Short Communication

Altered expression of UVB-induced cytokines in human papillomavirus-immortalized epithelial cells

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Keratinocytes can be induced to produce cytokines by exogenous stimuli, such as UVB, and dysregulation of this production has been described in various skin diseases, including cancer. In this study, we compared the effect of UVB on the secretion of several cytokines involved in inflammation by human keratinocytes immortalized or not with human papillomavirus (HPV)16 or HPV38 at the mRNA and protein levels. We show that expression of the HPV E6/E7 oncoproteins influences not only the basal cytokine secretion profile of keratinocytes, but also its modulation upon UVB irradiation. In particular, UVB upregulates interleukin (IL)-6, IL-8 and transforming growth factor (TGF)-β in HPV-immortalized cells to a higher extent than in control keratinocytes. Moreover, expression of other pro-inflammatory molecules such as S100A8/9 and interferon (IFN)-κ was downregulated in HPV-immortalized cells. These data support the functional similarity between HPV16 and 38, and suggest an active role of these viruses in modulation of the inflammatory process.

The association of chronic inflammation with the development of several forms of cancer is a well-accepted notion and has been the focus of several experimental tumour systems (Mueller, 2006). Persistent expression of pro-inflammatory cytokines at tumour sites may exert both protective and detrimental effects, ranging from an increase in malignant cell invasiveness to promotion of an anti-tumour immune surveillance that may counteract malignant cell growth (de Visser et al., 2006).

Keratinocytes can be induced to produce cytokines by several exogenous stimuli and dysregulation of this production has been described in several skin diseases, including cancer (van Kempen et al., 2003). One of the main exogenous inducers seems to be UVB irradiation. It stimulates keratinocyte cultures to secrete pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10 and IL-8. The release of these cytokines can trigger a cutaneous inflammatory response that develops in skin exposed to sunlight and sunburn (Kupper et al., 1987; Kondo et al., 1993; Strickland et al., 1997). The preferential localization of skin squamous cell carcinoma (SCC) and human papillomavirus (HPV) infection to chronically sun-exposed body sites strongly suggests that UV and HPV may synergistically cooperate in the development of skin lesions (Pfister 2003; Akgül et al., 2006). One possibility is that this synergism may partly act at the level of the skin inflammatory response, but the effects of UVB irradiation on cytokine production by HPV-infected cells has not been studied yet (Ruhland & De Villiers, 2001).

The differential expression profile of pro-inflammatory molecules comparing the conditions of mucosal (alpha genus) and cutaneous (beta genus) HPV genotypes (de Villiers et al., 2004) has been reported (Woodworth & Simpson, 1993; Smola-Hess et al., 2001; De Andrea et al., 2007), but much less is known about the synergistic effect of
E6/E7 oncoproteins and UVB on induction of inflammation (Ruhland & De Villiers, 2001; Akgül et al., 2005).

The aim of this study was to investigate the effect of UVB irradiation on secretion of the cytokines TNF-α, IL-1β, IL-6, IL-8 and transforming growth factor-β (TGF-β) involved in the inflammatory process by human keratinocytes immortalized with HPV16 or HPV38 (Kupper et al., 1987; Kondo et al., 1993; Strickland et al., 1997). In addition, expression of the pro-inflammatory molecules S100A8, S100A9 and keratinocyte-specific interferon-κ (IFN-κ) was also evaluated.

Keratinocyte cell lines, grown in KGM-2 serum-free medium (Lonza), were immortalized by infection of pooled primary human keratinocytes derived from neonatal foreskin (HFK) with an amphotropic LXSN retrovirus (Pear et al., 1993) expressing the open reading frames of the E6 and E7 genes from HPV16 (HPV16-HFK) or HPV38 (HPV38-HFK) (Caldeira et al., 2003). As negative control, HFK (passage 2) were infected with the empty viral vector (LXSN-HFK), selected with G418, pooled and used at the very early passages (De Andrea et al., 2007). To exclude variation due to the specific genetic background of donors, HFK from two different pools were used independently. RT-PCR analysis was performed to confirm that the transgenes were expressed at similar levels (data not shown).

Since it has been previously reported that the immortalization process subsequent to the expression of HPV16 E6 and E7 may modify UVB sensitivity (Jackson et al., 2000; Simbulan-Rosenthal et al., 2002), and to determine a UVB dose that would stimulate the cells, but not be so toxic that mRNA would be degraded, the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide dye] assay was used to assess viability of HFK cell lines 24 h after exposure to increasing doses of UVB. As shown in Table 1, viability was lower in HPV38-HFK than in HPV16-HFK and LXSN-HFK at the highest UVB doses (i.e. 400 and 500 J m⁻²) (P<0.05, one-way ANOVA test). In contrast, survival of both HPV16-HFK (62 %) and HPV38-HFK (57 %) was not significantly different from that of LXSN-HFK (68 %) at the 300 J m⁻² UVB dose (P>0.05, one-way ANOVA test).

Table 1. Dose-dependent effect of a single UVB irradiation on normal keratinocytes and HPV-immortalized cells

<table>
<thead>
<tr>
<th>Target cells</th>
<th>UVB irradiation intensity (J m⁻²)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>LXSN-HFK</td>
<td>100</td>
</tr>
<tr>
<td>HPV16-HFK</td>
<td>100</td>
</tr>
<tr>
<td>HPV38-HFK</td>
<td>100</td>
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<tr>
<td>P value</td>
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Results are illustrated in Fig. 1. UVB irradiation strongly upregulated IL-6 mRNA in both HPV16-HFK and HPV38-HFK, especially at 24 h post-irradiation (4.7- and 3.1-fold, respectively; P<0.05), whereas its induction in LXSN-HFK was minimal. Consistent with the mRNA expression analysis, IL-6 secretion was increased in both HPV-immortalized cell lines, but not in the normal counterpart, although maximal levels were observed with HPV16 48 h after UVB irradiation. According to our previous report (De Andrea et al., 2007), the basal level of IL-6 was higher in both HPV-immortalized cell lines compared with LXSN-HFK. A similar induction pattern was detected with IL-8; its mRNA was strongly upregulated by UVB in both HPV16-HFK and HPV38-HFK, especially at 24 h after irradiation (22.5- and 3.1-fold, respectively; P<0.01), whereas its induction was weak in LXSN-HFK. According to our previous report (De Andrea et al., 2007), the basal level of IL-8 mRNA was higher in HPV38-transduced cells and lower in HPV16-transduced cells compared with the normal counterpart. When the levels of secreted IL-8 were assessed in cell supernatants, there was a consistent increase in its production in all cell lines 48 h after UVB irradiation. Analysis of IL-1β mRNA expression levels revealed no significant (P>0.05) variation in the basal condition.

To assess the effects of HPVE6/E7 on the inflammatory response of primary keratinocytes to UVB, cells were irradiated with UVB and analysed at different time points after irradiation for the expression of inflammatory molecules at both the mRNA and protein levels. The differential mRNA expression pattern of inflammatory molecules in irradiated keratinocyte cell lines was analysed by means of real-time RT-PCR at 6 and 24 h post-irradiation with commercial assays from Applied Biosystems, and shown in Fig. 1(a). The housekeeping gene hypoxanthine phosphoribosyltransferase 1 was used to normalize for variations in cDNA and PCR was performed in triplicate for each sample. The results were analysed with a relative expression software tool (REST®) (Pfaffl et al., 2002). To verify expression at the protein level, supernatants from the same cultures were collected at 24 and 48 h after stimulation and analysed by the Human Inflammation kit (BD Biosciences) or ELISA assay (TGF-β1) (R&D Systems). For the latter, the supernatants were treated by acidification before processing. Statistical significance was determined by one-way ANOVA, followed by Bonferroni’s multiple comparison post-hoc test.

Analysis of IL-1β and IL-6 supernatant levels showed that both were strongly induced by UVB, whereas IL-8 mRNA and protein levels were lower. The protein level of IL-1β was similar between LXSN-HFK and HPV16-HFK at both 6 and 24 h after UVB irradiation (P>0.05), whereas its induction was weak in LXSN-HFK. The inflammatory cytokine IL-8 was upregulated by UVB in both HPV16-HFK and HPV38-HFK, especially at 24 h after irradiation (22.5- and 3.1-fold, respectively; P<0.01), whereas its induction was weak in LXSN-HFK. According to our previous report (De Andrea et al., 2007), the basal level of IL-8 mRNA was lower in HPV38-transduced cells and lower in HPV16-transduced cells compared with the normal counterpart. When the levels of secreted IL-8 were assessed in cell supernatants, there was a consistent increase in its production in all cell lines 48 h after UVB irradiation. Analysis of IL-1β mRNA expression levels revealed no significant (P>0.05) variation in the basal condition.
Interestingly, after UVB exposure, there was a significant induction ($P<0.01$) of its mRNA in control cells, but no relevant upregulation in HPV-immortalized cell lines. Production of IL-1β in the cell supernatant, measured as total cytokine, was induced by UVB only in control cells, as already observed at the mRNA level. In the case of TNF-α mRNA, we observed a significant ($P<0.05$) upregulation of its mRNA in both control cells (24-fold) and HPV38-transduced cells (31-fold), but not in HPV16-transduced cells, at 6 h post-irradiation. Its release into the cell supernatant was very low in all samples, below 4 pg ml$^{-1}$, and no variation was detected after UVB treatment. TGF-β mRNA and secretion were substantially upregulated by UVB irradiation in both HPV16-HFK and HPV38-HFK, but not in control cells. Basal levels of both mRNA and protein were higher in HPV38-HFK than in HPV16-HFK and control cells.

The Human Inflammation kit also includes evaluation of IL-10 and IL-12 protein levels, but their levels were always low, close to the detection limit of the assay, with no significant variation in either basal or stimulated condition (data not shown).

Several reports have suggested a direct link between the expression of two small calcium-binding proteins, S100A8 and S100A9, and inflammation in cancer. Both genes are highly upregulated in keratinocytes and infiltrating cells upon TPA (12-O-tetradecanoylphorbol-13-acetate)
treatment, as well as during tumour promotion in chemically induced skin carcinogenesis (Gebhardt et al., 2006). Therefore, we evaluated S100A8 and S100A9 by real-time RT-PCR in our HFK cell lines, with and without UVB irradiation. In parallel, the same technique was used to analyse the expression of IFN-κ, a cytokine belonging to the type I IFN family that is specifically expressed by keratinocytes (Scaponi et al., 2006; Buontempo et al., 2006). Results show that expression of S100A8, S100A9 and IFN-κ mRNA was high in LXSN-HFK, but low in both HPV16-HFK and HPV38-HFK. Moreover, UVB irradiation upregulated their mRNA in LXSN-HFK, but not in HPV16-HFK or HPV38-HFK (Fig. 2).

Inflammation follows infection, and is a key part of the defence mechanisms that protect the host from pathogens (Tindle 2002; Stanley 2006). While some studies show a lack of association between inflammation and epithelial skin cancer, predominant evidence clearly supports a tumour-promoting effect of inflammation on the development and progression of these cancers in both human and animal models (Coussens et al., 1996; van Kempen et al., 2003; Mueller 2006). Despite these findings, few reports are available on the modulation of cytokine expression by UV irradiation in HPV-immortalized keratinocytes (Ruhland & De Villiers, 2001). In this study, we show that immortalization by E6/E7 oncoproteins influences not only the basal cytokines secretion profile of keratinocytes, but also its modulation upon UVB irradiation. The influence on the cell response to UVB was detectable at the relatively toxic dose of 300 J m⁻², and not at 200 J m⁻² (data not shown), but it was not simply ascribable to differential cell death because toxicity was similar in all cell lines and the effect was heterogeneous on different cytokines. We demonstrate that UVB upregulates IL-6, IL-8 and TGF-β in HPV-immortalized cells to a significantly higher extent than in control keratinocytes. Since these cytokines display heterogeneous functions during the inflammatory process, including promotion of angiogenesis, neutrophil chemo- taxis, keratinocyte proliferation and matrix metalloproteinase (MMP) production (Apte et al., 2006; Gabay 2006; Kishimoto 2006; Li et al., 2006), they may play a key role in micro-environmental promotion of skin cancer development via the paracrine pathway. They may synergistically promote keratinocyte proliferation and inflammation by recruiting mast cells, neutrophils and monocytes/macrophages. In line with this hypothesis, a mouse model of HPV16-induced squamous epithelial carcinogenesis showed that both pre-malignant skin and cervix lesions display chronic infiltration of innate immune cells contributing to cancer development (Coussens et al., 1996). Furthermore, in our experimental system, the UVB-mediated effect was somehow selective, since it did not induce the release of other related cytokines, such as IL-10 and IL-12, for which involvement in the inflammatory process has also been demonstrated.

Inhibition of S100A8/9 expression in HPV16-immortalized or transformed cells has been previously reported (Tugizov et al., 2005), but this is the first report of S100A8 inhibition by the cutaneous beta-genotype HPV38. This inhibition has been associated with upregulation of cellular casein kinase II (CKII)-mediated E7 phosphorylation, and enhancement of its growth-promoting and oncogenic activity. Our finding of S100A8/9 inhibition in HPV38-immortalized cells indicates that both genotypes may exploit the same molecular pathway to escape the anti-proliferative activity of secreted S100A8/9, increase E7 oncogenic activity and possibly favour the progression of HPV-associated neoplasia.

IFN-κ has been shown to signal via the type I IFN-stimulated transcription pathway that produces an antiviral state in cells exposed to the recombinant protein (Buontempo et al., 2006). Several in vitro studies demonstrated the ability of HPV oncoproteins to control signalling pathways that lead to the expression of IFNs and IFN-inducible genes (Barnard et al., 2000; Koromilas et al., 2001; O’Brien & Saveria Campo, 2002). Expression of HPV16 E6 in human keratinocytes was able to diminish the induction of IFN-β gene expression by Sendai virus and, consequently, the expression of IFN-inducible genes (Ronco et al., 1998). In line with these findings, we observed that IFN-κ mRNA is significantly (P<0.01) downregulated in cells expressing E6/E7 from both HPV16 and 38. Thus, the capability of HPVs to limit IFN-κ production during infection may be responsible for reducing the host’s ability to mount an effective antiviral state.

**Fig. 2.** Inhibition of S100A8/9 and IFN-κ expression in HPV-immortalized cells. Expression of S100A8, S100A9 and IFN-κ mRNA levels in HFK expressing E6/E7 from HPV16 (16) and 38 (38) or infected with the empty viral vector (Ø), either mock-treated (hatched bars) or 6 h after UVB irradiation (white bars, 300 J m⁻²). mRNA transcript levels were determined by real-time RT-PCR. Expression was normalized to the endogenous control gene HPRT1 and is shown as mean fold changes relative to mock-treated control cells. Results are representative of three experiments, performed in triplicate (mean ± SD). *, P<0.05, for comparison of mock-treated, HPV-immortalized vs normal keratinocytes; **, P<0.005, for comparison of UVB-treated, HPV-immortalized vs normal keratinocytes.
It has previously been shown that HPV38 E6 and E7, similar to HPV16 E6 and E7, deregulate cell cycle control, increase the life span of primary human keratinocytes and induce cellular transformation (Caldeira et al., 2003; Accardi et al., 2006). Here, we show novel findings that further confirm the functional similarity between the two HPV types and suggest an active role of these viruses in modulation of the inflammatory process.

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References


