Human Papillomavirus vaccination: Immunological and epidemiological studies

Ryding, Janka

2008

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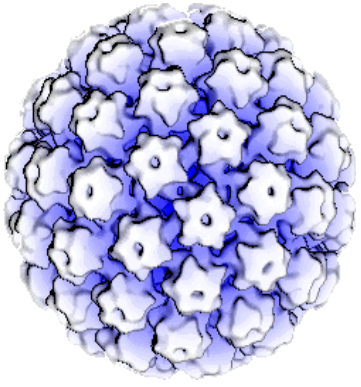

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Department of Laboratory Medicine, Division of Medical Microbiology, Lund University, Malmö University Hospital, Sweden

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Human Papillomavirus vaccination: Immunological and epidemiological studies

Janka Ryding

With the permission of the Medical Faculty of Lund University, to be presented for public examinatinon at the Pathology Lecture Hall, Entrance 78, University Hospital MAS, Malmö, on Monday, March 26th at 9.00 am.

Supervisor
Professor Joakim Dillner

Faculty Opponent
Patrick Olin, MD, Ph.D
Swedish Institute for Infectious Disease Control

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Abstract

This thesis has evaluated the immunological- and epidemiological aspects of human papillomavirus transmission dynamics, the effect on circumcision and the possible impact of vaccination in Sweden.

Effective prophylactic vaccines are based on type-specific neutralizing antibodies. A major neutralizing epitope is defined by the monoclonal antibody H16.V5. To investigate the importance of this epitope for overall immunogenicity of HPV16, we engineered HPV16 virus-like particles devoid of the H16.V5 epitope by site-directed mutagenesis of 10 non-conserved, surface exposed residues. Removal of the V5-defined epitope had only marginal effect on antigenic reactivity with antibodies in sera from infected subjects, but affected immunogenicity in experimental immunization of mice, with reduced induction of both antibody responses and CTL responses.

A serological survey of HPV16 antibody prevalence by age and sex in Sweden was performed and used it as a basis for modeling the optimal vaccination strategies in this population. By the year 2055, vaccination of females starting at age 12 in 2008 was most efficient, estimated to prevent 5.8 million cumulative HPV16 infections. Catch-up programs had a strong additional preventive effect. Vaccination also targeting males increased protective effect by about 4 percent, but had lower preventive effect per vaccination given. Addition of an HPV serosurvey to existing models and data has enabled us to estimate effect of different vaccination strategies, optimised to the HPV epidemiology in our population.

The age-dependent seroprevalence of HPV6, 11, 16, 18, and 52 infection was investigated and further used to asses the transmission dynamics in a representative Swedish population. Analyses of age-specific prevalence revealed different patterns for high- and low-risk HPV infections between females and males.

Circumcision has been reported to protect against infection with human papillomavirus in men, but results have been inconsistent. We followed males in a birth cohort born in Dunedin, New Zealand from age 3 to 32 years. Circumcision was not found to be protective, with the adjusted odds ratio (95% confidence interval) for HPV6/11/16/18 seropositivity among the circumcised compared with the uncircumcised being 1.4 (0.89-2.2).

Key words: HPV, antibody response, immunodominant epitope, V5, prevalence, mathematical modelling, circumcision

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LIST OF PAPERS

Deletion of a major neutralizing epitope of human papillomavirus type 16 virus-like particles.
J. Ryding, L. Dahlberg, M. Wallen-Ohman, J. Dillner
Journal of General Virology, 2007 Mar;88(Pt 3):792-802.

Seroepidemiology as basis for design of a Human Papillomavirus vaccination program.
J. Ryding, K.M. French, P. Naucler, R.V. Barnabas, G. P. Garnett, J. Dillner
Manuscript

Seroepidemiology of HPV types 6, 11, 16, 18 and 52 in Sweden.
J. Ryding, J. Dillner
Manuscript

Serologically determined human papilloma virus infection not related to early circumcision in a birth cohort.
N Dickson[1], J Ryding[1], T van Roode, C Paul, P. Herbison, J. Dillner, D. Skegg
Manuscript

ABBREVIATIONS

ATP	Adenosine triphosphate
BPV	Bovine papillomavirus
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CTL	Cytotoxic T-lymphocytes
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoabsorbent assay
EV	Epidermodysplasia verruciformis
HDAC	Histone deacetylase
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
HPV	Human papillomavirus
Ig	Immonoglobulin
LCR	Long control region
mRNA	Messenger ribonucleic acid
OR	Odds ratio
SIL	Squamous intraepithelial lesions
STD	Sexually transmitted disease
Rb	Retinoblastoma family of tumor suppressors
URR	Upstream regulatory region
VLP	Virus-like particle

INTRODUCTION

HUMAN PAPILLOMAVIRUSES

Classification

Papillomaviruses are highly diverse and widespread in nature. They have been

recognized primarily in higher vertebrates and are likely to occur in most mammals. Papillomaviruses are highly species specific and there is no firm evidence of a papillomavirus from one species causing productive infection in a second species. Over 100 human papillomavirus types (HPVs) have been described. A HPV type is defined as a genome whose major capsid protein nucleotide sequence differs from the homologous nucleotide sequence of every other HPV-type by at least 10 percent. Subtypes have 90 to 98 percent sequence similarity to the corresponding type and variants show more than 98 percent sequence homology to the prototype (de Villiers *et al*., 2004).

In an evolutionary perspective HPVs can be separated into a number of groups or genera (figure 1). Papillomaviruses are separated according to genotype i.e. differences in the open reading frame of the highly conserved L1 major capsid protein. L1 sequences that share less than 60 percent nucleotide sequence identity belong to different genera. Papillomaviruses within a species share between 71-89 percent nucleotide identity within the L1 open reading frame. The human papillomaviruses are found in the the *Alpha*-, *Beta*-, *Gamma*-, *Mu*- and *Nu*-genera. The other genera contain papillomaviruses isolated from various mammals and birds (de Villiers *et al*., 2004; Doorbar, 2006; Forslund, 2007). HPVs are highly trophic and infect basal epithelial cells of the skin or mucosa and can further be classified into cutaneous or mucosal types. *Beta* papillomaviruses are typically associated with inapparent cutaneous infections in humans; whereas the *Alpha* papillomavirus group mainly contains the genital/mucosal HPV types. Within each of these groups they can further be designated as high-risk or low-risk according to the propensity for malignant progression of the lesions they cause. Low-risk HPV types 6, 11, 42, 44, 53 and 83 are associated with benign warts in the genital tract (condyloma acuminata). High-risk HPV types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68 and 69 are usually found in malignant lesions (Munoz *et al*., 2006).

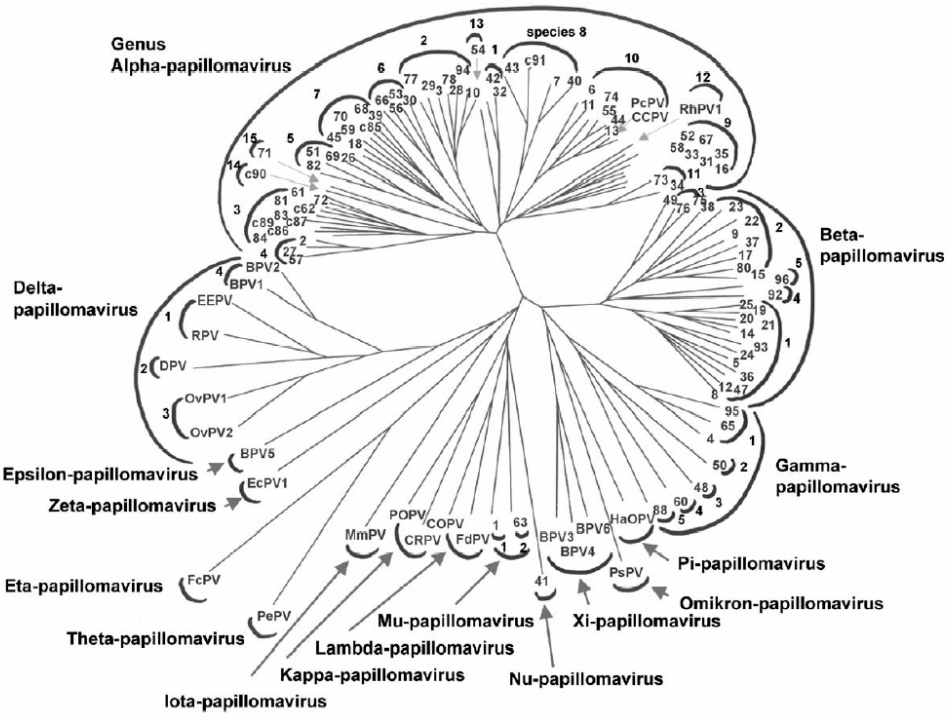

Figure 1: Phylogenetic tree illustrating the genus and species of 118 papillomavirus types (Adapted from de Villiers, 2004)

Associated diseases

Human papillomaviruses infect exclusively the basal cells of the skin and of mucosal epithelia adjacent to the skin such as the mouth, the upper respiratory tract, the lower genital tract and the anal canal. HPVs are found in a majority of human epithelia, and occasionally in cancer. In skin, infection causes a variety of benign proliferations which develop mostly on hands and soles. Various clinical presentations are described, including common warts, filiform warts, flat warts,

butchers' warts and mosaic warts. Most infections are self-limited and regress within 12-24 months and they are transmitted mainly by skin to skin contact (Doorbar, 2006).

Clinical, molecular and epidemiological evidence has established that HPV types are associated with anogenital neoplasms, including condyloma acuminata, cervical dysplasia and cervical carcinoma (Dillner *et al*., 2007; Parkin & Bray, 2006). These types are almost always transmitted sexually through various forms of sexual contact and are passed from skin to skin contact, not through bodily fluids like many other sexually transmitted infections. An individual's risk of acquiring HPV infection increases with number of sexual partners and with the risk behavior of sexual partners (Burchell *et al*., 2006). HPV infection also has an established role as the cause of cancers of the vulva, vagina, anal canal, perianal skin and penis. In addition, HPV is accepted as an aetiological factor for oropharyngeal cancers (Dillner *et al*., 2007; Parkin & Bray, 2006)

Condyloma Acuminata

Human papillomaviruses infecting the basal layer of genital epithelium often result in genital warts (condyloma). Condylomas are generally described as painless, flesh-colored or grayish-white warts on the vulva, penis or anus. The infection is transmitted through sexual contacts. Condylomas are caused by HPVs in the *Alpha* papillomavirus genus. HPV6 and 11 are the most frequent found types in genital warts and since they are not associated with genital cancer, they are designated as low-risk types. Similar to skin warts, condylomas are self limiting and regress spontaneously but treatments may be required. Although destructive therapy is often used to treat these lesions, topical treatments can be used for the clearance of genital warts (Ahmed *et al*., 2006).

Recurrent respiratory papillomatosis

HPV6 and 11 are also found in juvenile recurrent respiratory papillomatosis. This is a rare disease where wart-like growth occurs in the aerodigestive tract (Stamataki *et al*., 2007).

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) was the first evidence for the involvement of HPV infection in the development of skin cancer (Jablonska & Majewski, 1994; Jablonska & Orth, 1985). EV is a rare cutaneous heritable disease that persists through life. The individuals with EV are susceptible to persistent HPV infection because of a deficiency in cutaneous immunity (Orth, 2006). In early childhood, the disease results in the development of warts which occur predominantly on the face, dorsa of the hands and legs. These usually persist through life without a tendency to regress. About 30-60 percent of the patients develop carcinoma in situ or squamous cell carcinoma at sunlight exposed bodily sites (Akgul *et al*., 2006).

Anogenital HPV-associated Disease

HPV infection of the anogenital skin and mucosa results in lesions with two morphologies - anogenital warts (condyloma) and squamous intraepithelial lesions (SILs). Condylomas are as mentioned previously polypoid growths that generate infectious virus and have a low-to-negligible risk of malignant progression. SILs are classified histologically and form a distinct spectrum of histologic atypia. In the cervix, these lesions are graded on the degree to which they have lost cytoplasmic maturation and exhibit cytologic atypia. In Europe, three grades are recognized cervical intraepithelial neoplasia (CIN): CIN 1 - mild, CIN 2 - moderate, and CIN 3 - severe. The Bethesda classification for cytology used in the United States recognizes two classes - low-grade SILs (LGSILs) (CIN 1) and high-grade SILs (HGSILs) (CIN 2/3) (Schiffman *et al*., 2007).

Association with cervical cancer

Human papillomavirus is a highly prevalent, sexually transmitted infection and has been shown as an aetiologic agent for cervical cancer (Munoz *et al*., 2006). With approximately 500,000 newly diagnosed cases each year and a 50 percent mortality rate cervical cancer is the second most common cause of cancer-related death in women worldwide (Stanley, 2006b). Oncogenic HPV infection is a

necessary but not a sufficient cause of cervical cancer . There are several other factors that may modulate the risk of transition from HPV infection to cervical cancer development in conjunction with HPV. Risk factors for tumor development include: persistent infection with high-risk viral types, a large number of lifetime sexual partners, coinfection with human immunodeficiency virus, immunosuppression, parity, long term use of oral contraceptives and cigarette smoking (Dillner & Brown, 2004; Steben & Duarte-Franco, 2007). While most cases of cervical cancer are caused by HPV infections, not all women that are infected with high-risk HPV will develop cervical cancer. In most cases, the immune system is able to fight off the infection. Cervical cancer development is a multistep process. It often takes many years from HPV infection to cancer development and requires both the presence of oncogenic HPV genotypes and the interaction of many host factors (Munoz *et al.*, 2006). The latency period between initial HPV infection and the cancer can be more than 10 years (Frazer, 2007). The premalignant stages can be identified both clinically by speculum examination and in the laboratory by Papanicolaou smear analysis. Even though HPV infections are common among younger women of the age group 19-35 years, cervical cancer is more common in women over the age of 35 years (Dunne *et al.*, 2007; Schiffman *et al.*, 2007; Stone *et al.*, 2002; Wright *et al.*, 2006).

A subset of HPV types have been established as the causative agents of cervical cancer, since 99 percent of tumors are positive for HPV DNA (Munoz *et al.*, 2006). More than 35 HPV types regularly or sporadically infect the genital tract. Among these types, HPV16 is the most prevalent and accounts for 50 to 60 percent of the cervical cancer cases in most countries, followed by HPV18, who accounts for 10 to 12 percent. The four most common oncogenic HPV types (HPV16, 18, 31, and 45) were found in together about 80 percent of squamous cell carcinomas and HPV types 16, 18, 45, 59 and 33 together account for 94 percent of HPV-types found in adenocarcinomas (Dillner & Brown, 2004).

Genomic organization

HPVs are DNA viruses with a double-stranded closed circular genome of approximately 8 kilobases (Seedorf *et al.*, 1985). All papillomaviruses have a similar genomic organization. Viral genes can be divided into early or late categories dependent on the time of expression. The HPV genome (figure 2) encodes six early proteins (E1, E2, E4, E5, E6 and E7) and two late proteins (L1 and L2). The late genes encode the viral capsid proteins whereas the early genes encode proteins involved in viral DNA replication, transcription and cellular transformation (Hebner & Laimins, 2006).

The genome can be divided into three major regions: i) a ~1.0 kb non-coding long control region (LCR) that contains a variety of *cis* elements that regulate gene expression and viral replication. LCR has also been referred to as the upstream regulatory region (URR), ii) a ~4.0 kb early (E) region, that encodes nonstructural proteins, iii) a ~3.0 kb late (L) region, that encodes the two capsid proteins (Munger *et al.*, 2004). The HPV16 genome contains two major promoters. The early promoter, p97, initiates transcription upstream of the HPV16 E6 open reading frame, while the differentiation dependent late promoter, p742, located in the HPV 16 E7 open reading frame is activated during the late phase of the HPV life cycle (Doorbar, 2006; Klumpp & Laimins, 1999; Ozbun & Meyers, 1997).

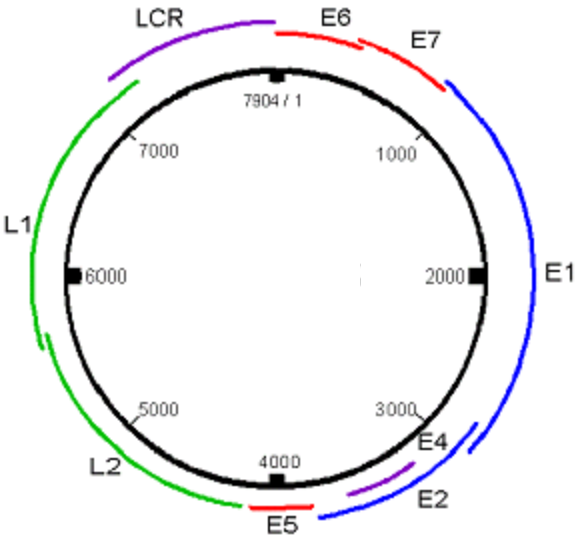

Figure 2: Genomic organization of human papillomavirus type 16

The viral Proteins

E1 and E2

E1 and E2 are important for viral replication and maintenance of papillomavirus DNA. The E1 protein is highly conserved among HPV types, and binds to specific recognition sequences in the viral origin of replication, located in the LCR. E2 is a site-specific DNA binding protein that helps to recruit E1 to the origin. By itself, E1 weakly binds origin sequences, but this binding is facilitated by complex formation with E2 proteins. E2 binding sites are located adjacent to E1 recognition sequences, and E2 acts by loading E1 onto the origin of replication. Once bound, E1 proteins form a hexameric ring and initiate its helicase ability allowing for the separation of the viral DNA strands ahead of the replication complex. The role of E2 is to catalyze the assembly of hexameric E1 complexes specifically at the origin. Adenosine triphosphate (ATP) hydrolysis is also required for E1 hexamers to distort the origin and encircle single strands of

DNA. ATP binding stimulates the E2-dependent association of E1 with the origin of replication (Sedman *et al.*, 1997). The E2 proteins bind to the E1 and stimulate viral DNA replication. During early stages of viral infection, the E2 protein represses transcription of the oncogenes E6 and E7 (Thierry & Howley, 1991). Integration of the viral genome, which often takes place during carcinogenic progressing, disrupts the E2 and E1 ORFs and results in elevated expression of E6 and E7 (Jeon & Lambert, 1995).

E2 is also important for preserving a stable episome of the viral genome in the nucleus during cell division. To prevent loss of the episome, the E2 protein can bind to a cellular protein called Brd4 which attaches the viral genomes to mitotic chromosomes, leading to maintenance of the viral genome. However, the interaction of E2 and Brd4 is not required for genome partitioning of all papillomaviruses since a number of papillomavirus E2 proteins associate with mitotic chromosomes independently of Brd4 binding (Baxter *et al.*, 2005; McPhillips *et al.*, 2006).

E4

E4 is synthesized primarily in the late phases of the HPV life. The E4 origin of replication lacks the initiator codon AUG and is therefore translated from a spliced transcript resulting in an E1^E4 fusion protein, which consist of the first five codons of E1 fused to the open reading frame of E4 (Nasseri *et al.*, 1987; Wilson *et al.*, 2005). The E1^E4 is the most highly expressed protein in the productive life cycle of HPVs. Initial studies indicated that E1^E4 induces the collapse of the cytokeratin network when it is overexpressed in transient assays, and they suggested a role for the protein in facilitating viral egress (Doorbar *et al.*, 1986; Doorbar *et al.*, 1991; Roberts *et al.*, 1993). However, no such collapse is seen in lesions induced by many high-risk HPV types (Doorbar *et al.*, 1997; Doorbar *et al.*, 1996; Pray & Laimins, 1995). In HPV lesions, the expression of E1^E4 occurs in the upper layers of stratified epithelia, coordinating with the onset of genome amplification but preceding the expression of L1. This pattern of E1^E4 expression suggests a possible role in regulating late viral functions.

However, the function of E1^E4 in viral life cycle is not completely understood (Wilson *et al*., 2005).

E5

The E5 proteins are small hydrophobic polypeptides that localize to membranes of the Golgi apparatus and endoplasmic reticulum (Conrad *et al*., 1993). Carcinogenic HPV types code for an E5 protein, whereas most low-risk types either lack a definable homologous E5 ORF and/or a translation start codon for E5 (Schiffman *et al*., 2005). The E5 proteins encoded by the human papillomaviruses display weak transforming activity (Conrad *et al*., 1993). Expression of the E5 gene of the high risk HPV types is often terminated by integration of the viral genome during the progression to cervical cancer, so if the HPV E5 proteins play a role in carcinogenesis, they must act at an early stage during carcinogenic progression (DiMaio & Mattoon, 2001).

E5 is capable of stimulating cell growth via interaction with cellular growth receptors such as the epidermal growth factor receptor (DiMaio & Mattoon, 2001). E5 can also bind to vacuolar ATPase. ATPase is involved in acidification of endosomes, who regulates receptor recycling from the cell membrane. E5 was shown to be able to inhibit acidification of endosomes, resulting in the prolongation of the active signal from growth receptors (Straight *et al*., 1993). However, another report suggests that HPV16 E5 affects endocytic trafficking rather than endosome acidification (Thomsen *et al*., 2000).

E6

E6 is one of the earliest expressed proteins but it can also be found throughout the layers of differentiated epithelial cells. E6 is together with E7 the main transforming proteins and they are consistently expressed in malignant tissues (Snijders *et al*., 2006). E6 proteins of high risk HPVs have been shown to function as oncoproteins (Huibregtse & Beaudenon, 1996; Oh *et al*., 2004). E6

can bind to multiple cellular targets that provide functions that alter the cellular environment, making it more amenable to production of new viral particles. The best characterized activity of the high-risk E6 is its ability to bind a cellular ubiquitin-ligase, forming a complex which is able to bind the tumor suppressor protein p53, resulting in p53 degradation mediated by the cellular ubiquitin proteolysis system. Because p53 is required for the growth arrest following cellular DNA damage, cells without functional p53 are not arrested appropriately in G1, but display genomic instability (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1990; Thomas *et al.*, 1999; Werness *et al.*, 1990). Although degradation of p53 by E6 is specific to high-risk HPV types, some evidence suggests that low-risk forms of E6 may be able to bind to p53 but with a lower affinity (Oh *et al.*, 2004).

E6 can also bind many other cell proteins, such as various PDZ domain containing proteins. PDZ proteins are involved in a variety of functions including cell signaling and cell-cell adhesion. The ability of E6 proteins to bind PDZ proteins have been shown to be required for the induction of epithelial hyperplasia in transgenic mice (Nguyen *et al.*, 2003a; Nguyen *et al.*, 2003b). Another important function of the high-risk E6 protein is the activation of telomerase in infected cells. In normal cells, DNA replication results in the shortening of telomeres, eventually producing genomic instability and cellular senescence. E6 has been shown to increase telomeric length in epithelial cells by activating telomerase (Klingelhutz *et al.*, 1996; Stoppler *et al.*, 1997; Veldman *et al.*, 2001).

E7

E7 is the main transforming protein. One of its main functions in the HPV life cycle is the binding and degradation of the retinoblastoma (Rb) tumor suppressor family of proteins. The Rb proteins are major regulators of the cell cycle. Hypophosforylated Rb controls the transition at the G1/S phase of the cell cycle through binding of the E2 family of transcription factors that activate transcription of many components involved in S-phase replication. In normal

cells, phosphorylation of Rb by cyclin-kinase complexes leads to a release of E2Fs and transcription of S-phase genes. E7 can override this normal cell cycle control through binding and degradation of hypophosphorylated Rb and thereby constituvely releasing E2F-complexes (Boyer *et al*., 1996; Hebner & Laimins, 2006; Jones *et al*., 1997). Both high-risk and low-risk E7 proteins have been shown to bind to the Rb protein, but low-risk E7 proteins bind with a lower efficiency. As a result the low-risk E7 proteins do no efficiently transform cells (Gage *et al*., 1990; Munger *et al*., 1989b). E7 can interact with many other cellular proteins, most which are important regulators of the growth, but the actual biological relevance is uncertain. Another consequence of E7 expression is the induction of genomic instability, as seen in many malignancies. The E7 has been shown to induce abnormal centrosome duplication resulting in multipolar, abnormal mitoses and aneuploidy. The low-risk E7 proteins do not induce similar abnormalities (Duensing *et al*., 2001; Duensing *et al*., 2000).

E7 can also interact with histone deacetylases (HDACs), which act as transcriptional repressors by remodeling chromatin through the deacetylation of histones. Actively transcribed genes show a high level of histone deacetylation while repressed genes do not. E7 binding to HDACs has been shown to increase the levels of E2F-family mediated transcription in differentiating cells, thus forcing cells into S-phase (Longworth *et al*., 2005).
E7 can also interact with various transcription factors such as the AP1 family, who mediate mitogenic effects and are involved in cell differentiation (Antinore *et al*., 1996). HPV E7 has an elaborate mode of action where it can modulate both the process of cell cycle progression as well as cell differentiation.

L1 and L2

The major (L1) and the minor capsid proteins (L2) are structural proteins that are expressed in the upper layers of infected tissue once viral genome amplification has been completed. L2 together with L1 packages the viral DNA into the virion and spontaneously form capsids (Doorbar, 2005). The capsid contains 360 copies of the L1 protein, and 12 copies of L2, organized into a capsomere icosohedral

shell (Chen *et al.*, 2000; Modis *et al.*, 2002). Compared to other papillomavirus genes, the amino acid sequences of most portions of L1 are well-conserved between types. However, the surface loops of L1 can differ substantially, even for different members of a particular papillomavirus species (Carter *et al.*, 2006). Both linear and conformational epitopes have been identified on the surface of L1 proteins (Christensen *et al.*, 1990), and it is now well established that conformational epitopes are responsible for the activity of neutralizing antibodies (Christensen *et al.*, 1994; White *et al.*, 1998; White *et al.*, 1999).

L2 has also been shown to interact with a number of cellular proteins during the infectious entry process and has been found to be essential for efficient infection (Richards *et al.*, 2006). L2 mediates egress of the viral genome from late endosomes and further mediates translocation of the viral genome into the nucleus (Kamper *et al.*, 2006). During assembly L2 mediates co-localization of L1 and E2 proteins (Day *et al.*, 1998). Small sections of L2 are well-conserved between different papillomavirus types, and experimental vaccines targeting these conserved domains offer some protection against a broad range of HPV types (Day *et al.*, 2008; Pastrana *et al.*, 2005; Slupetzky *et al.*, 2007).

Life cycle

Human papillomavirus enters the basal epithelial cells of the skin and mucosa. Infection is thought to occur through microwounds of the epithelium that expose cells in the basal layer (the only cell layer in an epithelium that is actively dividing) to viral entry. The HPV life cycle is tightly linked to the differentiation program of the infected host cell, the keratinocyte, with production of mature virion particles restricted to the differentiated suprabasal cells (figure 3) (Munoz *et al.*, 2006). At present there is some controversy as to the precise nature of the receptor for virus entry, but it is thought that heparan sulphate proteoglycan plays a role in initial binding and/or virus uptake (Joyce *et al.*, 1999; Patterson *et al.*,

2005; Shafti-Keramat *et al.*, 2003). Virus particles are taken into the cell relatively slowly (Culp & Christensen, 2004) and, for HPV16, this occurs by clathrin-coated endocytosis. This mode of entry may not be conserved among all HPV types. Virus particles disassemble in late endosomes and/or lysosomes, and the viral DNA is transported to the nucleus by the minor capsid protein L2 (Day *et al.*, 2004; Day *et al.*, 2003).

Infection leads to the establishment of the viral genome as a stable episome (without integration into the host cell genome) in cells of the basal layer and the early promoter is activated. In these infected cells, low levels of viral DNA synthesis occur. Viral episome replication occurs synchronously with host cell chromosome replication. Since HPVs do not encode enzymes for DNA replication, production of viral genomes is critically dependent on the host cellular DNA synthesis machinery (Schiffman *et al.*, 2007).

After division of basal cells one daughter cell remains in the basal layer while the other one move up and starts to terminally differentiate. HPV episomes are also replicated and distributed evenly among daughter cells. Differentiated cells contains little or no replicative machinery, therefore the virus will be unable to propagate if cells are permitted to terminally differentiate. Therefore the virus expresses proteins that stimulate G1/S progression. However, the virus still requires a certain level of differentiation because the shift from early to late promoter, which transcribes capsids protein mRNA, is mediated in differentiated cells. As HPV positive cells diffentiate, the late promoter is activated leading to expression of late genes and new virions are produced. The factors determining promoter shift have not yet been determined. In the uppermost layers of the epithelium DNA is packaged into newly formed virus capsids and shed into the environment as a cargo within epithelial squamae (Munger *et al.*, 2004; Munoz *et al.*, 2006).

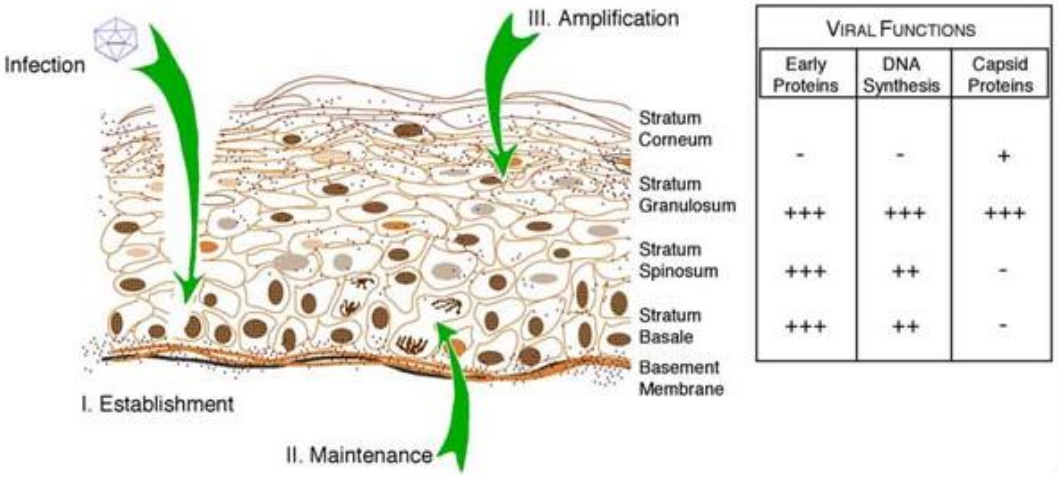

Figure 3: Differentiation dependent life cycle of HPVs
(Adapted from Ozbun, 2007)

Pathogenesis

Why do some HPV types cause cancer? Both benign and malignant viruses have to stimulate G1/S progression for successful replication of their genomes. They use the same pathways but the oncogenic viruses have a greater potential for chromosomal accidents, which can lead to malignant conversion. Expression of the early gene products, E2, E6 and E7, determine whether an HPV infection is active, latent or leads to malignant transformation. The E6 and E7 proteins of the high-risk HPV types act as viral oncoproteins, but no such functions are associated with the corresponding proteins from the low-risk types (Munoz *et al.*, 2006).

One of the key events of HPV induced carcinogenesis is the integration of the HPV genome into a host chromosome. The integration sites are widely distributed all over the human genome and there are no apparent hot spots and no evidence for insertional mutagenesis (Thorland *et al.*, 2003). When the papillomavirus genes are expressed in the non-differentiated, replicating cells of the basal and parabasal cell layers, the E6 and E7 gene products interfere with the ordered sequence of the cellular replication machinery, leading to genetic instability and subsequent neoplastic transformation (Ziegert *et al.*, 2003). The frequency of HPV integrations seems to differ. In low-grade lesions, the majority of HPV genomes persist in episomal state, whereas in high-grade lesions and carcinomas, an increasing number of HPV genomes is found to be integrated into the host genome (Durst *et al.*, 1985; Lehn *et al.*, 1988; Schwarz *et al.*, 1985). Although chromosomal instability is tightly linked to high-risk HPV integration, it is still unclear whether integration represents the cause or simply the consequence of genomic instability (Hebner & Laimins, 2006; Pett *et al.*, 2004). While HPV integration equates the end of the viral life cycle due to the loss or functional inactivation of large parts of the viral genome, expression of the viral oncogenes E6 and E7 is maintained and even enhanced upon integration, favoring neoplastic transformation and progression (Jeon *et al.*, 1995; Jeon & Lambert, 1995; von Knebel Doeberitz *et al.*, 1991).

The E6 and E7 oncogene protein expression is modulated by the E2 gene. During the HPV cell cycle the E2 and E1 proteins form a complex that binds to sequences at the viral origin of replication and acts to recruit cellular polymerases and accessory proteins to mediate replication. At low levels the complex activates and at high levels it represses expression. Integration of HPV into the host chromosome leads to the disruption of the E2 gene and therefore leads to increased expression of E6 and E7 (Frazer, 2004). High-risk E6 binds the p53 tumor suppressor protein as part of a trimeric complex with the cellular ubiquitin ligase, E6AP, leading to the rapid turnover of p53 thus causing loss of the role of p53 controlling the cell cycle (figure 2). E6 protein of low-risk HPVs does not affect p53 levels. E7 protein of the high-risk HPVs binds to the Rb family of tumor suppressors, as well as other proteins involved in cell cycle regulation (zur Hausen, 2002). These proteins control the activity of the transcription factor E2F. E7 liberates active E2F from an inactive pRb-E2F complex by targeting Rb. E2F then drives the cell into S phase and proliferation (figure 4). The low-risk HPV E7 protein binds to the RB proteins with much lower efficiency (Munger *et al.*, 2004).

It has been demonstrated that only the E6 and E7 genes of high-risk types can immortalize human cells in tissue culture (Hawley-Nelson *et al.*, 1989; Munger *et al.*, 1989a) so the primary role of malignant transformation can be assigned to the E6 and E7 genes and their proteins, which are consistently expressed in malignant tissue.

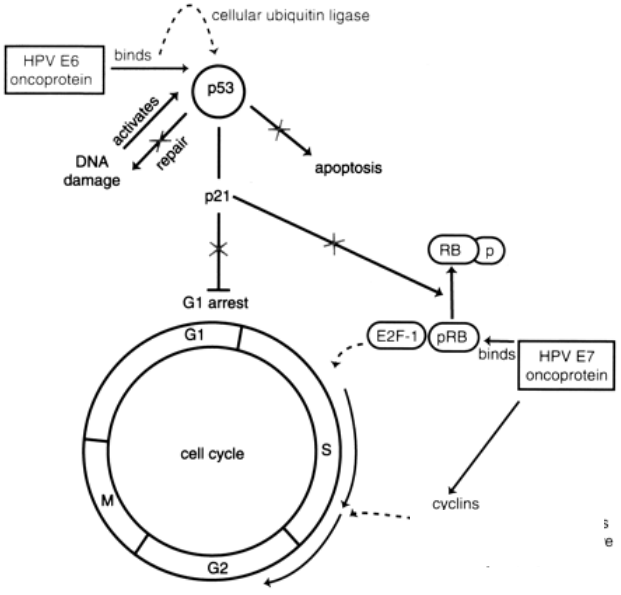

Figure 4: A summary of the functions of the E6 and E7 proteins

Structure

Papilloma virions are small nonenveloped virions approximately ~55nm in diameter. They contain two capsid proteins, the major capsid protein L1 and the minor capsid protein L2. The outer shell of the virion contain 360 molecules of the major capsid protein L1, arranged as 72 pentamers or capsomers in a T = 7 icosahedron lattice where sixty of the pentamers are hexavalent and 12 are pentavalent (Baker *et al.*, 1991). The minor capsid protein L2 is associated with these 12 vertex capsomers in a ratio of 12 molecules of L2 to 360 (72 pentamers) molecules of L1 (Kirnbauer *et al.*, 1993). Each capsomer contains five monomers of L1, which is the most conserved of the papillomavirus proteins. Structural analysis of papillomavirus particles has been limited by an inability to grow large amounts of virus in culture and by a lack of success in obtaining suitable crystals of virions purified from warts (Chen *et al.*, 2000). The capsid proteins will, when

expressed, self-assemble to form virus like particles (VLPs) i.e. particles morphologically indistinguishable from the authentic virions but lacking the potentially oncogenic viral genome (Kirnbauer *et al.*, 1992; Zhou *et al.*, 1991).
The secondary structure of L1 was determined from the crystal structure of N-terminal truncated HPV16 L1. The N-terminal region of the L1 (residues 1-27) and the β-sheets and helices h3, h4 and h5 are highly conserved whereas the C-terminal region is very variable (Chen *et al.*, 2000).

The L1 monomer (figure 5a) folds in a jelly roll sandwich that is made up of the residues 20 – 382 (of 504 residues) with helices on the C-terminus. The C-terminal lateral projections (residues 383 – 475) are significantly α-helical. Three helices (h2–4) form the surface of contact with other monomers, while a short strand and a final helix (h5) anchor the projection back to the jelly roll (Chen *et al.*, 2000). The C-terminal arms form the major interpentamer contacts, by extending away from their subunits of origin and invading a subunit in the adjacent clockwise pentamer. The h5 helix anchor the C-terminal arms back to the jelly roll (Modis *et al.*, 2002). This interaction is termed invading arm since each pentamer receives five invading arms. Several amino acids in this C-terminal region are divergent among HPV types and may be important for recognition by type-specific antibodies (Modis *et al.*, 2002).
The outer surface of each L1 pentamer (figure 5b) has five broad pockets, created by the hypervariable BC-, EF-, and FG-loops. While the rim of the L1 pocket is extremely variable, the floor is somewhat more conserved. These pockets are all putative receptor pockets (Chen *et al.*, 2000). The crystal structure of HPV16 L1 pentamers shows that the hypervariable loop domains extend toward the outer surface of the capsid. Regions within the FG- and HI-loop have been proposed as receptor sites for the monoclonal antibody H16.V5, which is a monoclonal antibody that reacts with a major neutralizing epitope on HPV16. It has been demonstrated that hybrid VLPs that have transplanted the FG- and HI-loops into the HPV11 L1 protein have gained significant binding of the H16.V5 antibody (Christensen *et al.*, 2001). The loops are elaborately intertwined: the HI loop of one monomer extends outwards and inserts between the FG and EF loops of the

anticlockwise neighbour. The five points of the star-shaped cap of the pentamer are created by part of the EF loop projecting outwards to the edge of the pentamer.

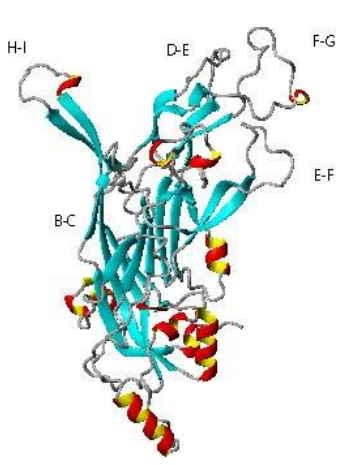

Fig. 5a

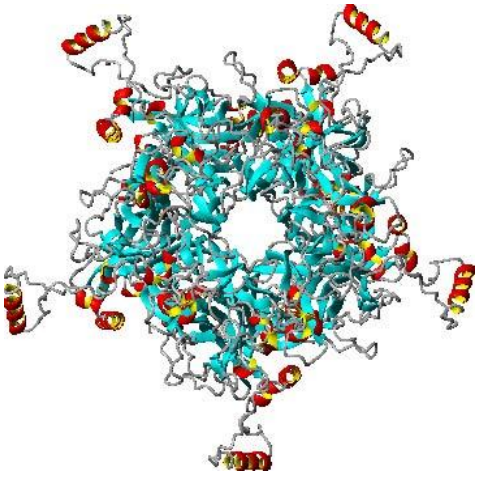

Fig. 5b

Figure 5: a) HPV16 L1 Monomer and b) HPV16 L1 Pentamer (Graphic was created in Molmol 2k.2)

Cell surface receptors

Very little is known about the initial steps in papillomavirus uptake. Although papillomaviruses can bind to a variety of cell types their productive tropism is generally limited to keratinocytes and fibroblasts (Muller *et al.*, 1995). Some time ago a α6-integrin was proposed as the epithelial cell receptor for HPV6. This was based on the observation that laminin, a substrate for α6-integrin receptors, was effective in preventing binding of HPV6 VLP to the α6-integrin (Evander *et al.*, 1997). Further studies revealed that α6-integrin was not the obligatory receptor for papillomaviruses (Giroglou *et al.*, 2001a; Shafti-Keramat *et al.*, 2003; Sibbet *et al.*, 2000). Cell surface glycosaminoglycans (heparin, heparan sulphate etc) have been found to be of importance and are primary candidate receptors for papillomaviruses (Drobni *et al.*, 2003; Giroglou *et al.*, 2001a; Joyce *et al.*, 1999). There are seven types of glycosaminoglycans: heparin sulfate; chondroitin sulfate A, B and C; hyaluronic acid; and keratin sulfate. Some of the glycosaminoglycans are highly sulfated and they are found on the cell surface, extracellular matrix and intracellular vesicles. For viruses, heparan sulfate seems to serve as primary receptor (initial attachment receptor) but it is believed that a secondary receptor is needed to facilitate internalization (Drobni *et al.*, 2003; Shafti-Keramat *et al.*, 2003). Joyce *et al* suggested that the C-terminus 15 amino acids are responsible for the interaction with the heparan sulphate and identified a conserved region within the last 15 amino acids of most HPVs that could conceivably serve as or part of a heparin sulfate-binding motif (Bousarghin *et al.*, 2003; Joyce *et al.*, 1999). However, this has later been questioned (Giroglou *et al.*, 2001a). More recent results by Rommel *et al* demonstrate that the interaction between the papillomavirus capsid and heparan sulfate requires an intact outer surface structure (Rommel *et al.*, 2005).

HOST CONTROL OF HPV INFECTION

The natural first line of defence against any pathogen is provided by the skin and mucosal surfaces through the innate immunity. The second line of defence is specific (acquired immunity) for each pathogen and comprises of humoral immunity and cell-mediated immunity. The time required for clearance of the high-risk HPV types, particularly HPV16, averages 8–14 months, whereas the low-risk HPV types only require 5–6 months (Stanley, 2006a). Why the immune system ignores or fails to detect a HPV infection for so long is a central question. This could be due to several structural and physiological characteristics of papillomaviruses: i) HPV is a double-stranded DNA virus with no RNA intermediate to stimulate an innate immune response, ii) during the early phase of infection, HPV primarily produces non-secreted nuclear proteins that are not cross-presented by infected cells, iii) most HPV proteins are expressed at very low levels in the basal epithelium, which is not very accessible by the host immune system, iv) HPV infects only cells of the skin and does not induce cell death, a major activator of innate and acquired immunity and v) systemic HPV antigen presentation is limited (Frazer, 2007). HPV infections are characterized by a very high rate of spontaneous clearance. Several studies have been consistent in their estimates of a 70 percent clearance rate during a 12-month follow-up. After 18 months there is a 80 percent clearance and after 5 years the clearance rate is 90 percent (Dillner & Brown, 2004). How the infected individual clears the infection is not fully understood but it is believed that cell-mediated responses by the host are important aspects in controlling the infection. Antibodies against HPV are type-specific and are effective for prevention of infection while T-cell responses are involved in the clearance of established infections (Dillner *et al.*, 2007; Frazer, 2004).

The humoral response

Serological assays using VLPs indicate that a high proportion of individuals that are exposed to HPV develop systemic antibodies to the L1 major capsid protein (Carter *et al.*, 2000; Carter *et al.*, 1996; Ferguson *et al.*, 2006; Kirnbauer *et al.*, 1996). The intensity of the antibody response depends on viral load and persistence (Ho *et al.*, 2004). Antibody responses specific for the L1 major capsid protein develop approximately four months to five years after the first infection although 10 to 20 percent of infected individuals develop antibodies at the same time that HPV DNA is first detected (af Geijersstam *et al.*, 1998; Andersson-Ellstrom *et al.*, 1996; Andersson *et al.*, 2001; de Gruijl *et al.*, 1997; Lehtinen *et al.*, 1996). About half of the women who test positive for HPV DNA or have HPV associated lesions do not have detectable type-specific anti-HPV antibodies (Carter *et al.*, 1996; Dillner *et al.*, 1995; Eisemann *et al.*, 1996). The major isotypes of serum antibodies against HPV capsids are IgG1 and IgA. Other IgG subclasses are only occasionally detected (Wang *et al.*, 2000). The serum IgA response is also HPV type-specific and correlates mainly with the recent number of sexual partners, suggesting that the IgA response may be a marker of recent or active infections (Wang *et al.*, 2000). Secretary IgA antibodies to HPV capsids are detectable in cervical mucus and it has been demonstrated that cervico-vaginal lavage fluid harvested from immunized primates neutralizes HPV11 particles (Bontkes *et al.*, 1999; Lowe *et al.*, 1997; Rocha-Zavaleta *et al.*, 2003; Wang *et al.*, 2000). IgG is the principal immunoglobulin in cervical secretions but systemic IgG levels are substantially higher than those in cervical secretions. The assumption at present is that protection is mediated by serum IgG (predominantly neutralizing IgG) that can transude across the cervical epithelium in high enough concentration to bind to the virus particles and prevent an infection (Nardelli-Haefliger *et al.*, 2003). It is possible that potential sites of infection on cutaneous and mucosal epithelial surfaces may have access to systemic antibodies. This situation may be possible due to the fact that establishment of infection requires that the virus come into direct contact with keratinocytes in the basal layer of the epithelium. The epithelial microtrauma that can occur during intercourse,

increases the likelihood of such exposure and possible access to systemic IgG (Stanley *et al.*, 2006). During natural infection antibody levels are generally stable for several years (af Geijersstam *et al.*, 1998).

Studies in animal models have shown antibody mediated protection against animal papillomavirus infection when VLPs were used as antigens (Roden *et al.*, 2004). Antibody neutralization of papillomaviruses has been proposed to occur by two distinct mechanisms: either blocking of cell binding receptor sites on the virus (presumably by steric interference) or by inhibition of uncoating (Booy *et al.*, 1998; Christensen *et al.*, 1995; Roden *et al.*, 1994). Difficulties in the propagation of stocks of infectious HPV in tissue cultures have hampered the examination of neutralizing antibody responses against authentic HPVs. Initially it was thought that the protection offered by neutralizing antibodies was only mediated by the neutralizing antibodies that recognize conformational epitopes (Breitburd *et al.*, 1995; Kirnbauer *et al.*, 1992). However, recent reports suggest that linear epitopes in some cases also can elicit neutralizing antibodies (Combita *et al.*, 2002; Roden *et al.*, 2000). Serological studies have demonstrated that antibodies cross-reactive with multiple HPV VLP types recognize type-common epitopes and are, in general, linear and non-neutralizing. Neutralizing antibodies are on the other hand highly type specific, despite the significant sequence conservation in the L1 genes of different genotypes, and not cross-neutralizing (Christensen *et al.*, 2001; Giroglou *et al.*, 2001b; Slupetzky *et al.*, 2001; White *et al.*, 1999). VLPs such as HPV6/11 (Orozco *et al.*, 2005), HPV31/33, HPV18/45 (Giroglou *et al.*, 2001b) and HPV16/31 (Fleury *et al.*, 2006) share one or more cross-neutralization epitopes although these seem to be less immunogenic than the type-specific epitopes (Combita *et al.*, 2002; Fleury *et al.*, 2006).

Antibodies specific to other types of papillomavirus antigens in patients with acute or persistent HPV infection or cervical cancer are less common. Antibodies to E7 develop at the onset of invasive cervical cancer, suggesting that anti-E7 antibodies may be useful as a marker for this malignancy (Baay *et al.*, 1995; Jochmus-Kudielka *et al.*, 1989)

The cellular immune response

Cell-mediated immune responses to HPV infection have also been intensively studied but are not completely delineated. Cellular immunity plays a key role in the control of HPV infection. It has been shown that spontaneous regression of HPV induced genital warts is associated with an infiltration of lymphocytes into the lesions (Coleman & Stanley, 1994). This has also been shown in animal models (Rudolf *et al.*, 1999; Wakabayashi *et al.*, 2002). Foreign viral antigens are recognized on the surface of virally infected cells in the form of short peptides bound to human leukocyte antigen (HLA) class I molecules These antigenic viral peptides are recognized by cytotoxic T lymphocytes (CTL) and trigger the secretion of different cytokines (Dillner *et al.*, 2007; Stanley, 2006a; Tindle & Frazer, 1994). The foreign peptides are often 8–10 amino acids long and are generated by the proteolysis of viral proteins in the cytoplasm of an infected cell. For CTL recognition of HPVs, it is envisaged that expression of the early HPV genes (E1–E7) within the cytoplasm of infected cells will generate HPV derived peptides that are able to bind to HLA class I molecules. HPV infected or HPV transformed antigen presenting cells of the epithelium then migrate to the draining lymph node, processing HPV antigens en route, and present antigens to naive T-cells in the node. The T-cells then differentiate into armed effector cells and migrate back to the infected site, and destroy the infected keratinocytes (Stanley, 2006a).

HPV VACCINES

For many years it was difficult to develop papillomavirus vaccines because in these viruses replication occurs only in differentiating epithelial cells, and thus, conventional method of growth was not efficient in cultured cells (Hagensee, 1993). Even if they did grow, to generate a killed or attenuated vaccine is not practical. An attenuated vaccine would potentially expose healthy people to viral oncogenes and, thus, not be acceptable. The breakthrough for prophylactic HPV vaccines came with the discovery that VLPs spontaneously assemble when the papillomavirus L1 protein is overexpressed in several different cell types, including mammalian, insect, and yeast cells (Hagensee, 1993; Kirnbauer *et al.*, 1992; Zhou *et al.*, 1991). Recombinant DNA technology was used to generate VLPs capable of mimicking the natural virus and eliciting high-titers of virus neutralizing antibodies (Breitburd *et al.*, 1995; Kirnbauer *et al.*, 1992; Lowe *et al.*, 1997; Suzich *et al.*, 1995).

VLP-based prophylactic vaccines

The aim of prophylactic vaccination is the generation of neutralizing antibodies against HPV L1 and/or L2 capsid proteins. (Brown *et al.*, 2004; Harper *et al.*, 2006; Koutsky *et al.*, 2002; Villa *et al.*, 2005). L1 VLP vaccines are delivered by intramuscular injection, which allows them to gain access to the draining lymphatics and small vessels at the vaccination site (Stanley *et al.*, 2006). Neutralizing antibodies elicited after vaccination will block the binding of virus to the cellular receptors, and thus prevent infection. Administration of either a quadrivalent HPV (types 6, 11, 16, 18) L1 VLP vaccine or a bivalent HPV (types 16, 18) L1 VLP vaccine in HPV naive women have demonstrated almost complete protection against persistent infection with the targeted HPV types (Harper *et al.*, 2006; Hildesheim *et al.*, 2007; Koutsky *et al.*, 2002; Villa *et al.*, 2005; Villa *et al.*, 2006). The various trials confirmed the safety of the vaccine and the generated antibody response was found to be greater than or equal to that observed during a natural infection and is maintained up to 4.5 years (Harper *et al.*, 2006; Olsson *et al.*, 2007; Paavonen *et al.*, 2007; Villa *et al.*, 2006). The type-

specific antibody response reach maximum titres at month 7, i.e. 1 month after administration of the third dose, and then decline until month 24 and remain stable thereafter (Dillner *et al.*, 2007). Recent mathematical modeling suggests that the antibody response for vaccines will remain at least above detectable levels in 50 percent of women for over 32 years, with a range of persistence from 12 years to near life-long (Fraser *et al.*, 2007).

Interestingly, the immune response to the quadrivalent HPV vaccine is inversely correlated with age. The antibody responses induced were higher in males and females aged 10 to 15 than those observed in 16 to 23 year olds (Villa *et al.*, 2005; Villa *et al.*, 2006). The demonstrated high efficacy found in these trials applies primarily to HPV naive women aged 16 to 26. For women in the general population, who are sexually active, efficacy is expected to be much lower. HPV vaccination does not accelerate clearance of the virus in already infected individuals and should not be used to treat prevalent infections (Hildesheim *et al.*, 2007). To achieve maximum effectiveness, vaccination will need to occur prior to the onset of sexually activity, which means vaccinating young adolescents. Some concerns have been raised about the propriety of vaccinating adolescent girls for a sexually transmitted infection. The fact that the HPV vaccines are likely to be perceived as cancer prevention vaccines also presents unique challenges since it may be difficult for parents to reconcile vaccinating 9–14 year old girls for a cancer that they are unlikely to develop for at least two to three decades. This problem is exacerbated by the fact that the duration of protection afforded by vaccination is not yet known (Wright *et al.*, 2006). Some of the more pressing issues that need to be resolved include who should be vaccinated, at what age should vaccination start and the implication of vaccination on current screening programs. Mathematical models that reflect transmission dynamics have been used to predict the impact of vaccination over time (Barnabas *et al.*, 2006; French *et al.*, 2007; Garnett *et al.*, 2006; Goldie *et al.*, 2003; Hughes *et al.*, 2002; Kulasingam & Myers, 2003; Lehtinen *et al.*, 2006; Taira *et al.*, 2004). For instance, French *et al.* found that vaccinating 12 year olds delays the prevention gains, compared to vaccinating older adolescents and implementing catch-up

vaccination will significantly contribute to infection control (French *et al.*, 2007). Barnabas *et al* found that vaccinating males as well as females has an added benefit over vaccinating females alone; however, successively smaller additional benefits were seen in scenarios with high population coverage (Barnabas *et al.*, 2006). Despite the use of different base models or assumptions the results show consistency in supporting the added benefit of papillomavirus vaccination. However, there is a theoretical concern for type replacement i.e. emergence of other HPV types as the predominant cause of disease after the reduction in the prevalence of HPV infection from the types included in the vaccine. Even though papillomaviruses are DNA viruses that are genetically very stable and have co-evolved with their specific vertebrate host over millennia, the possibility of replacement can not be completely ruled out (Stanley *et al.*, 2006). Viral type replacement can occur only if two conditions are fulfilled: i) there exists partial competition of different types during natural infection and ii) no cross-protection from the vaccine against types competed against. There exists some evidence to date as to why this scenario is unlikely. Data from vaccine efficacy studies have found varying degrees of cross-protection and results from several epidemiological studies to date indicate that the presence of type-specific antibodies for one HPV type is associated with a strongly increased risk for also being seropositive for other HPV types. Thus, the opposite of what is expected had there been competition. However, it should be noted that the studies investigating type competition have had limited statistical power (Dillner *et al.*, 2007).

The neutralizing antibodies elicited by VLPs are highly type-specific. Immunization with intact VLPs derived from heterologous genotypes do not offer protection (Breitburd *et al.*, 1995). Limited cross-reactivity can occur between L1s of different genotypes with > 85 percent amino acid sequence identity (Roden *et al.*, 1996). There are at least 15 oncogenic HPV genotypes and it has been suggested that they may represent an equivalent number of serotypes (Roden *et al.*, 1996; White *et al.*, 1998). However, some evidence has emerged suggesting that administration of HPV vaccines offers protection against

additional types of HPVs (HPV31 and 45, the third and fourth most common types of HPV associated with cervical cancer) not included in the vaccine. However, the clinical significance of cross protection for cervical lesions of any grade has not yet been established (Harper *et al.*, 2006). Creating a vaccine that would offer almost full protection against cervical cancer would require a highly polyvalent vaccine.

Therapeutic vaccines

Although vaccination with preventive HPV vaccines is able to generate high titres of serum-neutralizing antibodies in animals and humans such immunization may not be able to generate significant therapeutic effects for established infections that have escaped antibody mediated neutralization. Due to the considerable burden of HPV infections worldwide, it would take decades for preventive vaccines to affect the prevalence of cervical cancer. This is because of the long latency period from HPV infection through dysplasia to cervical cancer, and evidence that a prophylactic vaccine will be of little or no benefit to women already infected and on this pathway of disease. The aim of therapeutic vaccines is to reduce or eradicate existing disease or infections by targeting cells expressing tumour-associated or tumour-specific antigens on their surface (Roden *et al.*, 2004).

There are many different types of therapeutic vaccine candidates e.g. those based on viral gene-derived peptides and proteins, DNA, and various viral and bacterial vectors. They all aim to induce specific cell-mediated immunity and, in most cases, the targets are the E6 and E7 proteins. Therapeutic protein vaccines have generally proven safe and immunogenic although proof of significant clinical efficacy is less evident (Hung *et al.*, 2008; Rudolf *et al.*, 1999; Slupetzky *et al.*, 2001; Winters *et al.*, 2006). In theory the capsid component of a chimeric vehicle would induce a protective humoral response, while the foreign antigen would be delivered to and processed by antigen presenting cells and elicit a CTL response against HPV induced tumor cells. Efficient immunization using HPV VLPs

carrying foreign antigens has been demonstrated in several systems, e.g. E6/E7, melanoma antigens and human immunodeficiency virus antigens (Liu *et al.*, 2000; Nieland *et al.*, 1999; Sadeyen *et al.*, 2003). Peptide vaccines have the advantages of safety and ease of production; however, their weak immunogenic properties and the need for HLA matching must be overcome. Peptide fragments of the E6 and E7 proteins have been tried out as a vaccine and were found safe and relatively immunogenic. Since 40 percent of caucasians carry the HLA-A2 alleles, the peptides presented by this allele have been the most widely studied (Muderspach *et al.*, 2000; Ressing *et al.*, 2000; Zwaveling *et al.*, 2002). DNA based vaccine approaches often focus on enhancing presentation of HPV antigens via the HLA class I or HLA class II pathways (Brinkman *et al.*, 2007). Many candidate vaccines with therapeutic potential that use a variety of delivery systems have been trialed and are the subject of ongoing trials; there are however low expectations that any of the current therapeutic vaccines will have a substantial public health impact in the near future.

PROJECT OBJECTIVES

Paper I

To provide more detailed knowledge of the immunodominant type-specific epitope structure and characterize VLPs deficient for the H16.V5- defined epitope for CTL inducing ability.

Paper 2

To explore the impact of different vaccination policies in Sweden.
To perform an age-specific HPV16 seroprevalence survey in Sweden.
To illustrate a strategy by which any county with access to suitable serum biobanks can devise a scientifically based vaccination policy.

Paper 3

To assess the sex- and age-specific cumulative incidences of HPV6, 11, 16, 18, and 52 in Sweden.

Paper 4

To evaluate the hypothesis that circumcised men are less likely to acquire HPV16 and 18 and/or HPV6 and 11.

SUMMARY

Paper I

Effective prophylactic vaccines are based on type-specific neutralizing antibodies. A major neutralizing epitope is defined by the monoclonal antibody H16.V5. We have investigated the importance of this V5-defined immunodominant type-specific epitope for overall immunogenicity of HPV16. HPV16 virus-like particles devoid of the H16.V5 epitope was engineered by site-directed mutagenesis of 10 non-conserved, surface exposed residues. We have designed a strategy that successfully removed this major type-specific neutralizing epitope. Removal of the V5-defined epitope had only marginal effect on antigenic reactivity with antibodies in sera from infected subjects, but affected immunogenicity in experimental immunization of mice, with reduced induction of both antibody responses and CTL responses. These findings may have important implications for the continued design of VLP vaccines as well as for monitoring of functional antigenicity of VLP preparations and associated antibody responses.

Paper 2

The availability of effective HPV vaccines necessitates investigations of the vaccination strategies that would have the greatest effectiveness. Basic questions that need to be addressed are: at what age should subjects be vaccinated? What is the benefit of a catch-up program and what age groups should it target? Should both males and females be targeted? To address these questions the effects of vaccination in populations, in addition to individuals, has to be estimated. This is possible through the use of models of the transmission dynamics of the infection and disease. The detection of serum IgG antibody to HPV provides a straightforward method for assessing cumulative HPV exposure, as many countries routinely perform serosurveys and/or have comprehensive serum repositories that can be exploited. We have performed a serological survey of HPV16 antibody prevalence by age and sex in Sweden and used it as a basis for modeling the optimal vaccination strategies in this population. We illustrate a strategy by which any county with access to suitable serum biobanks can devise a scientifically based vaccination policy.

Our data suggest that little exposure to HPV16 occurs before early adolescence and generally that the HPV seroprevalence were higher among females than among males. The observed data from the Swedish model argue that a vaccination program will have the greatest impact in the long run if it targets 12 year old females. Strategies involving vaccination of males as well as females can prevent at least 99.5 percent of infections, approaching extinction of infection, but at the cost of approximately doubling the required numbers to vaccinate. To speed up impact, catch up vaccinations are important and yield comparatively large additional health benefits at least up to 18 years of age. Pre-vaccination HPV seroprevalence surveys in different countries could thus be of interest to design optimal vaccination strategies. Furthermore, data on the pre-vaccination age-specific spread of HPV infections may help evaluating the effect of the programs.

Paper 3

Investigating the transmission properties of sexually transmitted infections is important for designing preventive strategies. We have determined the age-dependent seroprevalence of HPV6, 11, 16, 18, and 52 infections, using a multiplexed luminex assay, in a representative Swedish population aged 9 to 26. The findings document that HPV6, 11, 16 and 18 infection is high among 16 to 26 year olds. Analyses of age-specific prevalence revealed different patterns for high- and low-risk HPV infections between females and males. Among the female population HPV16 is seen to accumulate most rapidly with a steady increase, followed by HPV6 and 11. Among males HPV6 accumulates most rapidly followed by HPV16 and 11. HPV6 and 11 peak around age 19 and then decline sharply. Our data offer an additional viewpoint on the epidemiology of vaccine related HPV types in Sweden. This data can also be used in simulation models in which different vaccination and screening strategies can be compared.

Paper 4

Circumcision has been reported to protect against infection with human papillomavirus in men, but results have been inconsistent, with one of the suggested reasons being variability in methods for taking genital samples from men. Serological assays of HPV have been extensively validated as a marker of cumulative HPV exposure. Using serology has advantages over DNA sampling for detecting cumulative exposure to HPV, because it also shows the effect of HPV exposures in the past, whereas detection of DNA reveals only current infection. Moreover, sampling of serum is readily standardized and reflects the exposure of the subject, without bias related to the exact bodily location sampled. HPV serology, however, will underestimate the total number of men who have ever been infected with these HPV types. We have tested the hypothesis that circumcised men are less likely to acquire HPV16 and 18 and/or HPV6 and 11. Our finding of no relationship between circumcision and seroprevalence of HPV6 and 11, which commonly cause genital warts, is consistent with three large population based cross-sectional studies in the USA, United Kingdom and Australia that all found no relationship between circumcision and self reported genital warts. However, a small protective effect cannot be ruled out

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vad är HPV och livmoderhalscancer?

Livmoderhalscancer (cervixcancer) uppstår i den del av livmodern som sticker ner i slidans övre del. Där möts två olika slemhinnor och i skarven kan en mycket vanlig virusinfektion ibland orsaka cellförändringar som i sällsynta fall kan utvecklas till cancer. Livmoderhalscancer är en vanlig sjukdom globalt, och är den näst vanligaste orsaken till cancerdöd bland kvinnor. I Sverige har förekomsten minskat kraftigt under de senaste årtiondena. Varje år diagnostiseras knappt 500 fall, cirka hälften så många som i början av 70-talet. Minskningen anses främst bero på att man med gynekologisk cellprovskontroll kan upptäcka och behandla förstadier till sjukdomen. Livstidsrisken för en kvinna i Sverige att få livmoderhalscancer var före införande av gynekologisk cellprovskontroll 2 procent, men är idag ungefär 0.5 procent.

Den viktigaste orsaken till livmoderhalscancer är att man någon gång har smittats av ett vanligt sexuellt överförbart virus, som kallas humant papillomvirus eller HPV. Det finns mer än hundra olika typer av detta virus som kan uppträda på olika ställen i kroppen. En del av dem klassas som lågrisktyper t ex papillomavirus 6 och 11, och är inte cancerframkallande. Lågrisktyperna kan orsaka vårtor i underlivet, så kallade kondylom. Dessa sjukdomar leder inte till utveckling av cancer. Högrisktyper med namn som papillomavirus typ 16 och 18 återfinns nästan alltid i fall av livmoderhalscancer. De ger inga synliga förändringar och orsakar inga symtom. Det bör också nämnas att humant papillomvirus också kan orsaka flera andra cancerformer, bl a i penis, anus, vagina och vulva.

Av alla som blir smittade är det endast en bråkdel som drabbas av cancer. Oftast bekämpas viruset av immunförsvaret och infektionen läker ut. Vissa kvinnor kan dock få en bestående infektion som ibland kan leda till cellförändringar och cancer. Det tar i medeltal ungefär 20 år från infektion till utveckling av cancern.

Livmoderhalscancer är ovanlig hos kvinnor som är yngre än 25 år, vanligast är sjukdomen hos kvinnor i åldrarna 40 till 45 år och över 70 år.

Hur kan man skydda sig?

HPV överförs bl a genom vaginalt, oralt och analt sex. De kulturella normerna för sexuellt beteende (fler personer har sex vid yngre ålder och med flera olika sexpartners) verkar ha lett till en ökad förekomst av HPV-infektioner. Eftersom symptomen kanske visar sig sakta eller inte alls kan smittade personer sprida HPV utan att de känner till det. Alltså är preventivmedel som kondom att rekommendera för att motverka att sjukdomen sprids. Kondom erbjuder dock inte absolut skydd. Livmoderhalscancer går också att förebygga i hög utsträckning med gynekologisk cellprovskontroll, där alla kvinnor mellan 23 och 60 år kallas regelbundet.

Idag finns förebyggande papillomavirusvacciner som förhindrar infektioner som kan ge upphov till cancer. Dessa bör tas före sexualdebuten och skyddar mot de vanligaste virustyperna som kan orsaka livmoderhalscancer. De vacciner som finns idag består av s k viruslika partiklar, d v s virusprotein utan arvsmassa (benäms som VLPs i avhandlingen). Immunologiskt fungerar dessa partiklar som virus och har i både djurstudier samt patientprövningar gett ett mycket bra skydd. Ca 70 procents skydd mot cancer får personen som inte redan är infekterad. Den som redan har eller har haft HPV får ingen, eller mycket liten, effekt av vaccinet.

Flera forskargrupper gar konstruerat modeller som visar på goda möjligheter att uppnå skyddseffekt på populationsnivå, men det finns osäkerheter i beräkningarna; t.ex. måste modellerna anpassas till de olika HPV-infektionernas förekomst i olika länder. Räcker vaccination av målgruppen eller behövs också hög täckning i grupper där ett litet antal individer har ett mycket stort antal sexuella kontakter?

Vad har min avhandling visat?

Cancervacciner kan delas in i två huvudgrupper: förebyggande och terapeutiska, det vill säga behandlande. De förebyggande vacciner som finns på marknaden idag skyddar mot infektioner som orsakas av HPV av de typer som vaccinet innehåller. Vaccinet erbjuder bara skydd mot den specifika papillomavirustypen som man immuniserade mot. Detta beror på att på ytan av viruset så finns specifika områden som immunförsvaret känner igen och kan binda till. Syftet i det första arbetet var att definiera och karaktärisera de olika områdena som immunförsvaret kan tänkas reagera på. Mer kunskap inom detta underlättar för vaccinforskningen.

Förekomsten av HPV vaccin gör det nödvändigt att undersöka olika vaccinationsstrategier. Grundläggande frågor som bör besvaras med forskning är:

i) Vid vilken ålder bör man vaccinera individer?
ii) Vad finns det för fördelar med uppföljningsvaccinationer och vilka åldersgrupper bör de omfatta?
iii) Bör både pojkar och flickor vaccineras?

I arbete 2 fördjupar vi oss i dessa frågor. Vi undersökte förekomsten av av den absolut vanligaste cancerframkallande papillomavirustypen hos 9-26 åringar i Sverige m h a blodprover från Smittskyddsinstitutet samt södra Sveriges Mikrobiologiska Biobank. Dessa resultat användes som bas för att matematiskt kunna modellera optimala vaccinationsscenarion i Sverige. Sett över en tidsperiod på 50 år visade det sig vara mest effektivt att börja vaccinera 12-åriga flickor. Att vaccinera även pojkar gav en komplett skyddseffekt nästan 20 år tidigare. Generellt illustrerar vi en strategi hur olika länder med tillgång till blodprover från biobanker, med enkla medel vetenskapligt kan designa en vaccinationspolicy.

I arbete 3 valde vi att fördjupa oss ytterligare och gjorde en liknande immunitetslägesundersökning som i arbete 2. Vi undersökte förekomsten i Sverige av totalt 5 olika papillomavirustyper som förekommer både i kondylom och olika cancerformer. Analysen av den ålders-specifika förekomsten av

papillomavirus uppvisade olika mönster för infektion för hög- samt lågrisk typer hos män och kvinnor. För att göra kvalitativa beslut om prevention och vaccination så behövs det detaljerad information om förekomst och åldersdistribution i en population. Alltså är den här typen av pre-vaccination prevalensundersökning högintressant för myndigheter som bestämmer vaccinationsprogram i Sverige och utformar program för uppföljning av vaccinationsprograms effekt.

Det finns en del forskningsresultat som visar att omskärelse av män minskar risken för peniscancer hos männen och livmoderhalscancer hos deras partner. Omskärelse har rapporterats att skydda män mot papillomvirusinfektion. Dock har resultaten varit motsägelsefulla. I arbete 4 har vi följt omskurna män i Nya Zealand under 30 år. Vi har korrelerat förekomsten av papillomavirus infektion med deras sexuella- samt socioekonomiska bakgrund. Vi fann inte att omskärelse reducerade risken för papillomvirusinfektion, även om en liten skyddseffekt inte kan uteslutas. Baserat på nuvarande information är det svårt att förespråka manlig omskärelse som skydd mot papillomavirus-relaterade cancertyper.

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