Review Article


Human papillomavirus infection, cancer & therapy

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Infection with human papillomaviruses (HPVs) is a major public health burden worldwide and is associated with a variety of epithelial lesions, including benign warts and several types of anogenital tumours, particularly cervical carcinoma. From available data it is clear that members of the HPV family are important human pathogens. Prevention or elimination of these infections would not only benefit the numerous patients with benign lesions, but ultimately should reduce the incidence of cervical cancer and possibly other epithelial cancers as well. Although prophylactic vaccines to block genital HPV infection have become available, it is not certain if they would be of benefit to those already infected. Therefore, the enormous and growing population of infected individuals would benefit from papillomavirus-specific therapy. In this review, we will discuss the functions of the viral proteins that appear to be the most appropriate for the development of therapeutics aimed at the treatment of viral infection and virus-induced cancers.

Key words E6 - E7 - HPV - protein targets - therapy

HPV & cancer

The last 30 years have seen the discovery of many different types of human papillomaviruses (HPVs) as well as the demonstration of their role in human cancer and their significance as targets for diagnosis and therapy. The HPVs can be broadly grouped into cutaneous types and mucosal types based on their preferred tissue tropism. The cutaneous types are typically found in the general population and cause common warts. Other cutaneous types are found in individuals who are immunosuppressed. The mucosal HPVs are further classified into high-risk and low-risk types, based on their respective degree of association with cervical cancer. The most common low-risk types are HPV 6 and 11, detected most often in benign genital warts. HPV 16, 18, 31, and 45 are predominant types found in cervical squamous cell carcinoma (SCC), accounting for more than 90 per cent of cases, with HPV 16 alone accounting for about half the cases worldwide. HPV 18 is the most prevalent type in cervical adenocarcinomas (55%), followed by HPV 16 (32%) and HPV 45 (10%). Epidemiological evidence has convincingly demonstrated that infection with HPV is the greatest risk factor, its role in the progression of the precursor lesions to cervical cancer is well established.

The HPV life cycle

HPVs are exclusively epitheliotropic, and their replication is intimately linked to the differentiation process of the host cells. Normal squamous epithelial
cells grow as stratified epithelium, with those in the basal layers dividing as stem cells of transient amplifying cells. After division, one of the daughter cells migrates upward and begins to undergo terminal differentiation while the other remains in the basal layer as a slow-cycling, self-renewing population. Productive papillomavirus infection begins when infectious virions gain access to cells of the basal layer, probably through micro-wounds. The viral genome is maintained in these cells as a stable episome at low copy number, and it is these infected cells that form the reservoir for the development of a productive wart. The early HPV genes E1 and E2 support viral DNA replication and its segregation such that the infected cells can be maintained in the lesion for a long period. As infected daughter cells migrate towards the epithelial surface, viral late gene products are produced to initiate the vegetative phase of the HPV life cycle, resulting in the high-level amplification of the viral genome. In the outer layers of the epithelium, viral DNA is packaged into capsids and progeny virions are released to re-initiate infection (Fig. 1). As the viral DNA replication depends almost totally on host replication factors except for the viral helicase E1, the other early genes E6 and E7 are required to coordinate the host cell environment so that it is suitable for viral DNA replication. In these suprabasal post-mitotic cells, E6 and E7 induce unscheduled re-entry into S-phase of the cell cycle, activating the host replication machinery needed for amplification of viral genomes prior to virion synthesis. The mechanism by which these proteins act, and their normal function during the virus life cycle, is partially understood. The E7 protein drives cells into S-phase largely by associating with, and causing the degradation of, members of the Rb family. For the high-risk HPV types, this includes pRb itself and the p130 protein, which is involved in the regulation of terminal differentiation. As a result E7 disrupts the association between pRb and the E2F family of transcription factors, irrespective of the presence of external growth factors. E2F subsequently transactivates the expression of a large number of cellular proteins required for DNA replication, such as DNA polymerase and thymidine kinase.

For the low-risk types that cause warts, such E7-mediated degradation appears to be confined to p130, whose degradation through E7-binding leads to S-phase entry in the upper epithelial layers of infected tissue. E7 also associates with other proteins involved in controlling cell proliferation, including histone deacetylases, components of the AP1 transcription complex and the cyclin-dependent kinase inhibitors p21 and p27. Taken together, E7 is responsible for driving cells into an artificial S-phase, rendering them capable of replicating the viral DNA, and alters the differentiation capability of the infected keratinocyte. The function of the viral E6 protein complements that of E7 and, in the high-risk HPV types, the two proteins are expressed together from a single polycistronic mRNA species. One of the major

![Fig. 1. The HPV life cycle. Shown is the coordinate expression of the different viral proteins during the course of a productive infection.](image-url)
roles of the E6 protein during productive viral infection is to inhibit the cellular apoptotic response to E7-driven cell-cycle re-entry in the upper epithelial layers. Certainly, a key function of the high-risk HPV types is their ability to bind to the cellular p53 tumour suppressor protein and cause its degradation via the ubiquitin pathway, thereby inhibiting its apoptotic activity\textsuperscript{17,18}. A more general role of E6, as an anti-apoptotic protein, is emphasized further by the finding that it also associates with Bak\textsuperscript{19} and Bax\textsuperscript{20}. These antiapoptotic activities of E6 are of critical significance in the development of cervical cancer, as they compromise the effectiveness of the cellular DNA damage response and allow the accumulation of secondary mutations to go unchecked.

The E6 protein of the high-risk HPV types also plays a role in increasing cell proliferation independently of E7, through its C-terminal PDZ ligand domain [the name PDZ is derived from the first three proteins in which these domains were found: PSD-95 (a 95 kDa protein involved in signalling), Dlg (the \textit{Drosophila} discs large protein), and ZO1 (the zonula occludens 1 protein which is involved in maintaining epithelial cell polarity)\textsuperscript{21}]. E6 PDZ binding can mediate suprabasal cell proliferation\textsuperscript{22,23}, and this is thought to occur by uncoupling the cell proliferation and polarity control that exist in a differentiated epithelium.

There are two important features in the HPV life cycle that indirectly contribute to the development of cancer. First, the replicative phase of HPV is confined to differentiating epithelial cells that have exited the cell cycle and which are normally non-permissive for DNA synthesis\textsuperscript{24}. Since HPVs use cellular enzymes to replicate their genomes, they need to induce the cellular replication machinery while simultaneously maintaining differentiation, which as we have seen, is achieved by the combined activity of the viral E6 and E7 oncoproteins (Fig. 2). However, should this process be in any way perturbed, then events leading to cell immortalisation and malignancy can be initiated. This has been amply demonstrated in \textit{in vitro} assays, where E6 and E7 can efficiently cooperate to immortalize human keratinocytes\textsuperscript{25-27}. Additionally, the spatial and temporal differences between high and low-risk HPVs with respect to their sites of DNA replication within the epithelium are also likely to be critical. Low-risk HPVs tend to initiate DNA replication in the less differentiated cell population where elements of the cellular DNA replication machinery are still present. In contrast, high-risk HPVs replicate in the higher levels of the epithelium, and therefore require more vigorous priming of the cell division machinery\textsuperscript{24}.

Infection with high-risk HPV is associated with cervical intraepithelial neoplasia (CIN), and cervical cancers are thought to arise from these lesions after long persistent infection\textsuperscript{28}. CIN I (mild dysplasia) and CIN II (moderate dysplasia) lesions show relatively low levels of E6 and E7 expression in which the viral genomes replicate episomally, whereas CIN III (severe dysplasia, carcinoma \textit{in situ}) and invasive cancer lesions often display high-level expression

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Cellular protein targets of the E6 and E7 oncoproteins. Some of the important cellular targets are highlighted together with their respective positions in the progression to malignancy.}
\end{figure}
of E6 and E7, in most cases with the integration of viral DNA into the host cell genome\textsuperscript{29}. Integration of high-risk HPV genomes is believed to represent a significant event in the pathogenesis of cervical cancer associated with progression from pre-neoplastic lesions to invasive cancer\textsuperscript{30}. However, the frequency of integration shows marked differences in individual HPV types. HPV 16, 18 and 45 are found substantially more often in the integrated state compared with HPV types 31 and 33. Furthermore, pre-cancers induced by HPV types 18, 16, and 45 progress to invasive cervical cancer in substantially less time compared with pre-cancers induced by HPV types 31 and 33\textsuperscript{31}. However, integration is not a normal part of the HPV life cycle characterised by large deletions in the viral DNA coupled with the uncontrolled expression of the E6 and E7 oncoproteins\textsuperscript{32-34}. Thus, it represents a by-product of viral infection that may confer a selective advantage to the host cell without any apparent advantage to the virus.

The development of transgenic mouse model systems has allowed a series of elegant in vivo studies on HPV-associated carcinogenesis\textsuperscript{35-38} clearly establishing the oncogenic potential of the E6 and E7 proteins from HPV 16. Targeting the expression of E6 and E7 to the basal cells of the squamous epithelium using the keratin 14 promoter results in epithelial hyperplasia and skin tumors\textsuperscript{39}. Even the individual expression of E6 and E7 causes progressive epithelial neoplasia in skin and ear tissue, but whereas tumours induced by E7 are relatively benign, E6 tumours are predominantly malignant\textsuperscript{40,41}. Further experimental dissection of the individual effects of E6 and E7 suggests a role for E7 during promotion of tumour development and for E6 during both promotion and malignant progression\textsuperscript{40-42}. Taken together, the induction of cutaneous epithelial hyperplasia by E6 and E7 in vivo reflects cooperativity between the two oncoproteins. These data are consistent with early in vivo studies demonstrating that cooperativity between E6 and E7 is necessary for the efficient immortalisation of primary human epithelial cells\textsuperscript{35,26}. Chronic estrogen treatment of E6/E7 transgenic mice results in cervical carcinogenesis to model human disease in the context of the physiologically significant target tissue\textsuperscript{43,44}. Interestingly, there are substantial differences in the relative contributions of E6 and E7 to skin versus cervical phenotypes. Whereas the potency of E6 malignancy is emphasized in the skin, E7 appears to be the predominant oncogene in the cervical cancer model. E7 expression in cooperation with estrogen treatment induced high-grade dysplasia and invasive disease. In contrast, E6 expression only mediated the development of low-grade dysplasia in these experiments, even though E6 was able to stimulate E7 induced phenotypes. The observed differences in the relative importance of E6 versus E7 in the skin and cervical cancer models may be, at least in part, due to the particular cooperativity between estrogen and E7, as well as to differential apoptosis induction by E7 in the skin but not in the cervix\textsuperscript{45}, which could reflect differences in the expression of its respective target proteins in the different tissues.

**Therapy**

Given the worldwide burden of HPV infection (anogenital warts and neoplasia of several sites), prevention of infection could provide relief from an important public health threat. With the introduction of cervical screening in developed countries, the number of deaths from cervical cancer has declined dramatically\textsuperscript{46}, but in developing countries it still remains the number one female cancer, with approximately a quarter of a million deaths occurring each year. It is thus a major goal to develop safe and effective therapeutics to prevent and to treat HPV infections and their associated diseases. Because of the major role played by host defence mechanisms against HPV, major efforts have been made in the development of candidate prophylactic and therapeutic vaccines against cervical cancer and HPV-related infections in the last few years\textsuperscript{47}. These efforts have led to the approval of the HPV vaccine GARDASIL in several countries in 2006. Importantly, however, this vaccine does not appear to affect a current HPV infection, cervical cancer precursor lesions or genital warts pre-existing at the time of vaccination. Furthermore, because HPV infection is pandemic in humans and there is a long latency from HPV infection to the development of invasive cervical cancer in women, several decades will pass before cancer incidences in developing countries begin to decline, even if widespread vaccination is introduced immediately\textsuperscript{48}. Clearly there is a gap in the current treatment arsenal and, even if we currently had full vaccine coverage, we would still require alternative therapies for existing patients.

**E6 and E7 as therapeutic targets**

Human papillomaviruses encode at least six nonstructural proteins: E1, E2, E4, E5, E6, and E7. E4 is necessary for the productive phase of the HPV life cycle, but it is expressed at very high levels in the
infected cell, which may make it questionable as a therapeutic target. The function of E5 is still largely unknown, and further work is needed before it can be considered as a target for therapeutic intervention. In contrast, the E1 and E2 proteins represent excellent therapeutic targets in both mucosal and cutaneous HPV infections. Therapeutic agents that could disrupt the E1-E2 interaction, their binding to DNA, or the helicase ATPase activity of E1, are likely to be highly effective inhibitors of the viral life cycle, with minimal levels of toxicity. However, since these proteins are often lost during malignant progression, they would not be valid targets for treatment of HPV-induced malignancies. In contrast, the E6 and E7 proteins are constitutively expressed in cervical cancers and cells lines derived therefrom, are essential for maintenance of the transformed phenotype, and are necessary for the normal viral life cycle. Furthermore, they are both present in low amounts and both function in an enzymatic manner by recruiting elements of the ubiquitin proteasome pathways to some of their respective cellular target proteins (Fig. 2). All these considerations make these two proteins the targets of choice in the treatment of HPV infection and HPV-induced malignancy.

Numerous approaches have been directed against the polycistronic E6/E7 mRNA to block the expression of both E6 and E7 in HPV-positive cervical cancer cells. This includes selectively inhibiting viral transcription, or by using antisense constructs, ribozymes, or short-interfering RNA (siRNA), which lead to the degradation of the E6/E7 mRNAs. Similar inhibitory effects have been obtained following the reintroduction of the E2 transcription factor into cervical cell lines, such as SiHa and HeLa, where it can associate with the integrated viral regulatory region and suppress the continued expression of the transforming proteins. As E6 and E7 are expressed together from a bicistronic mRNA, such approaches generally lead to the reactivation of both pRb and p53, and to the attainment of a growth-arrested state that resembles the replicative senescence that is achieved by primary cells after they have reached their normal life span in culture. A superior strategy appears to be to inhibit the function of E6 alone, which exposes the cell to the pro-apoptotic activity of the E7 protein. This has been achieved using E6-binding peptides and an intrabody-based approach. Indeed, administration of peptide aptamers and intrabodies to E6 was found to induce apoptosis of HPV-positive cancer cells.

Thus, blocking the E6-mediated degradation of p53 is a major therapeutic goal, since there is strong evidence that the p53-responsive pathways are fully functional in cervical tumour cell lines, and reactivation of p53 could then bring about growth arrest and/or apoptosis of the HPV transformed cells. The mechanism by which E6 overcomes p53 activity is well established, and involves a tripartite complex between E6, the cellular ubiquitin ligase E6-AP, and p53. One of the most interesting aspects of the E6/E6-AP/p53 interaction is that it is specific to HPV infected cells. p53 levels are normally regulated by Mdm2, but this pathway does not function in HPV transformed cells, where p53 is subject to the E6/E6-AP induced degradation. Therefore any antiviral which could block this activity of E6 is likely to be highly specific. Many approaches have been tested to block the E6/E6-AP/p53 complex. The use of antisense oligonucleotides against E6-AP resulted in upregulation of functional p53 in certain HPV-positive tumour cells, and the use of small peptides to block the ability of E6 to degrade p53 also resulted in up-regulation of p53 and induction of apoptosis. However, this is unlikely to be universally applicable, since inhibition of E6-induced degradation does not always lead to increased p53 levels. In several cervical cancer cell lines p53 can be stabilised only after additional genotoxic insult, indicating a lack of intrinsic p53 activation, despite the presence of the viral oncogenes. Furthermore, E6’s ability to transform cells does not always correlate with its ability to cause p53 degradation.

A striking feature of all E6 proteins derived from the high risk HPV types is the presence of a highly conserved carboxy-terminal domain, a PDZ-binding domain, which is not involved in p53 binding and degradation, but which nonetheless contributes to E6 transforming activity, since its deletion impairs E6’s ability to transform rodent cells. Furthermore, transgenic mice expressing a mutant of E6 lacking the six amino acids at the carboxy terminus, E6Δ146-151, demonstrate that the PDZ-binding domain is necessary for E6’s induction of epithelial hyperplasia in vivo. It has been shown that interactions between viral E6 proteins and PDZ domain-containing proteins constitute a general mechanism for virus-induced oncogenesis, hence this class of interactions might represent ideal therapeutic targets for the later stages of virus-induced disease. Because the structures of a number of PDZ domains have been solved, and the binding motif of E6 is small and exposed, the rational design of
relatively non-toxic chemotherapeutic agents, capable of specifically inhibiting the interaction between E6 and this class of targets should be possible. Indeed the utility of this approach has been shown by the use of synthetic peptides. These peptides show high affinity for the E6 protein and efficiently abolish the ability of E6 to target p53 and the PDZ-domain containing proteins hDlg and MAGI-1 for proteolytic degradation in vitro. The ability of these peptides to block E6-induced degradation of cellular proteins known to be important in cellular transformation, suggests that they may have considerable potential in the chemotherapy of HPV-induced disease.

Conclusions

The need for effective antiviral agents that can clear HPV infections is widely acknowledged as HPVs are associated with significant morbidity and mortality. The development of prophylactic vaccines utilizing virus-like particles to initiate immune responses holds great promise for reducing the prevalence of HPV-mediated disease in the long term. However, vaccines will not help those already infected and more research is necessary to develop new therapies and treatments. Conventional drug discovery programs have not yet produced a specific inhibitor of HPV protein function, and there is a need to fully explore less conventional approaches. These include the use of oligonucleotide-based therapies (antisense oligonucleotides, ribozymes and siRNA), as well as protein-based approaches. However, while success in vitro and in small animal models has been demonstrated, clinical trials have not yet been initiated. The issue of delivery is the major stumbling block. However, from the experiences with antisense and ribozymes there is a sense that success will be achieved in not too distant future.

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References


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