Cutaneous HPV5 E6 causes increased expression of Osteoprotegerin and Interleukin 6 which contribute to evasion of UV-induced apoptosis

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Human papillomaviruses (HPVs) are small DNA viruses, which specifically infect keratinocytes at different body sites. The association between cutaneous squamous cell carcinoma (SCC) formation, ultraviolet (UV) irradiation and infection with a high-risk subset of cutaneous HPVs has been postulated although the underlying molecular mechanisms by which HPV may play a role in SCC development are not yet fully elucidated. Expression of the viral E6 oncoprotein has been shown to interfere with DNA damage responses and inhibit UV-induced apoptosis, suggesting HPV can contribute to early stages in tumorigenesis. However, cutaneous SCCs, in contrast to HPV-associated anogenital cancers do not harbor HPV DNA in every tumor cell. Here, we show that expression of E6 from the prototypic skin-cancer-associated HPV type 5 induced the secretion of factors that were able to inhibit UV-induced apoptosis in non-HPV-expressing cell lines and primary human keratinocytes. The anti-apoptotic effect of HPV E6 expression was found to be mediated in part by upregulation of osteoprotegerin (OPG) and interleukin 6 (IL6). Purified OPG and IL6, when added to cells together, but not individually, reduced apoptosis following UV irradiation. We provide evidence that OPG and IL6 inhibit the extrinsic and intrinsic apoptotic pathways, respectively. Furthermore, we show by immunohistochemistry of HPV-typed SCC sections that IL6 protein is upregulated in HPV-positive tumors compared with HPV-negative cancers. These findings support the hypothesis that a small number of HPV-infected cells influence UV-induced apoptosis in the skin and contribute to tumorigenesis.

Introduction

Non-melanoma skin cancer is the most commonly diagnosed cancer in Caucasians with increasing incidence worldwide. Squamous cell carcinoma (SCC) accounts for ~20% of cutaneous malignancies and occurs predominantly on ultraviolet (UV) exposed body sites. Cumulative UV radiation is the main etiological agent, with UV-induced cellular damage and immune suppression involved in development and progression (1,2). UVA causes production of damaging reactive oxygen species, however, DNA is the main chromophore for UVB irradiation (290–320 nm) making this the most carcinogenic part of the UV spectrum. UVB irradiation generates both cyclopurimidine dimers and 6–4 photoproducts that activate the DNA damage response, leading to cell cycle arrest and DNA repair. As these pathways are error-prone, mis-incorporation of DNA bases during repair can lead to the generation of deleterious mutations that can contribute toward carcinogenesis (3). If the DNA damage is too great to repair, apoptosis is induced, with reactive oxygen species, mitochondrial and death receptor pathway activation all contributing to the crucial removal of damaged cells, termed ‘sunburn cells’ in the epidermis (4).

Abbreviations: CPT, camptothecin; HPV, human papillomavirus; IL6, interleukin 6; mRNA, messenger RNA; OPG, osteoprotegerin; PBS, phosphate-buffered saline; PI, propidium iodide; RM, Rheinwald Media with growth factor supplements; SCC, squamous cell carcinoma; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; UV, ultraviolet.

The intrinsic pathway, induced by intracellular stress, causes depolarization of the outer mitochondrial membrane by pore formation, leading to the release of pro-apoptotic factors and activation of caspase 9 (5). The death receptor pathway is triggered by binding of ligands such as tumor necrosis factor-related apoptosis-inducing factor (TRAIL) to cell surface receptors that leads to activation of caspase 8. Activation of the initiator caspases 8 and 9 leads to activation of executioner caspases 3 and 7, which specifically cleave many cellular targets leading to the controlled dismantling of the cell (6).

There is increasing evidence that human papillomaviruses (HPVs) contribute to SCC formation along with UVB. HPVs are a large family, numbering >100 types, of epitheliotropic DNA viruses associated with a number of important diseases. Anogenital HPV types are the causative agents of cervical cancer, but the link between potentially high-risk cutaneous types and SCC is poorly understood (7). This association was first studied in patients with the rare genetic disease Epidermodysplasia verruciformis, who are predisposed to infection with a group of cutaneous HPVs (now termed β-HPVs), who develop multiple SCC on sun-exposed sites that harbor HPV DNA, typically types 5 or 8 (8). In addition, immunosuppressed renal transplant patients have a ~200 times increased risk of SCC, with ~75% of tumors containing β-HPV DNA (9). Several epidemiological studies have also shown an association between SCC and β-HPV in the immunocompetent general population (10,11). Additionally, the higher viral loads found in actinic keratoses (SCC precursor lesions) suggest a role for HPV in early stages of SCC formation (12). However, several studies (13,14) showing the high prevalence of multiple HPV types in normal skin of the general population has made the exact role of β-HPV in SCC formation difficult to delineate. This contrasts with anogenital high-risk HPVs that cause cervical cancer, where expression of the major HPV oncoproteins, E6 and E7, is required to maintain the transformed cellular state through the degradation of cellular proteins, primarily p53 and pRb by E6 and E7, respectively (15). The E6 and E7 of β-HPVs do not share the overall transforming potential of the anogenital high-risk HPV types yet still display oncogenic activities. For example, UVB-induced p53-dependent transcription of specific pro-apoptotic genes is inhibited by expression of E6 from the cutaneous HPV 77 (16). Although β-HPV E6 does not target p53 for degradation, expression of E6 from β-HPV types (including type 5) protects cells from UVB-induced apoptosis irrespective of p53 status (17). UVB-induced apoptosis is also reduced in keratinocytes-expressing β-HPV E6 (18). UVB irradiation leads to activation of the pro-apoptotic mediator protein Bak causing mitochondrial pore formation and release of apoptotic factors, a process which is inhibited by the E6 of several β-HPV types, including 5, 8, 10, 20, 38 and 77 (19–21) through the proteolytic degradation of the pro-apoptotic mediator protein Bak. The E6 protein from the β-HPV type 5 can also delay the repair of UV-induced DNA damage and allow the cells to progress through the cell cycle despite harboring this damage (22). Together, these effects of β-HPV on DNA damage and apoptosis in combination with UV could lead to the accumulation of mutations, which predispose to SCC formation. While anogenital HPV types are present in every cervical cancer cell, in SCC of immunocompetent patients viral load determinations estimate the copy number as one HPV genome per 20–5000 cells, with HPV DNA being undetectable by in situ hybridization in the majority of tumor cells (23). However, HPV-positive SCC display greatly reduced overall apoptotic rates when compared with HPV-negative SCC, despite similar proliferation rates (24). This suggests that persistent viral infection may potentiate the effects of exogenous factors by eliciting a ‘ bystander effect’ on neighboring cells that permits cell survival and facilitates the accumulation of UV-induced DNA damage. We have tested this hypothesis in both HT1080 cells and primary human epidermal keratinocytes-expressing...
HPV E6 proteins. We show that conditioned media from either HT1080 cells or primary human adult keratinocytes-expressing HPV5 E6 protects non-E6 cells from apoptosis. Furthermore, we identify using cytokine arrays that this effect is in part mediated by increased expression and secretion of osteoprotegerin (OPG) and interleukin 6 (IL6) by E6-expressing cells, which act together to reduce UVB-induced apoptosis in non-E6-expressing cells. We provide evidence that OPG protects via the extrinsic pathway, whereas IL6 inhibits the intrinsic pathway. Upregulation of IL6 in HPV-positive versus HPV-negative SCC biopsies supports the idea that β-HPV may exert a bystander effect that may contribute to SCC formation.

Materials and methods

Polymerase chain reaction

Messenger RNA (mRNA) was extracted using RNEasy (QIAGEN, Crawley, UK) according to the manufacturer’s instructions. One microgram RNA was used as template for reverse transcription with Promega’s (Southampton, UK) kit using random primers. Quantitative real-time polymerase chain reaction was performed on an Opticon 2 (MJ Research, Waltham, MA) with SYBRGreen (Finnzymes, Espoo, Finland) using 0.5 μg template per reaction. Results were normalized to the housekeeping gene GUS (glucuronidase) and the linear fold change ratio calculated by 2ΔΔCt. Primers were: GUS fwd 5’-AACAGGTGCAGGTTTAC-A-3’ and GUS rev 5’-CTCTCTGCCTGTTACTGTTCA-3’; IL6 fwd 5’-TACATCTCCGACGGCATCTC-3’ and IL6 rev 5’-TTTCCAGGAAGTCTCT-3’ and S6b fwd 5’-GTTAAATTTTCAAGTCTTCTG-3’.

Cell culture

HT1080s, a p53 wild-type fibrosarcoma line, were maintained in Dulbecco’s modified Eagle’s medium + glutamine supplemented with 10% fetal calf serum at 10% CO2. Primary human adult cutaneous keratinocytes were purchased from Cascade Biologies (Mansfield, UK) and routinely maintained in serum-free defined media (EpiLife) from the same at 5% CO2. To allow more differentiation of primary keratinocytes, they were exchanged into the Rheinwald–Green system (25), referred to as RM supplemented with dialyzed fetal calf serum was used. With 0.1% bovine serum albumin as a carrier. In cytokine assays, media and cytokine assays, fresh conditioned media/cytokines were replaced on the 1:2 ml conditioned media (normalized for cell number) overnight at 4°C. Membranes were washed three times for 5 min each in Wash Buffer I and twice in Wash Buffer II at room temperature and then incubated in biotin-conjugated primary antibody mixture overnight at 4°C. Membranes were washed as described and incubated with horseradish peroxidase-conjugated streptavidin overnight at 4°C; washed again and developed to film. Image J software (W.Rashbass, National Institutes of Health) was used to quantify pixel density, which was normalized according to positive and negative control spot density. For full array maps, see http://www.raybiotech.com/map_all_m.asp#11.

Western blotting

Total cell lysates were separated on 12% sodium dodecyl sulfate acrylamide gels and transferred to membranes. Membranes were blocked in 10% non-fat milk in Tris-buffered saline-0.1% Tween and incubated with anti-OPG antibody (Imgenex, San Diego, CA; IMG-103A) 1/500 in 1% milk or blocked in 5% Blocker BSA (Pierce, Rockford, IL) and incubated with anti-glyceraldehyde 3-phosphate dehydrogenase (Abcam, Cambridge, UK; ab9484) 1/5000 in 1% Blocker BSA. Secondary antibodies were horseradish peroxidase conjugates from DAKO (Ely, UK) used 1/1000.

Flow cytometry

Apoptosis was measured with Annexin V/propidium iodide (PI) flow cytometry. Cells (including floating cells) were harvested by trypsinization, washed in phosphate-buffered saline (PBS) and resuspended with AnnexinV-AlexaFluor 647 ( Molecular Probes, Invitrogen, Paisley, UK) and PI in AnnexinV-Binding Buffer (BD Biosciences, Oxford Science Park, Oxford). After gating to remove debris, AnnexinV and PI shifts are measured. Bak activation flow cytometry was performed as described (27); briefly, cells were washed with PBS then fixed in 0.25% paraformaldehyde/PBS on ice for 10 min and harvested by scraping. Cells are permeabilized by incubation in PBS/0.01% saponin for 5 min, followed by incubation with anti-Bak Ab (Calbiochem, Merck, Nottingham, UK; # AM03) 1/50 in PBS/0.01% saponin or mouse IgG (Abcam) 1/400 overnight. The shift in fluorescence compared with IgG controls is used to calculate Bak activation.

Immunohistochemistry

For immunohistochemistry, staining was performed with Vectastain ABC kits (Vector Labs, Peterborough, UK; anti-rabbit and anti-mouse Elite) according to the manufacturer’s instructions. Briefly, 5 μm sections of paraffin embedded SCCs were dewaxed and hydrated through graded xylene and industrial methanol spirit. There was no antigen retrieval, and sections were blocked with serum. Anti-OPG and anti-IL6 (Abcam; ab6672) were incubated, 1/400 overnight at 4°C. All subsequent steps were at room temperature. After three washes in PBS, endogenous peroxidase was quenched with 30 min incubation with 0.3% H2O2 in water. After a further three PBS washes, the biotinylated secondary antibody is incubated for 30 min. Further washing and processing with ABC and DAB (Vector Labs) detection reagents was performed. The keratinocyte cells in at least three fields of view for each SCC were scored for staining intensity, with the percentage scored as negative (1), weak (2), moderate (3) and strong (4) recorded, and the overall score was percentage multiplied by intensity.

The immunofluorescent Bak activation assay was performed on cells grown and treated in chamber slides as described (27). In summary, cells were fixed for 10 min in 4% paraformaldehyde in PBS. Anti-Bak Ab1 was incubated 1/50 in PBS with 0.05% digitonin (Calbiochem) at 4°C overnight. After washing, the Alexafluor488-conjugated anti-mouse secondary antibody (Molecular Probes, Invitrogen) was incubated 1/1000 in PBS for 30 min at room temperature. Slides were mounted in FluorountM (Biorad) (product of Birmingham, AL) with 4′,6-diamidino-2-phenylindole. An Axioskop 2 plus (Zeiss, Welwyn Garden City, UK) microscope and software was used.

For all assays, significance was calculated with non-paired two-tailed T-tests, except the immunohistochemistry staining scores, which were compared with one-way analyses of variance (PASW statistics software).
Results

Conditioned media from HPV E6-expressing cells confers protection from apoptosis

Previous work showed that HPV E6 can inhibit UV-induced apoptosis in different cell types. Considering the observations that HPV-positive cancers show decreased overall apoptotic rates compared with HPV-negative SCCs, we hypothesized that E6 may protect neighboring non-E6-expressing cells. To investigate this possibility, we tested if conditioned media from HPV5 E6-, HPV8 E6- and HPV18 E6-expressing cells could protect non-E6 cells from apoptosis. The HT1080 cell line was used for initial experiments as we have previously used this well-characterized system to show that UV-induced apoptosis can be inhibited by HPV E6 expression. HT1080s were transfected with either E6 or empty pcDNA vectors and drug selected as described. Media was conditioned and harvested from empty vector and HPV5 E6-, HPV8 E6- and HPV18 E6-expressing cells, used to culture normal HT1080 cells, and apoptosis was induced with UV and assayed with Annexin V/PI flow cytometry as described. These experiments were expanded to include primary keratinocytes as these are the natural host of the virus. In addition primary keratinocytes grown in different media were used, as different culture conditions are known to affect the growth, differentiation state and morphology of the cells. Here, two different media types were tested, the serum-free EpiLife and media containing serum and defined growth factors (RM-), that allows the keratinocytes to undergo limited differentiation. Keratinocytes were retrovirally infected with either HPV5 E6 recombinant plLXSN viruses or empty vector and drug selected and then used to produce conditioned medium as described. Primary keratinocytes were cultured in the HPV5 E6-conditioned EpiLife or RM-, exposed to UV and apoptotic rates determined. Different UV doses were used to induce apoptosis in different cell types, as previous work has shown that keratinocytes are more resistant to UV, so a higher dose (40 mJ/cm2) induces apoptosis in an equivalent proportion of cells. Figure 1 shows that media conditioned with empty vector control cells provides some protection from apoptosis compared with non-conditioned media in both HT1080s and keratinocytes, presumably due to normal growth factors secreted by the cells. HT1080 cells are more sensitive to UV irradiation than keratinocytes; this could explain the larger protective effect of empty vector conditioned media in HT1080 cells when compared with keratinocytes. Importantly, Figure 1A shows the protection from apoptosis is significantly increased with HT1080 HPV5 E6- and HPV18 E6-conditioned media compared with empty vector-conditioned media 16 h post UV. While HPV8 E6 also appeared to have an effect, this did not reach the same level of statistical significance (P = 0.14).

Figure 1B shows a reduction in apoptotic keratinocytes cultured in HPV5 E6-conditioned EpiLife which did not reach statistical significance; however, Figure 1C shows a significant reduction in apoptosis in keratinocytes cultured in HPV5 E6-conditioned RM+. This observation that HPV E6 expression modulates the profiles of factors secreted from both HT1080 cells and primary keratinocytes

The conditioned media swap experiments suggested that secreted soluble factors from HPV E6-expressing cells modulated UV-induced apoptosis in cells lacking E6 expression. As a primary screen to rapidly identify changes in secretion of factors that could mediate the anti-apoptotic effect, cytokine antibody arrays were incubated with conditioned media to identify E6-specific changes. One hundred and seventy-four cytokines and growth factors are included along with control spots on each array to allow comparison. Pixel density (an indicator of protein level) of each spot was calculated with Image J. Although no significant inhibition of UV-induced apoptosis by HPV8 E6-conditioned media was observed in Figure 1, HPV8 E6-conditioned media was included on the arrays as this type is closely associated with SCC formation. Figure 2A shows part of Array VI in which the upregulation of IL 6 in media from HT1080 versus HT1080...
Fig. 2. Results of the arrays showing changes in cytokine secretion relative to the equivalent normal HT1080/keratinocyte-conditioned media. (A) An example of part of Array VI, wild-type and HPV5 E6 HT1080-conditioned media, with the spots showing IL6 upregulation highlighted and positive controls indicated. (B) Diagram representing changes in media from HT1080s and keratinocytes-expressing HPV5 E6 or HPV8 E6. Non-significant changes are white (where $P > 0.05$), significant upregulation is shown in blue ($>2$-fold) and orange ($>1$-fold). Significant downregulation is shown in blue ($<0.5$-fold) and pale blue ($1$- to $0.5$-fold). IL6, Array VI M 5,6, shown in yellow, is significantly upregulated in all cases. OPG, Array VII I 7,8, shown in yellow, is significantly upregulated in HPV5 E6 HT1080s and HPV8 E6 keratinocytes.
HPV5 E6-conditioned media can be seen in the highlighted box. Figure 2B shows the cytokines tested and their expression levels relative to conditioned media from non-E6-expressing cells, with up-regulation represented by orange and red and downregulation by pale and dark blue. It is noticeable that the profiles of secreted factors from HT1080 versus keratinocytes-expressing HPV5 E6 or HPV8 E6 are markedly different, even though both types of conditioned medium can inhibit UV-induced apoptosis in the respective cell type. This may be reflective of the different growth conditions required for these cell types. Also apparent are the different profiles generated by HPV5 E6 versus HPV8 E6 on Array VI, where more factors are upregulated by HPV5 E6 than HPV8 E6. While HPV5 E6 or HPV8 E6 gene expression levels are probably to be about the same in the different cell types, as they are driven by the same promoter (cytomegalovirus in pcDNA for HT1080 cells and the retroviral promoter in pLXSN for keratinocytes) as monitored by polymerase chain reaction (data not shown), the lack of equivalent E6 antiserum means that we cannot rule out minor differences in protein expression that may account for different profiles. However, the similar pattern of downregulated factors in media from either HPV5 E6 or HPV8 E6 on Array VII suggests that differences in the cytokine profiles may not be due to marked differences in E6 protein levels. The different pattern of secreted factors from HT1080-E6 cells compared with keratinocytes-expressing E6 indicates that the altered profile is cell type dependent. However, the fact that both types of conditioned medium protect from apoptotic stimuli suggests that either each cell type has a different requirement for a complex cocktail of factors or alternatively, that the anti-apoptotic effect is mediated by a smaller number of factors common to both types of conditioned medium.

Effects of HPV5 E6 expression on IL6 and OPG production

Inspection of the induced factors common to both E6 types, but that were also independent of cell type, revealed that IL6 was the only cytokine to show significant upregulation in all the E6 media types, by factors of 2.19, 2.43, 1.73 and 5.57 in HT1080 5E6, HT1080 8E6, keratinocyte HPV5 E6 and keratinocyte HPV8 E6 media, respectively, whereas OPG was upregulated by factors of 1.83 (1.23 and 0.99—not statistically significant) and 1.79, respectively. These cytokines have also displayed anti-apoptotic activity in various other systems [for example (28,29)] and could be important in keratinocyte apoptosis and are known to act in secreted form. This is in contrast to some of the other aberrantly secreted cytokines seen on the arrays, for example Fas, which although an important apoptotic factor, is thought to function on the cell surface as a death receptor with less known about its activity in media. Additionally, the chemokine growth-related oncogene (CXCL1) is also upregulated in three of the conditioned media types but not in keratinocyte HPV5 E6. The effect of downregulation of fibroblast growth factor 4 and 9 on apoptosis is also less well defined. Therefore, the involvement of IL6 and OPG in HPV5 E6-mediated protection of non-E6-expressing cells from apoptosis was investigated further. A full list of the fold changes and statistical significance in the expression of each cytokine can be found in supplementary Figure 2, available at Carcinogenesis Online.

Firstly, the effect of HPV5 E6 expression on transcription of OPG and IL6 was investigated by quantitative real-time reverse transcription-polymerase chain reaction, as previous studies have shown that E6 from other HPV types can directly modulate transcription (30). Primers specific to HPV5 E6 were used to verify E6 expression in the different cell types (Figure 3A). Figure 3B shows the increasing levels of fluorescence as the products are amplified; the point at which this crosses the threshold indicated by the dotted line (the cycle threshold) indicates the amount of mRNA present in the original sample, with more abundant messages being amplified earlier and having a lower cycle threshold. In both HT1080s and keratinocytes, the levels of the housekeeping gene GUS are the same; however, in EpiLife and RM+ HPV5 E6 keratinocytes, but not in HPV5 E6, HPV8 E6 or HPV18 E6 HT1080s, the levels of IL6 are higher as shown by the lower cycle threshold. For further quantification, after normalizing expression in different samples to the housekeeping gene GUS, the expression level...
of IL6 mRNA in pLXSN keratinocytes was set as the baseline and the fold change in IL6 in HPV5 E6 cells calculated. Figure 3C shows IL6 expression was increased in HPV5 E6-expressing keratinocytes. Figure 3C also indicates the media used affects the amount of IL6 mRNA in the keratinocytes-expressing HPV5 E6, with the greatest increase in cells grown in RM+, which allows more differentiation and more closely mimics the cutaneous environment with respect to serum levels. No significant difference in IL6 or OPG mRNA was seen in HT1080 cells nor was any significant difference in OPG mRNA levels in keratinocytes was observed under any of the growth conditions tested (supplementary Figure 3 is available at Carcinogenesis Online). However, western blots of total cell lysates for OPG show an increase of protein in HPV5 E6-expressing cells, where an increase in a 55 kDa protein band corresponding to OPG in lysates from both HT1080s and keratinocytes was observed (Figure 3D). This suggests an effect on the stability or translation of the message or alternatively on the processing or secretion of the protein in HPV5 E6-expressing cells.

**OPG and IL6 together are required to reduce UV-induced apoptosis**

The increased levels of IL6 and OPG produced and secreted by the HPV5 E6-expressing keratinocytes suggested that they may, in part, mediate the anti-apoptotic effects of the E6-conditioned medium. To test the effect of these cytokines on UV-induced apoptosis, recombinant purified human OPG and IL6 were added to HT1080s or keratinocytes 24 h prior to UV exposure and apoptosis levels measured 16 h later. Pilot experiments were performed to find appropriate doses of cytokines, which ranged initially from 1, 10, 50, 100 and 150 ng/ml, as these have shown previously inhibition of apoptosis in the literature, for example 100 ng/ml of IL6 inhibits Fas-induced apoptosis of multiple myeloma cells (31) and 100 ng/ml of OPG decreased osteoclast apoptosis caused by withdrawal of stimulatory factors (32). When added separately to HT1080 cells or primary keratinocytes OPG or IL6 had no effect on UV-induced apoptosis as measured by AnnexinV/PI flow cytometry (shown in supplementary Figure 4, available at Carcinogenesis Online). However, addition of both OPG and IL6 together significantly reduced UV-induced apoptosis in both HT1080 cells (Figure 4A) and primary keratinocytes grown either in EpiLife or RM+ media (Figure 4B). Cells grown in RM+, which permits a greater degree of keratinocyte differentiation, are more resistant to UV-induced apoptosis than keratinocytes grown in EpiLife medium. While apoptotic rates were reduced slightly by the combined addition of IL6 and OPG to keratinocytes grown in RM+, a greater relative degree of protection was observed in keratinocytes grown in EpiLife, although the overall apoptotic rates were still higher in cells grown in EpiLife compared with RM+. The observation that both cytokines are required for protection from apoptosis implies they have differing yet complementary roles in the inhibition of different apoptotic pathways. As discussed above, activation of the intrinsic or extrinsic pathways is dependent on relay of apoptotic signals through mitochondrial protein interactions or death receptor interactions, respectively. Therefore, the possibility that the cytokines are involved in inhibiting these different pathways was investigated. As OPG is a secreted decoy receptor, which prevents binding of TRAIL to its cell surface receptor (33), its role as an inhibitor of TRAIL-induced apoptosis was studied in HT1080 cells that are known to be sensitive to killing by TRAIL. Pre-incubation with OPG for 30 min prior to apoptosis induction increased the number of viable cells remaining after TRAIL treatment whereas incubation with IL6 for 4 h before induction had no significant effect on the number of AnnexinV/PI-positive cells (Figure 5A). An alternative apoptosis assay measuring executioner caspase 3/7 activation with a fluorogenic substrate also showed that growth of HT1080 cells with increasing doses of OPG decreased the amount of active caspases after TRAIL treatment (Figure 5B). These results support the idea that OPG inhibits the death receptor/extrinsic apoptotic cascade by reducing binding of the death-inducing ligand TRAIL. TRAIL has been found to induce apoptosis in primary keratinocytes, to a greater extent in transformed or dividing cells, meaning that OPG expression could be relevant to SCC formation (34).

**In contrast to OPG, IL6 is a multifunctional proinflammatory cytokine and has been shown to influence cell survival through activation of anti-apoptotic and proliferative signal transducers and activators of transcription 3, mitogen-activated protein kinase and phospho-inositol 3 kinase pathways (35). In view of our results showing OPG is involved in inhibition of the extrinsic apoptotic pathway, we reasoned that IL6 may inhibit apoptosis by affecting activation of the intrinsic pathway. This was also supported by previous reports of IL6 affecting levels of Bcl2 family proteins, important regulators of mitochondrial apoptosis (36,37). As HPV E6 proteins have been shown to target the key mitochondrial apoptotic effector Bak, we investigated mitochondrial apoptosis by measuring active Bak detected by a specific antibody (AbI) using flow cytometry after treatment with the DNA-damaging agent camptothecin (CPT). CPT binds to DNA and the enzyme Topo1, blocking its activity that leads to the generation of double-strand breaks in genomic DNA. This is particularly relevant as double-strand breaks generated by the processing of cyclopyrimidine dimers are believed to be the most genotoxic lesion in skin produced after UV irradiation (38). HT1080 cells grown in the presence of IL6 showed a significant reduction in the amount of active Bak observed 4 h after CPT treatment (Figure 5C). OPG was also...
able to reduce Bak-specific fluorescence although this did not reach statistical significance. This reduction may be in part due to the inhibition of initiator caspase 8 activation, which would cleave Bid, generating tBid that may activate Bak (39).

Fig. 5. OPG and IL6 mediate anti-apoptosis by HPV5 E6. (A) Growth with OPG but not IL6 significantly decreases apoptosis in HT1080s 16 h post TRAIL treatment; at 20 ng/ml TRAIL, the difference for IL6 P = 0.08, for OPG P = 0.0003. (B) Apoptosis of HT1080s 6 h post TRAIL was measured with a fluorogenic executioner caspase substrate. Incubation with increasing doses of OPG decreases active caspases. (C) Bak activation measured by FACS is reduced in HT1080s grown overnight plus treatment time in IL6, 4 h post CPT *P = 0.034. (D) Fold change in Bak activation 6 h post CPT measured by FACS in HT1080s grown overnight in media conditioned for 24 h growth with the cell type indicated. (E) Bak activation measured by FACS is reduced in keratinocytes grown in EpiLife overnight plus treatment time in IL6, 6 h post CPT *P = 0.016. (F) Examples of Bak activation in keratinocytes grown in RM+ with IL6 measured with Ab1 immunofluorescence. Negative control was incubated with serum in place of the primary antibody; nuclei are blue (4',6-diamidino-2-phenylindole) and Ab1 is green (AlexaFluor488 secondary). Strong punctuate staining indicates active Bak. (G) Quantification of active Bak staining by calculating the average percentage of Ab1-positive cells in three fields shows growth of keratinocytes in RM+ with IL6 reduces Bak activation significantly at 4 h post CPT treatment, *P = 0.021.

Fig. 5. Continued

fluorescence was used to detect the active Bak conformation in CPT-treated keratinocytes grown in RM+. Following CPT treatment strong green punctuate staining with Ab1 is indicative of Bak activation (Figure 5F). Quantification of active Bak staining showed that treatment of keratinocytes with IL6 significantly reduces the percentage of cells with active Bak 4 h after CPT treatment (Figure 5G). This indicates that IL6 affects the intrinsic apoptotic pathway involving an inhibition of Bak activation that in turn can affect release of apoptotic factors such as cytochrome c from the mitochondria and commitment to apoptosis.

IL6 protein levels are increased in HPV-positive SCCs

To investigate whether the observed increase in OPG and IL6 in HPV5 E6-expressing keratinocytes and the subsequent protection of cells from UV-induced apoptosis could be involved in HPV-associated SCC formation, sections from a panel of HPV-positive SCCs were
analyzed for OPG and IL6 expression patterns. Sections from 18 different SCCs that had been characterized and HPV-typed by the dermatology and pathology departments of the Royal London Hospital (London, UK) were used. Sections were stained for OPG and IL6 as described and a minimum of 3 fields-of-view of each section were scored blind as described in Materials and Methods, counting only the keratinocyte portions of the section (for example rather than connective tissue). As no nuclear staining was observed, cytoplasmic staining was scored with a protocol assigning higher scores for both increased intensity of staining and higher prevalence throughout the field-of-view. Nine of the SCCs were HPV-negative and nine contained HPV of various types, shown in Figure 6A. Figure 6B illustrates the staining for OPG and IL6 with an example of both HPV-positive and -negative SCCs, along with the negative with no primary antibody controls, which show specificity of the staining by the absence of brown staining. While a generally uniform staining pattern was seen for IL6 in HPV-negative tumors, in HPV-positive tumors stronger cytoplasmic staining was observed in regions of the suprabasal cell layers. Quantification of the data and analysis for statistical significance with a one-way analysis of variance test showed that the HPV-positive tumors express significantly more IL6 than HPV-negative tumors (Figure 6C, *F* = 14.5, *P* = 0.0003). There was no significant difference in the staining scores for OPG in SCC sections that was associated with HPV status (*F* = 0.016, *P* = 0.9).

Discussion

The E6 proteins of several high-risk HPVs of both cutaneous and mucosal types have been shown to inhibit apoptosis of host cells (18,40). These observations have been proposed to confer a survival advantage on virally infected cells. Such inhibition of apoptosis may be an important factor in tumorigenesis, with increased survival of DNA damaged keratinocytes leading to proliferation of mutant clones and progression to cancer (41). The overall inhibition of apoptosis in HPV-positive SCC compared with HPV-negative SCC (24) along with the low copy number of HPV in SCC (12) led to the hypothesis that E6 expression could reduce apoptosis in neighboring non-HPV-infected cells, possibly an important contributing factor in the early stages of tumorigenesis.

By analyzing conditioned media from HPV5 E6-expressing cells, we have identified a mechanism by which E6 affects secretion of factors that are capable of inhibiting UV-induced apoptosis in non-infected cells. The upregulation of OPG could have an important role in protecting neighboring keratinocytes from apoptosis as keratinocytes have been shown to be susceptible to TRAIL (42). In contrast, anogenital high-risk HPV has been shown to inhibit TRAIL-induced apoptosis via the degradation of intracellular apoptotic signaling mechanisms (43,44). However, our finding that IL6 is upregulated by HPV5 E6 expression is in agreement with previous studies (45) showing that expression of E6 and E7 from HPVs 5 and 16 increased expression of IL6 in primary keratinocytes. Noteworthy also are findings that persistent DNA damage leads to an increase in IL6 secretion in fibroblasts (46); as previous work by this lab has shown that HPV5 E6 expression impairs the repair of thymine dimers induced by UV (22) and this could therefore contribute to the increased production of IL6. IL6 is a pleiotropic inflammatory cytokine that has many functions, including an involvement in barrier formation in the skin (47). IL6 binding to cell surface receptors activates JAK tyrosine kinases that in turn can activate the mitogen-activated protein kinase, phospho-inositol 3 kinase and signal transducers and activators of transcription pathways. These lead to proliferation and cell survival by transactivation of anti-apoptotic and growth factors and inactivation of pro-apoptotic factors. In particular, constitutive activation of signal transducers and activators of transcription 3 is linked to many types of cancer (35). One of the anti-apoptotic pathways induced by IL6 is the upregulation of Bcl-2 family proteins (28) and therefore, we tested the effect of IL6 on the intrinsic apoptosis pathway and observed that IL6 reduced mitochondrial apoptosis as indicated by a reduction in Bak activation. Further studies elucidating the mechanism of the effect of IL6 on Bak activation and therefore commitment to mitochondrial apoptosis are underway. In addition to the cell culture models tested, we also probed IL6 and OPG expression in HPV-positive and -negative SCC biopsies. There were no apparent differences observed in OPG between HPV-positive and -negative samples.
The IL6 staining pattern revealed that suprabasal cells in both HPV-positive and -negative samples expressed IL6. However, closer inspection revealed pockets of cells expressing higher IL6 levels in the HPV-positive samples that were not observed in HPV-negative biopsies. This nucleated IL6 expression may be the result of localized E6 expression, and further experiments investigating HPV gene expression would be warranted to test co-expression of IL6 and HPV. However, as the signaling pathways activated by IL6 can lead to further expression of IL6 over a wider area, with the paracrine effect of IL6 recently observed in the skin (48), HPV and IL6 would not necessarily be co-expressed in every cell. Similarly, both OPG and IL6 may be important in several stages of SCC formation and further studies utilizing different grades of SCC and precancerous lesions would be of interest. This could also explain our observation that there is no difference in OPG levels in HPV-positive SCCs, if OPG plays a greater role in the early stages of SCC formation. Additionally, the expression of OPG we observed in the SCC, irrespective of HPV status, would still allow cooperation with upregulated IL6 to reduce apoptosis.

We provide evidence here that the increased secretion of OPG and IL6 by HPV E6 may serve to protect the infected host cell from UV-induced apoptosis via an autocrine mechanism, whereas a paracrine effect may protect neighboring non-HPV-infected cells through a bystander effect. A small number of keratinocytes exposed to higher cytokine concentrations may preferentially survive UV-induced apoptosis, contributing to tumorigenesis. As noted in Figure 2, several other cytokines were significantly changed, and these could be involved in the protection from apoptosis. For example, growth-related onco-gene and IL10R were significantly upregulated, and Fas, fibroblast growth factor-4 and fibroblast growth factor-9 are significantly down-regulated, in three of the conditioned media types. It is also possible that factors present in the conditioned medium but not included on the arrays are also significantly affected by HPV5 E6 expression and play a role in protection from apoptosis. Our initial study has not yet included comprehensive testing of the effect of conditioned media of other β-HPV types in our assays; however, it is possible that they also affect cytokine secretion and protect neighboring non-HPV-infected cells in a paracrine manner. A recent study (49) showed that HPV5 E6 and HPV8 E6 downregulate IL8 expression in primary keratinocytes, in agreement with our finding that IL8 is significantly downregulated in HPV5 E6 keratinocyte-conditioned media, although this downregulation in HPV8 E6 keratinocyte-conditioned media did not reach significance in our assay. In addition, a study of cytokine expression in keratinocytes-expressing E6 and E7, presented results concurring with our own (45), with the upregulation of IL6, inter-cellular adhesion molecule and monocyte chemotactic protein in HPV5 E6/E7 keratinocytes and additionally the upregulation of inter-cellular adhesion molecule in HPV38 E6/E7 keratinocytes. That study also highlighted the differences in cytokine expression between α and β HPVs. Much work has shown that high-risk α-HPVs cause cervical cancer with expression of oncogenic viral proteins in every cell. Consequently, the mechanisms of tumor formation are probably to be different from the cutaneous scenario, where the expression of E6 in only a few cells means the paracrine anti-apoptotic bystander effect may be important for tumorigenesis. A related tumorigenesis model involving a bystander effect has been proposed for hepatocellular carcinoma arising from chronic infection with hepatitis C virus, where hepatitis C virus inhibits Fas-induced apoptosis of infected hepatocytes but also impairs the immune response by affecting the CD8(+) T cells (50).

The significance of our results showing that both cytokines together, but not alone, reduce UV-induced apoptosis in primary keratinocytes is that the HPV5 E6 protein is able to affect intrinsic and extrinsic apoptotic pathways, both of which are activated by UV irradiation of keratinocytes. It has been established that the E6 protein of both high-risk and low-risk mucosal and cutaneous types have multiple binding partners and affect many cellular pathways (51). Our results provide an example of how different activities of HPV5 E6 work in combination to reduce UV-induced apoptosis. The finding that IL6 is increased in HPV-positive SCC sections imply that the protection from UV-induced apoptosis it provides may be important in tumor formation and is evidence that the bystander effect hypothesis merits further investigation.

**Supplementary material**

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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**References**

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