Biological properties of the oncoproteins E6 and E7 from mucosal and cutaneous HPV types

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Introduction

Although several epidemiological studies show that the cutaneous genus beta HPV types are present in non-melanoma skin cancer (NMSC), their direct involvement in this disease is still under debate. Multiple infections without a prevalence of specific HPV types have been detected in NMSC, in addition DNA of genus beta HPVs are often found in healthy skin. The characterization of the *in vitro* properties of the viral proteins, E6 and E7, is a valid approach to predict the potential carcinogenicity *in vivo*. In this thesis, we described a novel method to determine the efficiency of E7 protein in targeting the tumour suppressor protein, a key event in cellular transformation induced by the mucosal high-risk HPV types. In addition, we have characterized the biological properties of E6 and E7 of mucosal and cutaneous HPV types using *in vitro* and *in vivo* models.

The affinity of E7 for pRb is a indicator for the carcinogenic potential of a HPV type

The carcinogenic activity of HPV16 E7 is explained in part by its ability to associate with and induce degradation of pRb, the product of tumour suppressor gene retinoblastoma, which is a central regulator of cell cycle (Tommasino and Jansen-Dürr, 1997). The pRb neutralization by HPV16 E7 results in a loss of the G1/S checkpoint and uncontrolled proliferation. Three amino acids located at position 22, 24 and 26 in HPV16 E7 form the core of the pRb-binding site, LXCXE (Tommasino and Jansen-Dürr, 1997). Moreover, a second pRb binding site with much lower affinity than the LXCXE motif has been identified in the C-terminal region of HPV16 E7 (Patrick *et al.*, 1994). So far all identified high-risk HPV types can associate with pRb with high efficiency and target it for degradation. In addition, the E7 proteins of low risk HPV types 6 and 11 weakly interact with pRb and have much less activity than HPV16 E7 in several *in vitro* transformation assays (Storey *et al.*, 1988; Munger *et al.*, 1989). Therefore, the ability of E7 to associate with pRb appears to correlated with its *in vivo* carcinogenic activity.

We developed an *in vitro* plate-binding assay, which allows quantification of the affinity of different E7 proteins for pRb (Chapter 2), in contrast to other methods such as yeast two-hybrid or GST-pulldown assays. We also showed that this assay offers the possibility to perform a simultaneous analysis of several proteins and can be easily adapted to the analysis of other protein-protein interactions.

We tested the assay using E7 proteins from several HPV types, which have a different pRb binding motif. The fact that HPV10 E7 has low affinity for pRb, whereas HPV16 and 38 E7 have high affinity for pRb, correlates with the primary

structure of their pRb-binding motif (Chapter 2). As the binding of E7 with pRb can be efficiently inhibited by competition with a short peptide containing the LXCXE motif it is clear that this domain is the main player in the interaction of E7 with pRb.

It has been shown that mutations or deletions in the core pRb-binding site abolish the ability of HPV16 E7 to associate with pRb and to induce cellular transformation *in vitro* (Phelps *et al.*, 1992). Our data with the E7 proteins with a different amino-acid sequence at the pRb binding site show that indeed loss or substitution of one of the amino-acids L, C or E a positions 22, 24 and 26 respectively, leads to a reduction of affinity for pRb. This is further confirmed by the inability of peptides with a substitution of the central C to A to efficiently inhibit HPV16 E7-pRb interaction. Interestingly, although both HPV60 E7 and HPV48 E7 have a different amino-acid instead of the C in their pRb binding motif, HPV60 E7 is able to associate with a higher efficiency to pRb than HPV48 E7. HPV60, however, has a less conservative substitution (C->A) than the latter (C->S) in the pRb-binding domain. This would indicate that other E7 domains might play a role in the association with pRb and that HPV48 E7 lacks these domains. It is likely that, as described for HPV16 E7, the other E7 proteins also have the low affinity pRb-binding domain located at the C-terminal region. Further analysis of the C-terminal region of the different E7 proteins is required to support this hypothesis.

Degradation of pRb and inactivation of p53 are necessary for the transforming activity of a high risk HPV type

Using the assay describe above, we have determined the pRb binding affinity of E7 from HPV32, a benign mucosal type (Chapter 3). The E7 protein of HPV type 32 displays a high affinity for pRb, but, in contrast to HPV16 E7, is not able to induce its degradation. Giarré *et al.* have reported that degradation of the retinoblastoma protein by HPV16 E7 contributes to an efficient overcoming of the cell cycle arrest imposed by overexpression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ (Giarré *et al.*, 2001). In agreement with these data, we have observed that HPV32 E7 does not promote G1/S progression in the presence of ectopic levels of $p16^{INK4a}$ (Chapter 3).

Although HPV32 E7 can successfully transform immortalized rodent fibroblasts (Caldeira *et al.*, 2000), it is not able to promote proliferation of primary human fibroblasts. Thus, the ability of a specific E7 to bind pRb with high affinity does not necessarily correlate with its *in vitro* transforming activity. Similar conclusions were reached in previous studies using E7 from other HPV types (Ciccolini *et al.*, 1994; Schmitt *et al.*, 1994; Giarré *et al.*, 2001). Several studies on HPV16 have shown that the transforming activity of E7 is greatly increased in primary cells by

the presence of E6 (Halbert *et al.*, 1991; Watanabe *et al.*, 1989). The E6 protein from the high risk HPV type 16 is able to bind and degrade p53, and thereby abrogate the p53-mediated apoptotic and quiescent events (Mantovani and Banks, 2001). HPV32 E6 is unable to associate with p53 and promote its degradation. In agreement, we observed that induction of cellular stress by Actinomycin D led to cell cycle arrest and apoptosis in cells expressing HPV32 E6, while HPV16 E6 POFs continued to proliferate. These phenomena observed in HPV32 cells correlates with high levels of p53 and the cell cycle inhibitor $p21^{CIP1/WAF1}$, a p53 transcriptional target. Together, these data show that HPV32 E6 is not able to alter the functions of p53.

In agreement with their inability to target pRb and p53, HPV32 E6 and E7 were not able to immortalize primary human keratinocytes. It has been described that the HPV32 induces benign lesions, which do not progress to malignancy (Syrjanen and Syrjanen, 2000). Thus, our *in vitro* data correlate with the *in vivo* benign features of HPV32.

The genus beta HPV type 38; a high risk cutaneous HPV type?

Non-melanoma skin cancer (NMSC) is the most common malignancy worldwide. Several findings from experimental systems *in vitro* and *in vivo* have shown that ultraviolet light (UV) plays a direct role in skin carcinogenesis (Almahroos *et al.*, 2004; Hussein, 2005). In addition, a subgroup of the epithelio-tropic human papillomaviruses that belong of the genus beta of the HPV phylogenetic tree may co-operate with UV in the development of NMSC (Pfister, 2003). The potential carcinogenic role of these HPV types was initially suggested by studies on individuals with a genetic disorder termed Epidermodysplasia verruciformis (EV). The EV patients are susceptible to genus beta HPV infection and squamous cell carcinoma development (SCC). However, very little is still known about the oncoproteins of the EV HPV types. Initial studies on HPV5 and 8, the types most frequently detected in EV patients, showed lower *in vitro* transforming activity of their E6 and E7 compared with the oncoproteins of the high-risk mucosal HPV types (Iftner *et al.*, 1988, Yamashita *et al.*, 1993). However, transgenic mice expressing the entire early region of another EV HPV type, HPV8, under regulation of a keratin 14 (K14) promoter develop tumours spontaneously (Schaper *et al.*, 2005).

Recently it was shown that the EV HPV type 38 displays *in vitro* transforming properties (Caldeira *et al.*, 2003). HPV38 E7 inactivates pRb and induces loss of G1/S transition control with efficiency similar to HPV16 E7. In addition, HPV38 E6 and E7 can immortalize primary human keratinocytes that are naturally infected by the virus, suggesting a role of HPV38 in skin carcinogenesis (Caldeira *et al.*,

2003). To confirm the carcinogenic potential of HPV38 in an *in vivo* models, we have generated transgenic mice expressing the HPV38 E6 and E7 genes under the control of the bovine promoter-enhancer region homologous to the human K10 promoter (Blessing *et al.*, 1989). The transgenic animals, expressing HPV38 E6 and E7, display small hyperplastic and dysplastic areas on their skin. In addition, imunohistochemistry revealed that cells in the epidermis were more proliferative. A similar phenomenon was also observed in HPV16 E6/E7-Tg mice, in which E6 and E7 expression was under the control of the same promoter as in the HPV38 E6/E7 animals (Auewarakul *et al.*, 1994). Despite the increased proliferation, the HPV38 E6/E7 animals, in contrast to HPV16 E6/E7-Tg mice, do not have a diffuse increase of epidermis thickness. Thus, it appears that HPV38 E6 and E7 proteins are less efficient than HPV16 E6 and E7 in promoting morphological alterations of the epidermis when expressed by the K10 promoter. As the E7 proteins of HPV16 and HPV38 *in vitro* have similar efficiency in degrading pRb and deregulating the G1/S transition (Caldeira *et al.* 2003), other E6 and/or E7 intrinsic properties may be responsible for the difference observed in the skin of the transgenic mice. HPV16 E6, but not HPV38 E6, has a PDZ-binding motif at the C-terminus, which mediates interaction with several PDZ partners, including hDLG, hSCRIBBLE, MUPP1, and MAGI (Mantovani *et al.*, 2001). Thus the difference between HPV16 and HPV38 transgenic mice may be explained by the inability of HPV38 E6 to target these cellular proteins. In agreement with this hypothesis, it has been previously shown that Tg mice expressing an HPV16 E6 mutant that lacks the PDZ-binding motif did not show any epithelial hyperplasia, in contrast to mice expressing wild-type HPV16 E6 (Nguyen *et al.*, 2003).

As UV exposure and especially UVB is considered a risk factor for the development of skin cancer (Lee *et al.*, 2003) the interplay between UVB and HPV has to be considered in the development of skin cancer. UV exposure can cause DNA damage, leading to a rapid accumulation of p53 and cell cycle arrest. Indeed, we observed a strong up-regulation of p53 and decrease of proliferative cells in non-transgenic animals upon UVB treatment. Interestingly, we found that cultured human keratinocytes expressing HPV38 E6E7 and the keratinocytes in the skin of HPV38 E6E7 transgenic animals have high levels of p53 before UVB treatment (chapter 5 and data not shown). Despite these high levels of p53, the cells in the epidermis of transgenic mice and cultured keratinocytes expressing E6 and E7 from these HPV types appear to be unaffected and continue to proliferate. This indicates that p53 function has been impaired in cells expressing HPV38 E6E7. Indeed, we show in Chapter 5 that HPV38 is able to alter the p53 transcriptional functions and thus prevent apoptosis or cell cycle arrest. In addition, the p53 regulated CDK inhibitor $p21^{WAF1}$, is not accumulated in the epidermis of HPV38 E6/E7-Tg mice before or after UVB treatment (Chapter 4). This $p21^{WAF1}$ down-regulation in HPV38

E6/E7-Tg mice may be due to p53 inactivation by the viral proteins. Alternatively, HPV38 E6 can repress $p21^{WAF1}$ transcription by altering p53-independent pathways, as was shown for HPV16 E6 (Malanchi *et al.*, 2002; 2004).

It has been proposed by Jackson *et al.* (2000) that accumulation of the pro-apoptotic protein Bak plays an important role in the induction of apoptosis in UV damaged keratinocytes. The E6 proteins from several cutaneous HPV types can bind and target Bak for degradation, a feature shared by the high-risk mucosal HPV types 16 and 18 (Thomas and Banks, 1998; 1999 Jackson *et al.*, 2000), and thus prevent Bak induced apoptosis. We show that transgenic mice expressing the E6 and E7 from HPV38 do not accumulate Bak upon UVB irradiation (Chapter 4).

In summary, HPV38 E6 and E7 oncoproteins appear to overcome any anti-proliferative effects induced by UVB irradiation. These properties are beneficial for guaranteeing viral DNA replication in sun exposed cells and, more importantly, it might lead to accumulation of DNA damage and facilitate the development of skin cancer.

HPV 38 alters p53 functions via accumulation of ΔNp73

Although HPV38 E6 and E7 together are able to immortalize primary keratinocytes the mechanisms employed are clearly different from high-risk mucosal HPV types. The primary carcinogenic potential of the E6 protein of the high-risk HPV type 16 is considered to correlate with its ability to target and degrade the tumor suppressor protein p53 (Scheffner *et al.*, 1990). HPV38 E6 however, like the E6 proteins from HPV5 and 8 (Steger and Pfister 1992; Jackson *et al.*, 2000), does not induce degradation of p53 (Caldeira *et al.*, 2004). Instead, expression of HPV38 E6 and E7 leads to stabilization of the cellular protein in primary human keratinocytes as well as in transgenic mice (Chapter 5). In tumour cells, p53 mutations often result in protein stabilization. Therefore, to exclude the possibility that p53 stabilisation observed in HPV38 keratinocytes might also result from an accidental mutation in the TP53 gene, we sequenced exons 2 to 9 and confirmed a wild-type sequence. The p53 accumulated in HPV38 E6E7 expressing cells have a specific phosphorylation pattern and appears to have impaired transcriptional functions. Accumulation of wild type p53 (Chapter 5) would normally result in up-regulation of several cellular genes and cell cycle arrest or apoptosis. None of these events were observed in HPV38 keratinocytes (Chapter 5). Interestingly, this form of p53 is able to selectively activate the transcription of a gene encoding ΔNp73, an isoform of the p53-related protein p73 that inhibits the capacity of p53 to induce the transcription of genes involved in growth suppression and apoptosis. This up-regulation of p53 and ΔNp73 is direct consequence of

HPV38 E6 and E7 expression. In fact, inhibition of HPV38 E6 and E7 expression by shRNA leads to downregulation of both p53 and ΔNp73 (Chapter 5).

ΔNp73 has been shown to be able to antagonize the transactivation functions of p53 by competing with p53 and bind p53 responsive elements in the promoters of p53 responsive genes (Melino *et al.*, 2002). Indeed, in HPV38 E6E7 keratinocytes, ChIP analysis shows that ΔNp73 is able to bind the promoters of PiG3, a p53 responsive pro-apoptotic gene. Interestingly, ΔNp73 down-regulation by an antisense oligonucleotide leads to transcription reactivation of p53-regulated genes and apoptosis (Chapter 5).

Clearly, the role of ΔNp73 in altering the suppressive effects of p53 in HPV38 E6/E7 cells is important for malignant conversion and protects the cell against p53 mediated apoptosis. This hypothesis is supported by the fact that ΔNp73 displays *in vitro* transforming activities (Petrenko *et al.*, 2003) and is up-regulated in many human cancers (Concin *et al.*, 2004; Zaika *et al.*, 2002).

In summary these findings illustrated a novel mechanism of HPV-mediated p53 inactivation. However, many questions remain to be answered. It is not clear yet how HPV38 E6 and/or E7 stabilise p53 and modulate its activity and why p53 specifically transactivate the expression of ΔNp73. Most likely the answers to these questions lie in the particular status of p53 upon stabilization by HPV38 E6 and E7, which indeed significantly differ from that of p53 upon stabilization in response to DNA damage.

Cutaneous HPV types and their role in skin cancer

The causal role of HPV in skin cancer has been under debate due to the difficulty in identifying a potential high-risk cutaneous HPV types. The presence of multiple HPV types in any given skin tumour and the weaker activity of the oncogenes E6 and E7 of the cutaneous HPV types in targeting p53 and pRb, respectively, have been major obstacles in the identification of a potential cutaneous high risk HPV type. We and other groups have identified some HPV types from the Beta group that can alter and influence the cell cycle and thus contribute to the development of cancers. In addition, the data available also show that the mechanism employed by the cutaneous Beta HPV types for immortalization is different from the well studied mucosal high risk HPV types.

Indeed, studies on HPV lesions and skin cancers indicated that the presence of the viral genome may not be required in the later stages of carcinogenesis in these tumours (Weissenborn *et al.*, 2005). This mode of action is clearly different from that established for mucosal high-risk HPV in cervical cancer, where E6 and E7

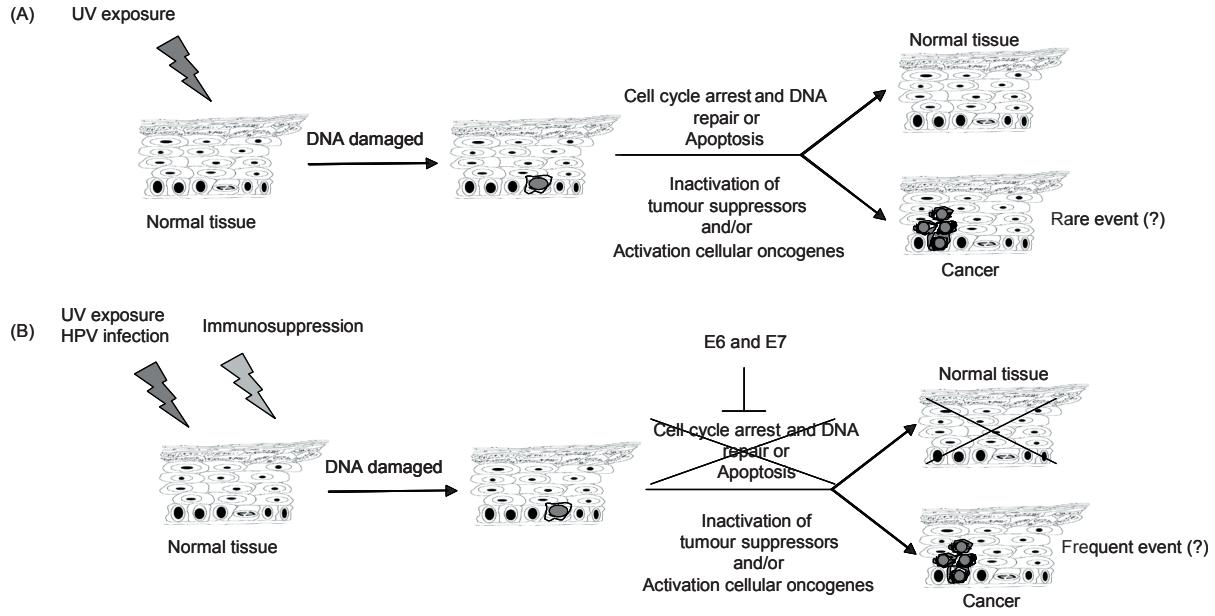

Figure 1 Model for UV and HPV co-operation in the development of non-melanoma skin cancer.

A. Exposure of normal skin to UV leads to accumulation of DNA damages that in turn activate several defence mechanisms. Cell-cycle arrest can be immediately induced in order to allow a cell to repair its damaged DNA before subsequent replication. Alternatively, if the UV-induced DNA damage is too large to be repaired, apoptotic cascades are activated to remove damaged cells from the proliferating pool of the epidermis. In a small percentage of cases, UV exposure may results in accumulation of mutations in key genes encoding tumour suppressors and/or oncoproteins leading to the establishment of neoplastic cells.

B. When UV-mediated DNA damage occurs in presence of HPV infection, cell cycle and apoptosis regulation is corrupt by the viral oncoproteins E6 and E7 in order to guarantee the completion of the viral life cycle. These virus-mediated events greatly increase the probability that skin infected cells will evolve towards malignancy. It is possible that, after the establishment of a neoplastic cell, the presence of the viral E6 and E7 oncoproteins is not necessary for the maintenance of the transformed phenotype. In addition, immunosuppression will allow the persistence of viral infection increasing the probability of tumour formation.

expression is constantly required for maintenance of the neoplastic phenotype of the infected cell.

As the skin is also constantly exposed to a number of stresses and possible carcinogens it is also probable that cutaneous HPV types co-operate with these factors in the malignant conversion of skin cells. We have shown that HPV38 E6 and E7 alone are not as efficient as HPV16 E7 and E6 in inducing morphological alteration, yet it appears to co-operate better with chemical carcinogens in the induction of skin cancers (Chapter 4). In addition, our study provides several lines of evidence that HPV38 can co-operate with UV in the induction of NMSC.

From our and other studies it is evident that infection with HPV increases the risk for development of skincancer. The mechanism of transformation and other additional factors play an important role in the development of skin cancer and a new scenario for the role of EV HPV in NMSC pathogenesis, such as a "hit-and-

run" mechanism (Figure 1), needs to be considered. However, the development of skin cancer is a long and multi-step procedure and additional studies are required to fully understand the role of HPV and other factors in NMSC development.

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