Charles University in Prague

Gene Immunotherapy of Cancer: DNA Vaccines against HPV 16

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PhD. Thesis

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Prague, 2010

The submitted PhD. thesis was preformed in the Department of Experimental Virology, Institute of Hematology and Blood Transfusion in Prague and was supported by grants no. MZ0UHKT2005 and NR9246-3/2007 from the Ministry of Health of the Czech Republic, grants no., GD310/05H533, GD521/05/2092 and GA521/06/0973 from the Czech Science Foundation.

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning.

LIST OF ABBREVIATIONS

Aa = amino acid

Ab = antibody

Ag = antigen

APC = antigen presenting cell

APM = antigen-processing machinery

CC = cervical cancer

cFLIP = cellular FLICE-inhibitory protein

CIN = cervical intraepithelial neoplasia

CMV = human cytomegalovirus

CRT = calreticulin

CTL = cytotoxic T lymphocyte

CTLA-4 = cytotoxic T-lymphocyte antigen-4

DAC = 5-aza-2'-deoxycytidine

DC = dendritic cell

DNA = deoxyribonucleic acid

DNMT = DNA methyltransferase

EP = electroporation

ER = endoplasmic reticulum

FA = Freund adjuvant

FasL = Fas ligand

FrC = Fragment C of tetanus toxin

GM-CSF = granulocyte-macrophage colony-stimulating factor

GUS = *E.coli* β-glucuronidase

HIV = Human Immunodeficiency Virus

HPV =human papillomavirus

HR = high-risk

HSIL = high grade squamous intraepithelial lesions

Hsp = heat shock protein

ICS = intracellular cytokine staining

IFN = interferon

i.d. = intradermal

IL = interleukin

IRF = interferon regulatory factor

i.m. = intramuscular

KLH = keyhole limpet hemocyanin

LAK cells = lymphokine activated killer (LAK) cells

LAMP-1 = lysosome-associated membrane protein-1

LC = Langerhans cells

LCR = long control region

LN = lymph nodes

LR = low-risk

LSIL = low grade squamous intraepithelial lesions

MHC CIITA = Class II, major histocompability complex, transactivator

MIP-1α = macrophage inflammatory protein 1α

MSC = myeloid suppressor cell

NCR = non-coding region

NMR = nuclear magnetic resonance

ORF = open reading frame

pDNA = plasmid DNA

PI3K = phosphatdylinositol 3'-kinase

PMED = Particle Mediated Epidermal Delivery

pRb = retinoblastoma protein

PV = papillomavirus

s.c. = subcutaneous

SIL = squamous intraepithelial lesions

SCT = single chain trimer

SS = signal sequence

TAA = tumour-associated antigen

TAM = tumour-associated macrophages

TBK 1 = TANK-binding kinase-1

TCR = T cell receptor

TGF = tumour growth factor

TIL = tumour-infiltrating lymphocytes

TLR = Toll-like receptor

T_{reg} = regulatory T cells

TSG = tumour suppressor gene

VLP = virus-like particle

wt = wild-type

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1. PREFACE

Cervical carcinoma represents the second most frequent cancer in women. Today it is well established that cervical tumours are mostly associated with human papillomavirus (HPV) infection. More then 95 % of cervical cancer biopsies contain high-risk HPV genomes (zur Hausen 2009). Infections with certain genotypes of HPV can lead to other anogenital and head and neck cancers or can cause benign warts. However, 91% of HPV-related cancer deaths, on the global scale, are due to cervical cancer (Cutts *et al.*, 2007). Nowadays, two prophylactic vaccines, protecting against HPV 16 and HPV 18, are licensed. Nevertheless, development of therapeutic vaccines is desirable to eliminate current HPV infections and to treat progressing tumours.

Immunotherapy has become a common approach in cancer treatment due to advances in understanding cellular and molecular mechanisms of the immune system. One of the several strategies of immunotherapy is the induction of antigen-specific immune responses. As a source of antigens may serve vaccines based on vectors, dendritic cells, peptides and DNAs. The discovery of DNA immunisation in the early 1990s (Wolff *et al.*, 1990; Wang *et al.*, 1993; Ulmer *et al.*, 1993) brought new options into immunotherapy of cancer. The novel vaccines based on DNA carry the genetic material that encodes an antigen, rather than the antigen itself. Moreover, the administration of the DNA vaccines leads to the induction of both humoral and cellular immune responses (Coban *et al.*, 2008). The immunogenicity of DNA vaccines is well established in animal models. Several DNA plasmid products are licensed for veterinary application. Unfortunately, the immunogenicity of DNA vaccines in large animals and particularly in humans is significantly lower. New strategies developed to improve the immunogenicity of DNA vaccines show that DNA immunization can indeed induce antigen-specific immune responses in humans. Clinical studies with DNA vaccines against HBV, influenza virus, malaria, HIV-1 and various cancers were reported (Lu *et al.*, 2008). DNA vaccines have emerged as an attractive form also for therapeutic treatment of HPV-associated lesions. The ideal targets of therapeutic HPV vaccines are the viral E7 and E6 oncoproteins that are essential in cellular transformation and constitutively expressed in malignant cells. Currently, several ongoing studies (also clinical trials) are focused on strategies enhancing the efficacy and safety of DNA vaccines against HPV.

Objectives of the PhD. Thesis:

- The enhancement of immunogenicity of DNA vaccines against human papillomavirus type16 E7 and E6 oncoproteins: construction of plasmid DNA carrying *Escherichia coli* β-glucuronidase-fused E7 or E6 genes

- Modifications of the viral E6 oncogene in order to increase the production of the full-length E6 protein and to decrease its oncogenicity

- Observation of immune responses induced after administration of the prepared plasmid DNA constructs

- Evaluation of the efficacy of the E7- and E6-derived peptide vaccines delivered with a tattoo device

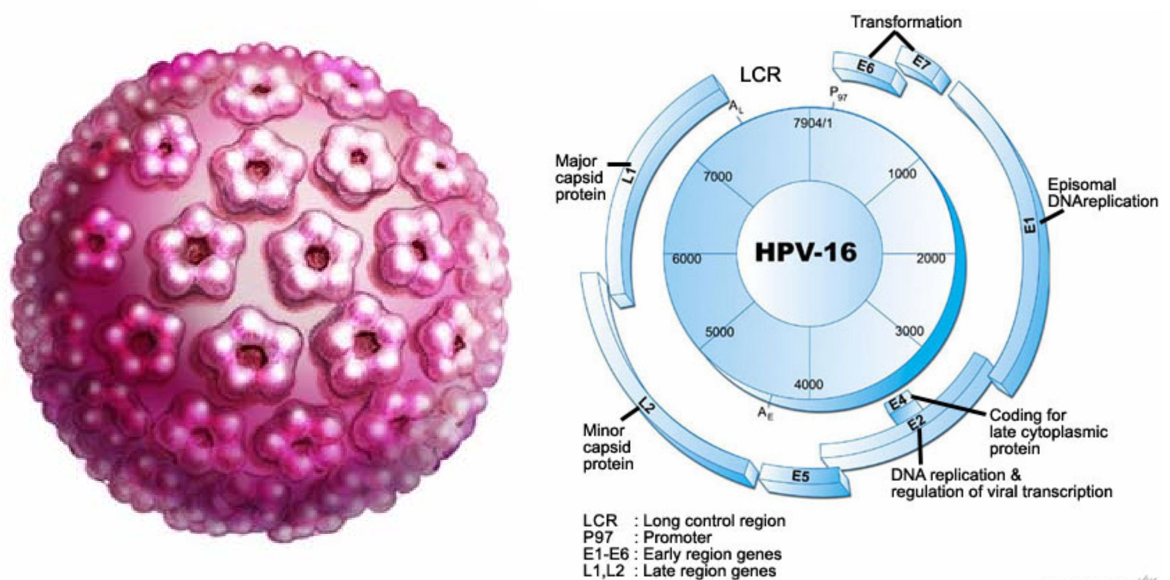
2. INTRODUCTION

2.1 Human papillomavirus

2.1.1 Genome structure and replication

Papillomaviruses (PVs) are a group of small non-enveloped icosahedral DNA tumour viruses with a virion size of ~55 nm in diameter and 72 capsomers (Fig.1). They form a distinct taxonomic family, the *Papillomaviridae.* All papillomaviruses contain a double-stranded, circular DNA genome approximately 8 kb in size that is generally divided into three major regions: early, late and a long control region (LCR or non-coding region [NCR]). The early region encodes six common open reading frames (ORFs; E1, E2, E4, E5, E6 and E7) that translate to individual proteins. Two other ORFs, E3 and E8, were also assigned to this region initially, but only the E8 (in bovine PV-1) has been proven to encode a protein. The late region lies downstream of the early region and contains L1 and L2 ORFs for translation of major L1 and minor L2 capsid proteins. The LCR region does not encode any protein, but bears the origin of replication and multiple transcription factor binding sites.

Figure 1. Model of papillomavirus capsid and genome of HPV 16



Papillomaviruses replicate and assemble exclusively in the nucleus of keratinocytes. Viruses infect the basal layers of a squamous epithelium and are in the phase of latent infection whereby the viral genome is maintained episomally at a low copy number without production of virions. The viral gene expression leads only to the expression of six non-structural viral regulatory proteins from the early region in the undifferentiated or intermediately differentiated keratinocytes. The function of these proteins is summarised in Table 1. In the upper layer, as the filial cells move towards the surface and undergo differentiation, two structural viral capsid proteins are expressed from the late region in terminally differentiated keratinocytes. Finally, the virions are released from the uppermost layer to search for new host cells (Fig. 2).

Papillomavirus DNA is frequently found to be integrated into host chromosomes in cervical cells. The integration sites are not distributed to hotspot areas and there is no evidence of insertional mutagenesis. Viral integration occurs downstream of the early genes E6 and E7, often in the E1 and E2 region; this disruption results in a loss of negative-feedback control on E6 and E7 oncogene expression by the viral regulatory E2 protein (Zheng & Baker, 2006; Woodman *et al.*, 2007; Yugawa & Kiyono, 2009).

Table 1. Papillomavirus proteins and their function

Protein	Functions
Early	
E1	Viral DNA replication, ATP dependent DNA helicase, DNA-dependent ATPase
E2	Viral DNA replication, regulation of transcription of viral genes
E3	Not known
E4 (late)	Disruption of cytokeratin filament network, virus maturation
E5	Transforming activity, downregulation of MHC class I expression
E6	Viral oncoprotein inducing cell immortalisation and transformation, binding to p53 protein
E7	Viral oncoprotein inducing cell immortalisation and transformation, binding to pRb protein
E8	Not known
Late	
L1	Major capsid protein: can form virus like particles
L2	Minor capsid protein: possible DNA packaging protein

2.1.2 Pathogenesis and epidemiology

Papillomaviruses infects various animals from birds to mammals, including humans. They are highly host-specific and are not transmissible between species. PVs are classified into genotypes based on the sequence of L1 ORF with more than 10% of difference. Up to the last year more than 100 different genotypes just of human PVs (HPVs) have been identified and sequenced. They are also strictly tissue-specific: they infect only epithelial cells of the skin and mucosa.

Although PV infections usually result in benign lesions, HPV infection may progress to the development of malignant lesions. According to their ability to induce malignancy, HPVs are classified as non-oncogenic low-risk (LR) or oncogenic high-risk (HR) types (Table 2). The human genital tract may be infected by about 40 HPV genotypes.

Table 2. Human papillomavirus genotypes

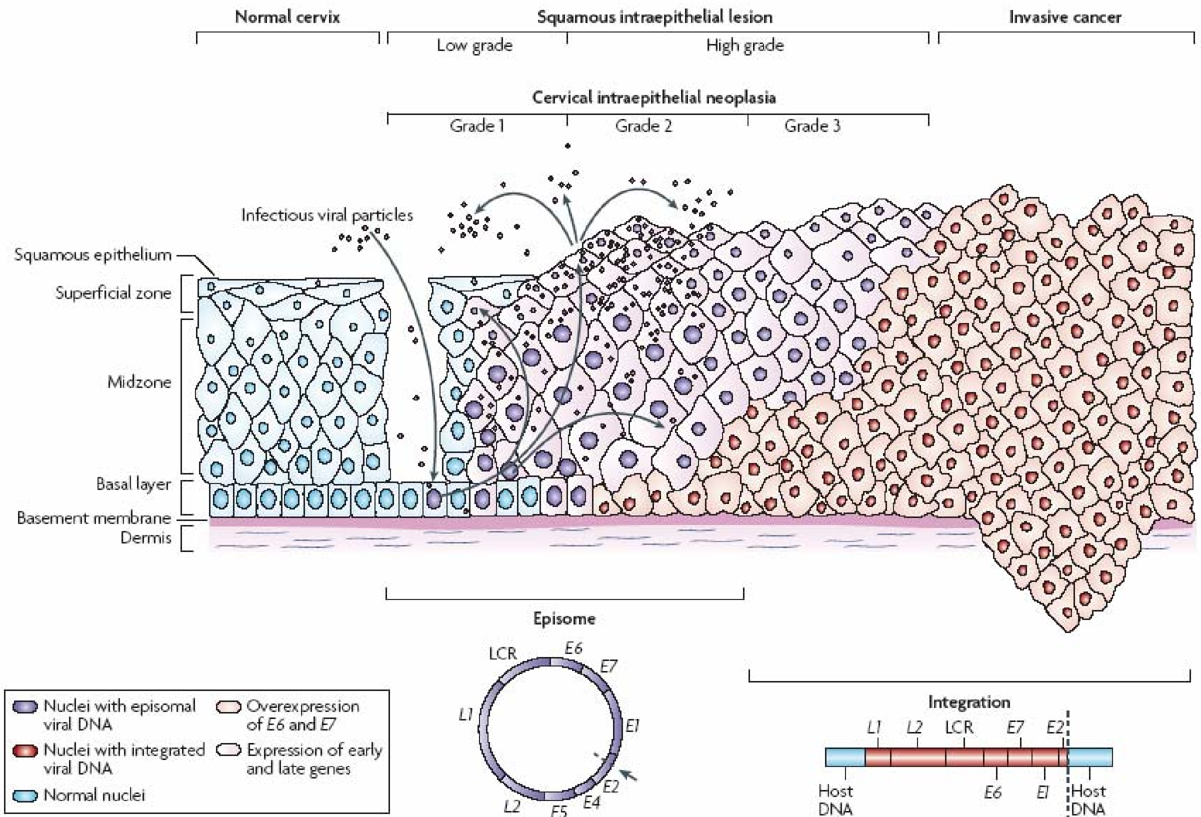
	Genotypes
High-risk HPVs	16,18,31,33,35,39,45,51,52,56,58,59,68,73,82
Probably high-risk HPVs	26,53,66
Low-risk HPVs	6,11,40,42,43,44,54,61,70,72,81,cand89

Genital HPVs are primarily transmitted by genital skin-to-skin contact, usually, but not necessarily, during sexual intercourse. High-risk HPV genotypes can lead to cervical cancer (CC) and are associated with other mucosal anogenital and head and neck cancers. Infections with LR HPVs can cause benign or low-grade cervical tissue changes and genital warts (*condylomata accuminata*), which are growths on the cervix, vagina, vulva and anus in women and the penis, scrotum or anus in men.

Cervical cancer is the second most common cancer among women worldwide affecting approximately 500 000 women each year with mortality rate about one-third of these cases. There are over one thousand new cases of cervical carcinoma only in the Czech Republic. Most women are infected shortly after beginning their first sexual relationship. In young women the infection is mostly asymptomatic, with only mild changes in the epithelium, and transient. In serious cases, the ongoing virus replication may induce abnormal growth of squamous cells called squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade (Fig.2). Cervical intraepithelial neoplasia (CIN) is a term for abnormal cells in the cervix; grades from 1 to 3 describe the proportion of thickness of the cervical epithelium.

The final stage of the pathological process is CC. The most common types of HPV detected in LSIL are the HPV 16 (26%), 31 (12%) and 51 (11%) (Cutts *et al.*, 2007; Vonka & Hamsikova, 2007; Woodman *et al.*, 2007; Tachezy & Rob, 2007).

Figure. 2 HPV-mediated progression to cancer



Woodman *et al.*, Nat Rev Cancer. 2007; 7:11-22.

2.1.3 Carcinogenesis

More than enough evidence exists about the connection between HPV infection and cervical cancer: the presence of the viral DNA in the infected cells, serological findings and the most important of all, the experimental confirmation of the transformation ability of the viral E6 and E7 oncoproteins.

2.1.3.1 Oncoproteins of HPVs

E5 protein

The HPV 16 E5 protein is a small hydrophobic membrane protein located downstream of the E2 ORF. Although this gene is not well conserved at the DNA level among HPVs or animal viruses, the expressed proteins are always hydrophobic and membrane-bound (Tsai & Chen, 2003). The E5 presents as a dimer and is distributed predominantly in the endoplasmic reticulum, the Golgi and the cytoplasmic membrane (Oetke *et al.*, 2000).

The E5 protein is under-expressed (because of the fragment deletion of the E5 ORF) in cervical carcinoma cells, which suggests that E5 may play a critical role in the genesis of CC but not in the persistence or progression and maintaining the malignant phenotype (Yang *et al.*, 2003). There are well recognised cellular targets for the E5 protein due to E5 may contribute to cell transformation: interaction with a subunit of vacuolar ATPase induces enhanced epidermal growth factor receptor signalling and so cell proliferation (Genther Williams *et al.*, 2005). Moreover, E5 causes the retention of MHC class I in the Golgi apparatus and restrain its transport to the cell surface (Kim & Yang, 2006), enhances the MAP kinase activation (Crusius *et al.*, 1997), stimulates the nuclear oncogenes, such as c-jun and c-fos (Jin *et al.*, 2001), and down-regulates the expression of the p21 tumour suppressor gene (Tsao *et al.*, 1996).

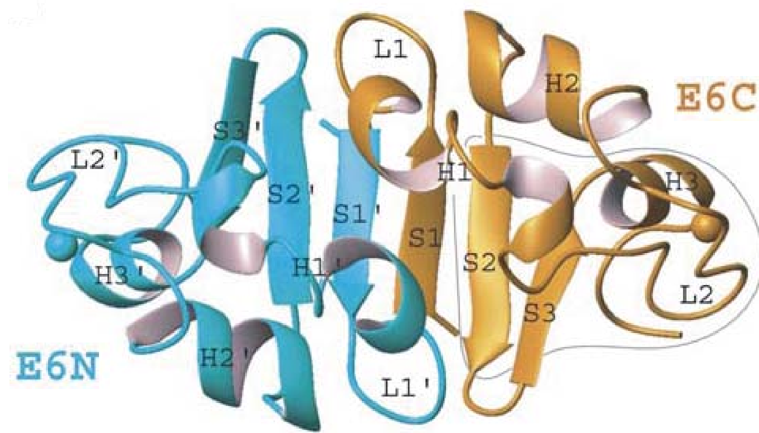
E6 protein

The E6 protein is nuclear and cytoplasmic protein of about 18 kDa. Most PVs have tandem ATGs of which the second one (151 aa form) usually, but not always, is the start. In HPV 16, a protein of 158 residues can be generated (Androphy *et al.*, 1987; Barbosa & Wettstein, 1988; Neary & DiMaio, 1989). The protein contains two CX_2C-X_{29}-CX_2C zinc-fingers joined by an interdomain linker of 36 amino acids and flanked by short amino (N) and carboxy (C) terminal domains (Howie *et al.*, 2009). Recently, the solution structure of the C-terminal half of HPV 16 E6 was solved by nuclear magnetic resonance (NMR) and a model for the whole protein was proposed (Fig. 3) (Nomine *et al.*, 2006).

Analysis of the genome of HR HPVs (but not LR HPVs) reported spliced isoforms of the E6 gene that lead to the expression of truncated E6 proteins denoted as E6*I and E6*II. The studies detected the E6*I transcript as the most abundant one in HPV 16 transformed cells, CC cell lines and clinical samples (Smotkin *et al.*, 1989; Cornelissen *et al.*, 1990; Griep

et al., 1993). E6* binds to the interface of the N- and C-terminus of the full-length E6 protein and thus inactivates its function (Nomine *et al.*, 2006).

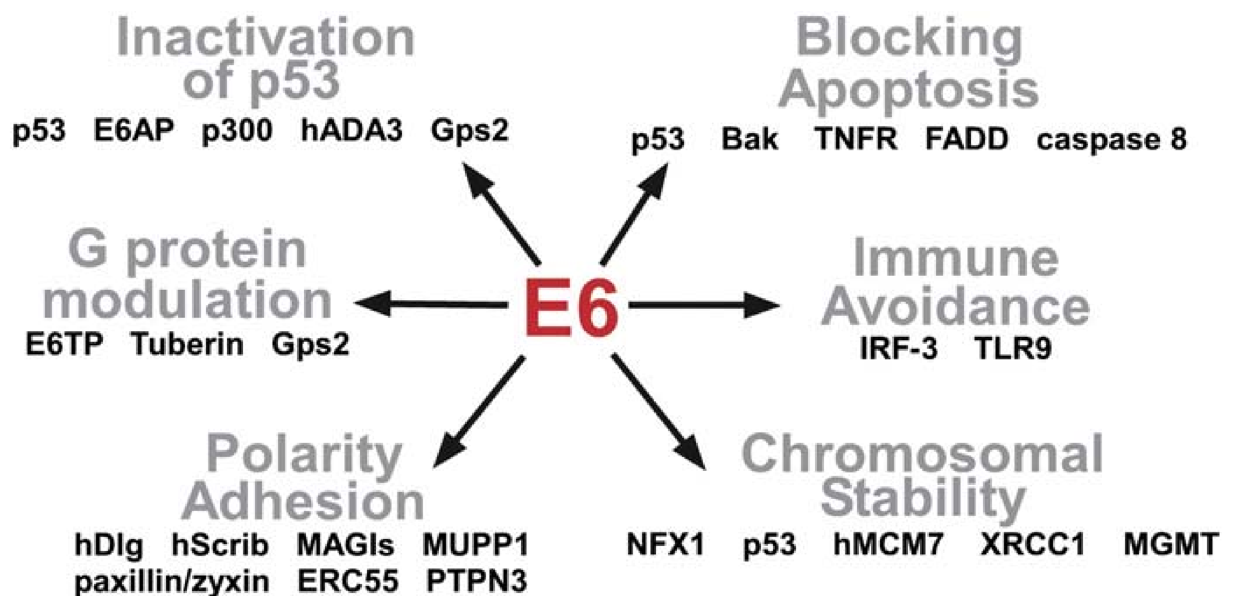
Figure 3. Proposed model of the E6 protein; pseudodimeric arrangement of E6N and E6C



Taken and modified from Nominé *et al.* Mol Cell. 2006; 21:665-78.

E6 interacts with a number of different proteins that mediate the apoptotic pathway, regulate transcription and mediate chromosomal stability, differentiation, cell-cell adhesion etc. The cellular proteins affected by the E6 protein are represented in Fig. 4.

Figure 4. Binding partners of E6



Howie *et al.* Virology. 2009; 384:324-34.

There are two main binding motifs for the E6 protein, the well characterized LXXLL motif and the specific domain on cellular proteins known as PDZ. The LXXLL motif was firstly described on the E6 associated protein (E6AP) that forms a complex with both E6 and target proteins leading to ubiquitination of the target protein and subsequent proteasome mediated degradation (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993; Be *et al.*, 2001). Other proteins binding to E6 by way of this motif are E6BP (ERC55), IRF3, paxillin and tuberin (Tong & Howley, 1997; Elston *et al.*, 1998; Ronco *et al.*, 1998). Binding to the LXXLL motif is highly conserved in PVs and E6AP binds to both HR and LR HPVs (Chen *et al.*, 1998). Only HR HPV E6 proteins (Kiyono *et al.*, 1997) have the ability to bind to the PDZ domain containing proteins such as hDLg1 and 4 (Lee *et al.*, 1997), hScrib (Nakagawa & Huibregtse, 2000) and MAGI 1, 2 and 3 (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002). The p53, Bak p300/CBP, Gps2, FADD proteins and procaspase 8 have been reported to bind various E6 proteins, but they lack both LXXLL and PDZ domains (Howie *et al.*, 2009).

One of the first identified and best characterised interacting partner of the E6 protein is the p53 tumour suppressor. Once activated, p53 induce DNA repair, cell cycle arrest and/or apoptosis, based upon the extent of damage (reviewed by Murray-Zmijewski *et al.*, 2008). During the carcinogenesis, to overcome this obstacle, the E6 protein causes the degradation of p53. The principle mechanism is through the ubiquitin-proteasome pathway (Scheffner *et al.*, 1990). Interestingly, both HR and LR HPV E6 proteins have been shown to be able to bind the p53 C-terminus but only the HR E6 proteins are capable of binding to the core region of p53 that is required for its degradation (Li & Coffino, 1996). There are some other mechanisms described how the HPV can block the function of the p53 independently on the protein degradation: (i) The interaction of E6 with p53 can inhibit the binding of p53 to its site-specific sequences, what correlates with the affinity that each E6 has for p53. This association may also cause conformational change in the p53 protein (Lechner & Laimins, 1994; Thomas *et al.*, 1995). (ii) Binding of the E6 to p53 may lead to sequestration of the p53 in the cytoplasm (Mantovani & Banks, 2001). (iii) Interaction of HR E6 with p300 inhibits the p53 acetylation and decreases its ability to bind to the DNA (Zimmermann *et al.*, 2000).

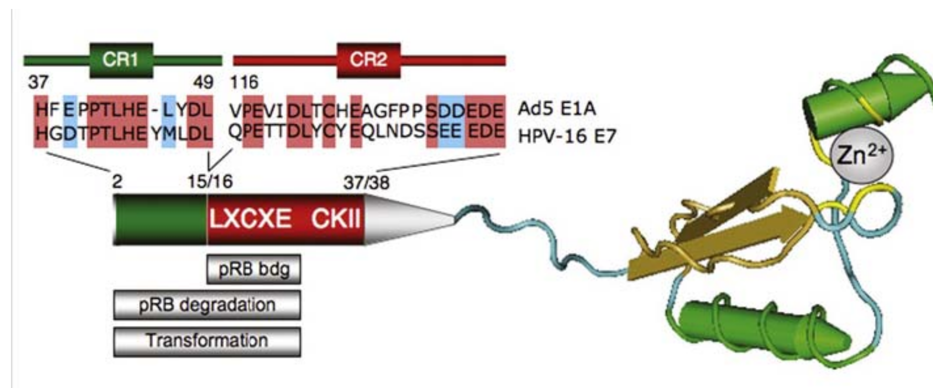
E7 protein

The E7 proteins are small, acidic proteins composed of about 100 amino acids (Fig 5). The N-terminus of E7 contains two conserved regions, CR1 and CR2, with sequence similarity to adenovirus E1A and polyomavirus SV40 T antigen (Phelps *et al.*, 1988; Vousden & Jat, 1989). The C-terminal part of E7 contains a zinc-binding domain composed of two

Cys-X-X-Cys motifs that function as a dimerization domain (Barbosa *et al.*, 1989; Clemens *et al.*, 1995).

Although the predicted molecular weight of HPV E7 is ~11 kDa, in polyacrylamide gels migrates with a molecular size of 18 to 20 kDa (Smotkin & Wettstein, 1986), which is mediated by the CR1 domain or the high content of acidic residues (Munger *et al.*, 1991; Armstrong & Roman, 1993). HPV 16 E7 is located mainly in the cytoplasm but also exists in the nucleus, and its half-life is about one hour (Smotkin & Wettstein, 1987; Greenfield *et al.*, 1991).

Figure 5. Schematic structure of HPV E7 oncoprotein



Taken and modified from McLaughlin-Drubin *et al.* Virology. 2009; 384:335-44.

The biological activities of the E7 protein are linked to its ability to associate with and disrupt the normal activities of cellular regulatory complexes (see Table 3).

HPV E7 proteins associate with pRb and the related pocket proteins that regulate G1/S entry and modulate the E2F transcription factors (Munger *et al.*, 1989). The pRB/E2F complex controls cellular processes such as cellular differentiation, apoptosis and genomic instability (Dyson, 1998). High-risk HPV E7 binds the E2F-bound pRB, destabilises it through proteasomal degradation and thus activates E2F-mediated transcription and uncontrolled S-phase entry (Jones *et al.*, 1997). This pRb-binding ability of the E7 protein leads to several alterations in cellular processes (see Table 3) and the outcome is to retain the differentiating keratinocytes in a DNA synthesis competent state.

Table 3. Cellular protein targets of the E7 protein (summarised from Zwerschke & Jansen-Durr, 2000; McLaughlin-Drubin & Munger, 2009; Yugawa & Kiyono, 2009)

E7 target	Cellular function	E7 domain	Result of E7 binding
pRb/E2F complex	transcriptional repressor, G1 arrest	CR2, C-terminus	proteasomal degradation of pRb, uncontrolled G1 exit
p107, p130	transcriptional repressors, G1 arrest	CR2	disruption of E2F-p107 and E2F-p130 complexes, derepression of E2F
cyclin A, E	subunits of cdk2	CR2	increased levels of cyclin A,E; cell cycle dysregulation
$p27^{KIP1}$,$p21^{CIP1}$	cdk inhibitors	C-terminus	inactivation of $p27^{KIP1}$,$p21^{CIP1}$, activation of cdk2
p600	? chromosome segregation, synaptic transmission, MAP	CR1	inactivation of p600, deregulation of anoikis (a form of apoptosis)
pyruvate kinase	glycolytic control enzyme	C-terminus	weakly active pyruvate kinase, increased glycolytic processes
S4 ATPase subunit	subunit of 26S proteasome	C-terminus	increased ATPase activity
AP-1	transcriptional activator	C-terminus	increased AP-1-dependent transcription
TBP	transcriptional activator	Ser31/32 of N-terminus	? modulation of transcription , interaction with TAF110
HATs	transcriptional co-activators	C-terminus	inactivation of HATs
HDACs	transcriptional co-repressors	C-terminus	increased E2F2-mediated transcription
IGFBP-3	regulation of cell survival	C-terminus	proteasomal degradation of IGFBP-3
IRF-1	transcriptional activator, IFN signaling	CR1, CR2	inhibition of activity

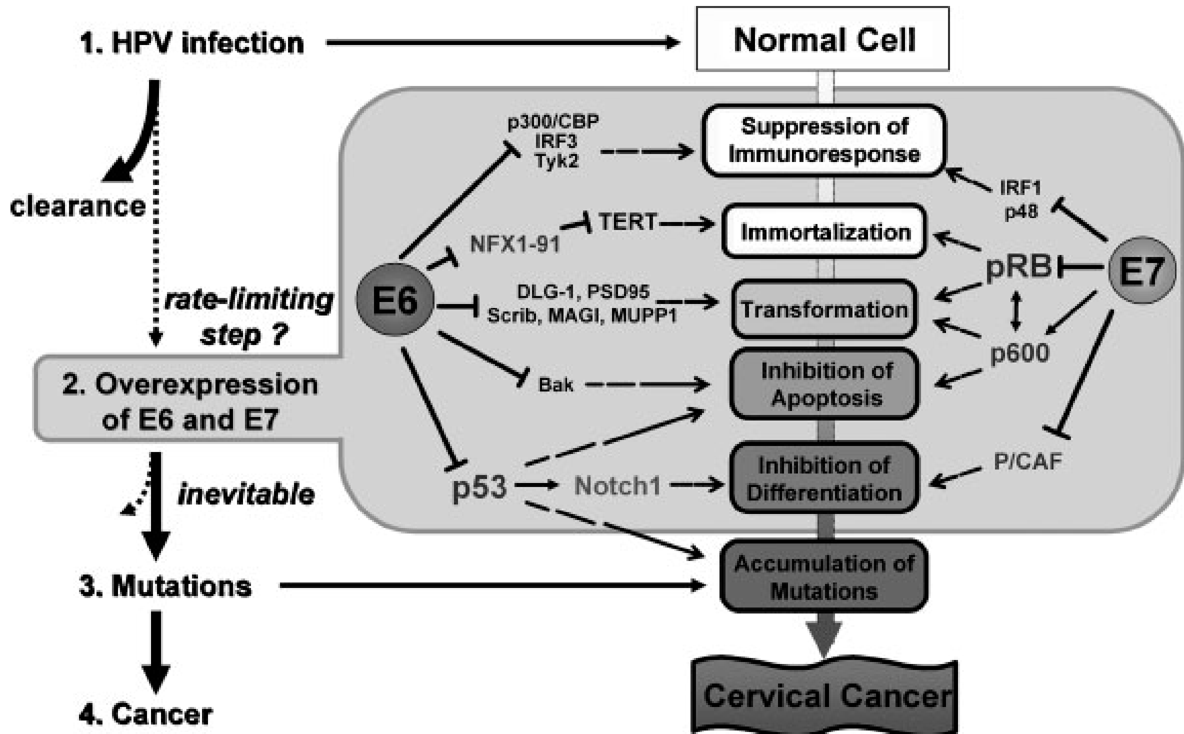
KIP1 – kinase activating protein 1, cdk – cyclin-dpendent kinase, p600 – pRb associated factor, AP-1 – activating protein 1, TBP – TATA binding protein, HATs – histone acetyl transferases, HDACs – class I histone deacetylases, IGFBP-3 – insulin-like growth factor binding protein, IRF-1 – interferon regulatory factor 1

2.1.3.2 Mechanisms in HPV-mediated carcinogenesis

HPVs have been characterised as causative agents for CC. Their DNA is found to be frequently integrated into the chromosomes in cancer cells. As the E6 and E7 viral oncoproteins are constantly expressed in high levels in the CC cells, these proteins play an important role in carcinogenesis and maintenance of transformed phenotype. However, several studies confirmed that the expression of E6 and E7 itself is not sufficient for cancer

development, but it seems to be involved in every stage of multi-step carcinogenesis (Fig. 6) (reviewed in Yugawa & Kiyono, 2009).

Figure 6. Multi-step carcinogenesis for HPV-induced cervical cancer



Yugawa *et al.* Rev Med Virol. 2009; 19:97-113.

Cooperation of E6 and E7 does not merely immortalise normal human epithelial cells but confers tumourigenic properties to transformed cells. One of the supposable epigenetic changes that can also contribute to carcinogenesis, is the aberrant methylation of CpG islands in tumour suppressor genes (TSGs) by DNA methyltransferases (DNMTs), which are over-expressed in several malignancies (Esteller, 2006). There is no evidence for HPV-induced methylation of TSGs so far, but HPV 16 E7 has been shown to bind DNMT1 and stimulate its enzymatic activity (Burgers *et al.*, 2007). Moreover, the transcription of the DNMT1 is under the control of E2F transcription factor that can be stimulated (as mentioned before) by the E7 oncoprotein. There are several other genes, which are commonly found methylated in increased levels in women with invasive disease and are reviewed in (Woodman *et al.*, 2007).

2.1.4 Immunology

Papillomaviruses are very successful infectious agents; the infection they induce is generally long lasting with a risk of progression to malignancy and the host immune response to the infection is weak. There are several reasons why HPVs are poor natural immunogenes (summarised by Frazer, 2004; Stanley, 2009):

- HPVs are dsDNA viruses, with no dsRNA intermediate to induce innate immune responses.
- Infection is strictly intra-epithelial: the virus is not lytic, there is no antigen (Ag) release and also no inflammation to alert the innate immune system.
- Infection has no viraemic phase and therefore less systemic Ag presentation occurs leading to weak humoral immune response.
- Viral non-structural proteins are expressed at low levels compared with more immunogenic proteins from other viruses.
- Additional mechanisms are provided for evading the induction of immune response: interaction of E6 with IRF3 and E7 with IRF1 etc.

Cell mediated immune response is the most important effector mechanism for the control and clearance of viral infections. Both in the regressing genital warts and in the cervical tissues, HPV specific cytotoxic T lymphocytes (CTLs) against early non-structural proteins can be detected. $CD4^+$ and $CD8^+$ are involved in these responses (Nakagawa *et al.*, 1999). HPV 16 E2 and E6 specific $CD4^+$ T cells are measurable in patients with regressing cervical disease and are not seen in persistent infection, what suggests that CTL response to E6 is important for viral clearance (Nakagawa *et al.*, 2000; van Poelgeest *et al.*, 2006).

The cell-mediated immune response is accompanied or closely followed by seroconversion – generation of serum neutralizing antibodies against the major L1 viral capsid protein. Antibodies to the minor L2 capsid protein are not detectable in natural infections with HPVs (Dillner, 1999). Antibody concentrations are low (absence of a viraemia) (Kirnbauer *et al.*, 1994) and 20-50% of women with HPV DNA do not have detectable specific anti-HPV antibodies, which may happen due to the fact that current serological assays are relatively insensitive. Anti-HPV L1 antibodies persist for many years; however, it is still unclear whether these low levels of antibodies protect sufficiently against reinfection with the same HPV type.

2.1.5 Vaccines against HPVs

The well-characterised features of HPV infections provide unique opportunities for development of vaccines aimed against these oncogenic viruses. Two different types of HPV vaccines can be designed, the prophylactic (preventive) vaccines for prevention of HPV infection and the therapeutic vaccine inducing regression of lesions evoked by the virus infection.

2.1.5.1 Prophylactic vaccines

Traditionally, prophylactic vaccines are aimed against virus-specific neutralising antibodies; however, the modest production of anti-HPV L1 antibodies complicated the development of HPV vaccines. The problem was solved after the recognition that the L1 capsid protein, if produced in large amounts in recombinant systems, is able to self-assemble to so-called virus-like particles (VLPs) (Hagensee *et al.*, 1993; Vonka & Hamsikova, 2007; Stanley, 2007).

Two HPV prophylactic vaccines have been developed: Cervarix™, a bivalent HPV 16/18 VLP vaccine, and Gardasil™ also known as Silgard, a quadrivalent HPV 16/18/6/11 vaccine (Table 4). The vaccines are subunit vaccines consisting of L1 VLPs and do not contain any live biological product or DNA, so they are non-infectious (reviewed in Cutts *et al.*, 2007; Stanley, 2009). Vaccines are injected intramuscularly in a three-dose immunisation scheme. The inoculated antigens access the local lymph nodes and thus circumvent the immune avoidance strategy of viral intra-epithelial life cycle. These antigens are highly immunogenic with ability to activate both innate and adaptive immune responses (Harro *et al.*, 2001). VLPs induce high concentrations of neutralizing antibodies to L1 (Harper *et al.*, 2004; Villa *et al.*, 2006) and there is also evidence that HPV L1 VLP vaccines generate not only type specific but also cross-neutralizing antibodies (Smith *et al.*, 2007).

Both vaccines show high efficacy in the Phase II and Phase III randomised control trials achieving over a 5 year period 100% protection against high-grade cervical lesions in 15-26 years old women naïve for HPV 16 and 18 at trial entry (Paavonen *et al.*, 2007; Ault, 2007). The vaccination had only weak effect on lesion development in women who had evidence of past HPV infection and women with persistent HPV infection were not protected at all (Hildesheim *et al.*, 2007). The optimal time for immunisation with VLP vaccines is

before puberty. Studies with the quadrivalent vaccine show that antibody levels are higher in 9-15 year-old girls than in 16-23-year-old women (Block *et al.*, 2006).

Table 4. Characteristics of HPV L1 VLP vaccines (modified from Cutts *et al.*, 2007; Stanley, 2007)

Manufacturer and trade name	Quadrivalent vaccine Merck /Gardasil, Silgard/	Bivalent vaccine GlaxoSmithKline /Cervarix/
L1 VLP antigens	HPV 6, 11, 16, 18	HPV 16, 18
Expression system	Yeast (*S. cerevisiae*)	Baculovirus
Adjuvant	Proprietary aluminium hydroxyphosphate sulphate (225µg)	ASO_4 (500 µg aluminium hydroxide + 50 µg 3-deacylated monophosphoryl lipid A)
Injection volume	0.5 ml i.m.	0.5 ml i.m.
Immunisation schedule	0, 2 and 6 months	0, 1 and 6 months
Adolescent safety and immunogenicity bridging trials	Children 9-15 years Women 15-26 years	Females 10-14 years Males 10-18 years

Future generations of preventive vaccines should possess increased number of HPV types to maximize the protection against HPV malignancies. A polyvalent L1 vaccine containing VLPs for nine HPV types is in Phase II clinical trial at present (http://clinicaltrials.gov/ct2show/NCT00943722). Also an L2-based vaccine can be employed, which is less immunogenic than the L1 vaccine, but this may be overcome by using strong adjuvants such as Toll-like receptor agonists (Alphs *et al.*, 2008).

2.1.5.2 Therapeutic vaccines

The existing preventive HPV vaccines targeting L1, as mentioned before, have no therapeutic effect and are unable to eliminate pre-existing HPV infection. Therefore, women already infected with oncogenic HPVs are at risk of developing cancer. It is estimated that it would take ~ 20 years from the mass preventive vaccination to affect the cervical cancer rates.

In order to treat currently infected patients, it is important to develop therapeutic vaccines against HPV (reviewed in Hung *et al.*, 2008; Cid-Arregui, 2009).

Recently developed therapeutic vaccines are aimed to induce cellular immune responses against HPV early antigens. The ideal targets are the E6 and E7 proteins, which unlike capsid proteins are constitutively expressed in HPV precancerous lesions and tumours and are important for induction and maintenance of cellular transformation.

Therapeutic HPV vaccine approaches include live-vector-based, peptide- and protein-based, nucleic-acid-based and cell-based vaccines, each with advantages and disadvantages. These vaccines control HPV infection through cell mediated immunity, mainly through $CD8^+$ T cells, which requires the collaboration of $CD4^+$ helper T cells to get them completely effective (Cid-Arregui, 2009).

The success of therapeutic vaccines may be decreased by the tumour microenvironment. For instance, T regulatory cells release immunosuppressive cytokines that can paralyse T cell functions (Lin *et al.*, 2010a). Therefore, depletion of T regulatory cells from the tumour microenvironment significantly enhances the potency of therapeutic HPV DNA vaccines (Chuang *et al.*, 2009).

The Table 5 summarises the current therapeutic HPV vaccines in clinical trials.

Table 5. Therapeutic HVP vaccine clinical trials (summarised from Hung *et al.*, 2008; Cid-Arregui, 2009; Lin *et al.*, 2010b)

Type of vaccine	Vaccine construct + adjuvant	Antigen(s)	Target subtype(s)	Phase of study/subjects	Sponsor	Reference(s)
Live vector based (bacterial, viral vectors)	Attenuated recombinant *Listeria monocytogenes* encoding antigen (Lovaxin C)	E7 protein fused to listeriolysin O	HPV 16	Phase I, CIN III	Advaxis	(Radulovic *et al.*, 2009)
	Recombinant Vaccinia virus (TA-HPV)	E6/E7 fusion protein	HPV 16, HPV 18	Phase I/II, CIN III	Xenova	(Kaufmann *et al.*, 2002)
	Attenuated recombinant Modified Vaccinia virus Ankara (MVA)	E2 protein	HPV 16, HPV 18	Phase II, CIN III	IMSS	(Garcia-Hernandez *et al.*, 2006)
Peptide based	Set of overlapping long peptides + Montanide ISA-51	E6 protein (9x) + E7 protein (4x) 25-35aa long	HPV 16	Phase I, CIN III	NA	(Kenter *et al.*, 2008)
	Lipopeptide	lipidated E7 (86-93aa)	HPV 16	Phase I	NCI	(Steller *et al.*, 1998)
Protein based	Fusion protein (SGN-00101/HSPE7)	HSP/E7 fusion protein	HPV 16	Phase II, CIN III	Nventa/ StressGen	(Roman *et al.*, 2007)
	Fusion protein (TA-CIN)	L2/E6/E7 fusion protein	HPV 16	Phase I	Xenova	(de Jong *et al.*, 2002)
	Fusion protein + ISCOMATRIX®	E6/E7 fusion protein	HPV 16	Phase I, CIN I-III	CSL Limited	(Frazer *et al.*, 2004)
Dendritic cell based	Autologous dendritic cells (DCs)	DCs pulsed with recombinant HPV 16 E7 or HPV 18 E7	HPV 16, HPV 18	Clinical pilot study, CIN II-III	NA	(Santin *et al.*, 2006; Ferrara *et al.*, 2003)
DNA based	*see under DNA vaccines against HPV*					
Prime-boost	Prime with: TA-CIN Boost with: TA-HPV	L2/E6/E7 fusion protein + E6/E7 fusion protein	HPV 16 + HPV 16, 18	Phase II, AGIN III	Xenova	(Fiander *et al.*, 2006)

aa – amino acid, AGIN – Anogenital intraepithelial neoplasia, CIN – cervical intraepithelial neoplasia, HSP – Heat shock protein, IMSS – Instituto Mexicano del Seguro Social, NCI – National Cancer Institute

2.2 Gene immunotherapy of cancer

2.2.1 Immunotherapy of cancer in general

Immunotherapy, beyond the conventional methods, chemotherapy, radiotherapy and surgery, turned out to be an effective strategy in the fight against malignancies. The purpose of tumour immunotherapy is to stimulate or restore the ability of the immune system, which is commonly weakened during tumour development, to fight. Generally, the goal is to provide either active or passive immunity against cancer. Table 6 gives a brief overview of several methods of anti-tumour immunotherapy.

Passive humoral or cellular immunotherapy consists in administration of components of the immune system to patients: anti-tumour antibodies or specific effector cells that are isolated from patients, activated *ex vivo* and introduced to the bloodstream to affect directly the tumour. However, the application of monoclonal antibodies has some limitations. They do not provide long-lasting effect and therefore repeated delivery is needed. Moreover, these antibodies are potentially immunogenic, which may be a problem for repeated administration (King *et al.*, 2008). The main limitation of autologous effector cells is their preparation – not all cells grow well enough in culture to generate the quantity of cells that is required to produce a useful anti-tumour effect.

Active immunotherapy may be specific or non-specific depending on the properties of the induced immune response. Active **specific** immunotherapy makes efforts to activate effector mechanisms, generally the cytotoxic T cells, which are specific against tumour-associated antigens (TAAs). In comparison with passive immunotherapy, active immunotherapy with vaccines has the potential to induce besides tumour-specific effectors also memory T cells (Disis *et al.*, 2009). Several strategies can be used to induce the cellular immunity and to stimulate the host response; they usually involve administration of peptides, proteins, DNA, DCs pulsed with antigens or tumour cells, which serve as a source of antigens. The purpose of the **non-specific** immunotherapy is to induce the global immune system by application of recombinant cytokines or parts of microorganisms. Clinical trials indicate the effectiveness of recombinant interferons and certain bacterial adjuvants (Table 6), usually simultaneously with chemotherapy or radiotherapy.

Table 6. Anti-tumour immunotherapy

Type of immunotherapy	Treatment principle
Passive immunotherapy	
Humoral	**Monoclonal anti-tumour antibodies (mAbs)** - conjugated with toxins/radioisotopes - linkage to second Ab reacting with CTL
Cellular	**Lymphokine activated killer (LAK) cells** - patient's T cells exposed to IL-2 *ex vivo* and returned to the bloodstream **Tumour-infiltrating lymphocytes (TILs)** - isolated from tumour tissue, exposed to IL-2 and injected to the patient's bloodstream **TCR gene transfer into T cells** - patients treated with autologous peripheral blood T cells transduced with the α and β chains of TCR by viral vectors
Active immunotherapy	
Specific	**Peptide-based vaccines** - short peptide segments from defined TAAs **DNA-based vaccines** - plasmid DNA encoding a specific Ag **Tumour-cell-based vaccines** - *ex vivo* treated autologous tumour cells or allogeneic tumour cell lines (irradiated, treated with neuraminidase, genetically modified *etc.*) **Vector- based vaccines** **-** recombinant bacterial vaccines or recombinant viruses expressing tumour Ags, immunostimulatory cytokines *etc.* **Dendritic-cell-based vaccines** - generated *in vitro* or *ex vivo* and introduced to patients - DCs pulsed with tumour lysates or peptide tumour epitopes, fused with irradiated tumour cells, transfected with nucleic acids encoding TAAs *etc.*
Non-specific	**Bacterial adjuvants** - BCG and its derivatives, killed suspensions of *Corynebacterium parvum* **IFN-α,β,γ**

Ab – antibody, Ag – antigen, BCG – bacille Calmette- Guérin, CTL – cytotoxic T lymphocytes, DC – dendritic cell, IFN – interferon, IL – interleukin, TAA – tumour-associated antigen, TCR – T cell receptor

As most types of immunotherapy, both active and passive, are designed to be targeted against specific antigens of cancer cells, this therapy may lead to one serious disadvantage: the tumour cells can mutate and thus avoid immune responses. Moreover, the same tumour may produce a slightly modified antigen in different patients. Therefore, the immunotherapy directed against a certain antigen might become ineffective.

A term **gene immunotherapy** of cancer was established for immunotherapies that apply methods of gene therapy trying to use the genetic pattern of tumour cells to fight with cancer. The strategies of gene therapy, such as gene modification of tumour or non-tumour cells and utilisation of therapeutic anti-cancer vaccines, induce anti-tumour immune responses or use the mechanisms of immune system to eliminate tumour cells.

2.2.2 Mechanisms of tumour escape from anti-tumour immunity

In early stages of tumour development, effective anti-tumour immune response occurs that persists even during the tumour growth. The concept of tumour immunoediting gives an explanation for the role of the immune system in tumour development. Three phases, the elimination phase, the phase of equilibrium and the escape phase, form this concept. During the phase of elimination, also called tumour immune surveillance, the immune system eliminates the detected tumour cells. This elimination can be complete, when all tumour cells are cleared, or incomplete. The phase of equilibrium represents a period of cancer persistence. During this phase, tumours accumulate changes that help them to escape from or to suppress the immune responses. The balance between the activation and suppression of immune responses determines the fate of the tumour. If the immune system fails to contain the tumour growth, the tumours progress and the tumour development leads to the escape phase (Khong & Restifo, 2002; Swann & Smyth, 2007).

The ability of tumours to evade the host immune system may affect the immunotherapy. Therefore, several factors, described in the following two chapters, have to be considered when designing therapeutic vaccines.

2.2.2.1 Tumour microenvironment and cellular mechanisms of tumour escape

The tumour microenvironment is a unique environment comprised primarily of tumour cells, immune cells, fibroblasts and the extra-cellular matrix. Inflammatory cells found in tumours contribute to the progression of tumours and their escape from the host immune system. Modification of the function of infiltrating cells by the tumour cells leads to creating a microenvironment suitable for tumour growth. The immune cells in tumours are represented generally with T lymphocytes, dendritic cells and macrophages and occasionally with B cells and natural killer (NK) cells (reviewed in Whiteside, 2008).

Lymphocytes in the tumours, also called tumour-infiltrating lymphocytes (TILs), are the major component of the tumour microenvironment. These cells are commonly $CD4^+$ and $CD8^+$ T cells and many of them are specific for TAAs but mostly ineffective in avoiding tumour growth (Whiteside, 2006). A subset of T cells with $CD4^+$ $CD25^{high}$ $Foxp3^+$ characteristics is a population of regulatory T cells (T_{reg}) with suppressing abilities affecting the proliferation of effective TAA-specific T cells thus contributing actively to tumour development. T_{reg} depletion or inhibition of their function belongs to the main strategies of cancer immunotherapy (Colombo & Piconese, 2007).

Tumour-associated macrophages (TAMs) play a key role in tumour growth, dissemination and angiogenesis and, through releasing of inhibitory molecules, they suppress lymphocyte functions (al-Sarireh & Eremin, 2000; Martinez *et al.*, 2008). Furthermore, immature myeloid precursors, also known as myeloid suppressor cells (MSCs), are accumulated in the tumour site and peripheral blood of cancer patients. These $CD14^+$ $CD11b^+$ $HLA\text{-}DR^{neg/low}$ myeloid precursors influence the immune system by suppressing the development of specific T cell responses. They inhibit T cell immunity through TGF-β release or arginase I production (Serafini *et al.*, 2006; Filipazzi *et al.*, 2007).

When talking about tumour microenvironment, it is necessary to mention vesicular structures called microvesicles or exosomes, which are released by tumour cells. As the content of these recently re-discovered organelles involves tumour antigens, they were supposed to be potential anti-tumour vaccines. Unfortunately, it turned out that tumour microvesicles have negative effect on anti-cancer immune responses. They also retain molecules, which promote the tumour progression *in vivo* (reviewed in Iero *et al.*, 2008).

2.2.2.2 Molecular mechanisms leading to tumour escape

Tumour cells attempt to escape mostly from immune *recognition*. A number of molecular mechanisms enabling the tumour to become invisible for the immune system were described. Here is a brief summary of the well-known escape strategies:

MHC class I loss or down-regulation:

Several MHC class I phenotypes and changes in MHC class I expression were described in animal or human tumours. These alterations are mostly results of mutations and defects in the antigen-processing machinery (APM), which lead to down-regulation of MHC I molecules. The total loss of MHC I expression and the loss of MHC class I haplotype or allelic loss may occur through mutations in genes for the MHC I. Such abnormalities affect the MHC I-dependent antigen presentation and the tumour cells escape from recognition by $CD8^+$ T cells (reviewed in Ahmad *et al.*, 2004). Moreover, epigenetic modifications were reported to be an important factor in regulation of the APM. DNA methylation or histone deacetylation of genes encoding components of APM may lead to reduced MHC class I molecule expression on the tumour cell surface (Manning *et al.*, 2008).

Tumour antigen loss, down-regulation or mutation:

Modifications in TAA expression are another reason for the escape of tumour cells from the immune system, even in the presence of TAA-specific CTLs. Mutations in the immunodominant epitope of the antigen (antigenic drift) disable the MHC-peptide interaction and the binding to TCR (Ahmad *et al.*, 2004). The suboptimal expression level of the antigen is associated with the *in vivo* immunoselection of antigen-loss variants of the tumour (Lozupone *et al.*, 2003).

Alterations in signalling pathways:

Fas/Fas ligand (FasL) signal pathway plays a key role in tumour immune escape as the activation of this pathway leads to apoptosis of the immune cells. Suppression of the FasL expression in tumour cells, further blocking the TNF apoptotic signal pathway of the immune cells, can increase the survival of the cells of the immune system (Zhang & Xu, 2007; Xu & Zhang, 2008). Furthermore, the Fas/FasL complex generally activates the caspase-8 pathway that finally leads to cell death. Therefore, in many tumours, cellular FLICE-inhibitory protein (cFLIP), a caspase-8 inhibitor, is expressed (Medema *et al.*, 1999). Several human cancers escape from apoptosis by activation of the survival signal (antiapoptotic factors of the Bcl-2 family) with phosphatidylinositol 3’-kinase (PI3K) and protein kinase B (Osaki *et al.*, 2004).

Alteration in signal transduction molecules:

The CD3 ζ chain is a part of the TCR complex and is concerned in inducing the activation signal in T lymphocytes. The total loss of the CD3 ζ chain or its reduced expression is associated with elevated production of immunosuppressive cytokines and thus contributes to immune evasion (Ahmad *et al.*, 2004).

Lack of co-stimulation:

Cancers may progress even in the presence of TAAs due to the lack of expression of co-stimulatory molecules on a tumour cell surface. The insufficient co-stimulation induces anergy in the T cells (Abken *et al.*, 2002). Viral vectors co-expressing IL-12 and B7.1 could be used in the immunotherapy of cancer, which reverses the expression of co-stimulatory molecules and thus increases the immunogenicity of tumour cells (Wen *et al.*, 2001).

2.2.3 Tumour-escape mechanisms used by HPVs

Various reasons were described why the immune system fails to recognise the replicating HPV. As the virus is non-lytic, has no blood-borne phase and the early proteins are expressed at low levels, the production of the viral antigens is limited and insufficient to induce pro-inflammatory signals to activate the host immune system. Other reasons could be the different gene-codon usage and the ability of viral proteins to mimic the host proteins to take advantage of the host's self-tolerance (reviewed in Kanodia *et al.*, 2007).

The alteration of antigen presentation in HPV infected tumour cells represents an essential role in immune evasion. Several evidence exists suggesting that the immunogenic peptides from the E6 and E7 proteins are not efficiently processed by tumour cells, and a down-regulation of MHC class I molecules and TAPs was recorded (Bauer *et al.*, 2000; Evans *et al.*, 2001). The regulation of transcription of genes involved in antigen presentation is one of the many functions of the E7 protein, which leads to reduced protein presentation and the virus easily escapes from CTL attack. Moreover, the HPV 16 E5 protein affects the stability of the peptide-MHC class I complex by alkalinisation of the Golgi apparatus and endosomes (Ashrafi *et al.*, 2005).

HPVs, like many other viruses, also disrupt the IFN type I pathway, which has anti-viral and immunostimulatory activities. The E6 protein binds to IRF-3 to inhibit its trans-

activation function and to block the IFN-β gene transcription (Ronco *et al.*, 1998). The E7 protein also prevents the transcription of IFN-β due to IRF-1 binding (Park *et al.*, 2000).

Furthermore, HPVs up-regulate the PI3K pathway in Langerhans cells (LC) at the site of primary infection and thus inhibit LC from inducing immune response (Fausch *et al.*, 2005). The E6 protein contributes to inhibition of apoptosis of the infected cells – this protein prevents cells from p53-dependent cell death by binding to p53 (Howie *et al.*, 2009).

2.3 DNA vaccines

DNA vaccines are a relatively new vaccination strategy but the beginnings of their development date back to the early 1990s. A number of animal model studies indicated the success of vaccination with DNA preparations (Wolff *et al.*, 1990; Williams *et al.*, 1991) and with time DNA vaccines emerged as an attractive form of immunotherapy.

A DNA vaccine is composed of a plasmid DNA encoding the antigen of interest under the control of a mammalian promoter, traditionally the human cytomegalovirus (CMV) immediate-early or CMV-Chicken-β actin promoter, and can be easily produced in bacteria. Commonly utilized selectable markers are the antibiotic resistance markers. The expression is generally higher if an intron is present downstream of the promoter. However, a number of prokaryotic sequences, negatively affecting the gene expression in eukaryotic cells, have been identified. Therefore, it is important to evaluate all changes made in the composition and orientation of elements within the prokaryotic region of the plasmid (reviewed in Williams *et al.*, 2009). Moreover, there are several components build into the plasmid DNA that can affect the immunogenicity of the vaccines (see Chapter *Enhancement of DNA vaccine potency*).

2.3.1 Immune responses and vaccine delivery

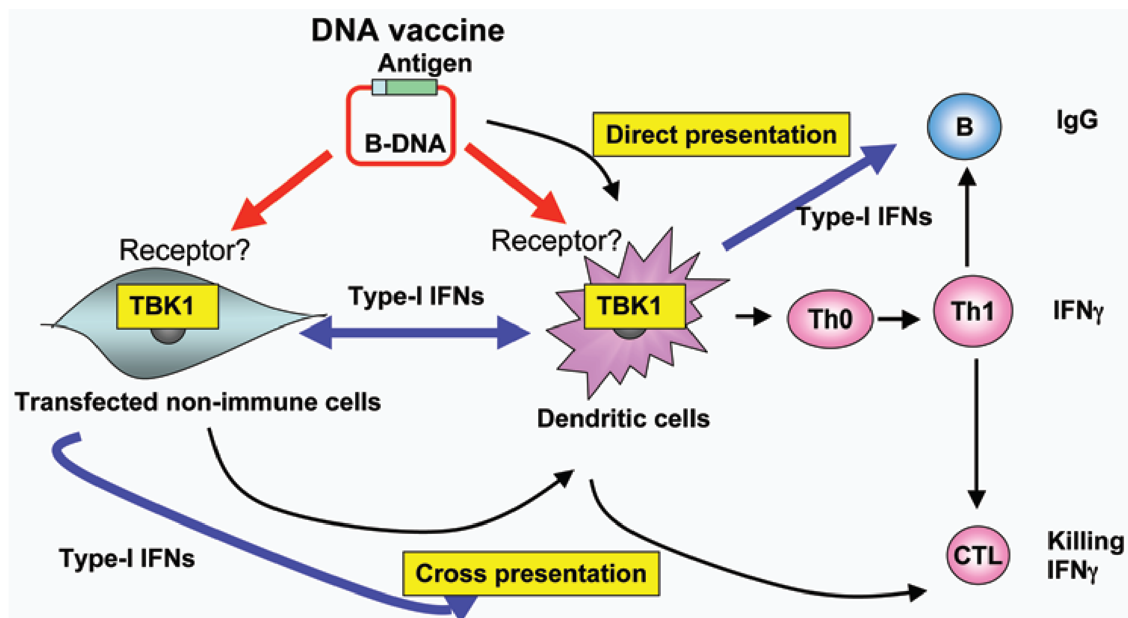
Once the plasmid DNA is administered *in vivo*, the encoded antigen is expressed in the host cells and presented by antigen presenting cells (APCs). This occurs mainly in the lymph nodes and leads to the induction of both the cellular and humoral immune responses that is a unique feature of the DNA vaccines (Coban *et al.*, 2008).

APCs, generally dendritic cells (DCs), play the key role in the activation of the innate immunity. They process and present the endogenously expressed antigens to class I and class II MHC molecules that leads to the priming of naïve $CD8^+$ and $CD4^+$ T cells. The activated $CD8^+$ cytotoxic T cells then kill tumour cells by inducing apoptosis in the target cells and the induction of the $CD4^+$ T cells can help to augment the $CD8^+$ T cell immune response (Lin *et al.*, 2010b). As the DNA vaccines are ordinarily injected into muscle or skin, the antigen is mainly produced in myocytes or keratinocytes, which are not professional APCs. The antigen must be transferred to DCs and this indirect process of presentation is termed cross-presentation. DCs may acquire the antigen from exogenous source into the MHC class I pathway also from dead or dying cells by phagocytosis (reviewed in Rice *et al.*, 2008;

Abdulhaqq & Weiner, 2008). The transfected myocytes and keratinocytes can contribute to immune activation through production of cytokines and chemokines and through the up regulation of the MHC class I expression (Larregina & Falo, Jr., 2000; Shirota *et al.*, 2007).

However, there are still many unclarities about the precise cellular and molecular mechanisms by which the DNA vaccine works *in vivo*. For instance: it has been thought that the CpG motifs, sequences of an unmethylated C followed by G, are the built-in immunostimulatory elements of the DNA vaccines. The addition of many CpG motifs into the plasmid DNA enhances the immunogenicity of the vaccines (Coban *et al.*, 2005). The Toll-like receptor 9 (TLR9) is the mediator for the induction of the protective immune response (Krieg, 2006). Nowadays, it is known that DNA vaccines can stimulate the innate immune system independently of TLRs. The double stranded structure of the DNA vaccine is essential for the activation of type I IFN-mediated immune response. The key signalling molecule in this process is the TANK-binding kinase-1 (TBK1, Fig. 7). This way of activation affects both the direct and the cross-presentation of an antigen (reviewed in Coban *et al.*, 2008).

Figure 7. Priming of the immunity after DNA vaccination



Coban *et al.* Hum Vaccin. 2008; 4:453-6.

Different administration methods may influence the immune response by the rate of transfection efficacy or by affecting the way of antigen presentation thus the immunogenicity of the DNA vaccines could be different depending on the way of delivery. Moreover, the high immune response relies not on the amount of administered DNA but on the ability of the

DNA to enter efficiently the targeted cells. There are four currently leading delivery methods for DNA vaccine administration and each of them has its advantages and disadvantages.

Intramuscular (i.m.) needle injection

The DNA vaccine designed for i.m. injection contains the plasmid DNA dissolved mostly in saline or other solutions (Lu *et al.*, 2008). The optimum dose for the vaccination is approximately 50-100 μg for mice. The predominant cells transfected after the inoculation of the vaccine are myocytes. A very recent observation characterised the distribution and presentation of the plasmid-encoded antigen *in vivo* as following: The injected DNA vaccine promptly enters the peripheral blood and lymphatics from the site of injection and reaches the lymphoid tissue as free DNA within 24 h. DNA in lymph nodes (LNs) is taken up by peripheral cells that then migrate deeper to the LN. pDNA and/or the expressed antigen is then transferred to DCs ($CD11c^+$) for presentation to naïve T cells. Simultaneously, DNA from the blood reaches the bone marrow and spleen where it is taken up by DC precursors. After three days, antigen-specific $CD4^+$ T cells are detectable in these tissues. In summary, the immune responses are induced by DNA vaccines within days and become systemic very rapidly (Rush *et al.*, 2010).

Intradermal (i.d.) delivery via gene gun

The commercial name used for the gene gun technology is Particle Mediated Epidermal Delivery (PMED) that presents a needle free device (Fig. 8). The plasmid DNA is coated onto gold particles and delivered via the gene gun under pressure into the epidermal layer of a skin. Thus the DNA penetrates directly into the cytoplasm (Tang *et al.*, 1992) of keratinocytes and DCs (Langerhans cells) present in the epidermis. Generally, only a small amount (1-2 μg for mice) of the plasmid DNA is delivered and the antigen expression persists 4-14 days in the site of administration. Transfected DCs migrate to regional LNs within 12-24 h and present antigen to naïve $CD8^+$ T cells (Porgador *et al.*, 1998).

Intradermal (i.d.) delivery via tattooing

Tattooing represents a method of solid vibrating needle (Fig.8) that repeatedly punctures the skin and wounds the epidermis and dermis. This procedure causes dermal haemorrhage and necrosis and induces cutaneous inflammation followed by healing (Gopee *et al.*, 2005). 50-100 μg of plasmid DNA dissolved in saline is usually delivered to the skin at

the dorsum of animals and is able to induce cellular and humoral antigen-specific responses (Baxby, 2002). Tattooing involves much larger area of the skin than other intradermal administrations, what potentially leads to more transfected cells (Bins *et al.*, 2005). However, the gene expression after tattoo delivery peaks after 6 h and vanishes within 4 days (Corder *et al.*, 1996). Moreover, studies with an *ex vivo* human skin model showed extremely low transfection efficiency of this technique that indicates a necessity to develop strategies for enhancing the *in vivo* transfection efficacy of tattoo-delivered DNA vaccines (van den Berg *et al.*, 2009).

Figure 8. Helios gene gun from Bio-Rad and tattoo machine from Bortech Tattoogrosshandel

Electroporation-mediated i.m. delivery

Electroporation (EP) is a method in which multiple electric pulses (20-30 ms) of low voltage (50-200 V/cm) are applied to the vaccination site to improve transfection efficacy in the tissues where the DNA vaccine was delivered before by conventional needle injection. The transfection efficacy is enhanced by electric pulse by two potential ways: either the electric pulse creates pores in a cell membrane, which facilitates the entry of naked DNA into a cell, or the tissue damage induces inflammation and recruits DCs and lymphocytes to the site of injection (reviewed in Abdulhaqq & Weiner, 2008). The doses of DNA applied in this vaccination method are comparable with those used by i.m. injection. EP elicits high cellular and humoral immune responses in various animals and also in humans. However, a special device is needed for human application. It is important to be proved that EP does not generate high risk of DNA integration into the host-cell genome (Lu *et al.*, 2008).

Several DNA delivery studies were published that compare the different DNA vaccine immunisation methods (Trimble *et al.*, 2003; Wang *et al.*, 2008). Based on these results, only

the gene gun-mediated approach is able to elicit protective levels of immune responses in humans. EP-mediated administration is also highly effective in inducing antigen-specific immune responses in animal models and in prime-boost combination protocols is successful in humans, too (Chiarella *et al.*, 2010). Indeed, both the gene-gun immunisation and the administration with EP are several times more immunogenic than the simple i.m. needle injection. A more recent study performed a comparison of EP and gene-gun delivery methods in ability to generate antigen-specific $CD8^+$ T cell responses and anti-tumour immune responses against the HPV E7 protein. The DNA vaccine administered with EP induced the higher number of E7-specific cytotoxic cells when compared to gen-gun delivery (Best *et al.*, 2009). Comparison of the efficacy of DNA vaccines delivered by a tattoo device or needle injection revealed that the humoral and cellular immune responses induced by tattooing are significantly higher than those after i.m. administration of DNA (Pokorna *et al.*, 2008). To the best of my knowledge, there is no published data about the comparison of gene gun or EP-mediated delivery of a DNA vaccine with tattooing.

2.3.2 Advantages and disadvantages

DNA vaccines have several advantages when compared to other forms of vaccines. First of all, they are relatively safe, safer than live attenuated vaccines or inactivated viral vaccines, which may cause infection *in vivo*. Moreover, DNA vaccines are unable to revert to viral forms, unlike live vector-based vaccines, and can be administered repeatedly to the same individual (Abdulhaqq & Weiner, 2008; Lin *et al.*, 2010b). Studies with DNA vaccines have shown that DNA plasmids themselves are not immunogenic and they do not generate anti-DNA antibodies, even after multiple administrations (Smith, 2000). Additionally, DNA vaccines are stable and relatively simple to design and prepare at high purity. As they are not really temperature sensitive, their storage and transportation is inexpensive thus DNA vaccines are highly suitable for mass production and distribution.

Potential integration of the plasmid DNA into the host cell genome may represent a risk in term of clinical application of DNA vaccines. Integration of the foreign DNA into the site of proto-oncogenes or tumour-suppressor genes could lead to tumour progression. However, there is still no evidence about connection between tumour development and the integration of the plasmid after DNA vaccination (Nichols *et al.*, 1995; Ramirez *et al.*, 2008).

Other important limitation of DNA vaccines is their low immunogenicity, particularly in large animals and humans, due to the inability to spread from transfected cells into surrounding cells *in vivo*.

2.3.3 Enhancement of DNA vaccine potency

As mentioned above, the suboptimal immunogenicity of DNA vaccines requires solutions. Nowadays, numerous strategies exist to enhance the DNA vaccine efficacy. Here is the summary of the main improvements:

1. *Modification of plasmid sequences*

Induction of a strong immune response after plasmid DNA immunisation depends on the high constitutive expression levels of the encoded antigen. Therefore, utilising strong regulatory elements, the promoter/enhancer, intron and polyadenylation signal, is a key parameter in plasmid DNA vaccine vector design. Unmethylated CpG motifs also contribute to immune system stimulation (Williams *et al.*, 2009). Additionally, differences between codon usage in a heterologous gene and host organisms may affect antigen expression. Therefore, codon adjustment of the plasmid expressing the antigen is considerable to maximise translational efficiency and consequently the immune response. For instance, the replacement of wild-type codons in the HIV-1 gp120 DNA vaccine increased the expression of the gp120 compared to the wild-type gene and significantly enhanced the $CD8^+$ T cell response. Such codon optimisation of the antigen increased the immunogenicity of DNA vaccines also against tetanus or malaria (Garmory *et al.*, 2003).

2. *Improved delivery methods*

The different routes of administration may determine or affect the induced immune responses. Various vaccine deliveries are described precisely in the Chapter *Immune responses and vaccine delivery*.

3. *Utilisation of adjuvants*

Nowadays, novel types of adjuvants, the molecular adjuvants, are used beyond the conventional ones (e.g. Freund adjuvant) including cytokines, chemokines and costimulatory molecules. Co-injection of DNA encoding cytokines IL-2 and IFN-γ stimulates the T_H1 immune response and thus the activation of $CD8^+$ T cells (Chow *et al.*, 1998). Co-administration of plasmids encoding GM-CSF (granulocyte-macrophage colony-stimulating

factor) and MIP-1α (macrophage inflammatory protein 1α) with a DNA vaccine recruits macrophages and DCs to the site of inoculation that leads to activation of $CD4^+$ and $CD8^+$ T cells (McKay *et al.*, 2004). However, an optimal combination of adjuvants with DNA vaccines is needed to be further proposed to significantly enhance the DNA vaccine potency (Ohlschlager *et al.*, 2009).

The co-delivery of "helper antigens" with weak antigens of interest may be an alternative strategy. Helper antigens, such as keyhole limpet hemocyanin (KLH), tetanus toxin or β-galactosidase, are foreign antigens that activate strong T cell immune responses. The gene encoding the helper antigen may be administered separately from the gene for the target antigen on a second plasmid but to the same site of immunisation, or the two plasmids may be co-coated onto the same gold particles when delivered by a gene-gun device (Leitner *et al.*, 2009).

4. *Modification of antigen*

Construction of DNA fusion-gene vaccines is another strategy to activate effective immune response. Co-expression of helper antigens by generating fusion proteins with the target antigen enhances immune responses, for example heat shock proteins (Hsp; Chen *et al.*, 2000; Qazi *et al.*, 2005) or *E.coli* β-glucuronidase (GUS; Smahel *et al.*, 2004) fused to target antigen induce high $CD8^+$ T cell response. Moreover, the linkage to Fragment C (FrC) of tetanus toxin results in tumour growth suppression (Stevenson *et al.*, 2004).

Several modifications of the plasmid-expressed antigens are focused on their targeting to antigen presentation pathways. Attachment of a signal sequence permits the antigen to entry the endoplasmic reticulum (ER) and subsequently, the antigen is presented on the cell surface through MHC class I molecules to $CD8^+$ T cells (Leifert *et al.*, 2004). Furthermore, antigens can be directed to the lysosome/endosome by linkage to lysosome-associated membrane protein-1 (LAMP-1) and thus induce the $CD4^+$ T cell responses via MHC class II molecules (Wu *et al.*, 1995).

The fusion of the antigen with VP22, a viral translocatory protein from HSV-1 or the Marek's disease virus, helps the spread of the antigen from cells where they are abundantly expressed into neighbouring APCs (Manoj *et al.*, 2004). Linking the antigen to cytotoxic T-lymphocyte antigen-4 (CTLA-4) targets the antigen directly to APCs through the recognition with the B7 costimulatory molecule (Boyle *et al.*, 1998).

2.3.4 DNA vaccines against HPV

DNA vaccines against HPV represent therapeutic vaccines focused on two, E6 and E7, viral antigens. The administration of the E6 and E7 genes may lead to cell transformation as the produced E6 and E7 proteins are oncogenic. Thus a modification is needed to turn E6 and E7 to proteins incapable of such transformation.

Limitation of HPV E6 and E7 DNA vaccines is definitely their low immunogenicity. Therefore, several strategies have been developed to overcome this obstacle (for review see Lin *et al.*, 2010a; Lin *et al.*, 2010b):

- Increasing the number of HPV antigen-expressing or HPV antigen-loaded DCs – including different routes of administration or utilisation of microencapsulated vaccines, increasing intercellular spreading of HPV antigens to DCs etc.
- Improving HPV-antigen expression, processing and presentation – codon optimisation, directing the antigen to MHC presentation pathways etc.
- Enhancing DC and T cell interaction – prolonging DC survival and increasing cytokine expression, priming helper T cells etc.

The following paragraphs detail some of these methods of enhancing the immunogenicity of therapeutic HPV DNA vaccines:

An effective strategy to enhance antigen expression is the employment of demethylation agents. It has been shown that DNA methylation, particularly the methylation of CpG motifs in the plasmid of DNA vaccines, silenced gene expression (Hirasawa *et al.*, 2006). Thus, application of a nucleoside analogue 5-aza-2'-deoxycytidine (DAC), which inhibits DNA methyltransferases (DNMTs), could prevent DNA methylation. The combination of the DNA vaccine encoding calreticulin linked to HPV 16 E7 (CRT/E7) with DAC treatment led to the up-regulation of CRT/E7 expression and enhanced E7-specific $CD8^+$ T cell response (Lu *et al.*, 2009).

CIITA (Class II, major histocompability complex, transactivator) is a regulator of the expression of MHC I and MHC II molecules on the DC surface. Therefore, the co-delivery of CIITA with HPV DNA vaccines leads to enhanced antigen presentation through both MHC pathways and subsequently to stronger $CD4^+$ and $CD8^+$ T cell responses (Kim *et al.*, 2008).

A technology called single chain trimer (SCT) represents a DNA vaccine encoding the antigenic peptide fused to β2-microglobulin and MHC I heavy chain. The fusion protein (antigenic peptide/MHC class I molecule) is expressed on the DC surface as MHC I

molecules already loaded with the antigenic peptide. Mice immunised with the HPV E6 SCT vaccine were completely protected from E6-producing TC-1 tumour cells and an increased E6-specific $CD8^+$ T cell response was detected (Huang *et al.*, 2005).

To disable DCs or T cells from undergoing apoptosis after their mutual interaction, the pro-apoptotic proteins and the pro-apoptotic signalling protein of the DCs are essential to be blocked. The co-administration of the anti-HPV 16 E7 DNA vaccine with siRNA silencing the expression of the pro-apoptotic Bak and Bax proteins or with DNA encoding shRNA blocking the pro-apoptotic signalling protein, the Fas ligand, resulted in significantly enhanced E7-specific $CD8^+$ T cell response and strong anti-tumour effect in vaccinated mice (Kim *et al.*, 2005; Huang *et al.*, 2008).

Several DNA vaccines have already been tested on humans and the following table summarises the finished or ongoing clinical trials with therapeutic HPV DNA vaccines (Table 7).

Table 7. Clinical trials with therapeutic HPV DNA vaccines (modified from Lin *et al.*, 2010b)

Vaccine construct + adjuvant	Antigen(s)	Target subtype(s)	Route of administration	Phase of study/subjects	Sponsor	Reference(s)
DNA (ZYC101)	E7 epitope (83-95aa)	HPV 16	i.m. injection with microencapsulation of the DNA vaccine	Phase I, anal HSIL Phase I, CIN II-III	MGI Pharma	(Klencke *et al.*, 2002; Sheets *et al.*, 2003)
DNA (ZYC101a)	E6, E7	HPV 16, HPV 18	i.m. injection with microencapsulation of the DNA vaccine	Phase II, CIN II-III	MGI Pharma	(Garcia *et al.*, 2004)
DNA (pNGVL4a-sig/E7(detox)/HSP70)	E7	HPV 16	i.m. injection	Phase I, CIN II-III	NCI	(Trimble *et al.*, 2009)
Prime: DNA (pNGVL4a-Sig/E7(detox)/HSP70) Boost: rVV (TA-HPV) ± imiquimod	E7 E6, E7	HPV 16 HPV 16, HPV 18	i.m. injection (DNA vaccine and rVV) topical (imiquimod)	Phase I, CIN III	NCI	(Lin *et al.*, 2010b)
DNA (pNGVL4a-CRT/E7(detox))	E7	HPV 16	i.d. injection via gene gun	plans for Phase I, CIN II-III	NCI	(Lin *et al.*, 2010b)
DNA (VGX-3100)	E6, E7	HPV 16, HPV 18	i.m. injection with electroporation	Phase I, CIN II-III	VGX Pharmaceuticals	(Lin *et al.*, 2010b)

aa – amino acid, CIN – cervical intraepithelial neoplasia, CRT – calreticulin, HSIL – high-grade squamous intraepithelial lesions, HSP – heat shock protein, i.d. – intradermal, i.m. – intramuscular, NCI – National Cancer Institute, rVV (TA-HPV) – recombinant vaccinia virus

3. PAPERS

3.1 Paper I:

Mutation in the immunodominant epitope of the HPV 16 E7 oncoprotein as a mechanism of tumor escape

Michal Smahel, Pavla Tejklova, Jana Smahelova, Ingrid Polakova,
Jana Mackova

Cancer Immunology, Immunotherapy. 2008; 57:823-831.

Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes for the ELISPOT assay and tetramer staining and carried out the ELISPOT assays.

3.2 Paper II:

Enhancement of T cell-mediated and humoral immunity of β-glucuronidase-based DNA vaccines against HPV 16 E7 oncoprotein

Michal Smahel, Ingrid Poláková, Dana Pokorná, Viera Ludvíková, Martina Dusková, Josef Vlasák

International Journal of Oncology. 2008; 33:93-101.

Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes and carried out the ELISPOT assays.

3.3 Paper III:

Vaccincvkqp with human papillomavirus type 16-derived peptides using a tattoo device

Dana Pokorná, Ingrid Poláková, Martina Kindlová, Martina Dušková,
Viera Ludvíková, Pavel Gabriel, Luďa Kutinová, Martin Müller, Michal Šmahel

Vaccine. 2009; 27:3519-3529.

Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes for the ELISPOT assay and tetramer staining and carried out the ELISPOT assays. Occasionally, I helped with tattooing.

3.4 Paper IV:

DNA vaccine against human papillomavirus type 16: Modifications of the E6 oncogene

Ingrid Poláková, Dana Pokorná, Martina Dušková, Michal Šmahel

Vaccine. 2010; 28:1506-1513.

Contribution of the author of this PhD. thesis:

I carried out almost all experimental work, from plasmid design, through *in vitro* and *in vivo* assays to immunisation experiments and evaluation of the results. The experimental work and the preparation of the manuscript were realized under the supervision and coordination of Michal Šmahel.

4. DISCUSSION

The submitted PhD. thesis represents a work focused on therapeutic DNA vaccines against HPV 16, especially on problems with low immunogenicity of the vaccines. The prepared DNA constructs were designed in an effort to induce strong immune responses targeted against the E7 or E6 oncoproteins. A gene gun delivery method, which belongs to the main administration methods of DNA vaccines, was chosen for immunisation. As immunoresistance was observed when immunisation against E7 was accomplished, a tumour escape mechanism was discovered and described. Moreover, immunodominant epitopes from the E6 and E7 proteins were employed in vaccination using a tattoo device.

4.1 Construction of plasmid DNA vaccines encoding fusion genes

The low immunogenicity belongs to important limitations of DNA vaccines. Their efficacy can be enhanced by using several strategies as described under Chapter *2.3.3 Enhancement of DNA vaccine potency*. The strategy of modification of the antigen was preferred in this work. However, this type of modification may lead to alterations in protein synthesis, protein stability and/or its cellular localisation (Manoj *et al.*, 2004).

Previously, in order to enhance the immunogenicity of the modified E7 protein (E7GGG), several plasmid DNA vaccines encoding E7 fusion genes were constructed in our laboratory. We fused the E7GGG gene with LAMP-1 (Smahel *et al.*, 2003), GUS (Smahel *et al.*, 2004) and Hsp70 (Pokorna *et al.*, 2005). Overall, the highest anti-tumour immune responses were recorded after immunisation with the GUS-fused constructs. Therefore, in the following experiments, the E7GGG.GUS was further modified to enhance even more its immunogenicity. *Paper II* demonstrates the performed modifications (see also below).

The steady-state level of antigen production that may be influenced by the sequence of the gene encoding the antigen, and the stabilisation of the antigen contribute to DNA vaccine efficacy (Manoj *et al.*, 2004; Bins *et al.*, 2007). Hence, to increase the steady-state level of resultant proteins expressed from plasmid DNA, the following modifications were performed with the E7GGG.GUS fusion gene: i) As the fusion of the full-length E7GGG with GUS evokes a decreased steady-state level of GUS antigen (Smahel *et al.*, 2004), portions of amino

acids from the C-terminus of the E7GGG were deleted (E7GGG41.GUS, E7GGG60.GUS and E7GGG75.GUS), ii) the GUS initiation codon was eliminated to abolish the production of GUS alone (E7GGG.EGUS), iii) E7GGG codon adjustment for enhanced expression in human cells was realized (hE7GGG.GUS), and iv) the E7GGG was fused also to the 3'-terminus of GUS (GUS.E7GGG) as the stability of the fusion proteins can be influenced by the position of fusion partners (Li *et al.*, 2006). The production of fusion proteins was compared *via* measuring the enzymatic activity of GUS and immunoblotting staining (*Paper II, Figures 2A, B and 4A, B*). The results showed that only the deletion mutants and the GUS.E7GGG had an increased antigen production when compared with E7GGG.GUS. Examination of protein stability by pulse-chase labelling followed by immunoprecipitation indicated that the stability of the proteins corresponded with their steady-state levels (*Paper II, Figure 7*).

Targeting the antigens to certain cellular location or compartment is another important factor of enhancing the DNA vaccine potency. However, it is dependent on the nature of the antigen. A signal sequence (SS) targets the antigen to the ER from where it may be retro-translocated to the cytosol for proteasome degradation (Bonifacino & Weissman, 1998; Golovina *et al.*, 2002) and subsequently, successful antigen presentation and a strong antigen-specific immune response may be induced (Leifert *et al.*, 2004). Fusion of the signal sequence from the adenoviral E3 gene to GUS.E7GGG led to the accumulation of the SS.GUS.E7GGG protein in the ER. The detection of GUS activity showed that the protein was not secreted from cells (*Paper II, Figures 4 and 5*).

According to the results achieved with the GUS-fused E7GGG protein, the E6 gene was joined to both termini of GUS (E6.GUS, GUS.E6; *Paper IV*). The expression of fusion proteins was verified by immunoblotting staining (*Paper IV, Figure 2*) and measuring the enzymatic activity of GUS (data not published). The unidentified bands found after the immunoblotting detection are supposed to be the products of expression from different initiation codons or the products of degradation or alternative splicing as it was found out after further modifications were performed with the E6 gene.

And how these results finally influence the immune responses induced after the immunisation with the corresponding constructs? These findings are described under Chapter *4.3.*

4.2 Modifications of the HPV 16 E6 oncogene

A low expression of the full-length E6 protein has been reported for the HR E6 genes as an alternative splicing occurs from the one 5' (donor) splice site and two 3' (acceptor) splice sites. The production of the spliced E6*I and E6*II transcripts give rise to two truncated proteins beside the full-length E6. Moreover, the E6*I transcript is the most abundant E6 mRNA in HPV-associated premalignant and malignant lesions (Smotkin *et al.*, 1989; Cornelissen *et al.*, 1990; Griep *et al.*, 1993). The fusion of the E6 gene with the 3'-terminus of GUS (GUS.E6) led to markedly increased level of the full-length E6 mRNA as the RT-PCR amplification and densitometric analysis of the amplified transcripts showed while the fusion to the 5'- terminus (E6.GUS) merely increased the production of the E6*II at the expense of the E6*I mRNA *(Paper IV, Figure 4, Table 1*).

To further increase the production of the full-length E6 protein and thus to enhance the immunogenicity of DNA vaccines, the 5' splice site was abolished in the E6 gene. From the resultant E6cc gene with two substituted nucleotides, a protein with the V49L mutation was produced (*Paper IV, Figure 1*). RT-PCR amplification demonstrated that the abolition of the donor splice site completely eliminated the expression of E6*I and E6*II. The corresponding spliced transcripts were also not expressed from the modified E6cc.GUS and GUS.E6cc fusion genes *(Paper IV, Figure 4*). Immunoblotting staining revealed substantially enhanced modified-E6cc-protein production when compared to the production of the unmodified E6 protein (*Paper IV, Figure 5A*). Densitometric analysis demonstrated also the difference for GUS fusion proteins – a higher production of proteins was detected after the fusion of the E6 or E6cc genes to the 3'- terminus of GUS (*Paper IV, Figures 5B, C and Table 2*). As it was predicted, the middle band of the GUS.E6 sample represents the product of the alternative splicing since this band is missing after the abolishment of the splice site (*Paper IV, Figure 5C*).

Chapter *2.1.3.1 Oncoproteins of HPVs* describes in details the role of the E6 protein in HPV associated carcinogenesis. When designing a DNA vaccine based on the expression of a protein that is originally an oncoprotein (wild-type E6), it is necessary to take in consideration the safety of the vaccine, especially when a clinical trial is expected.

One of the possible ways to decrease the oncogenic feature of the E6 protein is to reduce its ability to induce the degradation of the p53 tumour suppressor protein. Several studies reported mutations that affect the binding of the E6 protein with p53 or its α-helix partners, including E6AP (Smotkin *et al.*, 1989; Dalal *et al.*, 1996; Nguyen *et al.*, 2002). In

this work the E6 protein was altered with one or two substitutions to decrease its oncogenicity. The introduced C70G and I135T mutations (*Paper IV, Figure 1*) were reported to reduce E6-mediated p53 degradation (Dalal *et al.*, 1996; Nguyen *et al.*, 2002; Shai *et al.*, 2007). The ability of the mutated proteins (E6G, E6T, and E6GT) to induce degradation of the p53 protein was tested by an *in vivo* p53 degradation assay. Cotransfection of p53-null cells with plasmids encoding p53 and the modified E6 genes led to the following results: The proteins with a single mutation (E6G or E6T) were still able to induce p53 degradation similar to the wt E6 protein. Interestingly, the E6 protein modified with both mutations (E6GT) eliminated the degradation of p53 (*Paper IV, Figure 7*). These results suggest that the E6AP binding surface of E6 consists of residues from both the N- and C- termini (Nomine *et al.*, 2006). As the modified E6GT protein was the only one unable to induce degradation of p53, solely this protein was fused with GUS (GUS.E6GT) for immunisation experiments. The expression of the fusion protein was verified with immunoblotting staining and by measuring the GUS activity (data not published).

4.3 Immunisation with the prepared DNA vaccines

The prepared plasmid DNA constructs, containing the non-fused and fusion genes with or without modifications, were used in immunisation experiments to verify the vaccine efficacy. C57BL/6 female mice were immunised with DNA vaccines utilising a gene gun device. Administration of DNA vaccines by a gene gun is one of the main delivery methods of these vaccines that leads to effective induction of antigen-specific immune responses (see Chapter *2.3.1 Immune responses and vaccine delivery*). The immunisation scheme of animals, the doses of plasmid DNA and the inoculation of tumour cell lines are described under *Materials and methods of Papers II* and *IV*. To pursue the immunogenicity of the DNA vaccines, two methods were used for the detection of antigen-specific T cells – the intracellular cytokine staining (ICS), when IFN-γ was stained in $CD8^+$ T lymphocytes, and the more sensitive ELISPOT assay to detect IFN-γ-producing cells. The production of antibodies was examined by ELISA.

DNA vaccines against the E7 protein:

Comparison of the production of E7-specific antibodies after immunisation with the truncated E7GGG.GUS genes revealed a high production of antibodies against E7 while the primary E7GGG.GUS construct induced a weak humoral immune response (*Paper II, Figure 3A*). Furthermore, the level of antibody production correlated with the steady-state levels of the fusion proteins (*Paper II, Figure 2A*). On the contrary, about a twice higher cell-mediated immunity was observed by the ELISPOT assay after immunisation with E7GGG.GUS than with the truncated fusion genes (*Paper II, Figure 3B*). Immunisation with GUS.E7 and the truncated fusion genes showed a low number of the IFN-γ-producing splenocytes after restimulation with the immunodominant H-2D^b $E7_{49-57}$ epitope, despite the fact that the high steady-state level of the modified fusion proteins predicted a higher amount of the released E7 antigen and thus suggested an enhanced cross-priming. Moreover, the results from preventive immunisation against TC-1/A9 tumour cells with reduced surface expression of MHC class I molecules corresponded with the highest efficiency of the E7GGG.GUS gene in the ELISPOT assay: all mice developed a tumour except of two animals immunised with the E7GGG.GUS construct (*Paper II, Figure 3C*). The therapeutic immunisation against TC-1 cells led to elimination of tumour cells in about half of mice induced by both the original E7GGG.GUS and also the newly constructed GUS fusion genes (*Paper II, Figure 3D*).

Immunisation with the other modified genes, SS.GUS.E7GGG, E7GGG.EGUS and hE7GGG.GUS showed that none of the vaccines induced significant production of E7-specific antibodies and only immunisation with SS.GUS.E7GGG demonstrated higher antigen-specific CTL immune response in comparison to the original E7GGG.GUS fusion gene. Furthermore, the tumour development was significantly slower after immunisation with SS.GUS.E7GGG in a therapeutic manner of vaccination (*Paper II, Figures 6A, B*). As mentioned above, the SS.GUS.E7GGG protein was not secreted (*Paper II, Figure 4D*) and accumulated in the ER (*Paper II, Figure 5*). This vaccine might have induced the highest cell-mediated immune response due to the retrograde transport of the fusion protein from the ER into cytosol and its degradation with proteasomes as reported by Golovina *et al.* (Golovina *et al.*, 2002). The immunogenicity of the fusion gene with codon optimisation in the E7GGG part (hE7GGG.GUS) and also its protein production were comparable to E7GGG.GUS (*Paper II, Figures 4A and 6A*) though the hE7GGG gene alone produced about a 6-fold higher amount of the E7GGG protein when compared to the E7GGG gene (data not published). A reasonable assumption for this unaltered immunogenicity of the hE7GGG.GUS

fusion protein may be the fusion with the 6-fold longer GUS. This non optimised part may suppress the contributory effect of E7GGG codon optimisation.

The cell-mediated immunity may be influenced by the antigen stability. It has been reported that the destabilisation of the E7 antigen, for instance with a mutation, enhanced the CTL responses (Shi *et al.*, 1999). However, vaccination with fusion proteins with higher (E7GGG60.GUS) or lower (E7GGG.EGUS) stability than the original E7GGG.GUS protein did not enhance the $CD8^+$ T cell immune responses (*Paper II, Figures 3B, 6A and 7*). This may be in accordance with the data reported by Golovina *et al.* showing that mutations or deletions causing misfolding of the proteins did not impact on the epitope production (Golovina *et al.*, 2005).

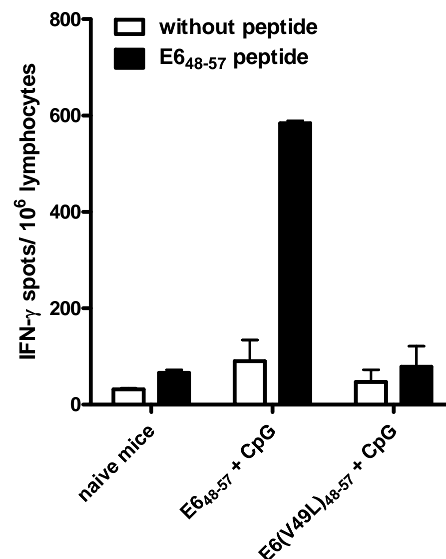
DNA vaccines against the E6 protein:

The fusion of the E6 gene with both termini of GUS produced a bit different immunisation results than the fusion of the GUS with the E7 gene. IFN-γ-producing $CD8^+$ T cells were detected by ELISPOT or ICS assays after overnight incubation with the H-$2K^b$ $E6_{48-57}$ epitope. While the E7GGG.GUS induced the strongest immune response after immunisation with E7-derived plasmid DNAs, the immunogenicity of the E6 fused to the 5'-terminus of GUS (E6.GUS) was comparable with that of the E6 gene alone. The highest number of E6-specific T cell was recorded after immunisation with the GUS.E6 construct (*Paper IV, Figure 3A*). Moreover, using a preventive immunisation scheme against TC-1 tumour cells the immunisation with GUS.E6 resulted in a significantly lower tumour growth (*Paper IV, Figure 3B*). The high efficacy of the GUS.E6 vaccine could be caused by the higher production of the unspliced E6 transcript (*Paper IV, Figure 4, Table I*) and subsequently the high full-length E6 fusion protein production.

The cell-mediated immunity was moderately decreased after immunisation with plasmids carrying genes with the abolished E6 splicing site (E6cc constructs) – a decreased number of E6-specific T cells was recorded by ELISPOT assay when compared with the unmodified genes (*Paper IV, Figure 6A*). Immunisation of animals against TC-1 cells also confirmed the reduced immunogenicity of the modified fusion genes (*Paper IV, Figure 6B*). An acceptable explanation could be the substitution of valine by leucine at position 49 (V49L) in the H-$2K^b$ immunodominant epitope of E6 (aa 48-57) as a result of the eliminated splice site. However, the comparison of the original E<u>V</u>YDFAFRDL epitope sequence with the mutated E<u>L</u>YDFAFRDL sequence by computer analysis did not reveal any significant difference in predicted binding to H-$2K^b$ molecules. For the analysis, two databases of MHC

ligands and peptide motifs were used, the BIMAS (www-bimas.cit.nih.gov/molbio/hla_bind) and the SYFPEITHI (www.syfpeithi.de). Furthermore, Peng *et al.* reported that the minimal core sequence required for the activation of $CD8^+$ T cells is the aa 50-57 region of the E6 protein (Peng *et al.*, 2004). Nevertheless, immunisation with synthetic peptides delivered together with CpG motifs (ODN 1826), using a tattoo device confirmed the reduced immunogenicity of the E6 peptide carrying the V49L mutation (data not published, Fig. 9). Supposedly, the abolishment of the splicing site, which led to the V49L mutation in the E6 protein impaired the immunodominant epitope for C57BL/6 mice and thus decreased the immunogenicity of the modified DNA vaccines.

Figure 9. Immunogenicity of the $E6_{48-57}$ and $E6(V49L)_{48-57}$ peptides



Mice (n=3) were immunised three times at a 1-week interval with the $E6_{48-57}$ or $E6(V49L)_{48-57}$ peptides (100 μg) supplemented with CpG motifs (50 μg) using a tattoo device. Splenocytes were isolated one week after the last immunisation, incubated overnight with the $E6_{48-57}$ peptide and IFN-γ-producing cells were detected by an ELISPOT assay.

Mutations C70G and I130T that were performed to reduce the E6-mediated p53 degradation evoked a slightly decreased immune response detected by ICS assay after vaccination with the mutated genes (*Paper IV, Figure 8A*). This moderate distinction was also confirmed after challenging the animals with tumour cells (*Paper IV, Figure 8B*). Moreover, the immunisation with plasmid DNA encoding the fusion protein with both types of modifications, the one eliminating the donor splice site in the E6 gene and that reducing the p53 degradation ability of the E6 protein (GUS.E6ccGT), did not lead to satisfactory results

(data not published). A recent proposal of the structure of the HPV 16 E6 protein indicates that many of the mutations made to find out the functions of this protein resulted in structure destabilisation (Nomine *et al.*, 2006). As the mutations performed in this work were chosen prior to these new structural findings, they are unfortunately all located at the key buried positions of the E6 protein. Therefore, the potential conformational changes induced particularly by the C70G and I130T mutations might have influenced the antigen processing and presentation that consequently moderated the immunogenicity of DNA vaccines.

4.4 HPV 16 E7 mediated mechanism of tumour escape

Chapter *2.2.2.2 Molecular mechanisms leading to tumour escape* describes the importance of the impaired presentation of the antigen epitopes by MHC class I molecules in tumour evasion from the immune system. The down-regulation of MHC class I and/or antigen expression or alterations in the epitope sequence can result in tumour escape. All these modifications may represent an obstacle in anti-tumour vaccine designing.

Recently, after vaccination of animals, we derived immunoresistant clones from their tumours developed from the challenged mouse TC-1 cells producing E6 and E7 oncoproteins. The clones were resistant to immunisation with a DNA vaccine against E7. The immunoresistance correlated with the N53S mutation in the immunodominant epitope of the E7 protein (Smahel *et al.*, 2005). *Paper I* demonstrates that this mutation is responsible for the evasion of TC-1 clones from the antigen-specific immune responses induced by vaccination.

The RANKPEP computer analysis for the prediction of peptide binding to MHC molecules revealed decreased binding of the mutated peptide to H-2D^b molecules. This prediction was confirmed after testing the ability of the E7 peptides (aa 49-57) to stabilise the H-2D^b molecules on the RMA-S cell surface (*Paper I, Figure 1*). The incubation of the cells with the RAHYSIVTF peptide carrying the N53S mutation showed the same result as the control RMA-S cells (with empty H-2D^b) while the original RAHYNIVTF peptide prolonged the presence of MHC I molecules on the cell surface. This finding suggested a reduced immunogenicity of the mutated E7$_{49-57}$ epitope as the immunogenicity of peptides correlates with their ability to stabilise surface MHC class I expression (Lipford *et al.*, 1995). Therefore, the N53S mutation was introduced to the E7GGG.GUS fusion gene (E7GGGS.GUS) to test its immunogenicity. The production of the fusion protein was verified by immunoblotting

staining and measuring the enzymatic activity of GUS (*Paper I, Figure 2A*). After the immunisation of animals with E7GGG.GUS and E7GGGS.GUS by a gene gun, the immunogenicity of the genes was detected by ELISPOT assay and tetramer staining after restimulation of the splenocytes with the RAHYNIVTF peptide (*Paper I, Figures 2B, C*). In both cases, a loss of immunogenicity of the E7GGGS.GUS gene was demonstrated even after restimulation with the mutated RAHYSIVTF peptide (data not published). Moreover, after the preventive immunisation against the TC-1 tumour cells, all mice vaccinated with E7GGGS.GUS developed a tumour (*Paper I, Figure 2D*). These findings show that the N53S substitution eliminates the $E7_{49\text{-}57}$ immunodominant H-2D^b epitope. Furthermore, the immunoresistance of the TC-1/F9 and TC-1/C6 clones, producing the E7 protein with the mutated epitope, is strictly E7-specific as the immunisation of mice against E6 prevented the growth of tumours from these clones (*Paper I, Figure 3*).

To prove finally that the N53S mutation was responsible for the immunoresistance of the TC-1 clones, the TC-1/F9 clone was transduced with the wt E7 signed with FLAG and the TC-1/F9/C1 clone was derived. The TC-1/F9/B5 control clone is a result of E7S.FLAG transduction. The clones produced the E7.FLAG or E7S.FLAG protein at a level similar to that of the E7 in TC-1 cells (*Paper I, Figure 4*). To test the immunosensitivity of the clones, mice were vaccinated with the E7GGG.GUS and E7GGGS.GUS gene and challenged with the appropriate TC-1 clones. Tumours developed in all immunised mice after the challenge with TC-1/F9/B5 or the parental TC-1/F9 cells. A reduced tumour growth was observed after the challenge with the TC-1/F9/C1 cells (*Paper I, Table 1*). The inhibition of the tumour development, though, was not significant as the tumour growth after the challenge with the TC-1/F9/C1 cells was also partly reduced in the pBSC- and E7GGGS.GUS-immunised animals. Furthermore, the oncogenicity of four TC-1/F9/C1-derived clones (A7, H10, G4, E12) was also decreased when compared with the TC-1/F9 cells while their immunosensitivity was high (*Paper I, Table 1*). This phenomenon of reduced oncogenicity of the tumour-cell-derived clones has been described previously. The transcriptional analysis of the examined cells revealed variability in the expression of immunomodulatory cytokines, namely MCP-1, osteopontin and midkine (Smahel *et al.*, 2005). These cytokines might influence the oncogenicity of TC-1 clones. Nevertheless, the summarised results showed significant inhibition of tumour development after the immunisation with the E7GGG.GUS gene and the challenge with TC-1/F9/C1 cell line or its clones and thus confirmed the responsibility of the N53S mutation for immunoresistance of TC-1/F9 cells.

Unfortunately, the frequency of the N53S escape mutation in the E7 oncogene was rare. The sequence analysis of the E7 gene in 52 clones derived from 10 cell lines isolated from TC-1-induced tumours did not detect any mutation in E7. However, the tested cell lines had reduced expression of MHC class I molecules (data not published). The previous study also showed reduced H-2D^b and H-2K^b molecules on TC-1 cells after immunisation against E7 (Smahel *et al.*, 2003). These data indicate that the principal escape mechanism of TC-1 cells from the host's immune system was the down-regulation of MHC class I molecules. However, the mutations in the HPV oncoproteins can contribute to the evasion from immune surveillance. For instance, the L83V substitution in a HPV 16 E6 protein variant can alter the activities of the protein important for its oncogenic potential (Lichtig *et al.*, 2006).

4.5 Vaccination against HPV 16 using a tattoo device for administration of E7- and E6-derived peptide vaccines

The $E7_{49\text{-}57}$ and $E6_{48\text{-}57}$ epitopes are considered to be the immunodominant H-2^b CTL epitopes of the appropriate HPV 16 oncoproteins (Feltkamp *et al.*, 1993; Peng *et al.*, 2004). Therefore, these epitopes may represent the key peptides employed in peptide-based vaccines against HPV 16. As described under the *Introduction* of *Paper III*, one of the disadvantages of peptide vaccines, similarly to the DNA vaccines, is their low immunogenicity. Several strategies were developed to enhance their efficacy like the modification of epitopes, administration with adjuvants, conjunction with helper proteins and different ways of application. Comparison of the administration methods of DNA vaccines revealed that tattooing induces higher cellular and humoral immune responses than i.m. needle injection (see Chapter *2.3.1 Immune responses and vaccine delivery*). The mechanical injuries caused by the tattoo procedure non-specifically stimulate the immune system and may partially compensate the need of adjuvants. As adjuvants approved for humans are rare and may cause adverse effects (Israeli *et al.*, 2009), a delivery method providing efficient immunisation without adjuvants is desirable. Tattooing for delivery of peptide vaccines and its comparison with s.c. needle injection of peptides was examined for the first time in our laboratory.

The immunisation experiments revealed that the cellular immune responses induced with the $E7_{49\text{-}57}$ peptide delivered by a tattoo device were comparable to those after gene gun DNA vaccination with pBSC/E7GGG.GUS (*Paper III, Figure 1A*). Further, all mice

immunised against TC-1 tumour cells with the E7 peptide in a preventive manner remained tumour free, which indicates a high immunogenicity of the tattooed peptide vaccine (*Paper III, Figure 1B*).

Subsequently, the ability of two different delivery methods for induction of efficient immune responses was compared using E7-derived peptide vaccines. The $E7_{49\text{-}57}$ and the $E7_{44\text{-}62}$ (carrying epitopes for CTL, $CD4^+$ T cells and B cells) peptides, supplemented with or without CpG motifs, were administered to animals by a tattoo device or s.c. needle injection. The highest cellular immune response was detected after immunisation with the $E7_{49\text{-}57}$ peptide and CpG motifs by a tattoo device. Similarly, high amount of E7-specific CTLs was detected in mice tattooed with the $E7_{44\text{-}62}$ peptide and CpG motifs (*Paper III, Figures 2A, B*). Humoral immune responses were examined only after immunisation with the $E7_{44\text{-}62}$ peptide (*Paper III, Figures 2C, D*). The production of E7-specific Abs was again higher after tattoo administration of the peptide in combination with or without CpG motifs than after s.c. injection of the same vaccine. Moreover, the results obtained from preventive immunisation of mice against TC-1 tumour cells showed that the protection of animals from tumour development corresponded with the immune responses induced by the peptide vaccines (*Paper III, Figure 2E*).

Surprisingly, the addition of CpG motifs to the s.c. injection of $E7_{49\text{-}57}$ peptide decreased the induction of cellular immune responses and the protection against tumour cells. The reason could be the mild precipitation of the water-dissolved peptide after addition of PBS. The peptide aggregation further increased after CpG motifs were added. The precipitation may influence the presentation of the peptide by APCs and may lead to decreased immune responses after s.c. delivery. On the contrary, the tattoo procedure may mechanically disrupt the peptide aggregates and then the induction of the immune system remains non-affected. In summary, tattoo delivery of peptide vaccines induced higher cell-mediated and humoral immune responses when compared with the s.c. injection, and the addition of CpG motifs further enhanced the efficacy of the vaccines. The comparison of these results with the previous results achieved with peptide immunisation used in other laboratories (Dileo *et al.*, 2003; Gendron *et al.*, 2006) where the vaccines did not protect the animals against tumour formation, indicates that the higher dose of the vaccines and the number of immunisations used in our laboratory and also the administration of lower dose of the challenging tumour cells could be the reason of the breaking through the detection limit.

In order to examine whether the tattoo delivery method is efficient also for peptide vaccines carrying different CTL epitopes, immunisation with the $E6_{48\text{-}57}$ peptide was

performed. The E6 peptide was administered to animals by tattooing or s.c. injection and the vaccines were supplemented with CpG motifs. Only the tattoo-delivered E6 peptide vaccine was able to induce CTL-mediated immune response (*Paper III, Figure 3C*) and to slow down the TC-1 induced tumour growth (*Paper III, Figure 3B*). However, all E6-tattooed mice developed tumours while two animals immunised subcutaneously remained protected against tumour formation (*Paper III, Figure 3A*).

Moreover, *Paper III* also demonstrated that the tattoo immunisation with KLH-conjugated $E7_{49-57}$ peptide (with or without CpG motifs) induced higher $CD8^+$ T cell immune responses in comparison with the s.c. injection of the same vaccine (*Paper III, Figure 4A*). However, the s.c. immunisation with the KLH-conjugated E7 peptide induced higher number of KLH-specific Abs than the vaccine administered *via* the tattoo device (*Paper III, Figure 4B*). This might be the result of the aggregation of the KHL-conjugated peptide that could have been less detrimental to the induction of humoral responses after s.c. delivery. Furthermore, the addition of CpG motifs enhanced the levels of KLH-specific Abs after immunisation by both delivery methods.

In conclusion, the comparison of the two delivery methods, tattoo device *vs*. s.c. needle injection, revealed that the administration of the E7- and E6-derived peptide vaccines by tattooing induced higher cellular and humoral immune responses than the s.c. injection.

5. SUMMARY

DNA vaccination has become an effective strategy for the development of vaccines against cancer including cervical carcinoma associated with HPV infection. DNA vaccines are proved to induce both humoral and cellular immunity. However, an enhancement of their low efficacy is required. The therapeutic DNA vaccines against HPV 16 are targeted against the viral E7 and E6 oncoproteins. In order to design safe vaccines, the oncogenicity of these targets has to be eliminated or properly reduced.

In this work, an enhanced immunogenicity of DNA vaccines against HPV 16 delivered by the gene gun was demonstrated after the fusion of the E7 and E6 genes with GUS.

DNA vaccines against the E7 protein:

The increased steady-state level of the E7GGG.GUS deletion mutants and the GUS.E7GGG fusion protein enhanced the production of E7-specific antibodies after immunisation with these vaccines but did not improve the CTL response. Joining of the signal sequence with GUS.E7GGG led to ER-localisation of the SS.GUS.E7GGG fusion protein, enhancement of the cell-mediated immune responses and slower tumour growth in immunised mice.

DNA vaccines against the E6 protein:

Enhanced immunogenicity was showed after immunisation with the E6 gene fused to the 3'-terminus of the GUS (GUS.E6). The abolishment of the splice site in the E6 gene resulted in complete elimination of the expression of the truncated E6 transcripts. However, this modification moderately reduced the immunogenicity of the non-fused (E6cc) or fused (GUS.E6cc) genes probably as a consequence of the V49L substitution in the immunodominant E6 epitope. The oncogenicity of the E6 protein was reduced by two point mutations and the modified E6GT protein was unable to induce p53 degradation. These substitutions in the E6 protein did not substantially influence the immunogenicity of the vaccines.

The infection with HPV can lead to development of malignant tumours. Several mechanisms may be responsible for the immune escape of the tumour cells infected with HPV. This work demonstrates one of the possible ways of this tumour escape. The N53S substitution in the RAHYNIVTF immunodominant epitope (aa 49-57) of the E7 protein was responsible for the immunoresistance of TC-1 clones derived from tumours of immunised

mice. The resistance of the clones was E7-specific as the tumour growth was inhibited after immunisation against E6. Transduction of the immunoresistant clones with the wt E7 gene restored their sensitivity to immunisation against E7.

Tattooing is another efficient delivery method of DNA vaccines beyond the gene gun administration and i.m needle injections. Nevertheless, it has not yet been tested for administration of peptide vaccines. This thesis reports the comparison of tattooing with s.c. injection using E7- and E6-derived peptide vaccines. Higher peptide-specific immune responses were observed after immunisation with the $E7_{49-57}$, $E7_{44-62}$ or $E6_{48-57}$ peptides administered by a tattoo device than after their s.c. inoculation. The addition of CpG motifs enhanced the induced immune responses after both types of vaccination.

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