Genital human papillomavirus infections: current and prospective therapies

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Infection with human papillomaviruses (HPVs) is very common and associated with benign and malignant epithelial proliferations of skin and internal squamous mucosae. A subset of the mucosal HPVs are oncogenic and associated with 5% of all cancers in men and women. There are two licensed prophylactic vaccines, both target HPV 16 and 18, the two most pathogenic, oncogenic types and one, additionally, targets HPV 6 and 11 the cause of genital warts. The approach of deliberate immunization with oncogenic HPV E6 and/or E7 proteins and the generation of antigen-specific cytotoxic T-cells as an immunotherapy for HPV-associated cancer and their high-grade pre-cancers has been tested with a wide array of potential vaccine delivery systems in Phase I/II trials with varying success. Understanding local viral and tumour immune evasion strategies is a prerequisite for the rational design of therapeutic vaccines for HPV-associated infection and disease, progress in this is discussed. There are no antiviral drugs for the treatment of HPV infection and disease. Current therapies are not targeted antiviral therapies, but either attempt physical removal of the lesion or induce inflammation and a bystander immune response. There has been recent progress in the identification and characterization of molecular targets for small molecule antagonists of the HPV proteins E1, E2 and E6 or their interactions with their cellular targets. Lead compounds that could disrupt E1–E2 protein–protein interactions have been discovered as have inhibitors of E6–E6-AP-binding interactions. Some of these compounds showed nanomolar affinities and high specificities and demonstrate the feasibility of this approach for HPV infections. These studies are, however, at an early phase and it is unlikely that any specific anti-HPV chemotherapeutic will be in the clinic within the next 10–20 years.

Introduction

As recently as 1970 it was assumed that there was only one human papillomavirus (HPV) and that infection with this was the cause of the various warty lesions that decorated different tissue sites. HPV was seen, except in rare instances (Jablonska et al., 1972), as causing unsightly but essentially trivial excrescences that, given time, would regress spontaneously. The advent of recombinant DNA technology overturned this simple view of the HPV world, and it became clear within a decade that there were multiple HPVs and that the warts on different tissue sites were caused by different HPV types with a tropism for mucosal or cutaneous squamous surfaces (Orth et al., 1978). It also became evident that HPV did not cause trivial disease but that some members of the HPV family, particularly a subset infecting the ano-genital tract, were true human carcinogens and were the cause of carcinoma of the cervix (zur Hausen et al., 1981), the second most common cancer in women worldwide (Arbyn et al., 2011).

HPVs are a very large family of dsDNA viruses comprising more than 180 types, numbered sequentially, that have been cloned from various clinical lesions (Bernard et al., 2010). These viruses are not classified as serotypes but as genotypes on the basis of DNA sequence and, since in vitro growth of these viruses is problematic, HPV infection is determined by detecting HPV DNA. The viruses have a predilection for either cutaneous or mucosal epithelial surfaces and fall into two groups – low-risk types that predominantly cause benign warts or high-risk types associated with malignant disease. This risk stratification is shown clearly in the ano-genital tract where about 30–40 HPVs regularly or sporadically infect the mucosal epithelium of men and women. The two most common low-risk mucosal HPVs are HPV 6 and 11, which together cause about 90% of genital warts and almost all recurrent respiratory papillomas (RRP) (Lacey et al., 2006) as well as a proportion of low-grade, cervical intra-epithelial neoplasms (CIN 1), vulval and vaginal intra-epithelial neoplasms grade 1 (VIN 1 and VAIN 1) and anal intra-epithelial neoplasia grade 1 (AIN 1) (Moscicki et al., 2006). There are about 15 high-risk mucosal HPV types of which the most important are HPV 16 and 18, which together cause more than 70% of cervical carcinomas in women (Arbyn et al., 2011). Cancers attributable to HPV are not confined to the cervix. In approximately 60% of cancers of the vagina, 40% of vulva and penis, 80% of anus (Parkin & Bray, 2006) and more than 60% of tonsil and base of
Prophylaxis
There are two commercially available prophylactic HPV vaccines, a bivalent HPV 16.18 virus-like particle (VLP) vaccine from Glaxo Smith Kline Biologicals and a quad-valent HPV 6, 11, 16, 18 VLP (qHPV) vaccine from MSD Merck. Both vaccines have shown very high efficacy against HPV 16/18 caused high-grade CIN 2/3 the end-point accepted as the ethically acceptable proxy for vaccine efficacy against cervical cancer (Kjaer et al., 2009; Paavonen et al., 2009). The qHPV has shown greater than 98% efficacy against HPV 6/11-associated genital warts (women and men), HPV 6/11/16/18-associated VIN, VAIN (women) and AIN (men) (Dillner et al., 2010). In the long-term 30–50 years, and with high vaccine coverage, the prophylactic vaccines will dramatically reduce the incidence of disease associated with the vaccine HPV types. However, the vaccines target only HPV 16 and 18 and in the case of the qHPV vaccine HPV 6 and 11; there is some cross-reactivity but this is partial (Paavonen et al., 2009). Despite the high efficacy of the vaccines only 70% reduction in cervical cancers could be expected. Further, unless populations are vaccinated and coverage exceeds 50%, a large unvaccinated population will remain with a huge burden of ano-genital disease requiring treatment. The global burden of genital HPV-associated disease is estimated as more than 60 million cases per annum (Koutsky, 1997). Cutaneous HPV infections are common, treatments are unsatisfactory and there is no prophylaxis. Effective, preferably virus-specific therapies remain a priority.

Infectious cycle of HPV
Progress in the development of effective therapies for HPV infection and disease has been slow, largely due to the difficulties in studying the biology and pathogenesis of these viruses that have a unique and complex replication cycle. HPVs are exclusively intra-epithelial pathogens with a replication cycle which is both time and differentiation dependent. The viral replication cycle is one in which viral infection is targeted to basal keratinocytes, but high level expression of viral proteins and viral assembly occur only in differentiating keratinocytes in the stratum spinosum and granulosum of squamous epithelium (Doorbar, 2005). The life cycle can only be reproduced experimentally in complex, technically demanding in vitro culture systems that generate small amounts of infectious virus (Meyers et al., 1997). There are no easily manipulable animal models for HPV and a mouse papillomavirus infection is at a very early phase of analysis (Ingle et al., 2011). The viral genome is small – 8 kb of dsDNA – and encodes a maximum of eight genes, six of which encode non-structural or early proteins E1, E2, E4, E5, E6 and E7 and two of which encode structural or late proteins L1 and L2 (Fig. 1). Viral genes are differentially expressed both temporally and spatially throughout the infectious cycle. (Fig. 2). Despite the small genome the number of gene products is much larger because of the complex use of splice sites within the genome (Schwartz, 2008)

A further complication of these infections is the phenomenon of latency. After infection, HPV DNA can remain latent within cells even though others have entered the productive cycle. Spontaneous wart regression is immune mediated, but this does not result in virus clearance and viral genomes can be detected in apparently normal epithelium (Abramson et al., 2004) many months and years after wart regression (Moore et al., 2002). However the strong, local, cell-mediated immunity that engenders regression of HPV-infected lesions probably controls latent infection and, in healthy immunocompetent individuals, recurrence of disease is unlikely. In contrast, immunosuppression predisposes to reactivation as is demonstrated by the high levels of genital HPV infection and neoplasia seen in immunosuppressed organ transplant recipients and those with human immunodeficiency virus (HIV) infection (Palefsky et al., 2006). Antiviral chemotherapies are essential for such patients.

Ano-genital HPV-associated disease
HPV infection of the ano-genital skin and mucosae results in lesions with two morphologies – ano-genital warts (condyloma acuminata) and squamous intra-epithelial lesions. Condylomata are associated predominantly, but not exclusively, with infection by low-risk types – HPV 6 and it’s relatives. These are frank, polyplloid growths that generate infectious virus and have a low to negligible risk of malignant progression. Squamous intra-epithelial lesions are classified histologically and form a distinct spectrum of histological atypia. In the cervix these are graded on the degree to which they have lost cytoplasmic maturation and exhibit cytological atypia. In Europe three grades of cervical intra-epithelial neoplasia (CIN) are recognized: CIN 1, mild; CIN 2, moderate and CIN 3, severe. In the vagina, vulva, penis and anus a similar but not identical spectrum of changes can be identified, VAIN, VIN, PIN and AIN. There is considerable confusion about the terminology used to define these lesions and to ensure clarity in this review the term high-grade intra-epithelial neoplasia (HGIN) includes: CIN 2/3, AIN 3, PIN 3, VIN 3 and VAIN 3; low-grade squamous intra-epithelial neoplasia (LGIN) encompasses CIN 1 and the equivalent lesions in other sites.

LGIN at any site can be associated with both high- and low-risk HPV types, although low-risk types predominate (Moschicki, et al., 2006). The majority of lesions maintain the virus as an episome, support a complete virus replication cycle and viral gene expression is tightly regulated. Late
genes are expressed and virus particles generated. Warts and low-grade squamous intra-epithelial lesion at all sites are likely to be amenable to the same therapeutic strategies. HGIN are associated almost exclusively with high-risk types. In general because of the defects in cellular differentiation that characterize these lesions, they do not support a complete viral infectious cycle. Late gene expression is either lost or significantly reduced, the viral DNA sequences may be integrated into the host genome and E6–E7 oncogene expression is deregulated. The genetic instability of these high-grade lesions and the changes in viral gene expression such as the loss of E2 and E1 expression that occur in association with this, means that they are unlikely to be amenable to the same therapeutic strategies as low-grade lesions.

Current therapeutic options

Ablative/excisional therapies. Current therapies for ano-genital HPV infections are mostly ablative and/or cytodestructive (von Krogh et al., 2000). Ablative therapies for genital warts include cryotherapy, scissor excision, laser therapy and electrosurgery (Sonnex & Lacey, 2001). Physically ablative therapies such as cryotherapy are often highly effective in the short term, with clearances of 70–80 %, but recurrence rates can be as high as 25–39 % despite multiple treatments (Maw, 2004).

CIN 3 arises almost exclusively at the squamo–columnar junction. Disease therefore is localized even though infection may be regional and excisional therapies such as loop excision of the transformation zone are highly effective (Prendiville, 1995). However, AIN, PIN, VAIN and VIN are often multi-focal, both disease and infection are regional and ablation may not be feasible or, if attempted, ineffective (Jones, 2001).

Non-surgical treatments

Cytotoxic agents. Cytotoxic agents are widely used in the treatment of genital warts (for review see Viera et al., 2010). They are topical preparations that kill cells on contact, irrespective of HPV status, by anti-proliferative or chemo-destructive modes of action.

Podophyllotoxin. Podophyllotoxin, as a cream (Europe) or a gel (USA) that can be self applied by the patient, is the first line treatment for genital warts (Lacey, 2005) achieving 50 % clearance, but with recurrence rates of 25–30 % (Maw, 2004). The mechanism of action is thought to be due to the binding of lignans to microtubule proteins with cell cycle arrest at metaphase. Podophyllotoxin is contra-indicated in pregnancy.

Trichloracetic acid (TCA). TCA is a clinic-based topical therapy that has a local caustic action effectively generating a chemical burn of the wart. It is as effective as podophyllotoxin
Experimental studies in intra-epithelial lesions infected with high-risk HPV types. The secretion of type I IFNs should be used with caution on immunomodulators such as Imiquimod that induce the inflammatory response. Imiquimod, a pharmacological agent that can modulate innate immune responses, is an agonist for Toll-like receptor 7, ligation of which activates dendritic cells, macrophages and keratinocytes to release type I interferons (IFNs) and other pro-inflammatory cytokines (Stanley, 2002). Randomized clinical trials (RCTs) with Imiquimod 5% cream applied topically to genital warts have shown efficacy and safety and a reduced recurrence rate (12%) compared with placebo (30%) (Beutner et al., 1998). It is feasible that Imiquimod will have a therapeutic effect on intra-epithelial disease and small trials on VIN (Terlou et al., 2011) and AIN (Fox et al., 2010) have demonstrated efficacy, but the drug is not licensed for this use. The inflammatory side effects of Imiquimod (erythema, oedema, itching and pain) have limited its use on mucosal surfaces.

Immunomodulators such as Imiquimod that induce the secretion of type I IFNs should be used with caution on intra-epithelial lesions infected with high-risk HPV types. Experimental studies in vitro have shown that both exogenously or endogenously derived IFN-α and IFN-β are highly effective in cells containing episomal HPV DNA, inducing growth arrest and episomal loss, but have no growth inhibitory effects on cells containing integrated HPV DNA species and thus select for cells with integrated genomes (Herdman et al., 2006; Pett et al., 2006). Repeated and protracted use of preparations that generate locally high concentrations of type I IFNs could result in the selection of cells with integrated HPV, thus driving progression to high-grade intra-epithelial and/or malignant disease.

Polyphenon E. Polyphenon E is a standardized extract of green tea leaves from Camellia sinensis that, self administered as a topical ointment, has shown efficacy against genital warts in RCTs with lower recurrence rates and greater clearance of warts in the treated (54%) compared with placebo groups (35%) (Tatti et al., 2010). The main catechin in Polyphenol E is (−) epigallocatechin gallate (ECGC), a molecule that impacts on multiple signalling pathways, inducing cell cycle arrest or apoptosis via caspase activation, altered Bcl-2 family member expression and inhibition of telomerase activity (Beltz et al., 2006; Yang et al., 1998). ECGC has been shown to have antioxidant properties, blocking nitric oxide production by suppressing inducible nitric oxide synthase via blocking nuclear translocation of the transcription factor nuclear factor-κB as a result of decreased IκB kinase activity (Ahmad et al., 2000; Khan et al., 2006).

Fig. 2. Papillomaviruses are absolutely species-specific and tissue-specific. HPV will only infect and replicate in a fully differentiated squamous epithelial cell. The virus infectious cycle is rather complex and can explain the duration of an HPV infection. It involves both temporal and spatial separation of viral protein expression. The virus first infects a keratinocyte in the basal layer of the epithelium as a consequence of microtrauma, i.e. an abrasion of the epithelium that exposes the basement membrane and basal cells. In the proliferative compartments of the epithelium, there is a phase of plasmid maintenance and the virus, the cells replicate together and viral copy number is maintained at around 50–100 in the daughter cells. For the oncogenic viruses in particular, viral gene expression is very tightly controlled during this phase. As long as the cell is dividing, the high-risk HPVs control the expression of their viral proteins very tightly. The oncogenes E6 and E7 are thus expressed at very low levels. When the host cell stops dividing and begins to differentiate into a mature keratinocyte, this provides a signal to the virus to activate all its genes to increase viral genome copy number to the thousands. In the case of incipient malignancy, control of E6 and E7 expression is lost and gene expression in the cell becomes deregulated. In the top layers of the epithelium, all the viral genes, including those encoding the L1 and L2 proteins, are expressed and many thousands of viral genomes are encapsidated. They exit the cell as infectious virus particles. The time taken from infection to the generation of infectious virus is at least 3 months. HPV thus has a very long infectious cycle, has no blood-borne phase and does not cause cell death.
Cidofovir. Cidofovir is a monophosphate nucleotide analogue of deoxycytidine (dCTP). After undergoing cellular phosphorylation, it competitively inhibits the incorporation of dCTP into viral DNA by viral DNA polymerase. Incorporation of the drug disrupts further chain elongation. Small trials in CIN have been reported (Van Pachterbeke et al., 2009) with promising efficacy. Cidofovir is currently being used off-licence to treat different viral infections, such as benign low-risk human HPV-related RRP but concerns about safety have been raised (Donne et al., 2009).

**Future prospects**

**Antivirals for HPV proteins**

At present there are no virus-specific therapies for papillomavirus infection, but such therapies are necessary for several reasons. Antiviral therapy has the potential to treat both inapparent HPV infection as well as visible clinical disease. There is a significant population of HPV-infected, immunosuppressed individuals who, at the present time, cannot be treated with immunotherapy – drugs are their only option. Multifocal lesions such as VIN and AIN are not amenable to ablation, may not respond to immunotherapy but could be targeted by chemotherapy and furthermore antivirals, unlike immunotherapies, may not be HPV-type restricted in their efficacy.

Traditionally, antiviral therapies have targeted viral enzymes. The papillomaviruses encode only one enzyme, the E1 helicase, rather limiting traditional approaches. The remaining early genes E2, E4, E5, E6 and E7 function largely by macromolecular interactions with host DNA and proteins and these interactions have not been readily amenable to drug design and the high-throughput screening necessary to identify candidate molecules.

**E1 and E2 proteins as antiviral targets.** E1 and E2 are the most highly conserved of the papillomavirus proteins and are essential for viral DNA replication. E1 is the only viral enzyme with ATPase and helicase activity. The protein is divided into an N’ domain (Amin et al., 2000), a sequence-specific DNA-binding domain (DBD) (Titolo et al., 2003) and a C’ helicase domain (Seo et al., 1993; Titolo et al., 2000). E2 is a sequence-specific DNA-binding protein with roles in DNA replication, transcriptional regulation (Hegde, 2002) and partitioning of viral genomes to daughter cells during mitosis (Ilves et al., 1999). The protein is organized into two functional domains, an N’ transactivation domain (TAD) involved in transcriptional regulation and direct association with E1 and a C’ DBD/dimerization domain (Hegde, 2002). Both domains are separated by a hinge region whose function remains poorly characterized.

HPV genome replication requires both E1 and E2 (Chiang et al., 1992; Yang et al., 1991). E1 monomers are specifically recruited to the viral ori through interaction with the E1 DBD as well as protein–protein interaction with the E2 TAD bound also to the viral ori via the E2 DBD (Sanders & Stenlund, 1998). The E1 DBD dimerizes on the ori, additional E1 monomers are recruited, a double hexamer forms and in the process melts the duplex DNA, resulting in the assembly of a hexamer around each strand of the ori DNA (Fouts et al., 1999). ATP plays an important role in these processes. ATP-binding enhances the E1–E2 interaction with the viral ori (Titolo et al., 1999), but also changes the conformation of the E1–E2 protein interaction (White et al., 2001) and as the E1 double hexamers are formed E2 dissociates and DNA pol-α is recruited to the viral ori (Masterson et al., 1998) via p70 and E1 (Lusky et al., 1994), the full replication complex assembles and viral DNA replication proceeds. In theory any one of these interactions could be targeted by small molecule inhibitors.

Small molecule inhibitors targeting the ATPase activity of HPV 6 E1 were identified by high-throughput screening of a large collection of in house compounds by scientists working for Boehringer–Ingelheim Canada (White et al., 2011). The lead molecules from this screen were biphenyl-sulfonacetic acid analogues (Faucher et al., 2004) whose mode of action probably affected ATP binding by an allosteric mechanism (White et al., 2005). Unfortunately this class of inhibitors were not active in cell-based assays. These workers also screened for inhibitors of helicase activity but were unsuccessful, an outcome shared by many research groups both in academia and industry.

**Inhibitors of E1–E2 interactions.** The conventional wisdom is that small molecules will be unable to inhibit protein–protein interactions because the interfaces are large, relatively flat and without pockets into which small molecules can bind (Fradet-Turcotte & Archambault, 2007). White and colleagues addressed these issues in a series of detailed studies to identify inhibitors of the cooperative assembly of HPV E1 and E2 on the ori, focussing on HPV 6 and 11 (White et al., 2011). They identified two series of inhibitors that bound to overlapping sites at the E1-binding interface on the E2 TAD (Yoakim et al., 2003). Both series of compounds, the indandiones and repaglinides, were optimized for binding by medicinal chemistry approaches and in both series the best compounds had nanomolar activity against HPV 11 E1–E2 activity (Goudreau et al., 2007). These studies demonstrate that druggable pockets can be found at protein interfaces even though the apo crystal structure may not appear favourable and, as the authors of these studies comment, ‘binding of a ligand can be necessary to induce formation of a pocket’ (White et al., 2011). Unfortunately when the high efficacy of the prophylactic vaccines in randomized control trials was demonstrated, these studies on E1–E2 inhibitors were halted by the pharmaceutical company.

**Inhibitors of E2 binding to cellular proteins.** Other targets for small molecule inhibitors of E2 are in the frame. The bromodomain and ET domain (BET) proteins that bind acetylated histones are of considerable contemporary interest as potential anticancer and antiviral targets (Smith
et al., 2010). Brd4, one of the most studied of the BET proteins, binds to bovine papillomavirus E2 to tether the viral episome to the mitotic spindle and permit equal partitioning of viral genomes at mitosis (Abbate et al., 2006; McBride et al., 2004). There is accumulating evidence that Brd4 is a cellular-binding partner for the E2 protein of both low and high-risk HPVVs (Gagnon et al., 2009), and that these interactions are important for the maintenance of viral episomes and transcriptional regulation of viral oncopogenes (Smith et al., 2010). Inhibition of Brd4–E2 interactions are potential drug targets; recently structure and function studies of a small molecule competitive inhibitor of Brd4, JQ1, that displaces Brd4 from chromatin were reported (Filippakopoulos et al., 2010).

E6 and E7 as antiviral targets. The combined actions of the high-risk E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype and the evasion of apoptosis (Moody & Laimins, 2010). Abrogation of either E6 or E7 function (or both) in neoplastic cells by targeting gene expression or protein–protein interactions should be an effective strategy. They are therefore the targets of choice for high-grade disease and a number of approaches have been employed: siRNA, antisense RNA, ribozymes and peptide aptamers (Govan, 2005). In vitro data using HPV-expressing cells suggest that targeting E6 and/or E7 is a feasible approach, but to date, unfortunately, these proteins have not been amenable to inhibition by small molecules, although some progress has been made with E6.

The high-risk HPV E6 gene encodes a small protein of approximately 150 aa. This protein deregulates the cell cycle and one of the key molecular interactions by which this is achieved is through inhibition of p53 function. E6 binds to p53 to form a stable complex, which then undergoes proteolytic degradation. This E6-mediated degradation requires a third protein, E6-AP, which in combination with E6 acts as a ubiquitin ligase (Talis et al., 1998). E6 mediates degradation not only of p53 but also the PSD95/Dlg/ZO-1 domain-containing proteins that modulate signalling pathways deregulating differentiation and E6-AP appears to be the primary partner in these E6 degradation functions (Nomine et al., 2006). Inhibition of the E6–E6-AP complex should reactivate p53 function in infected cells, resulting in growth arrest and/or apoptosis. Small peptides that inhibit E6–E6-AP binding by competing with E6-AP have been designed (Be et al., 2001; Liu et al., 2004) and there are some candidate inhibitors that have potential for optimization for potency and selectivity, but these studies are at a very early stage and at least a decade from the clinic.

Protecting p53 from E6-mediated degradation. Small molecules that target p53 and protect it from E6-mediated degradation rather than a direct inhibition of E6 may be more tractable strategies. In this regard the small molecule activation of p53 and induction of apoptosis (RTA) was shown to suppress the growth of cervical cancer cells in vitro and also of carcinoma xenografts in vivo (Zhao et al., 2010). RTA blocks p53 ubiquitination by preventing E6-AP binding to p53 with the consequent upregulation of proapoptotic p53 targets Noxa, PUMA and BAX and downregulation of pro-proliferative factors cyclin B1, CDC2 and CDC25C.

Selective inhibition of the proteasome is a property of HIV proteases, raising the possibility that the drugs that inhibit this function might also affect the ability of E6 to mediate proteasomal p53 degradation. This notion has been explored by Hampson and colleagues in a series of studies that have shown that Lopinavir, an anti-retroviral protease used in a fixed-dose combination therapy (KALETRA) in HIV infections, can stabilize p53 and induce apoptosis of HPV-positive cell lines (Hampson et al., 2006; Kim et al., 2010). They have shown further that the mechanism of action of Lopinavir is related to its ability to block viral proteasomal activation and induce upregulation of RNASEL in HPV-containing cells (Bateman et al., 2011). These are interesting and promising data, but HIV patients receiving Lopinavir do not show enhanced clearance of HPV-associated lesions (De Vuyst et al., 2008). However, the concentration of drug required to mediate the in vitro growth inhibition is higher than that achieved by oral administration (Hampson et al., 2006) and cervico-vaginal concentrations in patients are probably sub-optimal. If a topical preparation of Lopinavir can be formulated then clinical trials to test the safety and efficacy of the drug against both low- and high-grade intra-epithelial lesions would be indicated. Since Lopinavir is a licensed drug a successful outcome to such a trial could result in a topical treatment for high-grade HPV-associated disease being in the clinic within years rather than decades.

Inhibition of virus entry

Prevention of virus entry by microbicides or virocides is an alternative approach to targeting viral proteins after exposure. Virus entry for HPV 16, 18 and related types is a complex process that requires epithelial microabrasion and wound healing (Sapp & Bienkowska-Haba, 2009). The microabrasion results in full thickness epithelial denudation but retention of the basement membrane (BM) (Roberts et al., 2007). HPV binds initially to heparin sulphate proteoglycans (HSPGs) in the BM via the L1 protein before attaching to and entering the wound keratinocyte (Kines et al., 2009). Carrageenan, a sulphated polysaccharide extracted from red seaweed, is a potent inhibitor of the attachment of the virus particle to HSPGs on the BM and the cell surface. In an experimental cervico-vaginal challenge model in the mouse, carrageenan was highly effective at preventing HPV transmission to cervical epithelial cells even in the presence of nonoxynol-9, a product that enhances HPV infection in this model (Roberts et al., 2007). Carrageenan is a component of several gels used as lubricants in vaginal examination and the use of carrageenan-based gels during digital vaginal examination and Pap smear collection is advisable (Roberts et al., 2011).
Immunotherapy

Prophylactic HPV VLP vaccines are highly effective and in the long-term should control HPV infection and disease against the HPV types in the vaccine. In the interim, however, therapeutic intervention to enhance or induce effective immune targeting and clearance of established infections is an attractive strategy. Such therapies have the potential for treating inapparent infection and/or disease in addition to clinically visible lesions. A Th1 biased cell-mediated immune response is critical for regression of HPV-induced disease (Stanley, 2006). Agents, therefore, that enhance or induce strong cell-mediated immune responses would be predicted to be effective HPV therapies. In HPV-associated cancers and HGIN, oncogenic viral gene expression is deregulated and the E6 and E7 genes are constitutively expressed. The continued expression of these oncogenes is essential for progression to, and maintenance of, the malignant phenotype. In effect, therefore, there are only two possible antigenic targets, E6 and E7, since these are the only viral proteins that will be expressed in all cancers and HGIN.

The approach of deliberate immunization with E6 and/or E7 of HPV 16 and 18 predominantly, and the generation of antigen-specific CTL as an immunotherapy for HPV-associated cancer has been tested with a wide array of potential vaccine delivery systems in transplantable rodent tumour models (reviewed by Su et al., 2010). It turns out from these studies that HPV-expressing cancers in mice are relatively easy to cure, but human HPV-induced cancers and HGIN have been, to date, largely refractory to the approaches successful in rodents. All the vaccines tested in clinical trials in humans have been safe and well tolerated, they have induced vaccine-specific T-cell responses of varying magnitude, but these did not necessarily correlate with clinical responses (Trimble & Frazer, 2009).

Successful therapeutic HPV vaccines – what is needed

Recent trials in patients with VIN (Daayana et al., 2010; Kenter et al., 2009) provide reasons for cautious optimism and there is now the outline of a blueprint that could lead to successful therapeutic immunization of HPV-associated disease.

Immunogenicity

Firstly, vaccines must be highly immunogenic. Adjuvants will be central to enhancing the immunogenicity of HPV therapeutic vaccines. Early innate immune responses shape subsequent immune responses and the development of adjuvants that exploit innate signals specifically focusing and biasing the adaptive response to cytotoxic effector Th1 type will be critical for effective HPV therapeutic vaccines. The importance of robust immunogenicity and adjuvantation has been demonstrated in a recently reported Phase II trial (Kenter et al., 2009) in which patients with longstanding and refractory VIN 3 were immunized with a highly immunogenic vaccine comprising HPV 16 E6 and E7 overlapping synthetic long peptides with an oil in water adjuvant: 47% showed a durable and complete regression histologically and clinically. Importantly, responders had small lesions and regression was associated with a strong and broad HPV-specific Th1 type CD4+ and CD8+ systemic T-cell response that peaked after the first vaccination; non-responders on average had larger lesions and mounted a vaccine-induced HPV-specific regulatory T-cell response (Welters et al., 2010).

Manipulation of the local, lesional, immune milieu

Secondly, the local mucosal immune milieu must be manipulated. The many published studies show that systemic T-cell responses to HPV infections are weak and transient, but the presence of an intense T-cell infiltrate in regressing lesions implies that modulating the local immune micro-environment will be critical for successful immunotherapy. The cellular effectors in these local responses are still not unequivocally identified, but regression of CIN 1 in longitudinal studies has been shown to be correlated with the presence at study entry of functional cytotoxic CD8+ cells producing Granzyme B (Woo et al., 2010). Intraepithelial CD8+ T-cells in the cervix express the intraepithelial homing receptor α4/β7 and, significantly, in a retrospective analysis, CIN regression correlated with the expression on the stromal microvascular endothelium in dysplastic lesions of the mucosal addressin cell adhesion molecule 1, the ligand for α4/β7 (Trimble et al., 2010). This suggests that with increasing disease severity cytotoxic effector lymphocytes could not exit the lymphatics and vessels and enter the local lesion. Generating large numbers of circulating vaccine-induced HPV-specific cytotoxic effectors and antigen-specific CTL will be ineffective if their ingress into the lesion is prevented and/or they are disabled by regulatory T-cells when they arrive.

Immune trafficking and modulation of the local milieu could be modified by mucosal immunization. The route of administration significantly affects immunization outcome and a mucosal route would be desirable for both practical and target driven reasons, but immune induction at different mucosal surfaces requires specific signals. Vaccines delivered to mucosae will almost certainly require specific adjuvant formulations to efficiently target the mucosal inductive sites and induce strong cell-mediated Th1 type responses at these sites. Recent studies give some support to the notion of local immunomodulation. In a trial in patients with VIN 3 combining topical Imiquimod (functioning as a local immunomodulator/adjuvant) with systemic immunization with an HPV 16/18 E6/E7 protein delivered via vaccinia virus (TA-CIN) resulted in 32% of patients showing complete regression of lesions (Daayana et al., 2010).

Regulating the regulators

Therapeutic vaccines cannot cure large established malignancies or large persistent pre-cancerous lesions and the
evidence to date is that this is due, at least in a large part, to the dominance of T regs over cytotoxic effectors in the lesions (van der Burg et al., 2007; Welters et al., 2010). If therapeutic HPV vaccine success is to be improved then either T regs should be depleted or the pool of cytotoxic effectors increased, or both. T regs, probably because of their lower intracellular ATP levels, are more sensitive to cyclophosphamide than other T-cell subsets. Low-dose cyclophosphamide treatment has been used to modify the immune milieu in giant condyloma acuminata after laser surgery, resulting in a reduction in Foxp3+ T-cells and absence of recurrence (Cao et al., 2010). Potentially one could target the receptors and their ligands that regulate T-cell function either by expanding the pool of effector T-cells (Curran et al., 2010) or by reversing the T-cell anergy and immunosuppression (Sharma et al., 2009) induced by T regs. All of these strategies have been or are being tested in mouse models and have shown their potential, but transfer to the clinic remains to be achieved.

Conclusions
The fact that there are many therapies available for the treatment of HPV-associated lesions is a reflection of the reality that, on the whole, these are unsatisfactory. Current therapies are not targeted antiviral therapies, but either attempt physical removal of the lesion or induce inflammation and a bystander immune response. There has been recent progress in the identification and characterization of molecular targets for small molecule antagonists of the HPV proteins E1, E2 and E6 or their interactions with their cellular targets. Lead compounds that could disrupt E1–E2 protein–protein interactions have been discovered as have inhibitors of E6–E6-AP-binding interactions. Some of these compounds showed nanomolar affinities and high specificities and demonstrate the feasibility of this approach for HPV infections. These studies focussed on the mucosal low-risk HPV 6 and 11 and on the major oncogenic HPV 16 and 18 and their close relatives. However, once the efficacy of the prophylactic vaccines that target these HPV types was demonstrated in RCTs further development of the small molecule programmes, at least those in house in large Pharma, was halted in the expectation that the market for antivirals would be very significantly reduced.

The approach of deliberate immunization with HPV 16/18 E6 and/or E7 and the generation of antigen-specific CTL as an immunotherapy for HPV-associated cancer and their high-grade pre-cancers has been tested with a wide array of potential vaccine delivery systems in Phase I/II trials with varying success. Advances in our understanding of viral and tumour immune evasion strategies has emphasized that modulation of the local tumour immune micro-environment combined with the induction of strong systemic HPV E6 and E7 antigen-specific cytotoxic effector responses are prerequisites for successful therapeutic immunization.

The prophylactic vaccines are highly effective but focussed on a few types, broadly cross-protective L2 vaccines are in development pre-clinically, but have not entered clinical trials to date. The reality is that for the next 20–40 years at least there will remain a large unvaccinated population of women and men for whom treatment, whether antiviral drugs or immunotherapy or both, is needed. In addition, there is a need for effective antiviral therapy for the large population of immunosuppressed individuals and for the neglected cutaneous infections for which prophylaxis may not be effective or developed.

References
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