RNeasy spin column membrane was then washed with Buffer RW1 and centrifuged at 10 000 g for 15 s. The RNeasy spin column membrane was then washed with Buffer RPE twice at 10 000 g for 15 s and 2 min. The RNA was then eluted from the RNeasy spin column in RNase-free H₂O. A Qiagen One-Step RT-PCR kit was then used for PCR amplification according to the manufacturer’s protocol. PCR amplification was used to identify 300 bp amplicons with the designated E6 primers: 6E6 forward, 5'-ATGGAAGCTGCCAATTGCTC-3'/reverse 5'-TACCTCAACACGCGTTTGTGT-3'; 11E6 forward 5'-ATGGAAAAGTAAAGATGCGTCCT-3'/reverse 5'-TATTTCACACAAACGCGCTTGTT-3'; 16E6 forward 5'-ATGCACCAAAAGAGAACTGC-3'/reverse 5'-AGAAGCTGGTGTCTTCAATGGTAAATTC-3'. The primers for actin were acquired from QuantuRNA® β-Actin Internal Standards kit (ThermoFisher Scientific).

**Retrovirus production and infection.** Retroviruses were produced transiently in 293T cells by a vesicular stomatitis virus G-pseudotyped virus production protocol as described previously (Bart & Vodicka, 1997). Briefly, after concentration of the virus by ultracentrifugation, N-TERT-1 cells were infected at ~60 % confluence in a 10 cm plate with the addition of 5 µl Polybrene (10 mg ml⁻¹). At 4 h post-infection, cells were washed with PBS and the medium was replaced. The cells were allowed to recover for 24 h before the addition of selective media. N-TERT-1 cells were selected in G418 (50 µg ml⁻¹). Selection in G418 was usually complete within 7 days.

**UV-B irradiation.** Cells were allowed to reach 50–70 % confluence and inoculated with fresh medium 24 h before irradiation. For treatment with UV-B, cells were washed once with PBS and then irradiated through a thin film of PBS with UV-B (15 or 25 mJ cm⁻²). Fresh medium was replaced and lysisates were harvested at various time points, as indicated. The UV-B source was a parallel bank of two FS20T12/ UV-B bulbs (Solarc Systems) with an output range of 280–320 nm. The UV-B output was measured with an IL1400A radiometer coupled with a SEL240/UVB-1/TD UVB detector (International Light).

**Immunoblot assay.** Whole-cell lysisates were prepared by mechanically detaching cells in cold PBS and suspending in WE16th lysis buffer (50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 %
Immunofluorescence microscopy. N-TERT-1 cells (vector control, 6E6, 11E6 and 16E6) were grown on coverslips to 70 % confluence, and then either treated with UV (25 mJ cm\(^{-2}\)) or left untreated as a control. At the indicated time points the medium was aspirated and the cells were fixed for 5 min at room temperature in 4 % paraformaldehyde. The coverslips were then permeabilized with 0.1 % Triton X-100 for 5 min at room temperature. Coverslips were blocked in blocking solution: mouse anti-Bad (Cell Signalling), mouse anti-Bak (Ab-2; Calbiochem), mouse anti-p53 (Ab-6; Calbiochem), rabbit anti-Bcl-x\(_{l}\) (Cell Signalling), mouse anti-Bcl-2 (BD Transduction Laboratories), rabbit anti-Bax (Cell Signalling), rabbit anti-Bid (Cell Signalling), rabbit anti-Bim (Cell Signalling), rabbit anti-Mcl-1 (Cell Signalling), mouse anti-Noxa (Calbiochem), rabbit anti-Puma (Cell Signalling), mouse anti-GAPDH (Abcam) and mouse anti-nucleolin (C-23; Santa Cruz Biotechnology). For quantification of immunoblotting data, the membranes were scanned and bands were analysed by densitometry using ImageJ (http://imagej.nih.gov/ij/).

Statistical analysis. Statistical analysis was performed using the statistical package included in GraphPad Prism software. Paired t-tests, multiple t-tests and/or two-way ANOVA analyses were performed on experimental data where appropriate in order to determine statistically significant results.

ACKNOWLEDGEMENTS

We thank E.-M. de Villiers, Karl Munger and D. A. Galloway for the gifts of the HPV and E6 expressing plasmids. This work was supported by a National Institutes of Health grant NIDCD KO8DC010337 to M. P. U.

REFERENCES


