HPV: from infection to cancer

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Abstract

Infection with HPV (human papillomavirus) 16 is the cause of 50% or more of cervical cancers in women. HPV16 infection, however, is very common in young sexually active women, but the majority mount an effective immune response and clear infection. Approx. 10% of individuals develop a persistent infection, and it is this cohort who are at risk of cancer progression, with the development of high-grade precursor lesions and eventually invasive carcinoma. Effective evasion of innate immune recognition seems to be the hallmark of HPV infections, since the infectious cycle is one in which viral replication and release is not associated with inflammation. Furthermore, HPV infections disrupt cytokine expression and signalling with the E6 and E7 oncoproteins particularly targeting the type I IFN (interferon) pathway. High doses of IFN can overcome the HPV-mediated abrogation of signalling, and this may be the basis for the therapeutic effects on HPV infections of immune-response modulators such as the imidazoquinolones that induce high levels of type I IFNs by activation of TLR (Toll-like receptor) 7. Using the unique W12 model of cervical carcinogenesis, some of these IFN-related interactions and their relevance in the selection of cells with integrated viral DNA in cancer progression have been investigated. Our data show that episome loss associated with induction of antiviral response genes is a key event in the spontaneous selection of cervical keratinocytes containing integrated HPV16. Exogenous IFN-β treatment of W12 keratinocytes in which the majority of the population contain episomes results only in the rapid emergence of IFN-resistant cells, loss of episome-containing cells and a selection of cells containing integrated HPV16 in which the expression of the transcriptional repressor E2 is down-regulated, but in which E6 and E7 are up-regulated.

Introduction

The papillomaviruses are small double-stranded DNA viruses which infect squamous epithelia (or cells with the potential for squamous maturation), inducing proliferative lesions, of which the humble skin wart is a typical example. The viruses are absolutely species-specific, thus HPVs (human papillomaviruses) only infect humans, rabbit papillomaviruses only infect rabbits, and so forth. They are also exquisitely tissue-tropic, with a predilection for infection of either cutaneous or internal squamous mucosal surfaces. Papillomaviruses are not classified by serotype, but by genotype, and, to date, approx. 130 HPV types have been identified by sequencing the gene encoding the major capsid protein L1 [1]. The HPV genome can be divided into three domains: a non-coding URR (upstream regulatory region) of approx. 1 kb, an early region with ORFs (open reading frames) E6, E7, E1, E2, E4 and E5, and a late region encoding two genes, L1, the major capsid protein, and L2, the minor capsid protein (Figure 1). The functions of these ORFs are described in Table 1.

Within a species, the individual viruses show a predilection for either cutaneous or mucosal surfaces, and, within the groups of skin or mucosal viruses, they can be separated into high- or low-risk types, depending upon their oncogenic potential. This is shown most clearly in the genital tract in which there is regular or sporadic infection, with approx. 30–40 types. These can be divided into those predominately associated with benign ano-genital warts or condylomata (HPV types 6 and 11, and their relatives), and those associated with ano-genital cancers and the precursor (intra-epithelial neoplasia) lesions particularly of the cervix (HPV types 16, 18, 31, 33, 35 and 45, and minor types). Virtually 100% of cervical cancers, the second commonest cancer in women worldwide, contain the HPV DNA sequences from a high-risk oncogenic genital HPV. The most important players are HPV16, found in 50–70% of cases, and HPV18 found in 7–20% of cases [2]. Cervical and other ano-genital cancers are preceded by a spectrum of intra-epithelial abnormalities. In the cervix, these form a spectrum ranging from low-grade CIN (cervical intra-epithelial neoplasia) 1, moderate CIN2 and high-grade CIN3. High-grade CIN3 lesions are the obligate precursor lesions for cervical cancer and approx. 90% of high-grade CIN contain high-risk HPV DNA sequences, and, again, HPV16 is the major player [3].

High-risk genital HPV infection is very common, and the majority of individuals clear their infection with time, but a proportion of women, approx. 15%, cannot effectively clear the virus, and the persistence of a high-risk HPV is the major risk factor for the development of ano-genital malignancies [4]. To persist, HPV must escape the host immune system,

Key words: cancer, cervical cancer, cytokine, human papillomavirus (HPV), interferon (IFN), Toll-like receptor (TLR).

Abbreviations used: CIN, cervical intra-epithelial neoplasia; DC, dendritic cell; HPV, human papillomavirus; IFN, interferon; IRF, IFN regulatory factor; ISRE, IFN-specific response element; LC, Langerhans cell; ORF, open reading frame; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; URR, upstream regulatory region; VLP, virus-like particle.

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Figure 1 | **Genome organization of HPV16**

The papillomavirus genome is highly conserved across the types. The ORFs E6, E7, E1, E2, E4 and E5 encode proteins required for regulation of viral DNA replication and viral gene expression, the L1 and L2 ORFs encode the viral capsid proteins. Only one strand of the double-stranded DNA serves as the template for viral gene expression, coding for a number of polycistronic mRNA transcripts. Transcription is regulated by enhancer sequences located in the URR, which are bound by a number of cellular factors as well as the viral E2 product. The transcription start sites of viral promoters differ depending on the virus type, but, in all types, promoter usage is keratinocyte differentiation-dependent.

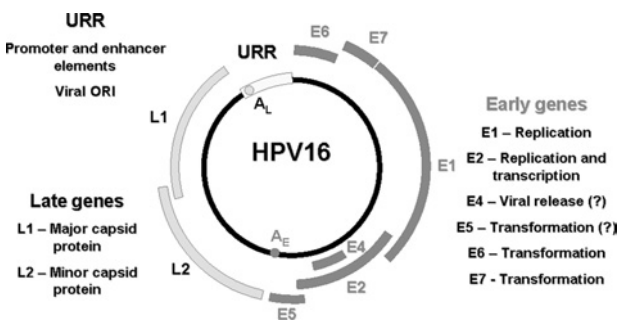


Table 1 | **Function of high-risk HPV ORFs**

ORF	Comments
E6	Binds p53, directs p53 ubiquitin-mediated degradation
	With E7 immobilizes primary keratinocytes
E7	Binds retinoblastoma protein, deregulates the G_1/S checkpoint
	Co-operates with E6 to immortalize primary cells
E1	Helicase, ATPase, ATP-binding protein essential for viral DNA replication
E2	Viral transcription factor
	Binds E1 to facilitate initiation of vital DNA replication important in genome encapsidation
E5	Weak transforming activity, up-regulates growth factor receptors
E4	Interacts with cytoskeletal proteins, allows viral assembly
L1	Major capsid protein
L2	Minor capsid protien

and this brief review will focus on the mechanisms by which high-risk HPVs evade the immune system.

Papillomavirus infectious cycle

The virus replication cycle is the key to understanding the pathogenesis and immunobiology of these viruses, since the infectious cycle is in itself an immune-evasion mechanism inhibiting host detection of virus. Our knowledge of this process is limited in several key areas mainly because of the inability to infect cells in tissue culture with virus and achieve a complete infectious cycle *in vitro*.

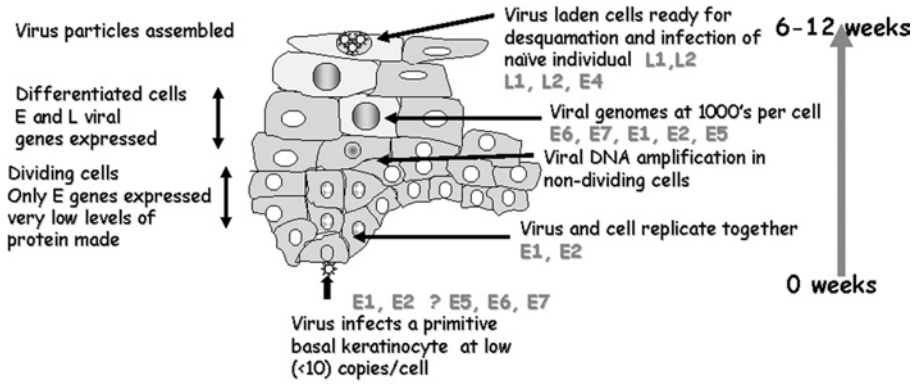
Infection and vegetative viral growth are absolutely dependent upon a complete programme of keratinocyte differentiation. Viruses infect primitive basal keratinocytes, probably targeting stem cells, but high-level expression of viral proteins and viral assembly occur only in the upper layers of the stratum spinosum and granulosum of squamous epithelia [5]. Viral gene expression is confined to the keratinocyte, and there is no evidence that viral genes are expressed in any cell other than keratinocytes. There is a spatial and temporal pattern of HPV gene expression in the infected epithelium (Figure 2). Virus infects a subset of primitive basal cells, probably stem cells, at low copy number. At some time after infection there is a round of viral DNA replication which appears to be independent of the cell cycle and which amplifies the viral copy number to approx. 50–100 copies/cell. The infected cell is thought then to leave this primitive stem-cell-like compartment to enter the proliferating compartment of the epithelium. There is then a phase of plasmid or episomal maintenance, viral gene expression is minimal and, in particular, the expression of oncogenes E6 and E7 is under very tight control with E6/E7 transcripts barely detectable. When the infected keratinocyte enters the differentiating compartment, exiting the cell cycle, there is a massive up-regulation of viral gene expression, viral DNA replication occurs, there is amplification of viral copy number to at least 1000 copies/cell, abundant expression of the early genes E6 and E7 and expression of the late genes from the late promoter [6].

It is important to recognize that these events occur in cells that are differentiating and have exited the cell cycle. The papillomaviruses encode only one DNA replication enzyme, E1, and, apart from this and the viral E2 protein, replication is totally dependent on the cellular DNA synthetic machinery. The problem for the virus is that the cellular DNA polymerases and replication factors are only produced in mitotically active cells. To solve this problem, the viruses encode proteins which, in the context of the viral life cycle, reactivate cellular DNA synthesis in non-cycling cells, inhibit apoptosis and delay the differentiation programme of the infected keratinocyte, creating an environment that is permissive for viral DNA replication [7]. The precise details by which this is achieved are imperfectly understood, but the viral genes central to these functions are E6 and E7, and an unfortunate, but rare, by-product of this role in high-risk HPV replication is the deregulation of growth control in the infected cell and the development of cancer.

The replication cycle takes a long time: even in the best-case scenario, the time from infection to release of virus will take approx. 3 weeks, since this is the time taken for the keratinocyte to undergo complete differentiation and desquamate. In reality, the period between infection and the appearance of lesions is highly variable and can vary from weeks to months. This virus is basically a hitchhiker joining the keratinocyte at the start of its journey as a primitive basal cell in the epithelium through to its end as a terminally differentiated squame. This is a replication strategy in which viral DNA replication and virus assembly occur in a cell already destined for death by natural causes; there is no viral induced cytolysis

Figure 2 | Infectious cycle of HPV16

The complete infectious cycle is absolutely dependent upon the differentiation programme of the keratinocyte. Virus infects keratinocytes in the basal layer of the epithelium, but only in terminally differentiated keratinocytes are viral capsid proteins and virus particles assembled.



or necrosis and therefore no inflammation. Thus, for most of the duration of the HPV infectious cycle, there is little or no release into the local milieu of pro-inflammatory cytokines that are important for DC (dendritic cell) activation and migration, and the central signals to kick start the immune response in squamous epithelia are absent [8]. There is no blood-borne phase of the HPV life cycle, and only minimal amounts of replicating virus are exposed to immune defences; in effect, the virus is practically invisible to the host. This is a viral strategy that results in persistent chronic infections as the host remains ignorant of the pathogen for long periods.

HPV infections are exclusively intra-epithelial, and, theoretically, HPV attack should be detected by the professional APC (antigen-presenting cell) of squamous epithelia, the LC (Langerhans cell). Virus capsid entry is usually an activating signal for DCs, but there is evidence that LCs are not activated by the uptake of HPV capsids [9]. LCs, when incubated with L1 VLPs (virus-like particles) of HPV16 do not initiate epitope-specific immune responses against L1-derived antigens. In contrast, stromal DCs are activated by VLPs and stimulate HPV-specific T-cells [10–12]. Studies in TLR (Toll-like receptor) 4-deficient mice suggests that TLR4 contributes to the recognition of HPV16 VLPs by stromal DCs [13]. There are some intriguing data that suggest that TLR activation may be secondary to VLP binding by cell-surface glycosaminoglycans [14,15].

Interference with IFN (interferon)

For most of the duration of the HPV infectious cycle, there is little or no release into the local milieu of pro-inflammatory cytokines that are important for DC activation and migration, and the essential signals to kick start the immune response in squamous epithelia are absent. However, even in the absence of viral-induced cytolysis and cell death, HPV-infected keratinocytes should activate the powerful generic antiviral defence system, type 1 IFNs. The type 1 IFNs, IFNα and IFNβ, have antiviral, antiproliferative, anti-angiogenic and immunostimulatory properties acting as a bridge between innate and adaptive immunity activating immature DCs [16]. Most DNA viruses have mechanisms for inhibiting IFN synthesis and signalling, and the papillomaviruses are no exception.

High-risk HPV infection down-regulates IFNα-inducible gene expression, and the HPV16 E6 and E7 oncoproteins interact directly with components of the IFN signalling pathways (reviewed in [17]). Thus E7 inhibits IFNα-mediated signal transduction by a binding to P48/IRF (IFN regulatory factor)-9, preventing translocation to the nucleus, thereby inhibiting the formation of the ISGF-3 (IFN-stimulated gene factor 3) transcription complex that binds ISRE (IFN-specific response element) in the nucleus [18]. E7 interferes with intermediate IFN-mediated signalling by also associating physically with IRF-1, inhibiting IRF-1-mediated activation of the IFNβ promoter, recruiting histone deacetylase to the promoter, thereby preventing transcription [19]. *In vivo* expression of HPV18 E7 results in reduced expression of IRF-1 target genes such as TAP1 (transporter associated with antigen processing 1), IFNβ and MCP-1 (monocyte chemotactic protein 1) by inhibition of the transactivating function of IRF-1 [20]. The E6 protein of HPV also targets the IFN pathway. E6 binds to IRF-3 and inhibits its transcript activation function, thereby preventing transcription of IFNα mRNA [21]. E6 binds to Tyk2, preventing binding to the cytoplasmic portion of the IFN receptor inhibiting phosphorylation of Tyk2, STAT (signal transducer and activator of transcription) 1 and STAT2, impairing JAK (Janus kinase)/STAT activation and therefore inhibiting specific IFNα-mediated signalling [22].

DNA microarray analysis of gene expression shows that HPV16 alters expression of three groups of genes:

IFN-response genes, NF-κB (nuclear factor κB)-stimulated genes and cell-cycle regulation genes [23–25]. E6 decreases expression of IFNα and IFNβ, down-regulates nuclear STAT1 protein and decreases binding of STAT1 to the ISRE. E6 and E7 therefore directly alter expression of genes that enable host resistance to infection and immune function.

IFN response and progression in cervical neoplasia

During the initial phase of infection, HPV exists as a nuclear episome, but the integration of high-risk HPV DNA into the host genome is an important step in neoplastic progression in the cervix [26]. Integration usually causes deletion or disruption of the viral regulatory E2 gene, while retaining a variable segment, including the E6 and E7 oncogenes and the upstream regulatory region. Overexpression of E2 from heterologous promoters in cells harbouring integrated high-risk HPV can repress the early promoter of the integrated virus, causing a sharp reduction in E6 and E7 expression [27]. High-risk HPV integration with consequent disruption/deletion of E2 leads to increased expression of the viral oncogenes [23,28,29]. Cells containing integrated high-risk HPV acquire a strong growth advantage over cells harbouring episomal high-risk HPV and undergo clonal expansion. These cells also show increased genomic instability [30,31] and therefore have a greater probability of acquiring the secondary genomic abnormalities that may drive malignant progression [32].

The unique HPV16-containing cervical keratinocyte cell line W12 was used to investigate some of these events. W12 was derived from a low-grade squamous intra-epithelial lesion infected with HPV16, and, at early passage, retains HPV16 as the episome at approx. 100 copies/cell [33]. In organotypic tissue culture, episome-containing W12 keratinocytes recapitulate the phenotypic characteristics of CIN1 [34]. Cells containing integrated HPV16 reproducibly emerge during long-term culture of W12 coincident with a rapid fall in episome numbers. During the period of emergence, single-cell clones containing integrants and reduced episome load were isolated. E2 expression was lower in such cells and associated with partial inhibition of E6/E7 transcription from the HPV16 integrant, but full deregulation of expression was not observed until there was complete loss of E2-expressing episomes. Microarray analysis showed that episome loss was closely associated with endogenous activation of antiviral response genes that are also inducible by the type I IFN pathway. Since overexpression of the viral oncogenes E6 and E7 requires loss of the transcriptional repressor functions of E2 following integration of HPV into the host genome, a key step in HPV-related carcinogenesis is therefore clearance of residual viral episomes which encode E2 [35], although the trigger for this activation of IFN-response genes in the absence of exogenous IFN is not known. Exogenous IFNβ treatment of W12 caused rapid reduction in numbers of HPV16 episomes and this was associated with the emergence of cells bearing previously latent integrants, in which there was increased expression of E6 and E7 [36].

These results indicate that episome loss, associated with induction of antiviral response genes, is a key event in the spontaneous selection of cervical keratinocytes containing integrated HPV16 and implies that loss of inhibitory episomes is a critical step in cervical carcinogenesis. IFNβ can dramatically hasten the transition from episomal to integrated HPV16 in naturally infected keratinocytes with clearance of episomes through non-cytolytic mechanisms. Integrated HPV16 can exist in a minority of cells in a polyclonal population for long periods with no selective growth advantage until there is loss of episomes with depletion of E2 and derepression of E6/E7. This has implications for treatment of HPV-infected cervical lesions by inducing an IFN response, since this may enhance lesion progression.

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