Human Papillomaviruses and Skin Cancer

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Abstract

Human papillomaviruses (HPVs) infect squamous epithelia and can induce hyperproliferative lesions. More than 220 different HPV types have been characterized and classified into five different genera. While mucosal high-risk HPVs have a well-established causal role in anogenital carcinogenesis, the biology of cutaneous HPVs is less well understood.

From patients with the rare genetic disorder epidermodysplasia verruciformis (EV) and animal models, evidence is accumulating that cutaneous PV of genus β synergize with ultraviolet (UV) radiation in the development of cutaneous squamous cell carcinoma (cSCC). In 2009, the International Agency for Research on Cancer (IARC) classified the genus β-HPV types 5 and 8 as "possible carcinogenic" biological agents (group 2B) in EV disease. Epidemiological and biological studies indicate that genus β-PV infection may also play a role in UV-mediated skin carcinogenesis in non-EV patients. However, they rather act at early stages of carcinogenesis and become dispensable for the maintenance of the malignant phenotype, compatible with a "hit-and-run" mechanism.

This chapter will give an overview on genus β-PV infections and discuss similarities and differences of cutaneous and genus α mucosal high-risk HPV in epithelial carcinogenesis.

Keywords

Human papillomavirus · HPV · E6/E7 oncogenes · Cutaneous infection · Carcinogenesis · Skin cancer · Keratinocyte carcinoma · Epidermodysplasia verruciformis · C/EBP · p63 · miR-203 · S100 · Immune escape

Introduction: Human Papillomaviruses and Cancer

Human papillomaviruses (HPVs) are double-stranded DNA viruses that infect epithelial cells of skin or mucosa in a species-specific manner and cause hyperproliferative lesions. More than 220 HPV types are classified into five genera on a genetic basis [1] with differences in biology and pathogenicity. Depending on the oncogenic potential of particular HPV types and body-specific sites of infection, lesions induced by HPVs range from benign warts to invasive carcinoma. The genus α mucosal high-risk HPV types has a well-established causal role in anogenital carcinogenesis. In particular, HPV16 and 18 are involved in about 70% of all cervical cancers. In

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2008 the discovery of this important link between viral infection and malignant disease was recognized with the Nobel Prize in Medicine to Prof. Harald zur Hausen. Benign anogenital condylomas are predominantly caused by the genus α-HPV types 6 and 11. Prophylactic vaccines against these most prominent high- and low-risk mucosal HPV types have been developed to prevent infection as well as HPV-induced diseases [2]. Recent epidemiologic studies have demonstrated the high efficacy of HPV vaccination on mucosal HPV-associated disease burden in countries with vaccination programs [3].

A link between HPV infection and skin cancer was first demonstrated in patients suffering from epidermodysplasia verruciformis (EV), a rare inherited disease. EV patients display a particular susceptibility to productive and persistent infection with cutaneous genus β-PV. As a consequence, they have a high risk to develop keratinocyte carcinomas at sun-exposed sites [4]. Two genus β-PV types, HPV5 and HPV8, were classified as "possibly carcinogenic" in patients with EV [5]. Studies in EV patients and animal models have provided evidence for the cocarcinogenic potential of HPV8 together with UV irradiation, and epidemiological studies suggest an association between β-HPV infection and keratinocyte carcinoma development also in the general human population. However, their "commensalic nature" and the fact that they are apparently dispensable for the maintenance of the malignant phenotype in skin cancer raise difficulties to proof this hypothesis [6, 7].

While the mucosal high-risk HPVs and their involvement of the microenvironment in carcinogenesis have been extensively studied [8], the biology of cutaneous genus β-PV is less well understood.

In order to gain more information of the mechanistic role of HPV in skin carcinogenesis, the International Agency for Research on Cancer (IARC) working group expressed the need for further research on potentially oncogenic cutaneous HPV types [5].

This chapter will give an overview on infections with genus β-PV and their roles in skin carcinogenesis in EV patients, in murine models, and in studies in vitro.

Human Papillomaviruses in Epidermodysplasia Verruciformis Patients

Epidermodysplasia verruciformis is a rare autosomal recessive genodermatosis first described by Lewandowsky and Lutz in 1922 [9–11]. EV-specific symptoms start early in life with the development of disseminated persisting flat warts or macular, red or brownish plaques, occasionally with a scaly surface. EV patients are at high risk to develop precancerous lesions and invasive cancer, particularly at sun-exposed areas of the skin [12]. Understanding fundamental mechanisms of β-PV during infection and skin carcinogenesis in EV patients may provide a better understanding of their potential impact on skin cancer in the general population.

Histology of EV Lesions and β-PV Genotypes

Histological analysis of EV lesions reveals large clear dysplastic cells with a granular cytoplasm and vacuolated nuclei found in clusters within the upper layers of the epidermis. This characteristic cytopathic effect is indicative of viral infection. Auto- and heteroinoculation experiments proved the infectious nature of the lesions [13]. Viral particles were then demonstrated in benign lesions by electron microscopy studies [14, 15]. Specific viral genomes were detected and later on characterized as human genus β-PV. In benign lesion of EV patients, the HPV types 5, 8, 9, 12, 14, 15, 17, and 19–25 were detected most frequently. Most cutaneous squamous cell carcinomas (SCC) in EV patients were shown to be associated with infection by the HPV types 5 and 8 pointing to a prominent role of these β-PV types in carcinogenic progression [16]. HPV types 5 and 8 were thus classified as "possibly carcinogenic" in patients with EV [5]. In contrast to earlier in situ hybridization data, highly sensitive techniques have allowed

the detection of viral genomes also in malignant lesions. β-PV positive nuclei were found heterogeneously distributed in the tumor tissues [17]. Also viral gene expression may still be detectable in atypical cells of SCC, albeit at a lower level than in the benign lesions [18]. It can be concluded that viral gene expression is strongest during early stages of skin carcinogenesis. However, in EV skin HPV may persist in the epithelium throughout the process of carcinogenesis.

Genus β-PV Epidemiology

Family transmission studies have shown that genus β-PV populate the skin of healthy individuals already very early in life. The majority of HPV types found in children were also detected in one or both parents indicating intrafamilial transmission [19, 20]. Due to their ubiquity and diversity, a "commensalic nature" of these viruses has been suggested [21]. Thus, β-PVs are found not only in SCC and actinic keratosis of non-EV patients but also in clinically normal skin and plucked hairs [16, 21, 22]. In the various studies, detection rates strongly depended on the methods applied.

Long-term immunosuppressed patients are at particular risk to develop keratinocyte carcinoma [23]. Of note, in plucked hairs from this patient group, HPV was detected more frequently and with increased probability of high viral loads [22, 24]. From this, it was suggested that higher viral loads may contribute to the risk of skin cancer development. Moreover, several recent (sero)epidemiological studies point to an association between genus β-PV infection, UV susceptibility, and skin cancer in organ transplant recipients as well as in the general population [25–28].

Quantification of viral loads in skin lesions in the general population revealed that precancerous lesions contain higher HPV copy numbers than keratinocyte carcinomas [29]. In addition, transcriptome analysis indicated that HPV is no more actively transcribed in non-EV SCC [30]. This suggested that genus β-PV might play an early role during the initiation phase of skin carcinogenesis rather than a role in sustaining the carcinogenic process at later stages of the disease. In fact, animal models using natural infection, conditional transgenic mice, and human explant cultures [31–33] support this hypothesis, compatible with a "hit-and-run" mechanism [34].

The β-PV Life Cycle

HPV infect keratinocytes of the basal layer, and it is assumed that they also reside within the hair follicle compartment comprising epidermal stem cells. As with other HPVs, the life cycle of genus β-PV is tightly linked to the differentiation program of the stratifying epithelium. To indicate the sequence of viral gene expression during the HPV life cycle, viral gene products have been classified as early (E) and late proteins (L). β-PVs encode E1, 2, 4, 6, 7 proteins but lack an E5 ORF. In benign EV lesions, the viral genome is actively transcribed in a differentiation-dependent manner, as demonstrated for HPV5 [35].

E1 and E2 transcription start in the basal cells and particularly E2 expression increases with differentiation in the middle layers of the epithelium. Both proteins play an important role in viral transcription and replication. The nuclear transcription factor E2 interacts with a variety of cellular factors involved in transcriptional regulation [36] and influences cellular gene transcription in favor of the viral life cycle [37]. β4-integrin is the first cellular gene shown to be transcriptionally regulated through specific E2-binding sites. It anchors basal keratinocytes to the basement membrane. Loss of β4-integrin leads to the detachment of keratinocytes from the underlying structures. HPV8 E2 was shown to downregulate β4-integrin transcription in human keratinocytes by displacing the cellular transcriptional activator AP-1 from its promoter [37, 38]. In vivo, β4-integrin downregulation in keratinocytes may initiate their egress from the basal to suprabasal layers. Thus, it is assumed that E2 expression pushes the virally infected cells into the transit-amplifying compartment, a prerequisite for differentiation [37].

In suprabasal cells cellular transcription factors of the CCAAT/enhancer-binding protein (C/EBP) family are expressed in a coordinated manner. HPV8 E2 is able to bind to these proteins and to enhance their transcriptional activity. This ensures the expression of a distinct profile of cellular differentiation-dependent genes in the middle layers of the epidermis [39, 40]. Notably, E2 exploits the same pathway to induce the differentiation-associated S100 proteins A8 and A9. Once they are released, S100 A8/A9 can recruit myeloid cells to the lesion contributing to an inflammatory microenvironment, which may support the viral life cycle and potentially also carcinogenesis [40]. In the nucleus, β-PV E2 proteins bind to pericentromeric regions of cellular DNA and tether viral DNA to host mitotic chromosomes [41]. Interactions of E2 with structural maintenance of chromosome 5 (SMC5) and SMC6 help to maintain viral episomal DNA [42]. E1 together with E2 initiates a DNA damage response [43]. As a consequence, DNA damage and repair proteins are recruited to viral replication foci, which may support vegetative viral DNA replication [44]. This may preferentially occur in suprabasal cells, where cellular DNA replication is normally shut down.

In benign HPV5-positive EV lesions E6 and E7 transcripts are abundantly detected. E7 expression is highest in the terminally differentiated epidermal layers [35]. Functions and roles of these putative oncoproteins in cutaneous HPV infection and skin carcinogenesis have been investigated in vivo as well as in vitro. In view of the viral life cycle, it is assumed that early proteins of cutaneous HPV ensure a cellular environment that allows viral DNA replication in differentiated layers [45].

Replication of the dsDNA genome of β-PV and viral transcription are controlled by the noncoding control region (NCR). This region is located between the 3′ end of the late gene region and the 5′ end of the early gene region. The NCR of EV-associated HPVs differs from that of other HPVs. It is characterized by its small size of about 400 bp. E2 and cellular transcription factors bind to the NCR and regulate its activity. UV irradiation, the major skin carcinogen, activates the NCR of several β-PV [46–48]. Of note, it was shown that UV light induces and activates nuclear expression of the cellular interferon regulatory factor-7 (IRF-7) [49]. IRF-7 then directly binds to the HPV8 NCR and transmits the UV-signal [48]. IRF-7 itself is induced by type I IFN and enhances IFN-α and IFN-β gene expression [50]. Thus, HPV8 utilizes a central part of the natural antiviral IFN pathway for its own gene expression. In contrast, IRF-3, another related interferon regulatory factor, strongly suppresses the HPV8 NCR. IRF-3-mediated suppression prevails over IRF-7-mediated activation of HPV8 transcription. Similarly, suppression is observed in keratinocytes treated with the potent IRF-3 activators, poly(I:C) or RNA bearing 5` phosphates [48]. Thus, local application of IRF-3-activating compounds might be a novel therapeutic concept against cutaneous β-PV infection particularly for EV patients [7].

The Genetic Defect in EV Patients

An important susceptibility locus of EV patients has been mapped to chromosome 17q25 comprising two adjacent genes EVER1/TMC6 and EVER2/TMC8 [51–53]. EVER genes are expressed in keratinocytes and leukocytes. EV patients are not generally prone to infection and EVER2 deficiency is associated only with mild changes in T lymphocytes [54]. Therefore, it is assumed that the EVER proteins function mainly as keratinocyte-intrinsic restriction factors for β-PV [55]. The transmembrane channel-like proteins are located in the endoplasmic reticulum [56], where they form a complex with one of the zinc transporters ZnT-1. However, it was controversially discussed whether EVER proteins regulate zinc homeostasis [57, 58].

Recently, a third EV susceptibility gene encoding the pleiotropic factor calcium- and integrin-binding protein 1 (CIB1) [59] has been identified [58]. In normal cells, CIB1 forms a complex with EVER1 and EVER2, while in EVER1- or EVER2-mutated keratinocytes, CIB1 protein levels are low. The E5 protein encoded by the α-HPV16 and the γ-HPV4 E8

protein were shown to interact with CIB1, and it is hypothesized that they interfere with CIB1-dependent restriction. β-PVs, however, are lacking an E5 ORF, and therefore CIB1 may specifically restrict β-PVs [58]. Conversely, keratinocytes with reduced levels of CIB1 or CIB1-specific defects may efficiently support β-PV replication.

EV-Like Disease

Common Gamma-c or Jak3 Deficiency

In 50% of patients with severe combined immune deficiency (SCID) due to gamma-c cytokine receptor subunit (gamma-c) or Jak3 mutations, EV-like pathologies ("atypical EV") can occur as a late-onset disease after successful hematopoietic stem cell transplantation [60]. They are either as a consequence of a natural killer (NK) cell or a keratinocyte-intrinsic defect.

Immunosuppression in Organ Transplant Recipients (OTRs), Inherited T-Cell Defects, and HIV

Molecular or seroepidemiological studies of OTRs who receive immunosuppressive treatments point to a crucial role of adaptive T-cell immunity for the control of β-PV infection and disease [27]. OTRs display infections with multiple β-PVs, higher viral loads than in the general population, and a more than 100-fold increased incidence of cSCCs [6, 61]. Although no overt EV-like disease is observed [55], β-PVs actively replicate in actinic keratosis and epithelium adjacent to cSCCs of these patients [62]. In addition, low penetrance of EV-like disease and infections with other pathogens are observed in patients with inherited primary T-cell deficiencies (summarized in [55]).

EV-like disease has also been described in HIV-positive individuals. Worsening of symptoms in these patients has been repeatedly observed during immune reconstitution associated with an inflammatory syndrome [63–65]. The relationship between the immune reconstitution syndrome and EV-like disease is, however, not yet fully understood.

Local Immune Control and Immune Escape in EV Patients

Although EV patients are able to mount a pronounced humoral response directed against the L1 major capsid protein [66], genus β-PV persists in the skin of EV patients for long periods of time. An important question is how these viruses, once expressed, manage to escape cutaneous immune control. It is assumed that cellular immunity against the virally infected cells is not efficiently elicited.

In this regard, a striking observation was the dramatic reduction of Langerhans cell numbers (Langerin-positive cells) in lesional areas of EV epidermis where viral replication and gene expression occurs [67]. This finding confirmed previous reports demonstrating the virtual absence of MHC class II or CD1a-positive cells in EV lesions [68, 69]. Skin immunity critically depends on the activity of Langerhans cells, specialized antigen-presenting cells residing in the epidermis. They locally take up antigen and migrate to local lymph nodes. In a homeostatic situation, they may dampen immune responses to self-antigens. However, depending on the microenvironmental stimuli, they will be able to cross-present soluble and cell-associated antigen from neighboring keratinocytes to $CD8^+$ effector cells [70]. Thus, Langerhans cells are key regulators of immune responses in the skin.

Upon UV-light exposure, Langerhans cells leave the skin, which is known as a part of UV-mediated immunosuppression [71]. Under normal conditions the epidermis will then be repopulated again with Langerhans precursor cells migrating along a chemotactic gradient toward the chemokine CCL20 [72–74]. CCL20 was found to be expressed in the most differentiated layers of human epidermis. Of note, lesional areas of EV epidermis devoid of Langerhans cells express only low or no CCL20 protein [67]. Chromatin immunoprecipitation of the CCL20 promoter and functional studies identified the differentiation-associated transcription factor C/EBPβ as a novel critical regulator of CCL20 gene expression in normal human

keratinocytes. In situ studies demonstrated that the expression patterns of CCL20 and nuclear C/EBPβ converge spatially in the most differentiated layers of human epidermis. Of note, the E7 oncoprotein of HPV8 was shown to co-localize and interact with C/EBPβ in the nucleus. The interaction site could be mapped to a FQELL motif within the putative C-terminal zinc-finger loop. Furthermore, it was demonstrated that the interaction between the viral and the cellular factor has important functional consequences. E7 interferes with the binding of C/EBPβ to the CCL20 promoter in vivo and specifically suppresses CCL20 gene expression. In fact, keratinocytes expressing the HPV8 E7 protein produce only very low amounts of the chemokine CCL20 and display strongly reduced chemotactic activity toward Langerhans cells [67]. As a consequence, EV lesions may not be properly repopulated with Langerhans cells after UV exposure resulting in impaired antigen presentation.

Thus, once expressed at sufficiently high levels, HPV8 is able to disrupt the epithelial immune barrier allowing viral persistence.

UV Light and β-PV as Cocarcinogens in EV Patients

Ultraviolet (UV) radiation and β-PVs cooperate as cocarcinogens in EV patients. Recently, investigations of EV lesions have shed light into the molecular mechanism underlying this multistep process, i.e., β-PV-mediated (1) expansion of the epithelial progenitor cell compartment, (2) enhancement of UV-mediated DNA damage, and (3) of chronic inflammation.

β-PV-Mediated Expansion of the Epithelial Progenitor Cell Compartment in EV Lesions

A seminal observation in skin lesions of EV patients was the HPV8-mediated expansion of the ΔNp63-positive stem cell compartment via suppression of the stemness-repressing microRNA-203 [75]. This was particularly interesting, since this compartment displays an enhanced susceptibility to carcinogenic progression [76]. As the underlying mechanism, the cellular differentiation-regulating transcription factor C/EBPα was identified as a novel regulator of microRNA-203. C/EBPα is strongly downregulated by the major β-HPV oncoprotein E6 and, like miR-203, potently suppressed in EV lesions [75]. In addition, β-HPV E6 also binds to Mastermind-like protein 1 (MAML1), thereby interfering Notch, another important regulator of keratinocyte differentiation [77, 78]. Notably, C/EBPα is not only a key regulator of epidermal differentiation, but it also suppresses UV-induced skin carcinogenesis in mice [79, 80]. Thus, this novel β-HPV E6-driven C/EBPα/microRNA-20/ΔNp63 profoundly disturbs epidermal homeostasis in EV patients and expands the stem cell compartment, a critical step paving the way for UV-mediated skin carcinogenesis.

UV-Induced p53 Mutations in EV Lesions

UVB displays significant mutagenic activity [81]. An important target gene of UV-induced mutagenesis is the tumor suppressor protein p53 [82, 83]. Upon genotoxic stress wild-type p53 activates cell cycle checkpoints in normal keratinocytes. This leads to growth arrest, which allows DNA repair or initiates the execution of programmed cell death [84]. UV-induced pyrimidine-pyrimidone photoproducts and unrepaired cyclobutane pyrimidine dimers may result in C to T or CC to TT mutations in the p53 gene [85, 86]. p53 mutations may arise causing the inactivation of p53 functions. As a consequence, this eventually results in genomic instability, a major step in carcinogenesis.

In a retrospective study of two EV patients during an 8-year period, p53 mutations were detected in five (62.5%) SCC, two actinic keratoses, and one benign lesion. These comprised UV-signature mutations as well as mutations that might correspond to DNA replication errors. It was speculated that unrepaired DNA lesions caused by other exogenous or endogenous mutagens such as reactive oxygen species might also play a role [87]. β-PV E6 interferes with the DNA damage response and UV-induced apoptosis in vitro [88, 89],

potentially allowing the accumulation of UV-mediated DNA mutations (summarized in [90]). Obviously, p53 mutations are common in HPV-associated skin cancer in EV patients. This is in strong contrast to cervical carcinogenesis, where p53 mutations are rarely detected. A major oncogenic activity of mucosal high-risk genus α-PV involves proteolytic degradation of p53 by the E6 protein forming a complex with the ubiquitin ligase E6-AP [91, 92]. Most β-PV E6 proteins, however, do not bind p53 or lead to p53 degradation [45, 93, 94]. This indicates that oncogenic mechanisms of human genus β-PV are distinct from those of mucosal high-risk genus α-PV. In genus β-PV-initiated carcinogenesis, rather the increased burden of critical mutations, which also affect p53, may substantially contribute to disease progression at later stages.

β-HPV-Mediated Amplification of Inflammation in EV Lesions

While mucosal HPVs suppress inflammatory cytokines and chemokines (summarized in [8, 95]) HPV8-positive skin of EV patients is infiltrated with myeloid cells, starting in the stroma of productive lesions. In the epithelium of EV lesions, S100A8/A9 proteins are tremendously upregulated in cells showing virus-induced cytopathic effects [40]. These differentiation-associated S100A8/A9 proteins form a calprotectin complex. Once released, calprotectin serves as a potent neutrophil chemoattractant [96]. Notably, the β-PV-encoded transcription factor E2 exploits the same C/EBPβ-dependent mechanism to upregulate S100A8/A9 [40] as previously shown for the premature enhancement of differentiation [39]. Also other neutrophil-attracting chemokines including interleukin-8 (IL-8), ENA-78, and NAP-2 are produced by keratinocytes co-expressing HPV8 E2 and C/EBPβ, which may further increase neutrophil infiltration [40].

The ability of β-PV E2 to promote differentiation thus appears to be intimately linked to the induction of inflammation [37–40], and the resulting inflammatory microenvironment may pave the way for tumorigenesis as observed in HPV8 E2 transgenic mice [97].

Functional Studies of Cutaneous PV in Animal Models

Transgenic Mouse Models

The oncogenic potential of β-PV has been explored in transgenic mouse models. Mice expressing the complete early region of HPV8 under the keratin-14 promoter, which directs transgene expression to the basal compartment, spontaneously developed skin tumors. In 6% of the mice, SCC arose without any need for physical or chemical carcinogens [98]. Of note, it was shown that the cellular signal transducer and activator of transcription 3 (STAT3) plays an important role in HPV8-induced skin tumor formation [99]. STAT3 is also activated in the epithelium and inflammatory infiltrate in preneoplastic lesions of the cervix uteri [100, 101]. Thus, STAT3 activation plays a major role in HPV-induced tumorigenesis.

Expression of the HPV8 E6 protein under the keratin-14 promoter generated essentially the same phenotype as seen in mice transgenic for the complete early region of HPV8 [102]. From these experiments it has been deduced that E6 is the major oncoprotein of HPV8 sufficient to induce skin cancer. Application of UV light or skin wounding strongly accelerated and enhanced tumor formation [102].

Under both conditions, UV exposure or wounding, tumors displayed a strong inflammatory infiltrate. Chronic inflammation has an important role in neoplastic progression [103]. This notion is compatible with the observation of EV-like disease during the immune reconstitution phase in HIV patients. Of note, chronic inflammatory infiltrates were also observed in lesional skin from EV patients, and a link between β-HPV E2, keratinocyte differentiation, and inflammation has recently been identified [40]. In vitro experiments have demonstrated that high E2 expression not only initiates premature differentiation of

keratinocytes [37–39], it also upregulates the differentiation-associated S100A8/A9 proteins and thereby leads to the recruitment of myeloid inflammatory cells [40]. Accordingly, in mice expressing the HPV8 E2 protein under the keratin-14 promoter, the epidermis was virtually thin predisposing to ulcerations, similar to a "chronic, non-healing wound" [104]. Lesions in these mice showed chronic inflammation, in 6% severe dysplasia, and some even progressed to skin cancer [97]. Thus, evidence is increasing that the β-PV E2 protein contributes to a chronic protumorigenic inflammatory response observed in vivo.

Expression of HPV38 E6 and E7 under the control of the keratin-14 promoter did not result in spontaneous tumor formation, but precancerous lesions and SCC developed after chronic UV irradiation [105]. Using a heterologous keratin-10 promoter directing HPV38 or HPV20 E6 and E7 transgene expression to the suprabasal compartment did not lead to spontaneous tumor formation, either [106, 107]. Comparison of the different models demonstrated that the oncogenic potency of genus β-PV is highest, if their major oncogene E6 is expressed in the basal layer of the epidermis. Chronic UVB irradiation of HPV20 transgenic mice increased papilloma formation and led to the rare occurrence of SCCs [107]. These animal models clearly demonstrated the oncogenic potential of genus β-PV in vivo when continuously expressed under the keratin-14 promoter. They also underscore a synergism between genus β-PV and UV light as well as the importance of inflammatory responses in β-PV-mediated skin tumor induction.

Evidence for a "Hit-and-Run" Mechanism

Using a novel mastomys coucha model with natural PV infection [108], conditional transgenic mice, as well as human explant cultures, the question has been investigated whether or not cutaneous PV are necessary throughout carcinogenesis [31–33]. Together, all these studies provided evidence that β-PVs have an early role in skin carcinogenesis, and at later stages, they become dispensable for the maintenance of the malignant phenotype, compatible with a "hit-and-run" mechanism [34].

Molecular and Functional Studies of Human Genus β-Papillomaviruses In Vitro

Comparative analyses demonstrated that several genus β-PV have transforming potential in vitro. For this, an oncogene (activated EJ-ras) cooperation assay in rodent cells was used [109, 110]. A subset of genus β-PVs was shown to extend the life span of primary human keratinocytes. For E6/E7 oncogenes of HPV49, HPV38, and HPV8, although weaker, immortalization of keratinocytes was demonstrated [111–113]. Thus, β-PV oncoproteins clearly have the potential to transform their natural host cells. However, the molecular mechanisms by which genus β-PV oncoproteins support the oncogenic process in skin can strongly differ from α-PVs. This part describes the major functional differences and similarities between mucosal genus α-PV and cutaneous β-PV.

The β-PV E6 Oncoprotein

There is evidence that β-PV E6 proteins have a profound impact on the regulation of epithelial homeostasis, UV-induced DNA damage responses and cell death in keratinocytes.

HPV oncoproteins lack enzymatic activity. Recent studies have unraveled important pathways targeted by genus β-PV E6 proteins. Thus, HPV8 E6 has been shown to transcriptionally suppress C/EBPα [75], a potent inducer of keratinocyte differentiation and suppressor of UV-induced carcinogenesis [79, 80]. C/EBPα was identified as a novel suppressor of this microRNA controlling the stemness factor ΔNp63 [75]. It directly binds to the microRNA-203 gene, and, via the novel C/EBPα/microRNA-20/ΔNp63 pathway, HPV8 E6 potently alters keratinocyte homeostasis. This leads to the

expansion of the ΔNp63-expressing epithelial progenitor compartment keratinocytes, which is highly susceptible to carcinogenic progression [76].

In addition, β-PV E6 proteins specifically bind to the Mastermind-like coactivator MAML1. As a consequence, this leads to suppression of Notch signaling [77, 114–116]. Notch is a key regulator of keratinocyte differentiation. Of note, MAML1 binding was highly specific for the cutaneous E6 proteins and was not observed for eight different genus α-PV E6 proteins. The latter E6 proteins neither interact with MAML1 nor with Notch1, Notch2, or RBPJ, a Notch-regulated transcription factor [45]. In mice, Notch also suppresses skin tumor formation [117]. Thus, interference of β-PV E6 with both, the C/EBPα/microRNA-20/ΔNp63 and the MAML1/Notch pathways, may contribute to tumorigenesis.

A key mechanism of the high-risk mucosal genus α-PV E6 oncoproteins is seen in its interaction with the tumor suppressor protein p53 as well as the ubiquitin ligase E6-AP, which targets p53 to proteasomal degradation [118–120]. In strong contrast, most genus β-PV E6 including HPV8 E6 do not bind to p53. Exceptions from this rule are HPV49 E6, as well as E6 proteins from two further β-PV, HPV38 and 92, which are able to interact with p53 [45, 93, 94, 113]. A comprehensive E6 interaction analysis, however, demonstrated that the p53 protein was rather stabilized by a posttranslational mechanism in keratinocytes expressing HPV38 or 92 E6 proteins. A similar effect was observed by HPV17a E6, a "p53 nonbinder" [45]. The functional significance of these findings and their consequences still has to be elucidated.

As outlined above, mutations of p53 are frequently found during skin carcinogenesis in the general population as well as in HPV-associated skin cancer in EV patients, which is a profound difference to cervical carcinogenesis. Moreover, several other ways might exist how β-PV E6 proteins interfere with p53 function. For example, HPV77 E6 selectively inhibits p53-dependent transcription of proapoptotic genes following UVB irradiation in cell lines [121]. HPV23 E6 was shown to prevent p53 phosphorylation through an interaction with the homeodomain-interacting protein kinase 2 [122]. In case of HPV38, the E6 protein was shown to affect p53 signaling indirectly, by inducing the expression of the deltaN isoform of p73 [123].

β-PV E6 proteins can extend the life span of human keratinocytes, and this was strongest for HPV38 and 8 [124, 125]. Particularly in cells expressing the latter E6 proteins, activation of telomerase was observed, and this occurred in an E6-AP-dependent manner [124], although no stable physical interaction of E6-AP and β-PV E6 proteins was observed in a different study [45]. Another important feature of different β-PV E6 proteins is seen in their ability to abrogate UV-mediated apoptosis. In vitro studies suggested that p53 degradation was not required, and inhibition of apoptosis was also observed in p53 null cells. One mechanism how β-PV E6 proteins exert their antiapoptotic activity is the proteolytic degradation of the proapoptotic molecule Bak [88]. This observation was later on confirmed for HPV5, 8, 20, 22, 38, 76, 92, and 96 in normal human keratinocytes [89].

β-PV E6 was shown to have variety of novel interaction partners including proteins containing PDZ motifs as well as proteins of the Ccr4-Not complex. Moreover, HPV5, 8, 20, and 25 E6 proteins specifically bind the acetyltransferases and transcriptional coactivators p300/CBP [45, 126, 127]. Several studies indicate that p300 binding by β-PV E6 affects important downstream signaling events most relevant for tumorigenesis, such as C/EBPα suppression [75], acetylation of p53, and p53-dependent transcription [128]. Thus, in HPV-associated skin carcinogenesis, p53 might either be mutated or inhibited at a functional level by β-PV E6 proteins.

P300 binding of E6 also contributes to suppression of keratinocyte differentiation and expression of the kinase ATR (ataxia telangiectasia, mutated and Rad3-related), a key regulator of the checkpoint pathway in the DNA damage response [127, 129]. Reduced ATR levels in β-PV HPV5 or 8 E6 expressing keratinocytes can increase the occurrence of UVB-induced double-stranded DNA breaks and thymine dimer

persistence [129] summarized in [90]. These data confirmed previous observations demonstrating a compromised repair of UV-induced thymine dimers in cell lines expressing β-PV E6 proteins [121]. The in vitro observations are also in line with the in vivo finding that HPV8 and 38 oncoproteins can significantly promote UV-induced tumorigenesis in transgenic mice [102, 105]. The fact that E6 enhances UV-induced mutagenesis may explain the accumulation of DNA mutations found in EV lesions including those within the p53 gene [87].

Thus, genus β-PV E6 proteins engage various strategies to promote tumorigenesis. At later stages of carcinogenesis, when E6 expression has promoted UV-induced genomic DNA alterations, p53 may itself be mutated and thereby inactivated. From this stage on, cellular mechanisms driving progression to cancer may dominate, and further persistence of the virus and maintenance of viral oncogene expression may become dispensable.

The β-PV E7 Protein

A key function of the mucosal high-risk E7 protein is seen in binding to and degradation of the retinoblastoma tumor suppressor protein pRb. The G_1-S phase checkpoint is bypassed, and cell cycle regulation is disrupted. This allows viral DNA replication in differentiating keratinocytes and contributes to the oncogenic activity. A recent systematic interaction analysis confirmed previous studies showing that genus α- and β-PV E7 proteins from different HPV species share the ability to bind to pRb as well as CUL3, a cullin-RING E3 ubiquitin ligase [130]. Most cutaneous E7 proteins bind pRb with lower affinities; however, HPV5 and 38 E7 were also shown to destabilize pRb [111, 112, 131].

β-PV E7 proteins may promote epithelial proliferation by further paracrine mechanisms altering the response to the local microenvironment. It has been demonstrated that the antiproliferative cytokine TGF-β is strongly upregulated in keratinocyte-fibroblast cocultures [132]. Keratinocytes expressing the E7 protein, however, showed strongly reduced responsiveness to TGF-β signaling. This was explained by their binding to Smad factors mediating the intracellular TGF-β signal. Again, this was a common feature of mucosal and cutaneous high- and low-risk HPV types [133, 134].

Another feature shared by the β-PV HPV8 and mucosal high-risk HPV16 but not the cutaneous low-risk HPV1 E7 protein is induction of the membrane-bound matrix metalloproteinase MT-1 MMP at mRNA and protein levels [135, 136]. There is a long list of MT-1 MMP substrates including MT-1 MMP itself, plasminogen, chemokines, cytokines, and growth factors promoting keratinocyte proliferation and angiogenesis (for review see [137]).

In addition, the genus β-HPV8 E7 protein may alter the microenvironment in a completely different manner. By binding to the transcription factor C/EBPβ in the granular layer, it specifically suppresses CCL20 expression and impairs Langerhans cell recruitment. This provides an explanation for the deficiency of Langerhans cells in EV lesions [67]. Thus, β-PV E7 proteins apparently do not directly promote carcinogenesis in vivo. However, it has been convincingly demonstrated that they can affect virus-host interactions critical for evading host immune defense and providing a microenvironment that is conducive for skin carcinogenesis.

Conclusions

Evidence is accumulating that cutaneous genus β-PVs are important cocarcinogens in UV-induced skin carcinogenesis. However, underlying mechanisms differ significantly from the carcinogenic process driven by high-risk mucosal genus α-PVs.

In the general population, β-PVs are found in the commensal skin flora. Their expression is tightly controlled by host restriction factors and extrinsic immunity. Patients with disturbed control mechanisms, i.e., mutations in restriction factors or impaired immune control, however, show higher disease penetrance, i.e., EV or

EV-like symptoms or development of skin cancer.

Once expressed, β-PV undergoes a life cycle that is highly adapted to the skin, UV exposure, UV damage, and an inflammatory host microenvironment. They expand the cutaneous epithelial progenitor cell compartment, which is highly susceptible to carcinogenic progression, disturb cutaneous immune homeostasis, fuel tumor-promoting inflammation, and lower the threshold to UV-induced DNA damage while promoting the life span of their host cells through preventing UV-induced apoptosis. This may lead to an enhanced accumulation of genomic mutations in infected cells. In vivo animal studies and ex vivo human studies imply that β-PV can act as powerful cocarcinogens at early stages of skin carcinogenesis. It is reasonable to assume that once genetic alterations, such as p53 mutations, have become established, the continuous presence of the virus may be dispensable for the maintenance of malignancy, compatible with a "hit-and-run" mechanism.

For the development of novel therapeutic strategies specifically interfering with β-PV at early stages of carcinogenesis, more research is needed to better understand the cross talk with their host keratinocytes and the local microenvironment.

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