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Persistence of human papillomavirus infections: keys to malignant progression

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Abstract

Human papillomaviruses (HPVs) are the etiologic agents of cervical and other epithelial cancers. Persistence of infections by high-risk HPV types is the single greatest risk factor for malignant progression. Although prophylactic vaccines have been developed targeting high risk HPV types, there is a continuing need to better understand the cell-host interactions that underlie persistent benign infection and progression to cancer. In this review, we summarize the molecular events that facilitate the differentiation-dependent HPV life cycle, how the life cycle is organized to facilitate viral persistence, and how the activities of HPV regulatory proteins result in malignancy.

Human papillomavirus infection leading to cancer

Viruses cause approximately 15% of human cancers, and of these nearly half are attributable to human papillomaviruses (HPVs) ¹. Infections by HPVs in the genital tract are the most common sexually transmitted viral infection ². Although progression to cancer is a rare event, the high prevalence of HPV infection makes HPV-related cancers, including cervical and other anogenital cancers, among the most common malignancies. It is estimated that over 450,000 new cases of cervical cancer are diagnosed each year making this the second most prevalent cancer in women worldwide ¹. The effective utilization of the Papanicolaou (Pap) smear to recognize cellular abnormalities associated with HPV infection has resulted in a reduction in the number of cases of cervical cancer in the US by 80% in the last 50 years. Despite this decrease, approximately 11,000 women are diagnosed with cervical cancer annually in the US and approximately 3700 die of this disease ². In addition, high-risk HPV infection is responsible for approximately 3,000 cases of anal cancer and over 3500 cases of oropharyngeal cancer annually in the United States ².

Papillomaviruses are small, non-enveloped DNA viruses that infect stratified squamous and cutaneous epithelia ³. Over 120 different types of HPVs have been identified and approximately one-third of these types specifically target epithelial cells in the genital tract. The remaining types infect epithelial cells in other tissues including cutaneous epithelia. The HPV types that infect the genital mucosa can be divided into two groups. The high-risk (HR) types, including HPV16, 18, 31 and others, are frequently found in cervical cancers. By contrast, the low-risk (LR) types, including HPV6 and 11, also infect the genital epithelia, but are rarely detected in malignancies ⁴. Two prophylactic vaccines (Gardasil (Merck) and Cervarix (GSK)) against the HR types HPV16 and 18 were recently approved

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by the FDA and act by blocking initial infection. However, these vaccines are unable to clear previously existing infections which retain the potential to progress to cancer². This underscores the need to continue Pap screening and to develop additional therapeutic strategies against ongoing infections.

HR HPV infections typically last from 12–18 months and are eventually cleared by the immune system⁵. Approximately 10% of women fail to clear HPV infections, resulting in long-term persistent infections⁶. Persistence by HR HPV over long periods is the single most important risk factor for the development of cancer^{2, 6, 7}. It is therefore important to understand the biology of persistent HPV infections and examine the features of lesions that progress to cancers as opposed to those that do not. In this review we will summarize the current knowledge of the factors regulating viral persistence, oncoprotein action, and malignant progression. To begin to understand how all these processes are linked, it is first important to discuss the unusual life cycle of HPVs.

HPV life cycle: maintenance of viral infection

The HR HPVs infect stratified squamous epithelia in the genital tract and link their productive life cycles to the differentiation of the host cell (Figure 1, Box 1). In normal stratified epithelia, the only actively dividing cells are located in the basal and parabasal layers adjacent to the basement membrane and consist of stem cells as well as cells of limited lifespan called transit amplifying (TA) cells (Box 2). As normal basal cells divide, one daughter cell becomes a new basal cell, while the other migrates away from the basal layer and begins to differentiate. Differentiating cells exit the cell cycle and undergo a complex series of changes in gene expression, eventually resulting in cell death and desquamation. Mucosal epithelia are not homogeneous, however, and different sites may be targeted by HPV in different ways though most cancers as well as precancers develop in a region of the cervix referred to as the transformation zone (Box 1).

Box 1

Why do high-risk viruses infect the cervix and cause cancers?

High-risk HPVs target the cervix for infection that can, in some cases, lead to the development of cancer. These viruses also infect penile epithelial cells with high efficiency yet cancers rarely develop in these tissues. The reason for this disparity is unknown. The cervix is unique among body tissues in the mechanical stresses and subsequent healing responses that it undergoes in the course of its normal function⁶⁶. HPVs are thought to require wounding of the epithelium to expose basal cells to viral entry, and HR-HPVs might be particularly adapted to the healing responses of cervical cells. Immunological and anatomical differences between mucosal and cutaneous epithelia might also influence the ability of the virus to infect or persist⁶⁷. Cervical cancers preferentially arise in the cervical transformation zone (TZ), the region that forms the boundary between the squamous epithelium of the ectocervix and the columnar epithelium of the endocervix⁶⁸ (Figure 1). Basal cells in the transformation zone retain the ability to differentiate, a property necessary for virion production⁶⁹. It is possible these basal cells are more readily accessible to HPV infections because of the presence of fewer overlying layers than in other locations. Hormones such as estrogen and progesterone, which are responsible for orchestrating cervical changes during menstruation and childbirth⁶⁶, potentiate both HPV infection⁷⁰ and cancer development⁷¹ in mouse models, and both are risk factors in the development of cervical cancer⁷². Intriguingly, anal cancer, which is also strongly HPV-associated, occurs predominantly at

the anal squamocolumnar transition zone which is anatomically similar to the cervical TZ^{67, 73}.

Box 2

Epidermal stem cells

Two classes of basal cells have been reported to exist in the basal layer of the cervix⁷⁴. The first type is the transit amplifying cells (TA) which are proliferating cells that are able to undergo terminal differentiation. TA cells divide and differentiate into the majority of cells in the suprabasal layers. The second class of basal cell is the stem cell, which has unlimited proliferation potential, but divides only rarely in order to replenish the TA pool. Stem cells serve as a reserve cell population to enable long-term maintenance of the tissue. Only one daughter cell of a stem cell division goes on to become a TA cell, while the other remains a stem cell. The existence of two distinct populations of cells remains controversial, and there may in reality be a continuum of proliferation potential in basal cells⁷⁴. It is unclear which cells in the basal layer are the targets of HPV infection and perhaps both can be infected. If this is the case, infection of stem cells could lead to one kind of outcome such as persistent infection while infection of TA cells leads to short term infections. Additionally, HPV might alter the nature of the cell it infects to more closely resemble one population or the other. This needs to be studied in greater detail in the future.

HPVs share a common genetic organization (Figure 2), and disparities between HR and LR HPVs are due to amino acid differences in common factors rather than the presence of additional proteins⁸. The early genes consist of E6 and E7, which are oncoproteins in HR HPVs. These factors act to alter the function of cell cycle regulators to allow for persistence of episomal genomes in undifferentiated cells as well as for productive replication of genomes or amplification in differentiated cells⁸. E1 and E2 are two virally encoded replication factors while E4 and E5 act to regulate late viral functions by mechanisms that are not well understood. L1 and L2 are the two capsid proteins that are synthesized following amplification.

Maintenance Phase

The life cycle of genital HPVs is closely linked to epithelial differentiation and extensive discussions of this process can be found elsewhere⁹. However, it is important to briefly discuss the organization of the life cycle because it is a major factor contributing to the persistence of HPV infections. HPV virions infect cells in the basal epithelial layer that become exposed through microwounds (Figure 1). Viral capsids bind initially to the basement membrane, and infection occurs as activated keratinocytes move into the wound, thus targeting the virus to basal cells¹⁰. Following entry and uncoating, HPV genomes are replicated in the nucleus to about 100 episomal copies per cell⁹. Maintenance of the viral episome in basal cells is the basic function of the early, or maintenance, phase of the viral life cycle. E6, E7, E1 and E2 are each necessary for continued episomal maintenance of whole genomes in cell culture^{11–14}. E1 and E2 cooperate to initiate viral DNA replication, while E6 and E7 modulate cell cycle regulators to maintain long term replication competence^{15, 16}. Viral proteins are expressed at very low levels in undifferentiated cells, and this contributes to immune evasion and persistence as discussed below.

Following this initial establishment phase, viral DNA is replicated coordinately with cellular chromosomes and viral genomes are distributed to the two daughter cells. For HPV genomes to persist in dividing basal cells, mechanisms must exist to allow episomal copies to be

retained in the nucleus and coordinately partitioned into daughter cells during mitosis. In the absence of such functions, extrachromosomal copies of HPV would be lost during cytokinesis. The E2 protein has been implicated as a major regulator of this process as it can bind to factors on mitotic chromatin and tether the viral genome to host chromosomes during mitosis¹⁷. In this manner, viral genomes are segregated equally into daughter cells along with the host DNA. The Brd4 protein has been implicated as a tethering factor for some HPV types while other tethering factors, such as ChlR1, are also used¹⁷.

Another requirement for viral DNA to persist in undifferentiated basal cells is the ability to retain a stable copy number. The E2 protein is likely a major regulator of this process as it has been shown to both positively and negatively control the early viral promoter that regulates expression of E6, E7, E1, as well as E2 itself¹⁸. In this way, E2 shares similarities to prokaryotic replication proteins that regulate copy number by autoregulating their own expression. In addition, a truncated form of E2 referred to as E8^E2C is a strong inhibitor of early gene expression and its mutation leads to loss of copy number control¹⁹.

Differentiation-dependent phase

Throughout the maintenance phase in undifferentiated cells, viral proteins are expressed at extremely low levels and this is important for escape from immune surveillance. When HPV-infected cells leave the basal layer they undergo differentiation and high levels of viral protein synthesis are induced. The restriction of high levels of viral protein synthesis to highly differentiated layers delays the expression of viral antigens to locations less susceptible to host immune surveillance²⁰. Compartmentalization of gene expression by HPVs is thus an important strategy to sustain long term infection, but it creates a conundrum for the virus. Because the virus does not encode its own DNA replication enzymes, cellular replication proteins are needed for productive viral replication but differentiating keratinocytes normally withdraw from the cell cycle and downregulate these factors⁸. To solve this problem, the virus forces the cell to remain active in the cell cycle thus enabling productive replication in differentiating cells. The primary viral protein responsible for maintaining replication competence in differentiating cells is E7, and this is accomplished in part by inactivation of pRb family members⁸. The consequences of E7 binding to pRb family members on transformation will be discussed in more detail in a subsequent section.

Activation of the late viral promoter in response to cellular differentiation occurs in approximately the spinous epithelial layer and is responsible for high levels of viral protein expression^{21–23}. Upon differentiation, the transcript levels of the E1, E2, E1^E4 (a fusion of the first amino acids of E1 onto the E4 ORF), and E5 genes increase dramatically^{24–26}. As a result, viral copy number amplifies from 50–200 copies to several thousands of copies per cell²⁷. Both E1^E4 and E5 contribute to the activation of late viral functions upon differentiation^{28–30}. The ATM-mediated DNA damage pathway is also activated upon differentiation and this is necessary for the formation of replication foci in differentiated cells, and amplification³¹.

Following genome amplification, the viral capsid proteins L1 and L2 are produced in the late phase and virions are assembled. Since L1 and L2 can be highly immunogenic, multiple layers of regulation control expression of the capsid proteins, serving to prevent expression until the most differentiated strata. These include differentiation dependent regulation of specific splice donors and acceptors, switching from the early to the late polyadenylation signals, regulation of transcript stability, and usage of rare codons³². Virions are shed into the environment with desquamated cells in the absence of lysis or necrosis, which further contributes to viral persistence by avoiding inflammation⁶.

Immune evasion by HPVs and persistence

As discussed above, the HPV life cycle is organized so to limit viral antigen synthesis in undifferentiated cells with high level expression restricted to highly differentiated cells. Furthermore there is no viremia, cell lysis, or induction of other signals to trigger an inflammatory response³³. Immune suppression frequently leads to the appearance of abundant HPV lesions, indicating that the immune system acts to limit HPV infections^{34, 35}. Thus in order to persist, HPV must actively suppress both innate and adaptive immune responses. One important innate immune pathway targeted by HPV in multiple ways is the interferon response^{6, 36}. Normal human keratinocytes constitutively express low-levels of interferon-inducible genes in the absence of added interferon. In contrast, cells expressing HR HPV E6 and E7 proteins repress the transcription of many interferon target genes including Stat-1, an transcriptional activator of interferon inducible genes^{37, 38}. E6 can bind and block the action of IRF-3, while E7 can bind IRF-1, both of which are regulators of the interferon pathway³³. E6 also blocks activation of protein kinase R (PKR)³⁹, as well as the kinase Tyk-2 responsible for activating Stat-1³³. HR E6 and E7 can also inhibit expression of the toll-like receptor (TLR9), which is important for sensing doubled-stranded DNA⁴⁰.

Keratinocytes constitutively express low levels of a variety of cytokines which can be upregulated following viral infection⁴¹. HPV-positive keratinocytes show significantly reduced expression of a wide range of inflammatory cytokines including IL-1, IL-6, TNF- α and TGF- β ^{42–44}. At the same time, expression of the anti-inflammatory IL-10 is increased^{40, 43}. These alterations in cytokine production reduce the ability of immune cells to infiltrate infected tissues. Keratinocytes constitutively express low-levels of interferons α , β , and κ , and expression of interferon κ is suppressed in HPV positive cells which could contribute to inhibition of expression of interferon-inducible genes⁴⁵.

Clearance of HPV-induced lesions occurs by cell-mediated immune mechanisms and is accompanied by infiltration of CD8⁺ and CD4⁺ lymphocytes⁶. Langerhans cells (LCs) are the major dendritic cell (DC) found in squamous epithelia and are likely responsible for triggering an anti-HPV immune response. The function of LCs is disrupted by HPV at several levels⁶. Infiltration of HPV-infected tissues by LCs and DCs is inhibited by HPV-induced changes in cytokine expression⁴⁶. Furthermore, HPV L2 proteins are able to suppress maturation, migration, and cytokine secretion by LCs⁴⁷. Finally, interaction between LCs and keratinocytes can be disrupted by the reduction of E-cadherin levels by E6⁴⁰.

Development of HPV-specific T-cell responses is repressed or delayed in HPV-infected patients, and this is more marked in HR as compared to LR infections⁴⁸. One reason for this impairment is likely the downregulation of major histocompatibility complex (MHC) I expression, and E7, E6 and E5 have each been implicated in this process⁴⁹. Furthermore E7 has been reported to downregulate the transporter associated with antigen processing (TAP), thereby interfering with presentation of antigens via the MHC I pathway⁴⁰. Finally, natural killer (NK) cell activity is also reduced in HPV infections^{33, 49}. HPVs have evolved numerous ways to suppress the activities of both the innate and adaptive immune responses and this plays a central role in persistence as well as in malignant progression.

Angiogenesis and persistence

As HPVs lesions persist, they become larger and exhibit increased requirements for nutrients. In proliferating lesions, there is competition between cells for nutrients and oxygen which can result in arrest of cellular proliferation or even cell death. Both benign and malignant HPV lesions overcome this constraint by promoting angiogenesis^{50–52}. Several studies have reported that HPV gene products can induce the production of

angiogenic factors by infected cells ^{53–56}. Angiogenesis can be triggered by hypoxia, which is a state of low oxygen and this is regulated through the activation of the transcription factor hypoxia inducible factor-1 (HIF-1) ⁵⁷. Both HR and LR E7 proteins can enhance levels of HIF-1 α as well as induce the increased expression of HIF-1 target genes under hypoxic conditions ⁵⁸. The enhancement of HIF-1 activity by E7 occurs through the displacement of histone deacetylases (HDACs) from HIF-1 resulting in enhanced transcription of a subset of genes that are normally responsive to HIF-1 (J. Bodily and L.A. Laimins, unpublished). The induction of angiogenesis is critical to both persistence and growth of HPV lesions and is regulated through modulation of the HIF-1 transcription factor.

E6, E7 and transformation

Cancers arise in persistent HPV lesions through the action of the two viral oncoproteins, E6 and E7, which also play key roles in maintaining HPV infections. Only the HR HPV E6 and E7 contribute to the development of cancers so it is important to discuss the similarities and differences between HR and LR E6 and E7, the cellular factors they bind, and the cellular changes they induce (Figure 3).

The E7 proteins from all HPV types examined to date bind to members of the pRb family of tumor suppressors but do so with varying affinities. The HR E7 proteins bind pRb family members p105 (pRb), p107 and p130 with significantly higher affinity than their LR counterparts ⁵⁹. This binding results in deregulated repression of E2F transcription factors that control expression of many S phase specific genes and consequent entry into S phase ⁵⁹. Abrogation of pRb function by E7 is important for maintenance of replication competence during differentiation. The HR E7 proteins also induce the degradation of pRb to more completely inactivate its function ⁵⁹.

Several additional cellular factors have been shown to bind to HR E7 proteins that are important for viral pathogenesis. These include HDACs 1,2 and 3 that are important for activating a number of cellular genes such as E2F2 and contribute to stable viral genome maintenance in undifferentiated cells and amplification in differentiated cells ⁶⁰. E7 can also bind to the cyclin kinase inhibitors p21 and p27, interfering with their cell cycle inhibitory functions, and to cyclins A and E to enhance their activities ⁵⁹. E7 binds to E2F6, a member of the polycomb complex ⁶¹, the DNA damage sensor ATM ⁶², and p600 to interfere with anoikis ⁶³. These activities are important to maintain cells active in the cell cycle, facilitating viral replication during differentiation.

One consequence of this highly efficient abrogation of pRb function by the HR E7 proteins is increased stabilization of p53 that can lead to apoptosis. As a result, the HR E6 proteins have evolved to induce degradation of p53 to block apoptosis ⁶⁴. HR E6 proteins are approximately 150 amino acids in size and act primarily as scaffolds for a number of cellular factors as they lack any known enzymatic activities. E6 binds the cellular E3 ubiquitin ligase E6AP to recruit p53 to a trimeric complex resulting in its ubiquitination and rapid turnover ⁶⁴. p53 is not a natural substrate of E6AP and is only targeted in the presence of HR E6. LR E6 proteins also bind E6AP but do not degrade p53, suggesting other factors could be targeted for degradation by LR E6. E6 also inactivates p53 through the binding of the histone acetyltransferases p300/CBP and ADA3 leading to reduced acetylation of p53. Interestingly, mutants of E6 that fail to abrogate p53 function are still able to immortalize indicating that other activities of E6 are also important ⁶⁴. Significantly, HR E6 proteins activate expression of hTert, the catalytic subunit of telomerase, through the binding and activation of a series of factors including c-Myc, E6AP, Max, Sp1, and X-box binding protein NFX1-91. No such activity is mediated by LR E6. Activation of hTert by E6 along

with inactivation of pRb function by E7 is necessary to immortalize human keratinocytes⁶⁴. E6 also binds a series of membrane-associated proteins through a PDZ domain at its C-terminus, which likely contributes to enhanced cellular proliferation and stable long term maintenance of viral genomes. The E6 and E7 proteins modulate the activities of a number of cellular factors that control the viral life cycle and, in persistent infections, mediate the development of cancers.

Consequences of persistence of HR HPV lesions

Cervical and other genital cancers are composed of cells that have lost the ability to differentiate, a process necessary for productive replication of HPVs. In addition, cervical cancers can often contain integrated HPV genomes rather than episomes and viral DNA needs to exist episomally in order to be productively replicated. Not surprisingly, cancer cells do not produce HPV virions. Since a primary goal of infection is to generate new virions, the induction of cancers by HR HPV types is a 'dead end' for the virus. In fact, it would seem to be in the interest of the virus to avoid promoting malignancy. However, the persistent nature of HPV infections allows cellular genetic changes to accumulate over extended periods of time until a combination of genetic abnormalities has occurred allowing cancer to develop.

Cells immortalized by E6 and E7 are not tumorigenic in nude mice. This is consistent with the observation that HPV infection is necessary but not sufficient for malignant progression⁸. Introduction of a second activated oncogene such as *ras* or extended passage in tissue culture results in secondary cellular mutations required for full transformation⁸. E6 and E7 themselves contribute to the accumulation of mutations in the cellular genome by promoting genomic instability during persistent infections⁶⁵. E6 and E7 induce centrosomal abnormalities resulting in abnormal segregation of chromosomes and aneuploidy. E7 causes centrosome reduplication leading to abnormal numbers of centrosomes⁶⁵. In addition, abrogation of cell cycle checkpoints through the targeting of p53 and pRb family members allows retention of cells with chromosomal abnormalities⁸. What advantage the virus gains by promoting cellular genome instability is not clear, but it could simply be a side-effect of inactivating the p53 and pRb surveillance pathways.

Conclusions

HPV biology is organized around the ability of the virus to persist, with each of the viral proteins contributing in some way to these processes. The mechanisms that permit viral persistence include the differentiation-specific organization of the viral life cycle, mechanisms to maintain genome copy number in undifferentiated cells, promotion of angiogenesis, and strategies to evade both innate and adaptive immune surveillance (Box 3). In most cases, the host is eventually able to prevail and HPV infections resolve. In some cases, however, HPV infections persist for longer periods, allowing the accumulation of additional cellular changes leading to cancer. For this reason, better understanding of the mechanisms of persistence will continue to be an important area of work in the future.

Box 3

Outstanding questions

1. Is viral persistence influenced by host genetic makeup?
2. Do mechanisms of infection or persistence differ in different tissues?

3. How is the cervix different from other epithelial tissues, and how do these differences potentiate cancer development? Why are the cervical and anal transformation zones particularly cancer prone?
4. To what extent does the outcome of infection depend on the identity of infected cells (TA versus stem cells)?
5. What controls differentiation-dependent genome amplification and late gene expression? What are the relative contributions of transcriptional, post-transcriptional, and translational mechanisms for expression of late genes? How are they linked to differentiation?
6. What causes integration of viral genomes? Why are some viral types found integrated more than others? Is integration a cause or a consequence of malignant progression?
7. What mechanisms are most important for HPV's evasion of the adaptive immune response? What viral activities are responsible for the suppression of host cytokine expression?
8. What role does induction of genomic instability play in the productive viral life cycle?

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Figure 1.

Structure of the cervical epithelium. **(a)** The location of the endocervical and ectocervical epithelia. **(b)** Schematic of the stratified ectocervical epithelia, the columnar endocervical epithelia, and the transformation zone (TZ). HPV infections and both benign and malignant lesions preferentially develop in the TZ. Infection initiates through microwounds that expose the basement membrane and basal keratinocytes. Viral episomes are maintained in the basal cells. As host cells differentiate, HPV gene expression is coordinated to result in capsid assembly and virion release in the uppermost differentiated strata. Nuclei are colored to indicate the viral proteins expressed during the various phases of the life cycle (also see Figure 2).

Figure 2.

Genetic organization of papillomaviruses. The circular HPV genome is shown linearized for simplicity. The replication origin and many transcriptional regulatory elements are found in the upstream regulatory region (URR). The viral early promoter, differentiation dependent late promoter, and two polyadenylation signals define three general groups of viral genes that are coordinately regulated during host cell differentiation. The E6 and E7 genes maintain replication competence. E1, E2, E4, E5, and E8 are involved in viral DNA replication, transcriptional control, and other late functions. L1 and L2 are the capsid proteins. ORFs are colored to indicate at which phase of the viral life cycle each is most highly expressed (see Figure 1).

Figure 3.

Targets of E6 and E7. Both HPV oncoproteins have a large number of interaction partners, including regulators of the cell cycle, gene expression, DNA replication, and cell signaling. Some binding interactions are result in inactivation or degradation of the target, and some result in activation.