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Short communication

Alpha- and betapapillomavirus E6/E7 genes differentially modulate pro-inflammatory gene expression

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Abstract

Keratinocytes, the target cell of human papillomavirus (HPV) infection, can produce numerous cytokines and pro-inflammatory molecules which are important for the generation of an effective immune response. How this biological response, which involves the tumor stroma, is affected by the HPV oncoproteins within the epithelial cell itself is not clear. Here it is shown that oncoproteins of different HPV genotypes (alpha- versus beta-HPV genus) alter the expression of pro-inflammatory molecules in early passage primary human keratinocytes and the immortalized cell line HaCaT. HPV5 E6/E7 oncoproteins significantly induced interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) expression. By contrast, the same molecules were down-regulated or not modulated in HPV16 E6/E7 transduced keratinocytes. Interestingly, HPV38 oncoproteins expression resulted in a lower induction of pro-inflammatory molecules, resembling the behavior displayed by the mucosal carcinogenic HPV16. Finally, inducible nitric oxide synthase (iNOS) expression levels and nitric oxide (NO) production were induced at similar levels by all the HPV genotypes tested. These results further emphasize the different biological activities among HPV genotypes, and offer new insights into HPV-associated skin diseases.

Keywords: Cytokines; Immune evasion; Inflammation; Nitric oxide; Papillomavirus; Skin diseases

Human papillomaviruses (HPVs) are small double-stranded DNA viruses found in a wide variety of proliferative lesions of epithelial origin, and can be grouped into either mucosal or cutaneous HPV types based on tissue tropism. Infection with a subset of high-risk mucosal HPV (HPV16, 18, 31, 33 being the most widespread) is the major risk factor for the development of cervical cancer (zur Hausen, 2002). Several experimental studies have indicated that much of the transforming potential of high-risk HPVs and their ability to stimulate proliferation arises from the biological effects of their E6 and E7 oncoproteins. However, HPV *per se* is not sufficient to permanently transform epithelial cells and to give rise to invasive cervical cancer. The host's cell-mediated immune response influences both susceptibility to and regression of HPV infections. Several studies have described a localized immune dysfunction accompanying cervical HPV infection, suggesting that the high-risk mucosal genotypes may interfere with recruitment or activation of lymphocytes and macrophages (O'Brien and Saveria Campo, 2002).

The cutaneous HPV types are phylogenetically distant from the mucosal types. Evidence for their involvement in non-melanoma skin cancer (NMSC) originated from studies of patients suffering from the rare hereditary disease epidermodysplasia verruciformis (EV), that results from abnormal susceptibility to infection with specific HPV types commonly referred to as EV-HPV types (Akgul et al., 2006). Although EV-HPV types were previously considered to be restricted to EV patients, it has became increasingly evident that they also occur at high frequency in the healthy general population, in patients with NMSC, and in patients with psoriasis. According to the most updated *Papillomaviridae* classification, the EV-HPVs and phylogenetically related types are grouped together into the *Betapa-*

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pillomavirus genus, while the mucosal genotypes belong to the *Alphapapillomavirus* genus (de Villiers et al., 2004). Attempts to identify mechanisms by which the beta-HPV genotypes can contribute to NMSC development revealed a rather weak transforming potential in vitro. No single HPV genotype predominates in skin cancers and so far, there has been no evidence of high-risk types analogous to cervical cancer for the beta-genus (Harwood and Proby, 2002; Pfister, 2003). Thus, the mechanisms of HPV induced skin carcinogenesis are still not fully established and differ substantially from the much better explored genital oncogenesis.

Keratinocytes, the target cell of HPV infection, can produce numerous cytokines which are important for the generation of an effective immune response and thus may contribute to controlling the development of squamous intraepithelial lesions. How the biological response, which involves the tumor stroma, is affected by the HPV oncoproteins within the epithelial cell itself is not clear (Balkwill et al., 2005).

The present study was undertaken to evaluate whether the expression of HPV oncoproteins from either alpha- or beta-genus could be associated with altered expression of pro-inflammatory molecules in human epithelial cells. To this end, normal human epidermal keratinocytes (NHEK) and the non-transformed spontaneously-immortalized cell line HaCaT were transduced with recombinant retroviruses containing the E6 and E7 genes from the indicated genotypes. The rationale for comparing NHEK with HaCaT cells lies in the fact that the immortal cell lines (such as HaCaT) and tumor-derived cell lines (such as HeLa, Caski, SiHa) used in other studies harbour alterations in various molecular pathways that may lead to abnormal responses following HPV infection. HPV5 and 38 were chosen for this investigation, as they broadly represent the beta-genotypes (ex EV-HPVs) in the *Papillomaviridae* family that are involved in skin cancer. HPV16, belonging to the alpha-genus, was included as the prototype of mucosal high-risk genotype. High-titer recombinant retroviruses were generated by transient transfection of Phoenix cells with pLXSN vector containing the E6/E7 open reading frame from HPV5, 16 and 38 and used to infect NHEK or HaCaT cells as previously described (Pear et al., 1993). After G418 selection, the presence of the transgenes was confirmed by RT-PCR analysis. As shown in Fig. 1, E6 and E7 genes were transcribed at similar levels in each cell culture. As negative control, cells were infected with the empty parental vector virus. NHEK from two different pools were used independently to exclude variation due to the specific genetic background. To investigate at the molecular level whether transduction of the E6 and E7 genes induced changes in mRNA expression of pro-inflammatory genes, real-time RT-PCR was performed. Gene expression was determined with commercial assays from Applied Biosystems. The housekeeping gene hypoxanthine phosphoribosyltransferase 1 was used to normalize for the variations in cDNA. PCR on each sample was performed in triplicate. The results were analyzed with a relative expression software tool (REST-384) (Pfaffl et al., 2002) and down-regulations were calculated as minus reciprocal expression ratio. In our culture conditions, IL-8 mRNA expression levels were increased 7.7- and 5.5-fold by HPV5

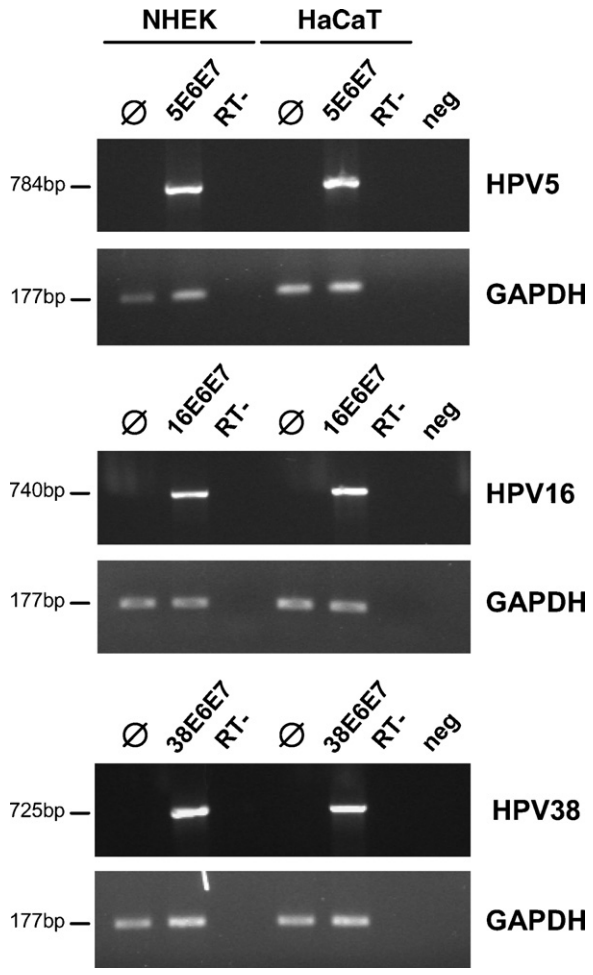

Fig. 1. Analysis of E6E7 transcription levels in NHEK and HaCaT cells transduced with E6/E7 from HPV5, 16 and 38. Ø, cells infected with the vector-only virus. RT-, control reaction in which reverse transcriptase enzyme was omitted in the RT step to exclude genomic contaminations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was performed to normalized variation in cDNA. The results shown are from a representative experiment of three independent infections from two separate pools of cells.

E6/E7 compared to the control in both NHEK and HaCaT cells, respectively, as shown in Fig. 2A. A different picture emerged with the other two HPV genotypes. HPV38 E6/E7 induced a slight increase (3.1-fold) in IL-8 mRNA expression in NHEK, whereas it did not significantly change its levels in HaCaT. By contrast, HPV16 E6/E7 decreased IL-8 mRNA levels in NHEK (4.4-fold), while it slightly up-regulated its expression in HaCaT cells. When the levels of secreted IL-8 were assessed by ELISA using the Quantikine Human IL-8 Immunoassay (R&D Systems) in cell supernatants (Fig. 2B), consistent with the mRNA expression analysis, HPV5 E6/E7 proteins significantly enhanced the release of IL8 in NHEK compared to control cells transduced with the empty vector (820 pg/mg versus 410 pg/mg, respectively). HPV16 E6/E7 proteins decreased IL-8 production when compared to empty vector-transduced NHEK. A similar pattern of IL-8 release was observed with HaCaT cells. Thus, IL-8, one of the most potent neutrophil chemoattractants involved in the early stages of the inflammatory response, was found to be significantly increased at