Human papillomavirus 5 and 8 E6 downregulate interleukin-8 secretion in primary human keratinocytes

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Human papillomaviruses (HPVs) of the genus Betapapillomavirus appear to be involved in the early stages of skin cancer development, since both the prevalence and viral load are higher in precancerous actinic keratoses than in skin cancers. Interleukin-8 (IL-8) is an inflammatory cytokine that serves to alert the surrounding tissue after UV-induced damage. We examined the effects of the E2, E6 and E7 proteins of HPV8 and the E6 proteins of various HPV genotypes on IL-8 secretion from primary keratinocytes. HPV5 and HPV8 E6 showed the highest downregulation of basal IL-8 secretion. HPV8 E6 also negatively modulated IL-8 mRNA expression and protein secretion upon UVB irradiation. The downregulation of IL-8 in actinic keratoses may weaken the response to UV-induced damage and thus favour the accumulation of UVB-induced mutations.
damage. The paracrine effects result in activation of endothelial cells, fibroblasts and melanocytes as well as neighbouring keratinocytes (Adachi et al., 2003; Bonner, 2001; Li et al., 2001; Moser et al., 1993).

In order to get insights into a possible effect of βHPV oncogenes on IL-8 secretion, we measured IL-8 secretion in culture supernatants of primary human adult keratinocytes (PHA K) expressing HPV8 early genes. For this purpose, pLXSN-based retroviral vectors coding for HPV8 E2, E6 or E7 (Akgül et al., 2005a; Leverrier et al., 2007; O’Shaughnessy et al., 2007) were transfected into PT67 cells. Two days after transfection, cells were plated into selection medium containing 500 μg G418 ml⁻¹. Resistant cells were grown to confluence, at which time retrovirus-containing cellular supernatants were collected. Primary keratinocytes were isolated from discarded abdominal skin, which was obtained with informed written consent from patients attending dermatology clinics at Barts and The London NHS Trust. Ethical approval was granted by the East London and City local research ethics committee. For infection of PHAKs, cells were seeded in defined keratinocyte serum-free medium (Invitrogen) at a cell density of 1 × 10⁵ cells cm⁻² in 6 cm dishes. Retroviral supernatants were added to the keratinocytes in the presence of 5 μg hexadimethrine bromide ml⁻¹ (polybrene; Sigma) and centrifuged for 1 h at 300 g. After 2 days, cells were selected with G418 (150 μg ml⁻¹) to generate stable cell lines. The use of pooled stable cell populations minimizes possible variations due to the apparent randomness of the viral integration site in the cellular chromosomes. After G418 selection, the expression of HPV genes was confirmed by RT-PCR analysis as described previously (Akgül et al., 2007). To assay cytokine production, culture supernatants were collected. Primary keratinocytes were retrovirally infected with HPV1 E6-, HPV4 E6-, HPV5 E6-, HPV8 E6-, HPV16 E6-, HPV20 E6- or HPV38 E6-encoding recombinant retroviruses. (b) IL-8 cytokine concentrations in cell-culture supernatants of keratinocytes retrovirally infected with HPV1 E6-, HPV4 E6-, HPV5 E6-, HPV8 E6-, HPV16 E6-, HPV20 E6- or HPV38 E6-encoding or empty retrovirus pLXSN.

**Fig. 1.** Strong reduction of IL-8 secretion by HPV8 E6. The results are means of three independent experiments with SEM. Concentrations were normalized to the total amount of secreted protein. (a) IL-8 cytokine concentrations in cell-culture supernatants of keratinocytes infected with the empty retrovirus pLXSN or HPV8 E2-, E6- and E7-encoding recombinant retroviruses. (b) IL-8 cytokine concentrations in cell-culture supernatants of keratinocytes retrovirally infected with HPV1 E6-, HPV4 E6-, HPV5 E6-, HPV8 E6-, HPV16 E6-, HPV20 E6- or HPV38 E6-encoding or empty retrovirus pLXSN.
Only small reductions of IL-8 were observed with the E6 proteins of the other HPV types. E6 of HPV1, HPV20 and HPV38 inhibited IL-8 secretion by 1.8-fold ($P<0.01$), HPV16 E6 by 1.3-fold ($P<0.01$) and HPV4 E6 by 1.15-fold ($P<0.05$) (Fig. 1b). The observed biological effects are likely to reflect qualitative differences between the E6 proteins, because no significant differences were observed in mRNA levels of early genes from various HPV types cloned in pLXSN (Westphal et al., 2009). The observed downregulation of IL-8 by HPV16 E6 is in line with previous observations demonstrating an inhibitory effect of HPV16 E6E7 on IL-8 secretion (De Andrea et al., 2007; Dell’Oste et al., 2008; Huang & McCance, 2002). However, the downregulation of IL-8 by HPV5 E6 is in contrast to the result of De Andrea et al. (2007), who showed an upregulation of IL-8 in HPV5 E6E7-expressing foreskin keratinocytes.

To analyse whether HPV8 E6 is also able to inhibit IL-8 in primary human foreskin keratinocytes (PHFK) and to clarify the effect of HPV8 E6E7 coexpression, we studied IL-8 expression by qRT-PCR in retrovirally infected PHFKs expressing E6, E7 and E6E7. Total RNA was extracted with the RNeasy Mini kit (Qiagen) and the cDNA synthesis kit from Fermentas. A dilution series of cDNA was used for qRT-PCR performed with the Fast Start DNA Polymerase kit (Roche), in which gene-specific primers for IL-8 (IL-8-fw, 5'-AGACAGCAGACACAGACG; IL-8-bw, 5'-ATGGTTCCCTCGGTGTTG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GAPGH-fw, 5'-CTGACTTC- AACAGGCACACC; GAPDH-bw, 5'-TGCTGTAGGCCAAA-TTCTGTGTTG) were used. The expression of GAPDH is not targeted by HPV early genes (Akgül et al., 2007). As shown in Fig. 2, HPV8 E6 also strongly inhibited IL-8 mRNA expression in foreskin keratinocytes, whereas E7 alone had a weak activating effect. When E6 and E7 were expressed simultaneously, the inhibitory effect of E6 clearly dominated the activation by E7. Similar repressing or activating effects of E6, E7 and E6E7 were observed for IL-6 and MCP-1 expression (not shown). The discrepancy between the results for β1 HPV E6E7 coexpression obtained by De Andrea et al. (2007) and in this study might be explained by slightly different relative amounts of E6 and E7 proteins in the two experimental settings. In line with findings for HPV16 and 38 (Dell’Oste et al., 2008), basal mRNA levels of TNFα were not significantly affected by HPV8 E6, E7 or E6E7 (not shown).

As UVB irradiation stimulates IL-8 production in keratinocytes, we were interested to analyse whether UVB irradiation may affect the production and secretion of IL-8 in HPV8 E6-expressing PHAKs. To perform this experiment, empty retrovirus (pLXSN-) and pLXSN-8-E6-infected PHAKs were seeded in 100 mm dishes and, once cells reached semiconfluence, the medium was aspirated and the cells were washed with PBS and irradiated in open dishes with 8 mJ UVB light cm$^{-2}$ (using a UVP CL-1000 UV cross-linker with F8T5 bulbs, giving a spectral peak at 312 nm) as reported previously (Akgül et al., 2005b). This sublethal UVB dose has a strong effect on PHAKs without killing them, as reported earlier (Li et al., 2001). After UVB exposure, cells were placed in fresh medium. Control cells were subjected to an identical procedure but were not irradiated. Cellular mRNA and culture supernatants were collected at specific time points and stored frozen in aliquots at $-80$ °C until further analysis. UVB exposure of control cells resulted in a 3.5-fold induction of IL-8 mRNA expression after 4 and 8 h, which returned to the baseline level at 24 h post-irradiation. The baseline level of IL-8 mRNA in HPV8 E6-expressing cells was much lower than the baseline level of control cells. Four hours after UVB irradiation, IL-8 mRNA expression was stimulated 6-fold in HPV8 E6-expressing cells compared with its baseline level. However, the IL-8 mRNA level had already returned to the baseline level at 8 h post-irradiation (Fig. 3a). Supernatants of the examined PHAKs were analysed in parallel by ELISA to measure IL-8 protein secretion. As presented in Fig. 3(b), in control cells, IL-8 secretion increased 2-fold after 8 h and 7-fold after 24 h of irradiation. In HPV8 E6-expressing cells, the IL-8 concentrations at 8 and 24 h were about half that for the control cells. These data indicate that IL-8 mRNA expression and cytokine secretion are attenuated in HPV8 E6-expressing cells after UVB irradiation.

Our results establish a correlation between E6 expression of the oncogenic HPV5 and HPV8 and downregulation of IL-8. We show that HPV5 and HPV8 E6 not only influence basal IL-8 secretion but also repress its transcription and secretion after UVB irradiation. The knockdown of the IL-8 cell-surface receptor CXCR2, which equates to IL-8
downregulation, delayed IL-8-induced replicative senescence and diminished the DNA-damage response after irradiation by reducing p53 protein levels and impairing ATM/ATR-regulated DNA-damage checkpoints (Acosta et al., 2008). Thus, it is tempting to speculate that these IL-8-regulated pathways are also disturbed in HPV5- or HPV8-positive actinic keratoses, which might be relevant to pathogenesis of these viruses in the early stages of skin cancer development. About 12% of actinic keratoses in the general population showed rather high viral loads, between 50 HPV DNA copies per cell and one HPV DNA copy in fewer than five cells (Weissenborn et al., 2005). These relatively high viral loads are likely to reflect enhanced HPV DNA replication and presumably enhanced gene expression. High E6 and/or E6E7 levels can therefore be reached in patients with actinic keratoses and cause IL-8 downregulation. In the normal human epidermis, the highest level of IL-8 immunolabelling was detected in the basal cell layer (Anttila et al., 1992). Clusters of HPV-positive cells in actinic keratoses harbouring higher viral loads might be able to reduce the IL-8 concentration in the local extracellular space. Downregulation of IL-8 in this compartment could then be responsible for the accumulation of UVB-induced DNA mutations in the epidermis over time as a consequence of a weak UV response of keratinocytes.

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References


