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Papillomavirus biology and therapeutic approaches

Review Article

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Abbreviations: bromodomain 4, (Brd4); E2 binding sites, (E2BSs); epidermodysplasia verruciformis, (EV); heat shock protein, (HSP); Human papillomavirus, (HPVs); long control region, (LCR); mitotic chromosome associated protein, (MCAP); nuclear domain 10, (ND10s); open reading frames, (ORFs); upper regulatory region, (URR); virus like particles, (VLPs)

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Summary

Human papillomaviruses (HPV) are ubiquitous in the population and typical infection of the dermis can result in development of cutaneous warts that usually clear on their own. However, infection of the genital tract can result in persistent infections lasting 12-30 months or in the development of cervical carcinoma decades after initial infection. 500,000 cases of cervical carcinoma are detected annually worldwide. This leads to an approximately 288,000 deaths yearly, with most of these cases occurring in developing nations. This review will describe the underlying mechanisms during HPV mediated pathogenesis, the role of the specific viral antigens and explore ongoing efforts towards development of therapeutic interventions for treatment of HPV associated cancers.

I. The impact of papillomavirus infection on society

Human papillomavirus (HPVs) infection is the most common sexually transmitted disease in the U.S., with approximately 5.5 million new cases per annum (Koutsky et al, 2004). HPV induces a variety of lesions classified as anogenital, nongenital cutaneous, or epidermodysplasia verruciformis (EV) (**Table 1**). The anogenital lesions that can progress to oncogenic dysplasia are typically caused by a subset of HPVs know as high-risk HPVs (Schlegel, 1988). These mucosal infecting viruses have been identified as the etiologic agents of cervical carcinoma, a major killer of women worldwide (Koutsky et al, 2004). Importantly the number of cervical cancer occurrences in the U.S. has been reduced by 70% with the development of Dr. George Papanicolau's screening commonly referred to as the "Pap smear" (Terry et al, 1993). The Pap smear test is readily available in the US and other western countries, but unfortunately is not effectively used in developing nations.

To date, cervical cancer is the second leading cause of death for women worldwide with a median age of 38 (Koutsky et al, 2004). Studies linking HPV to cervical carcinoma have demonstrated that 99.7% of these cancers are positive for specific HPV DNA (Wakabayashi et al, 2002). There are 500,000 cases of cervical cancer diagnosed worldwide and 288,000 deaths from the disease every year (Pagliusi, 2004). HIV/AIDS patients that are immunosuppressed (in particular low CD4+ cell counts) are at least three times more likely to have associated HPV infections (Palefsky et al, 1999; Ahdieh et al, 2000). This translates to rates of invasive cervical carcinoma that are 15 to 18 times higher in women with AIDS as compared to the general population (Franceschi et al, 1998; Serraino et al, 1999). Strikingly, the rate of HPV infection in HIV positive men with a history of anal cancer is also high, approximately 35 per 100,000, a number equivalent to that of cervical carcinoma before the advent of the Pap-smear screening (Sun et al, 1995; Piketty et al, 2003).

HPV sub-types that infect the genital mucosa resulting in pre-cancerous lesions, and the formation of condylomata are grouped as low-risk HPVs due to their low oncogenic potential (Storey et al, 1988). Non-genital mucosal infections by the low risk genotypes can result in benign disease such as conjunctival papillomas, or respiratory papilloma, whereas infection in non-genital mucosa with high-risk genotypes may lead to diseases such as laryngeal carcinoma, and oral carcinoma (Sewell

Table 1. Association of genotype and disease.

Anogenital disease	**HPV genotype**
cervical cancer	hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Howley and Lowy, 2001; Bousarghin et al, 2003
carcinoma of anus	Howley and Lowy, 2001; Bousarghin et al, 2003; Day and Lowy, 2003; Bossis et al, 2005
carcinoma of penis	hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Howley and Lowy, 2001
low-grade intraepithelial neoplasia	Palefsky et al, 1999, Sun et al, 1995; Munger et al, 2004
intermediate intraepithelial neoplasia	Scheffner et al, 1991; Funk et al, 1997; Steger and Corbach, 1997; Campo, 2002; Munger and Howley, 2002; Bousarghin et al, 2003; You et al, 2004; Bossis et al, 2005;
high-grade intraepithelial neoplasia	Dowhanick and McBride, 1995; hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Hou et al, 2000; Howley and Lowy, 2001
bowen disease	Piirsoo et al, 1996; hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Howley and Lowy, 2001; Bousarghin et al, 2003;
condylomata acuminata	Durst et al, 1985; Scheffner et al, 1991; Sun et al, 1995; Funk et al, 1997; Steger and Corbach, 1997; Palefsky et al, 1999; Campo, 2002; Munger and Howley, 2002; Selinka and Giroglou, 2002; Munger et al, 2004
Non-genital disease	**HPV genotype**
oral carcinoma	hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Howley and Lowy, 2001
conjuctival carcinoma	Howley and Lowy, 2001
laryngeal carcinoma	hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Howley and Lowy, 2001
squamous cell carcinoma of the lung	Sun et al, 1995; hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97; Palefsky et al, 1999; Howley and Lowy, 2001
respiratory papillomatosis	Sun et al, 1995; Palefsky et al, 1999
conjuctival papillomas	Sun et al, 1995; Palefsky et al, 1999
common warts	Schlegel, 1988; Scheffner et al, 1990; Terry et al, 1993; Zhou et al, 1995; Desaintes et al, 1997; McMillan et al, 1999; Sibbet et al, 2000; Wentzensen et al, 2002; Koutsky et al, 2004
plantar warts	Schlegel, 1988; Wakabayashi et al, 2002; Wentzensen et al, 2002; Koutsky et al, 2004
epidermodysplasia verruciformis	Shope and Hurst, 1933; Lowy et al, 1980; Baker and Howley, 1987; Schlegel, 1988; Storey et al, 1988; Terry et al, 1993; de Villiers et al, 1997; Knipe, 2001; Majewski and Jablonska, 2002; Piketty et al, 2003
	Olson Jr and Cook, 1951; Barksdale and Baker, 1993; Park et al, 1994; Bonne-Andrea et al, 1995; Daniel et al, 1995; Joyce et al, 1999; Klaes et al, 1999; Klumpp and Laimins, 1999; Chang and Laimins, 2000; Longworth and Laimins, 2004

et al, 2004). Additionally, infections with the low-risk HPV genotypes in non-genital mucosa results in cutaneous disease or lesions commonly known as warts, e.g., plantar warts, and butcher's warts, or in EV (a rare skin condition

that effects less than 1,000 people in the United States that manifests itself as small flat warts) (Majewski and Jablonska, 2002).

II. History and biology of papillomaviruses

In 1933, the first member of the papillomavirus family was identified by Dr. Shope as the etiologic agent responsible for warts in the cottontail rabbit (CRPV) (Shope and Hurst, 1933). Since the identification of CRPV, there have been over 100 family members identified in a wide range of organisms including bovines, felines, canines, parrots, catfinch, and humans (de Villiers et al, 1997; Howley and Lowy, 2001). Genomic analysis of the various papillomaviruses shows a high degree of similarity (Datab hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp97). The PV genomes typically contain 10 open reading frames (ORFs), which are all expressed from one strand. These ORFs are divided into two classes: early genes (E1-E8) that are expressed in non-productive infections and in transformed cells (Lowy et al, 1980), and late genes (L1 and L2) that are expressed in productive infections (Baker and Howley, 1987; Knipe, 2001). All PV have a non-coding region referred to as the long control region (LCR), or the upper regulatory region (URR), that contains the viral origin of replication as well as transcription regulatory elements (Knipe, 2001).

The life cycle of PV follows the differentiation of the epithelium, and is highly species specific. Moreover, the program for productive infection is only found in differentiated cells of the epithelium, or keratinocytes (Chang and Laimins, 2000). Interestingly, the only known genotypes to infect and induce tumors in multiple species are the bovine papillomaviruses (Olson Jr and Cook, 1951).

In the basal cells of the epithelium, where primary infection typically occurs, a low copy number of viral genomes is maintained (Klumpp and Laimins, 1999). Species specificity is thought to be established in these dividing cells by the cellular transcriptional regulators of the infected cell (Barksdale and Baker, 1993). Infection begins with the binding of the virus to the surface of the cells. The a6b4 integrin complex has been suggested as a potential receptor (McMillan et al, 1999). This receptor complex is expressed in epithelial cells, mesenchymal cells, and neurons. However, PVs can infect cells lacking this complex suggesting that alternative receptors can be utilized but the efficiency of infection may differ (Sibbet et al, 2000). In agreement with this notion, virions have been shown to directly bind heparin and surface glycosaminoglycans on human foreskin keratinocytes prior to internalization (Joyce et al, 1999).

A previous study indicated that BPV1 infects cells using a similar pathway as the polyomaviruses (Zhou et al, 1995). The virions in this study were seen in lipid raft-derived vesicles (Selinka and Giroglou, 2002). These vesicles are now known to be involved in caveolae-dependent internalization (Bousarghin et al, 2003). Interestingly, a recent study by Schiller and colleagues suggests that the internalization of BPV1 occurs via clathrin-coated vesicles (Day and Lowy 2003). Our recent work suggests that although binding to the cell membrane is a property of the L1 viral capsid protein, entry is likely mediated by the L2 capsid protein (Bossis et al, 2005). This recent study showed that the L2 capsid protein interacted with the intracellular vesicle transport protein syntaxin 18, resulting in movement of the viral particle from the cell membrane to the endoplasmic reticulum (Bossis et al, 2005). Following internalization, the viral DNA is transported into the nucleus by an as yet defined mechanism. The kinetics of infection are such that internalization of the bound virions occurs at 4 hours, and transcription of packaged viral DNA occurs after 12 hours (Day and Lowy 2003).

III. Stable infection of the basal cells by papillomavirus

After infection, the viral genome number is typically maintained at a low level in the basal cells where primary infection occurs (**Figure 1**). This genome maintenance is achieved by the basal cells ability to generate more basal cells, and by the ability of the early viral E2 protein to regulate the segregation of the viral DNA during cell division (Piirsoo et al, 1996). Recent studies have shown that E2 interacts with the cellular molecule bromodomain 4 (Brd4) tethering the viral DNA to the mitotic chromosome during cell division (You et al, 2004). In these dividing cells, the early gene products are expressed and replication of the viral genome is maintained by the ability of the viral antigens E1 and E2 to recruit the cellular replication complex to the origin of replication in the episomal viral genome (Park et al, 1994; Bonne-Andrea et al, 1995). While uninfected basal cells normally exit the cell cycle during differentiation, PV infected cells continue to actively divide despite ongoing cellular differentiation (Cheng et al, 1995). This provides the cellular machinery necessary for viral genome replication, translation of PV capsid proteins L1 and L2, and production of viral progeny (Hummel and Hudson, 1992).

The ability of the virus to induce an environment favorable for latent infection is primarily attributed to the E6 and E7 proteins (Thomas et al, 1999; Howley and Lowy, 2001). E6 and E7 have been demonstrated to interfere with the function of p53, and Rb respectively, two known regulators of the cell cycle (Scheffner et al, 1990, 1991). In addition, E6 and E7 expression is necessary for cellular transformation in the human papillomavirus infected cells and can result in the development of carcinoma (Munger et al, 2004). In cattle, the E5 oncoprotein is the primary transforming viral antigen (Campo, 2002). It may be more realistic to assume that the role of these proteins in carcinogenesis is secondary to their role in the viral life cycle (Munger and Howley, 2002; Longworth and Laimins, 2004).

As the basal cells differentiate, the level of viral genomes increases and mature virions are formed in the upper layers (Howley and Lowy, 2001). The spread of the infecting viruses occurs when the cells of the upper layer slough off and encounter an environment, such as the oral mucosa or the vaginal mucosa, in which to establish an

infection. Small abrasion or tears in the epithelium are believed to provide the route for the virus to infect the basal epithelium cells such as may occur during intercourse (Koutsky et al, 2004). Integration of the genome may occur resulting in deregulation of E6 and E7 gene expression. This loss of regulation can lead to the development of a number of associated malignancies (**Table 2**).

IV. Development of therapeutic and prophylactic approaches for treatment of papillomavirus associated disease

Typically, the development of cutaneous warts occurs in cells where the PV genome is maintained as an episome, whereas infections resulting in the integration of the viral genome into the host genome often lead to carcinomas (Cullen et al, 1991; Daniel et al, 1995; Pirami and Giache, 1997; Klaes et al, 1999). As an episome, the virus continues to replicate its DNA and the levels of the transforming genes E6 and E7 are maintained at a relatively low level by the expression of the viral transcriptional regulator E2 (Steger and Corbach, 1997). E7 expression induces the stability of p53 by interfering with the pRB repression of $p19^{arf}$ which is capable of deregulating mdm2, the ubiquitin ligase responsible for regulating p53. An increase in p53 level would result in apoptosis, but the virus avoids induction of apoptosis by replacing the function of mdm2 with the E6/E6AP complex (Scheffner et al, 1990). This results in the induction of a cellular program capable of maintaining DNA replication. This is also likely to be dependent on the role of E7 in inhibition of $p21^{cip1}$ which inhibits PCNA dependent DNA replication, and cyclin dependent kinases (Jones and Alani, 1997; Funk et al, 1997). The integration of the virus is random throughout the human genome, and it is only observed as a transforming phenotype when E6 and E7 expression is enhanced. As mentioned above, E2 protein is capable of regulating E6 and E7 expression, and loss of E2 expression as a result of integration has been shown to be involved in transformation (Durst et al, 1985; Jeon and Lambert, 1995). In fact, expression of E2 in the HPV positive cells such as the HPV-18 positive HeLa, results in cell cycle arrest that correlates with a decrease in expression of E6 and E7 (Dowhanick and McBride, 1995; Desaintes et al, 1997; Goodwin and DiMaio, 2000; Hou et al, 2000).

In view of the two different infection programs which result in either episomal maintenance or integration of the viral genome, there are two primary approaches for targeting associated diseases: 1) how to prevent infection and proliferation of the virus, and 2) how to treat infections once the viral genome has been integrated into the host genome. We will try to address and review the status of some of the current efforts, but first we'll expand on our description of the roles of the E2, E6, E7, L1, and L2 proteins, the main targets of papillomavirus vaccines.

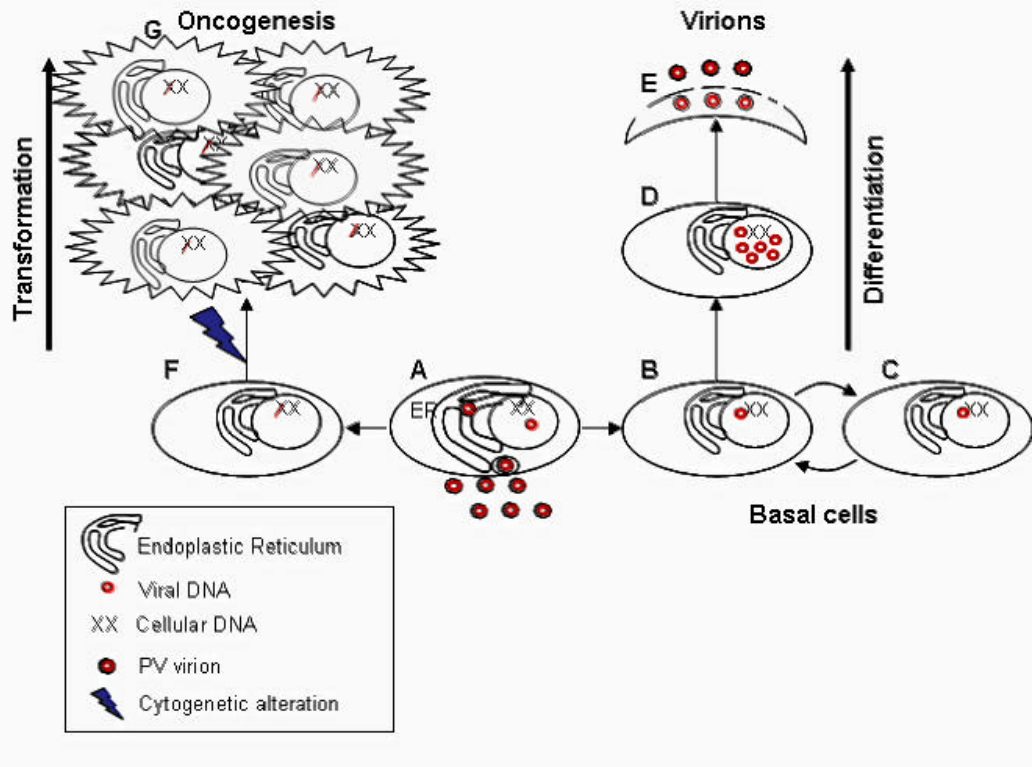

Figure 1. Papillomavirus (PV) infects basal cells of the epithelium (A). After binding to the plasma membrane, the virions are endocytosed by vesicles. These vesicles allow the passage of PV virions to the endoplasmic reticulum (ER). The viral DNA is translocated to the nucleus by an unknown mechanism. Basal cells maintain a low level of viral DNA copies as episomes (B and C). As the basal cells differentiate, the viral genome is amplified, late proteins are made, viral capsids are generated, and virus is released along with sloughing of the upper layer of the skin (D and E). Viral genome integration along with cytogenetic alterations can result in cellular transformation and oncogenesis (F and G).

Table 2. Vaccines in development

Vaccine antigen	Vaccine approach	Company/group	Vaccine type
HPV 16 L1	VLP	Merck	Prophylactic
HPV 16 L1	VLP	NCI/NIAID	Prophylactic
HPV 16, 18 L1	VLP	GSK/Medimmune	Prophylactic
HPV 16 L1	Recombinant Adenovirus	Wistar	Prophylactic
HPV 16 L1, E7	Chimeric VLP	MediGene	Prophylactic/ Therapeutic
HPV 16 L1, E7	Chimeric VLP	Univ. of Queensland	Prophylactic
HPV 16 L1, E7	Recombinant BCG	Univ. of Queensland	Prophylactic
HPV 16 E6, E7	Recombinant vaccinia virus with LAMP	Johns Hopkins Univ.	Therapeutic
HPV 16/18 E6, E7	Recombinant vaccinia	Cantab (TA-HPV)	Therapeutic
HPV 16 E7	peptide	NCI	Therapeutic
HPV 16/18 L2, E6, E7	Fusion protein/peptide	Cantab (TA-CIN)	Therapeutic
HPV 6 L2, E7	Fusion protein/peptide	Cantab/GSK	Therapeutic

A. E2 protein

The E2 proteins are approximately 50 KDa and are involved in DNA replication, viral genome maintenance, and transcription regulation (Howley and Lowy, 2001). There are E2 binding sites (E2BSs) in the URR of the genome of which three are next to the origin of replication (Stubenrauch and Lim, 1998). The two viral proteins needed for replication are E1 and E2 (Frattini and Laimins, 1994). E1 is a 68KDa protein which functions as a helicase (Bream and Ohmstede, 1993). Although there are E1 binding sites at the origin of replication, the affinity of E1 for these sites is not sufficient for self-loading of E1 onto the DNA. However, E2 has a higher affinity for viral DNA at E2BS, as well as E1 binding domain(s) and is able to efficiently load E1 onto the viral origin. E1 then hexamerizes, cellular DNA replication proteins are recruited, E2 is released, and replication ensues (Liu et al, 1995). The ability of E2 to bind DNA is therefore an important and likely critical function for the propagation of the viral genome as an episome. Additionally, E2 binds the genome at E2BS and binds the cellular protein bromodomain 4 (Brd4), also referred to as mitotic chromosome associated protein (MCAP), and results in the segregation of the viral DNA into daughter cells by tethering the E2/DNA complex to the Brd4/chromatin complex (You et al, 2004).

During early infection, cellular transcription factors are responsible for the induction of viral transcripts (Hummel and Hudson, 1992). Once E2 is expressed, it will then activate viral gene expression (Steger and Corbach, 1997). The concentration of E2 is critical for transcriptional regulation, as low concentrations of E2 induce early-gene expression, while at high concentrations it is able to repress gene transcription (Steger and Corbach, 1997). E2 may also be involved in regulating expression of genes involved in cellular differentiation and may be able to induce apoptosis independent of p53 (DeFilippis et al, 2003).

Studies of the status of the papillomavirus genome in cervical carcinomas suggest that virus integration does not result in insertional mutagenesis events that are responsible for the induction of oncogenes (Wentzensen et al, 2002; Wentzensen and Vinokurova, 2004). The integration events are thought to be induced by the ability of viral E6 and E7 oncogenes to affect chromosomal stability (Wentzensen and Vinokurova, 2004). The commonality among the various insertion sites is the ability to integrate at fragile sites in the genome (Wentzensen and Vinokurova, 2004). Interestingly, the observed site on the viral genome most frequently disrupted in integration is the E2 open reading frame (Schwarz et al, 1985; Matsukura et al, 1986; Romanczuk and Howley, 1992). The loss of E2 expression is indicative of a critical role for E2 in terms of viral gene regulation since loss of E2 expression is associated with the development of cervical carcinoma. The re-introduction of E2 from a heterologous expression system into HPV transformed cell lines such as HELA results in the accumulation of p53, suppression of E6 and E7 transcription, and leads to senescence (Dowhanick and McBride, 1995; Desaintes et al, 1997; Goodwin and DiMaio, 2000 Hou et al, 2000). Cumulatively, these studies indicated that E2 mediates regulation of transformation through E6 and E7.

B. E6 protein

E6 is approximately 160 amino acids in length, and has been shown to be a transcriptional regulator of minimal promoters (Sedman et al, 1991). E6 is also able to transform NIH 3T3 cells and can immortalize human mammary epithelial cells (Kiyono et al, 1998). When E6 is expressed in coordination with E7, human keratinocytes can be immortalized (Hawley-Nelson et al, 1989; Kiyono et al, 1998; Liu et al, 1999). One of the best-characterized roles of E6 is the inactivation of p53 function (Huibregtse and Scheffner, 1991). p53 has multiple functions including that of a cell cycle regulator which upon stress induces a series of events resulting in apoptosis (Ko and Prives 1996). Induction of apoptosis in HPV infected cells is inhibited by the E6 protein. E6 binds the cellular protein E6AP to form a p53 specific E3 ubiquitin ligase (Huibregtse and Scheffner, 1991). Ubiquitylation followed by proteasome degradation is one of the cellular mechanisms that regulate the turnover of proteins.

Typically three enzymes are involved in his process, and their role is to tag specific substrates for degradation by the proteasome (Howley and Lowy, 2001). The first protein in the cascade, ubiquitin activating enzyme (E1), activates the 76 amino acid ubiquitin and transfers it to an ubiquitin conjugating enzyme (E2). Then an E3 ubiquitin ligase is responsible for transferring the ubiquitin molecule from the E2 to the target protein(s). Thus the E6/E6AP complex transfers ubiquitin to p53, thereby targeting it for degradation, and in so doing changing the half-life of p53 from several hours to less than 20 minutes (Hubbert and Sedman, 1992; Huibregtse and Scheffner, 1993).

E6 can interact with a number of other cellular proteins including p73, IRF3, Bak, p300, paxillin, hScrib and several PDZ domain containing proteins (Howley and Lowy, 2001). These proteins have roles in regulating the integrity of the actin cytoskeleton (paxillin), regulation of apoptosis, (Bak) and inhibition of transcription (IRF-3 and p300) (Howley and Lowy, 2001). These interactions are likely to be involved in the regulation of cellular proliferation. E6 has also been shown to up-regulate telomerase activity through an as yet described mechanism (Klingelhutz and Foster, 1996).

C. E7 protein

The E7 protein is approximately 100 amino acids in length is known to be capable of immortalizing human keratinocytes although less efficiently as compared to that seen in combination with E6 (Munger et al, 1989). One of the most important roles of E7 is the ability to mediate the degradation of the tumor suppressor retinoblastoma family of proteins through the ubiquitin proteasome pathway (Dyson et al, 1989; Wang et al, 2001). E7 interacts with Rb by binding to the pocket domain through its LXCXE motif (Phelps et al, 1988). Rb, p107, and p130 are three 'pocket' domain family members whose role is to regulate transcription during apoptosis, S-phase progression including the regulation of cell cycle during epithelial differentiation (Edmonds and Vousden, 1989). E7 can also regulate Rb by enhancing the activity of cyclins and cyclin-dependent kinases involved in the phosphorylation of Rb (McIntyre and Ruesch, 1996; Zerfass-Thome et al, 1996; Jones and Alani, 1997), can bind histone deacelytases which are involved in viral and cellular activities such as maintenance of the viral DNA as episome (Longworth and Laimins, 2004), can regulate function of interferon regulatory factor 1 (IRF-1) (Park et al, 2000), and direct chromatin remodeling (Duensing et al, 2000).

D. L1 capsid antigen

The late protein L1 (55KDa) is the most abundant protein in the viral capsid (Lin et al, 1992) and is highly antigenic. Several studies have found L1 antibodies in sera of infected patients. L1 has been expressed in a variety of systems in order to produce viral like particles (VLPs) that are highly antigenic (Kirnbauer et al, 1992; Hagensee and Yaegashi, 1993; Rose et al, 1993). These viral like particles form spontaneously and do not require any other viral component. . Although L1 can bind DNA at low efficiency, the VLPs generated with L1 alone are unable to package DNA although have a morphology that resembles intact viral particles (Hagensee et al, 1994). The late protein L2 can be expressed simultaneously with L1 in order to make VLPs that can selectively encapsidate papillomavirus DNA (Zhou et al, 1993). Current evidence suggests that L1 mediates the primary binding of PV virions to the plasma membrane

E. L2 antigen

The role of L2 has been shown to be that of a multifunctional protein. L2 is required for viral DNA packaging, viral infection, and nuclear entry of the viral DNA (Holmgren et al, 2005). These various functions suggest that L2 may have various domains within its sequence responsible for the different roles. Previously published studies have identified two regions necessary for binding L1 and thus necessary for capsid formation (Day et al, 1998). Zhou and colleagues in 1994 showed that the N-terminal domain of L2 is involved in DNA binding, and that the C-terminal 9 residues were identified as a nuclear localization signal (Zhou et al, 1994). L2 has been shown to localize to nuclear domain 10 (ND10s) as well as recruit both L1 and the viral transcription/replication protein E2 (Day et al, 1998; Day et al, 2004) to these nuclear spots. Interestingly, the list of proteins associated with ND10s has increased dramatically in the past decade (Maul et al, 2000). Some of these proteins are up-regulated by interferon and this has implicated ND10s in the viral infection response (Negorev and Maul, 2001). Various viruses (e.g. Herpes Simplex Virus, Adenovirus, Epstein-Barr Virus) contain proteins that modify and/or associate with ND10s (Negorev and Maul, 2001). There is evidence to suggest that ND10s play a negative role in viral life cycle, as is the case of interferon-induced inhibition of HSV-1 immediate-early gene expression. There's also evidence that ND10s can play a positive role in viral infections, as some viruses have been shown to replicate their DNA and begin their transcription in ND10s or in the periphery of ND10s (SV40, Ad5, and HSV-1) (Maul, 1998). The ability of L2 to localize to ND10s may therefore be important for virus replication, packaging, and transcription of its open reading frames. L2 has also been implicated in the translocation of viral DNA to the nucleus during infection, and in the recruitment of the viral DNA to the capsid during encapsidation (Mallon and Wojciechowicz, 1987; Fay et al, 2004; Zhou et al, 1994; Sun et al, 1995). Recently our group has demonstrated that L2 is involved in the entry and intracellular trafficking of the viral capsid to the endoplasmic reticulum (Bossis et al, 2005).

V. Therapeutic approaches toward treatment of papillomavirus associated malignancies

There are two ways in which to deal with papillomavirus related disease: one is to prevent the onset of infection, and the other is to revert disease. To prevent infection, vaccines are been generated to target the capsid proteins that mediate viral attachment. The goal of the vaccine is to develop an immune response, primarily

antibodies, which will result in the neutralization of the virus. The current prophylactic antibodies are directed specifically at the L1 capsid protein (Jansen and Shaw, 2004). PV capsids in nature are composed of L1 and L2 viral proteins (Roden et al, 1994). Extensive *in-vitro* and animal data show that L1 is responsible for the binding of the viral capsid to the surface of target cells (Joyce et al, 1999). The development of antibodies against L1 results in the neutralization of this binding and loss of infection (Christensen and Kreider, 1990). In order to generate antibodies that are neutralizing in human subjects, current trials have generated virus like particles (VLPs) that contain only the L1 protein and are devoid of viral or other DNA (Kirnbauer et al, 1993; De Bruijn et al, 1998). VLPs are used as a specific strategy since making a live but attenuated vaccine is difficult due to the inability to efficiently grow the virus in culture. The VLPs are produced in recombinant baculovirus-infected insect cells (Unckell and Streeck, 1997; Marais et al, 1999). After immunization, the antibodies are generated systemically against the individual genotypes and have been shown to be 100% efficient in their prevention of HPV 16, even with infection occurring within the genital tract (Koutsky et al, 2002). This and other results are very encouraging that the vaccine may be able to prevent infection and have a tremendous effect on lowering the level of cervical carcinoma worldwide, which at this moment is approximately 500,000 cases annually with a 50% fatality rate (Parkin et al, 2005). A multivalent VLP vaccine being developed is geared towards preventing infection of the predominant genotypes found in cervical carcinoma, these are HPV 16 and 18 (MedImmune and Glaxo SmithKline) (GSK, 2004).

In a separate study, a pentavalent VLP vaccine directed at HPVs 16, 18, 31, 6, and 11 is being tested (Merck & Co.) (Koutsky et al, 2002). Of these, HPV 6 and 11 are non-oncogenic, but are strictly agents associated with cutaneous warts. Although the use of L1 VLPs as vaccines is very promising, it still needs to demonstrate long-term protection, and does not provide any therapeutic relief on existing infections.

One aspect of L1 VLP vaccine development that has not yet been overcome is the need for VLPs for each specific genotype targeted. These vaccines do not provide any cross genotype protection (Koutsky et al, 2002), and thus the vaccines being administered are really a combination of PV genotype specific vaccines. This brings up the question as to how many genotypes must be targeted? Most study have found that approximately 70% of the cervical cancer lesion contain HPV 16 or 18 (Bosch et al, 1995), thus the current efforts have the potential of reducing the level of carcinoma up the 70%. Although HPV 16 and 18 are the most commonly identified genotypes, thirteen HPV genotypes have been designated as oncogenic by the International Agency for Research on Cancer (IARC, 2005). To develop a broad acting vaccine, targeting the minor capsid L2 may be critical to achieve this goal. Data have shown that the ability of the virus to infect effectively depends on the presence of L2 and studies have demonstrated that a portion of L2 is involved in the binding/entry of the virus (Christensen and Kreider, 1991; Roden et al, 1994, 2001). Furthermore L2 antibodies are able to neutralize infection across species (Christensen et al, 1990; Pastrana et al, 2005). For example, an antibody made against the bovine papillomavirus type 1 L2 is capable of neutralizing BPV1 infection at 1:3400 dilution, as well as neutralizing HPV16 infection at 1:4700 and HPV18 at 1:7000 (Pastrana et al, 2005). These BPV1 L2 antibodies were made against the n-terminal 88 residues of L2, and our published data show that L2 residues 40-44 (DKILK) may be a common target for vaccine development (Bossis et al, 2005; Pastrana et al, 2005). This five-residue region is highly conserved through species and is most likely exposed on the outside of the capsid (Bossis et al, 2005). Data from our laboratory show that deletion of this region results in a non-infectious virus by preventing the intracellular sorting/entry of the virus from the plasma membrane to the endoplasmic reticulum (Bossis et al, 2005). DNA bases vaccines against L1 have been shown to protec rabbits from papillomavirus infection and wart formation (Donnelly, et al, 1996). The results of DNA vaccination in primates and nonhuman primates are discouraging since a very high dose of DNA is required to elicit an immune response. The high dosage required may result in other issues such as the induction of mutation and disruption of cellular cells. These safety issues need to be investigated, as is the possibility of inducing anti-DNA antibodies that may result in autoimmune disorders.

VI. Therapeutic options for existing papillomavirus associated malignancies

To treat existing infections, investigators must first decide which antigen to target, and then how to target this specific viral antigen. Stably infected cells produce several of the early viral gene products. Of these, E6, E7, E1, and E2 are most important. E1 and E2 are necessary for viral replication, and E2 is also a potent transcriptional regulator responsible for E6 and E7 expression. E6 and E7 are involved in directing the cell cycle of the infected cell. Several studies have used the early proteins to generate vaccines (Borysiewicz et al, 1996; Boursnell et al, 1996). These proteins are expressed throughout the life cycle of the virus, and E6 and E7 are the two proteins most directly involved in cervical carcinoma by regulating p53 and pRb expression. As described in this review, oncogenic progression with the high-risk genotypes results after integration of the viral genome has occurred (Wentzensen et al, 2002). The site of integration in the host genome is non-specific and most often results in the loss of expression of the E2 protein (Wentzensen and Vinokurova, 2004).

Several Studies have used the early proteins to generate vaccines. One of the first and most promising therapeutic vaccines is TA-HPV (Cantab Pharmaceuticals plc, Cambridge UK). This is a recombinant vaccinia virus that encodes the E6 and E7 gene products of HPV 16 and 18 (www.cantabuk/Pages/1science/hpv.html). This approach has resulted in greater that 10% of the cervical cancer patients in the study developing an HPV-specific immune response (www.cantabuk/Pages/1science/hpv.html). Other

prophylactic vaccines targeting E6 and E7 have been made by delivering the E6 and E7 ORFs in vectors such as BCG bacterium (Jabbar et al, 2000), Venezuelan equine encephalitis virus (VEE) (Zubritsky, 1998), and the Listeria bacterium (Sewell et.al, 2005).

Several studies are developing vaccines by fusing E6 and/or E7 to more antigenic proteins such as L1, L2, and heat shock protein (HSP) (Borysiewicz et al, 1996; Boursnell et al, 1996). The vaccination with a fusion protein of either L1 or L2 to E7 (by Medigene HG, and Glaxo SmithKline respectively) has not yielded therapeutic potential. It is of interest to point out that the portion of L2 protein fused to E7 in the GSK study did not consist of the n-terminal 90 residues, which we, and others have identified as important for infection, and for development of neutralizing antibodies (Bossis et al, 2005; Pastrana et al, 2005). Alternatively, vaccines against fusion proteins of L1 or L2 with E6 or E7 have yielded strong immune response when used to generate VLPs (Jabbar et al, 2000; Hussain and Paterson, 2005). Some of these approaches seem to be able to generate both a humoral and a cell-mediated immune response (Jabbar et al, 2000; Hussain and Paterson, 2005) and have resulted in reversal of skin warts growth and BPV disease (Campo and Jarrett, 1994; Campo, 1997; Greenstone et al, 1998; Jochmus et al, 1999; Leachman et al, 2002; Moore et al, 2002). It has yet to be demonstrated that the therapeutic vaccines will succeed in humans and non-primates. Lastly, generating an immune response with small regions of the antigens, i.e., using peptides, may eventually yield an inexpensive, low toxicity approach. Thus far peptides targeted to E6 or E7 have shown low immune response induction, and need to be further evaluated (van Driel et al, 1999). DNA vaccines have also been developed targeting E6 and E7 in order to induce an immune response (Cheng et al, 2001; Peng et al, 2004). Safety of DNA therapy has to be further analyzed including the possible complications with introduction of oncoproteins (such as E6 or E7).

VII. Assessment of the future

It seems clear that the best way to target HPV associated disease is to block infection. The vaccines developed against VLPs show the greatest level of promise (Koutsky et al, 2002). Attempts at delivering transgenes in viral vectors such as Adenovirus are ongoing and need further evaluation (Liu et al, 2004). The next decade will confirm if these vaccines can confer long-lasting protection against infection with oncogenic HPV genotypes, and will determine if there is a selection process that will lead to an increase in infection with HPV genotypes that are less oncogenic. Additionally, studies relating to the entry pathway of the virus can elucidate new strategies with which to prevent infection. Current therapeutic interventions have not been successful at reversing cellular transformation and or oncogenesis. Improvement of the current strategies, and new targets need to be identified if we are to reverse PV associated disease. On-going research into the structure, interaction function of proteins such as E6 or E7 should allow for the identification of new strategies. We must also overcome the stigma associated with vaccinating young pre-pubescent individuals who are not sexually active, against a sexually transmitted disease.

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