Specific betapapillomaviruses associated with squamous cell carcinoma of the skin inhibit UVB-induced apoptosis of primary human keratinocytes

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Epidemiological studies have shown an association between infections by specific betapapillomaviruses, such as human papillomavirus (HPV) types 5 and 8, and cutaneous squamous cell carcinoma (SCC). The role of betapapillomaviruses in the development of cutaneous SCC is, however, still enigmatic. The ability to inhibit UVB-induced apoptosis, as demonstrated for HPV5 in vitro, may be important in this respect, as survival of DNA-damaged and mutated cells increases the risk of transformation. The aim of this study was to assess whether inhibition of UVB-induced apoptosis is a general property of betapapillomaviruses and to identify apoptotic factors that are potentially involved in this process. Primary human keratinocytes transduced with E6 and E7 of selected betapapillomaviruses (HPV5, HPV8, HPV15, HPV20, HPV24 and HPV38) were characterized and subjected to UVB irradiation. HPV8- and HPV20-expressing keratinocytes in particular showed fewer signs of apoptosis, as demonstrated by lower levels of active caspase 3, less enzymic caspase activity and less DNA fragmentation. The observed inhibition of UVB-induced apoptosis was mediated by E6 and coincided with reduced steady-state expression of the pro-apoptotic protein Bax. In conclusion, E6 of HPV8 and HPV20 reduces the apoptotic responses upon UVB irradiation when expressed in primary human keratinocytes. Infections with HPV8 and HPV20 may therefore augment the carcinogenic effect of UV radiation and potentially contribute to oncogenic transformation of the skin.

INTRODUCTION

Papillomaviruses classified in genera, species and genotypes (de Villiers et al., 2004) can also be subdivided according to tropism into cutaneous and mucosal types. High-risk mucosal human papillomavirus (HPV) types belonging to the genus Alphapapillomavirus are the causative infectious agent of several anogenital cancers, such as cervical cancer (zur Hausen, 2002). The E6 and E7 proteins of high-risk HPV types, such as HPV16, have been identified as viral oncogenes (Mansur & Androphy, 1993). Simultaneous expression of high-risk HPV E6 and E7 induces efficient immortalization of primary human keratinocytes and is essential for maintenance of the transformed phenotype of cervical cancer cells. Crucial in this regard is the ability of E6 and E7 to deregulate host-cell apoptosis and cell-cycle pathways by binding and degrading the tumour suppressor proteins p53 and pRb, respectively (Steenbergen et al., 2005).

Cutaneous HPV types belonging to the genus Betapapillomavirus are epidemiologically associated with non-melanoma skin cancer, especially with cutaneous squamous cell carcinoma (SCC) (Boxman et al., 2000, 2001; Feltkamp et al., 2003; Harwood & Proby, 2002; Harwood et al., 1998, 2000; Karagas et al., 2006; Pfister et al., 2003; Struijk et al., 2003). In some of these studies, specific betapapillomavirus (βPV) types were associated with SCC, such as HPV5, HPV8 and HPV38 (Feltkamp et al., 2003; Karagas et al., 2006), suggesting the existence of high-risk βPV types analogous to the high-risk alphapapillomavirus (αPV) types. A direct role for βPV types in skin cancer development, however, has not been established.

Compared with αPV types, little is known about the molecular and cellular biology of the E6 and E7 genes of
βPV types. Attempts to identify the mechanisms contributing to cutaneous SCC development have revealed a weak transforming potential of the E6 gene product in rodent cells, and even less pronounced for the E7 gene product, in collaboration with an activated H-ras gene product (Nishikawa et al., 1991; Schmitt et al., 1994). HPV38 E6 and E7 were shown to have transforming activity in primary human keratinocytes (PHKs) (Caldeira et al., 2000). E6 and E7 of several βPV types (HPV5, HPV15, HPV17, HPV20 and HPV38) influence both growth and differentiation of PHKs in organotypic raft cultures (Boxman et al., 2001). Furthermore, invasive SCC was found to develop spontaneously in mice transgenic for the complete early region of HPV8 (Schaper et al., 2005), and after chemical carcinogen application in HPV38 E6E7-transgenic mice (Dong et al., 2005). Recently, a study showed that E6/E7 expression in UV-irradiated HPV20-transgenic mice affected proliferation and differentiation of the skin and promoted SCC development (Michel et al., 2006).

Concerning the possible mechanisms of transformation, it has been demonstrated that HPV38 E7 inactivates pRb almost as efficiently as HPV16 E7, indicating that cell-cycle control in HPV38 E7-expressing cells is disturbed (Caldeira et al., 2003). In addition, functional inactivation of p53 through activation of ΔNp73α has been detected in HPV38 E6E7-expressing human keratinocytes (Accardi et al., 2006). As the vast majority of SCCs are found on frequently UV-exposed skin and cumulative sun exposure is the most important known environmental SCC risk factor, the observation that HPV5 E6 eliminated UVB-induced apoptosis via degradation of Bak, thereby inhibiting the release of apoptosis-inducing factor, is particularly interesting (Jackson & Storey, 2000; Jackson et al., 2000; Leverrier et al., 2007). This suggests that UV radiation-induced genotoxic DNA damage is potentially facilitated by βPVs (Bouwes Bavinck & Feltkamp, 2004). Whether the ability to inhibit UVB-induced apoptosis can be considered a general property of βPVs is not known. So far, these observations have been based on a limited number of experiments, sometimes performed in irrelevant cell types (immortalized fibrosarcoma cells) and analysing only one βPV type (HPV5). In this study, we investigated whether PHK cells, HPV’s natural host cell, transduced with the E6 and E7 genes of six βPV types (HPV5, HPV8, HPV15, HPV20, HPV24 and HPV38) were hampered in their ability to undergo apoptosis following UVB irradiation.

**METHODS**

**Generation of retroviral constructs.** The E6 and E7 genes of HPV5, HPV8, HPV15, HPV16, HPV20, HPV24 and HPV38 were inserted into the XhoI and NotI restriction sites of the retroviral vector pLZRSneo (kindly provided by J. Collard, NKI Amsterdam). The selected βPV types were HPV types prevalent in skin lesions as well as on healthy skin (Harwood & Proby, 2002; Harwood et al., 1998, 2000; Pfister et al., 2003) and widely distributed over the genus Betapapillomavirus. The high-risk βPV type HPV16 was selected as a positive control for transformation as it is known to degrade p53 and pRb.

For HPV5, HPV8, HPV20 and HPV38, two sets of the pLZRS E6 constructs were generated containing a haemagglutinin (HA) tag (5'-TACCCATACGATTTCTCAGATTACGGT-3') at the N or C terminus of E6. For this purpose, two intermediate TOPO 2.1 constructs were generated (TOPO-TA cloning kit; Invitrogen). The 5’HA intermediate construct contained, consecutively, an XhoI restriction site, a Kozak sequence, an HA tag (5’HA), a Smal restriction site, the HPV5 E6 gene, a Smal restriction site and three stop codons in three different reading frames followed by a NotI restriction site. The 3’HA intermediate construct contained an XhoI restriction site, a Kozak sequence, a Smal restriction site, the HPV5 E6 gene, a Smal restriction site, an HA tag (3’HA) and three stop codons in three different reading frames followed by a NotI restriction site. The E6 genes of HPV8, HPV20 and HPV38 were inserted into the Smal and Smal restriction sites of the intermediate TOPO2.1 constructs. Similarly, HPV8 E6 and E7 were also expressed with a 5’ Flag tag. All intermediate TOPO 2.1 and pLZRS constructs generated were confirmed by DNA sequence analysis.

**Cell cultures.** The pLZRS-based constructs containing HPV E6E7 or E6 were introduced into the amphotropic packaging cell line Phoenix A using a Profection Mammalian Transfection System Calcium Phosphate kit (Promega). Transfected Phoenix A cells were selected with puromycin and the filtered supernatant containing the replication-deficient HPV-expressing recombinant retrovirus was used to transduce PHK cells. The PHKs were isolated from foreskin and cultured in keratinocyte serum-free medium supplemented with 2 ng human recombinant epidermal growth factor ml⁻¹ and 50 μg bovine pituitary extract (Life Technologies) ml⁻¹. PHKs from three different donors were used for the transduction experiments. Twenty-four hours after transduction, cells were continuously selected on geneticin (250 μg ml⁻¹). In all cases, the transduction efficiency was estimated to be 90–100 %, as only a few cells died after geneticin selection, whereas non-transduced geneticin-treated PHKs all died within 2 days. All transduced PHKs were grown under identical conditions to 70–90 % confluency and passaged 1:5 every week. HPV-transduced PHKs were passaged five times at the most, after which they were no longer used for experiments. The parental PHKs of the donors used did not contain any of the 25 characterized βPV types when tested in a broad-spectrum PCR specific for all known βPV types (data not shown; de Koning et al., 2006).

**HPV-specific (RT-)PCR.** Two weeks after transduction, DNA and RNA were isolated from approximately 3 × 10⁶ transduced PHKs using Trizol as described by the manufacturer (Invitrogen). Total RNA (1 μg) was transcribed with Thermoscript (Invitrogen) into cDNA with random nonamers at 50 °C for 1 h. DNA (5 ng) as well as cDNA were subjected to an HPV type-specific E6 and E7 PCR (see Supplementary Table S1 available in JGV Online and Struijk et al., 2003, respectively) and a β-actin RT-PCR (Ovstebo et al., 2003).

**UVB irradiation.** Transduced PHKs (passage 2 or 3) were seeded onto glass coverslips in six-well plates or grown in six-well plates. At a confluency of 70–90 %, cells were exposed to UVB irradiation (TL-12 lamp, peak emission 311 nm; range 270–400 nm; Philips). An irradiation dose of 600 J UVB m⁻² was used throughout the study, unless stated otherwise. Before UVB irradiation of the cells, the medium was replaced with 1 ml PBS (0.9 % NaCl) in each well. After exposure, cells were immediately re-fed with growth medium.

**TUNEL and caspase assays.** At given time points after UVB irradiation, cells grown on coverslips (10 mm diameter) were fixed in 3 % paraformaldehyde for at least 30 min, followed by treatment with...
1 % Triton X-100 for 5 min for permeabilization of the cells. For the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, apoptotic cells were detected using an In situ Cell Death Detection kit TMR Red (Roche) according to the manufacturer’s protocol. In the active caspase 3 immunofluorescence assay (IFA), apoptotic cells were detected using an antibody specific for active caspase 3 (Pharmingen) used at a 1:100 dilution, followed by Cy3-conjugated donkey anti-rabbit IgG (H+L) (Jackson Laboratory) used at a 1:1000 dilution. Nuclei were visualized with 0.01 % Hoechst staining in both assays. The percentage of apoptotic cells was calculated by scoring at least 500 cells counted in randomly chosen fields using a Zeiss immunofluorescence microscope (Axioskop 2 mot plus; Carl Zeiss) at ×200 magnification.

A quantitative enzymic caspase activity assay was performed as another marker of apoptosis, based on Asp–Glu–Val–Asp (DEVD) cleavage. For this assay, CHAPS whole-cell lysates were generated from pooled floating and adherent cells at 8, 12, 16 and 24 h after UV exposure and from unexposed cells. Cells from four to six wells were lysed in 50 μl CHAPS lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1 % CHAPS and protease inhibitors]. Protein concentrations were determined using a Bradford assay (Bio-Rad) with BSA used as a standard. CHAPS lystate (5 or 10 μg) was subjected to a fluorimetric caspase activity assay with fluorescent tetrapeptide substrate specific for active caspases 3 and 7 [Ac-DEVD-7-amino-4-trifluoromethyl coumarin (AFC); Alexis Biochemicals]. Free AFC was monitored over 1 h at 30 °C with a fluorimeter (Wallac Victor2 TM Multilabel Counter 1420) at excitation and emission wavelengths of 390 and 510 nm, respectively. The emission from each sample was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the caspase activity.

Western blot analysis. To detect HPV protein expression, 3 × 10^6 transduced PHKs were resuspended in 50 μl RIPA buffer (1 % NP-40, 0.5 % sodium deoxycholate and 0.1 % SDS in PBS containing 5 μg leupeptin ml⁻¹, 1 mM Pefabloc and 10 μM protease inhibitor ChzL–3). After centrifugation, the lysates were analysed using 15 % SDS-PAGE and blotted onto nitrocellulose. HPV16-specific monoclonal antibodies (mAbs) were used to detect HPV16 E6 and E7 protein expression. An anti-HA antibody was used to detect E6 protein expression in PHKs transduced with E6.

Nuclear and cytosolic extracts were generated from adherent non-irradiated and irradiated cells 16 h after UVB exposure. Cytosolic extracts (5 μg) were fractionated by 10 % SDS-PAGE for the detection of p53, and nuclear extracts (5 μg) were analysed by 8 % SDS-PAGE for the detection of pRb. CHAPS whole-cell lysates (5 μg) were analysed by 15 % SDS-PAGE for detection of the pro-apoptotic proteins Bak, Bax and Bcl-xL. The protein signals were detected with enhanced chemiluminescence (ECL; Amershams Biosciences).

Immunoprecipitation. Immunoprecipitation was performed with an Exactacruz C kit for detecting p53 (Santa Cruz Biotechnologies). For formation of antibody–matrix complexes, 40 μl of the matrix for mouse antibodies and 4 μg of mouse mAb anti-Bax 6A7 antibody (Pharmingen) were incubated for at least 1 h at 4 °C. After washing the complex, 100 μg cytosolic extract was added to the complex and it was incubated overnight at 4 °C. The complexes were pelleted, washed four times with RIPA buffer and resuspended in 40 μl 2 x Laemmlli buffer, and 20 μl was subjected to 15 % SDS-PAGE and immunoblotted with a rabbit polyclonal anti-Bax antibody (N-20; Santa Cruz Biotechnology). The Western blotting detection reagent from the Exactacruz C kit for detecting rabbit primary antibodies was used at a 1:2500 dilution.

Antibodies. Western blot analyses were carried out with the following antibodies: anti-p53 (DO-1) diluted 1:2500, anti-Bax (N-20) diluted 1:2000, anti-Bcl-xL and 4 diluted 1:2500 dilution.

RESULTS

Generation of HPV E6E7-transduced PHKs

PHKs from different donors were transduced with recombinant retroviruses containing the E6 and E7 genes from HPV types 5, 8, 15, 16, 20, 24 or 38, or the vector control pLZRS. Typically, about 90 % of the transduced keratinocytes survived genetic selection, indicating the polyclonality of these cultures as a result of high transduction efficiencies. Two weeks after selection, nucleic acids were extracted from the transduced PHKs to detect the presence of HPV type-specific DNA and mRNA. All transductants were found specifically to harbour the correct HPV DNA type (data not shown). With RT-PCR, using independent primer sets specific for E6 or E7, HPV RNA expression was found in all HPV-transduced keratinocytes (Fig. 1a). HPV16 E6 and E7 protein expression was demonstrated with HPV16 E6- and E7-specific mAbs, respectively (Fig. 1b). No specific antibodies are available for the E6 protein of βPV types; therefore, HA-tagged βPV E6 expression was detected, as described below.

p53 and pRb expression in HPV E6E7-transduced PHKs

Degradation of the tumour suppressor proteins p53 and pRb by HPV E6 and E7, respectively, is generally regarded as indicative of the ability of HPV types to transform cells. Therefore, we determined the expression of p53 and pRb in HPV-transduced PHKs. As expected, the steady-state levels of p53 and pRb expression in HPV16 E6E7-transduced PHKs, whereas p53 and pRb expression in βPV E6E7-transducing cells remained the same (data not shown).

UVB dose

To determine the appropriate UVB dose to induce and monitor signs of apoptosis in primary keratinocytes, a dose titration experiment was performed. Parental as well as pLZRS-transduced PHKs were exposed to 0, 300, 600 or
900 J UVB irradiation m$^{-2}$. A dose of 600 J UVB m$^{-2}$ was chosen for the following experiments, resulting in around 30% apoptosis of HPV-negative PHKs (Fig. 3).

Caspase 3 activation and DNA fragmentation in HPV E6E7-transduced PHKs

UVB-exposed and unexposed HPV E6E7-transduced PHKs were analysed for the presence of activated caspase 3, an important intracellular enzyme involved in the apoptosis cascade. Simultaneously, we analysed the induction of DNA fragmentation as a result of apoptosis by means of TUNEL staining. Non-exposed cells showed very little, if any, active caspase 3 activity or TUNEL staining, and no differences were found between unexposed HPV E6E7-containing PHKs and pLZRS-transduced PHKs (Fig. 4a).

After UVB exposure, 30–40% of the pLZRS-containing cells became positive for active caspase 3 as well as for TUNEL staining (Fig. 4a). In the HPV5, HPV8 and HPV20 E6E7-transduced PHKs, a reduction in active caspase 3 and TUNEL positivity was seen. In both assays, calculation of the percentage of apoptotic cells after UVB exposure showed a fourfold reduction in HPV8 and HPV20 E6E7-expressing PHKs compared with the vector control, and a twofold reduction in HPV5 E6E7-transduced PHKs (Fig. 4b). In the active caspase 3 assay, but not in the TUNEL assay, a slight reduction in the number of positive cells was observed in HPV15 and HPV38 E6E7-transduced PHKs. In contrast to the βPV types, the number of active caspase 3-positive and TUNEL-positive cells in HPV16 E6E7-expressing cells was almost twofold higher than in pLZRS-transduced cells (Fig. 4a and b).

Overall, the results shown in Fig. 4 were reproducible and were obtained in at least three independent experiments based on separate HPV transductions of PHKs isolated from a single donor. Similar results were obtained with PHKs derived from other donors (data not shown).
Quantitative caspase activity measurement in HPV E6E7-transduced PHKs

To compare the induction of apoptosis at different time points after UVB exposure in a quantitative manner, we added a substrate containing a caspase-specific cleavage site (DEVD) to the cell lysates and measured the release of a fluorophore-coupled cleavage product in real-time. In pLZRS-transduced PHKs, caspase activity increased up to 12 h after UVB irradiation and levelled off at 12–24 h (Fig. 5a). Compared with the vector control, HPV8 and HPV20 E6E7-transduced PHKs showed diminished cas-
pase activity at all time points (Fig. 5b and c). Except for the 24 h time point, this was also true for HPV15, which matched the slight reduction in active caspase 3-positive cells seen for HPV15 in Fig. 4(b). In line with the previous experiments, HPV16 E6E7-transduced PHKs reproducibly showed an increased caspase activity compared with the vector control, especially at the earliest time point (Fig. 5b).

Inhibition of UVB-induced apoptosis is mediated by E6

To investigate whether E6 is responsible for the observed apoptosis inhibition in HPV E6E7-expressing PHKs, as has been shown previously for HPV5 (Jackson et al., 2000), the induction of active caspase 3 and DNA fragmentation was analysed in Flag-tagged HPV8 E6- or E7-expressing PHKs. Following UVB exposure, in both the active caspase 3 and TUNEL assays, HPV8 E6-expressing PHKs showed a reduction in the number of positive cells, similar to HPV8 E6E7-expressing PHKs (Fig. 6a). HPV8 E7-expressing PHKs instead showed an increase in UVB-induced apoptosis in both assays, indicating that E6 is the main gene responsible for the observed reduction in UVB-induced apoptosis in HPV8 E6E7-expressing PHKs. Both HPV8 E6 and E7 expression were shown by Flag detection in a Western blot (Fig. 6b).

HPV E6 expression and signs of apoptosis

In order to rule out the possibility that differences in the apoptotic responses were attributable to differences in E6 expression, we generated sets of 5’HA- and 3’HA-tagged E6 constructs for selection of HPV types. These were used to transduce PHKs and to correlate RNA and protein expression with signs of apoptosis. HPV8 and HPV20 were selected as strong inhibitors of apoptosis. HPV5 and HPV38 were chosen as βPV types that affected the apoptotic responses weakly or not at all, as far as could be detected.

RT-PCR, using random primers for the reverse transcription reaction and HPV type-specific E6 primers for the subsequent PCR, confirmed HPV E6 RNA expression in all E6-transduced keratinocytes (Fig. 7a). RNA expression of 5’HA- and 3’HA-tagged HPV5 E6, 5’HA-tagged HPV8 E6 and 3’HA-tagged HPV20 E6 RNA was lower compared with the other tagged E6 constructs. In all transductants, E6 protein expression was detected using an anti-HA antibody, but expression in the 5’HA-tagged HPV5 E6 and 3’HA-tagged HPV20 E6 cells was relatively weak (Fig. 7b).

Subsequently, all of the HA-tagged HPV E6-transduced PHKs were UVB irradiated and monitored for signs of apoptosis. The 5’HA- and the 3’HA-tagged HPV8 and HPV20 E6-transduced PHKs showed the strongest reduction in the number of TUNEL-positive cells (Fig. 7c), comparable to what we observed for HPV E6E7-transduced cells (Fig. 4b). No signs of apoptosis inhibition were observed in the HA-tagged HPV5 and HPV38 E6-transduced keratinocytes. In the case of 5’HA-tagged HPV5, the lack of an apoptotic response reduction may be explained by low E6 mRNA and protein expression. In the case of HPV38, however, no effect on apoptosis was seen, despite proper E6 expression at both the transcriptional and the translational level. Inversely, a reduction in apoptotic responses was seen for 5’HA-tagged HPV20 E6, whereas mRNA and protein expression appeared weak. Taken with the results shown in Fig. 4, the HPV8- and HPV20-expressing PHKs appeared to be less sensitive to UVB-induced apoptosis than the other βPV-expressing PHKs, an observation that cannot be explained solely by a difference in detectable expression levels.
Expression of p53, pRb and Bcl-xL after UVB irradiation

To elaborate on the HPV-specific downregulation of UVB-induced apoptosis, we analysed the expression of specific pro- and anti-apoptotic proteins in a selection of the HPV E6E7-transduced PHKs. One of the main cellular responses to UVB-induced genotoxic damage is activation of p53, resulting in cell-cycle arrest, enhanced DNA repair or apoptosis. In all of the βPV-transduced PHKs, p53 was expressed (see Fig. 2) and expression increased after UVB irradiation comparable to the vector control (Fig. 8a). In HPV16 E6E7-transduced PHKs, p53 expression became detectable after UVB exposure but remained very low.

Expression of the anti-apoptotic proteins pRb and Bcl-xL decreased after UVB irradiation in all HPV E6E7-containing PHKs, comparable to the pLZRS-containing cells (Fig. 8a). As expected, the pRb levels in HPV16 E6E7-expressing PHKs were much lower than in the other PHKs. Similar to the results seen in Fig. 2, the steady-state pRb level was slightly reduced in HPV5-, HPV8- and HPV38-expressing PHKs. Overall, for p53, pRb and Bcl-xL, we did not observe expression patterns that correlated with the HPV typespecific reduction in apoptosis seen after UVB exposure.

Expression of Bak and Bax after UVB irradiation

After a death stimulus, Bak and Bax are activated in, or recruited to, the mitochondrial membrane to promote
apoptosis via the release of cytochrome c and apoptosis-inducing factor. In all HPV-containing PHKs, Bak was expressed constitutively, but in the HPV5 and HPV8 E6E7-transduced cells, Bak expression was lower (Fig. 8a). After UVB irradiation, all HPV-transduced PHKs showed an increase in Bak expression and obvious differences in expression were no longer observed between any of the HPV E6E7-transduced keratinocytes.

In contrast to Bak, the steady-state expression of Bax was reduced in HPV5 and HPV8 E6E7-transduced PHKs (Fig. 8a), whereas in HPV16- and HPV38-containing PHKs, Bax levels were comparable to the vector control. In all cases, including HPV5 and HPV8, an increase in Bax expression to similar levels as in the control cells was seen after UVB exposure. Because downregulation of Bax in the HPV5 and HPV8 cells may explain the reduction in UVB-induced apoptosis observed in these cells, we also looked for the presence of activated Bax. In βPV-expressing PHKs, activated Bax was only detectable after UVB exposure, and no differences in active Bax expression levels were observed between the βPV and vector control (Fig. 8a). In HPV16 E6E7-expressing PHKs, weak expression of active Bax could be detected without prior UVB exposure.

Finally, we monitored the expression of Bax after UVB exposure in a time-course experiment. As expected, a reduction in Bax expression was seen in the HPV5- and HPV8-transduced cells at the basal level (Fig. 8b). At 8 h post-irradiation, a reduction in Bax expression was still observed, but at 16 and 24 h after exposure, no apparent differences in Bax expression were observed between the different keratinocyte cultures (Fig. 8b).

**DISCUSSION**

Human keratinocytes located in the epidermis, the natural host cell of βPV, are frequently exposed to sunlight. Intact defence mechanisms against UV irradiation, such as apoptosis and DNA repair, are therefore crucial to prevent genotoxic damage. Recent publications have suggested that HPV5 and HPV8 are able to interfere with these defences and therefore putatively increase the risk of oncogenic transformation (Giampieri & Storey, 2004; Iftner et al., 2002; Jackson & Storey, 2000; Jackson et al., 2000). Elimination of UVB-induced apoptosis via proteolytic degradation of Bak promoted by HPV5 E6 has been proposed as a possible mechanism of transformation (Jackson & Storey, 2000; Jackson et al., 2000). Whether the ability to inhibit UVB-induced apoptosis is shared by all βPVs or could serve as a basis to identify potentially oncogenic βPVs is not known.

We investigated the ability of six frequently detected βPV types taken from species 1 and 2 (de Villiers et al., 2004) to inhibit UVB-induced apoptosis in primary human keratinocytes. PHKs expressing HPV8 and HPV20 E6E7 or E6 alone consistently showed reduced signs of apoptosis after UVB irradiation. PHKs transduced with HPV5 and HPV15 E6E7 or E6 displayed this phenotype to a lesser degree and not in all assays. The lack of apoptosis elimination, in particular in cells expressing HPV5 E6 alone, was not expected (Jackson & Storey, 2000; Jackson et al., 2000). In part, this finding may be explained by relatively low E6 expression, as demonstrated in Fig. 7. Alternatively, in the case of HPV5, the HA tag may have hindered the performance of E6, although this should then have been the case for both the 5' and the 3'-tagged protein. For HPV24 and HPV38, no apoptosis inhibition was observed.

Except for HPV15, the βPV types that showed inhibitory effects on UVB-induced apoptosis have been associated with non-melanoma skin cancer in epidemiological studies (Feltkamp et al., 2003; Karagas et al., 2006; Masini et al., 2003; Struijk et al., 2003). This might indicate that the
ability to inhibit UVB-induced apoptosis of keratinocytes is somehow associated with skin cancer development, and that particular βPV types may act as co-factors in carcinogenesis. For HPV38, a βPV type that has also specifically been associated with non-melanoma skin cancer (Feltkamp et al., 2003) and has been shown to have transformational properties (Accardi et al., 2006; Caldeira et al., 2003), we did not observe an apoptosis-inhibitory effect. Although HPV5, HPV8 and HPV20 all belong to βPV species 1, the number of βPV types tested in this study was too limited to conclude that this species in particular contains βPV types that potentially interfere with apoptosis.

The observed inhibition of UVB-induced apoptosis by βPV appeared to be independent of p53 and pRb. Only in HPV16 E6E7-transduced PHKs were p53 and pRb steady-state levels low or undetectable. In all of the βPV-transduced PHKs, both tumour suppressor proteins were readily detected, although pRb expression in HPV5-, HPV8- and HPV38 E6E7-expressing PHKs was less intense. For HPV38, this has been reported previously and accounted for by HPV38 E7-mediated binding and degradation of pRb (Caldeira et al., 2003). HPV5 and HPV8 E7 are known to bind pRb with some affinity, but pRb degradation was not described for these HPV types (Schmitt et al., 1994; Yamashita et al., 1993). In contrast to another report (Accardi et al., 2006), we observed no accumulation of p53 in HPV38 E6E7-transduced keratinocytes. This might be due to differences in culture conditions, such as the use of feeder cells, or to differences in passage number of the cell cultures tested.

The increased induction of UVB-induced apoptosis observed in HPV16 E6E7-expressing PHKs, despite down-regulation of p53, is remarkable and was reproducible in independent experiments using different keratinocyte donors. Previously, most studies have shown that HPV16 E6 deregulates pro-apoptotic proteins, resulting in a reduction in the rate of apoptosis (Alfandari et al., 1999; Filippova et al., 2002, 2004; Magal et al., 2005; Thomas & Banks, 1998, 1999; Vogt et al., 2006). None of these studies, however, used UV irradiation as a trigger of apoptosis. A study that did use UV irradiation also showed an increase
Expression of pro- and anti-apoptotic proteins in HPV E6E7-expressing PHKs following UVB irradiation. (a) Expression of p53, pRb, Bcl-xL, Bak, Bax and active Bax in HPV E6E7-expressing PHKs measured by Western blotting of lysates obtained prior to and 16 h after UVB irradiation. β-Actin was used as a loading control. Active Bax was immunoprecipitated with the conformation-specific antibody 6A7 and subsequently detected by human Bax antiserum and compared with the total amount of Bax in the lysates. It should be noted that central bleaching of some of the protein bands was caused by ECL overexposure, indicating a strong protein signal. (b) Bax expression in HPV5 and HPV8 E6E7-expressing PHKs at 0, 8, 16 and 24 h after UVB irradiation using Western blotting. β-Actin was used as loading control. Western blots of representative experiments are shown.

Fig. 8. Expression of pro- and anti-apoptotic proteins in HPV E6E7-expressing PHKs following UVB irradiation. (a) Expression of p53, pRb, Bcl-xL, Bak, Bax and active Bax in HPV E6E7-expressing PHKs measured by Western blotting of lysates obtained prior to and 16 h after UVB irradiation. β-Actin was used as a loading control. Active Bax was immunoprecipitated with the conformation-specific antibody 6A7 and subsequently detected by human Bax antiserum and compared with the total amount of Bax in the lysates. It should be noted that central bleaching of some of the protein bands was caused by ECL overexposure, indicating a strong protein signal. (b) Bax expression in HPV5 and HPV8 E6E7-expressing PHKs at 0, 8, 16 and 24 h after UVB irradiation using Western blotting. β-Actin was used as loading control. Western blots of representative experiments are shown.

in UVB-induced apoptosis in HPV16 E6E7 PHKs (Daher et al., 2006; Simbulan-Rosenthal et al., 2002). It concluded that immortalization of HPV16 E6- and E7-expressing PHKs, rather than expression of E6 and E7, is critical to the increased UVB-induced apoptosis. Although the HPV16 E6E7 PHKs used in our study finally become immortalized (R. D. M. Steenbergen and others, unpublished results), the cells we used were primary cells analysed at passage 3 or 4. Another report recently suggested that expression of a truncated form of HPV16 E6 (E6*) might increase the sensitivity to UV radiation in human keratinocytes (Mouret et al., 2005). In our study, we did not specifically look for different E6 transcripts in the HPV16-transduced PHKs and therefore cannot rule out this possibility.

Similar to our findings, a recent study in which leptomycin B treatment was used to induce apoptosis demonstrated increased active caspase 3 positivity in HPV16 E6E7-expressing cells, despite downregulation of p53 (Gray et al., 2007). To explain this finding, the authors speculated on a p53-independent mechanism involved in the induction of apoptosis. It is possible that the presence of activated Bax already present in non-irradiated HPV16 E6E7-expressing cells, as shown in Fig. 8(a), accounts for the HPV16 atypical apoptotic response when UVB is used as an apoptosis-inducing event. Considering the location of natural βPV infection, one could envisage βPVs being more suited to inhibit and consequently survive UVB-induced apoptosis, whereas this function is less relevant to mucosal HPV types, such as HPV16.

Constitutive Bax expression was reduced in HPV5 and HPV8 E6E7-containing PHKs. Although we have not tested this, it is most likely that E6 is responsible for this effect, possibly by promoting degradation of Bax, analogous to what has been described for Bak and HPV5 E6 (Jackson et al., 2000). Downregulation of Bax by HPV16 and HPV18 E6 in PHK and HeLa cells, respectively, has been reported by others (Magal et al., 2005; Vogt et al., 2006), but was not observed in the early-passage HPV16 E6E7-expressing PHKs we used. In contrast, as discussed earlier, we could already observe active Bax in the unirradiated HPV16 E6E7-containing cells, which may explain the ready increase in caspase activity in HPV16-transduced cells immediately after UVB exposure (Fig. 5).

Early (8 h) after UVB exposure, Bax expression still appeared reduced in HPV5 and HPV8 E6E7-transduced PHKs. The reduced constitutive Bax expression level and the slow induction of Bax in HPV8-expressing PHKs may be indicative of the slow increase in enzymic caspase activity observed for HPV8 and HPV20 (Fig. 5b). Overall, Bax downregulation cannot fully explain the inhibition of UVB-induced apoptosis observed in HPV5- and HPV8-expressing PHKs, as Bax expression normalized at 16 h post-exposure. At 16 h post-exposure, reduced levels of apoptosis were still observed in the other assays, but this may be a reflection of previous upstream events, such as Bax downregulation, that still have an impact on the number of apoptotic cells measured at later time points. Unfortunately, attempts to quantify and compare the number of apoptotic cells at time points later than 24 h post-irradiation failed because of too few viable cells in the culture.

Unlike the results of a previous study (Jackson et al., 2000), constitutive Bak expression was detected in all βPV-expressing PHKs without the addition of a proteasome inhibitor. We did observe a reduction in Bak expression in cells expressing HPV38 and especially HPV5 E6E7, which is in agreement with increased Bak degradation as described by others (Dong et al., 2005; Jackson et al., 2000). Unlike Jackson et al. (2000), no distinct differences in Bak expression levels were observed after UVB irradiation between the HPV E6E7-transduced PHKs and the vector control. This apparent discrepancy might be explained by the use of different cells and/or the use of different UVB doses: specifically, primary keratinocytes and 600 J m⁻² (this study) versus immortalized fibrosarcoma cells and 150 J m⁻² (Jackson et al., 2000).
In conclusion, expression of E6 of specific βPV types (HPV8 and HPV20) reduced the signs of UVB-induced apoptosis in monolayer primary keratinocyte cultures. Whether this phenomenon also occurs in naturally HPV-infected keratinocytes after UV irradiation, for instance when grown in organotypic raft cultures or present in the human epidermis, should be the subject of further studies. As HPV8 and HPV20 frequently infect the skin (de Koning et al., 2007), the ability to inhibit UVB-induced apoptosis in theory may potentiate the carcinogenic effect that UV radiation imposes on the human skin.

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