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**INTRODUCTION**

**HPV AND CANCER**

Human papilloma viruses (HPV) are non-enveloped DNA viruses that infect human skin and mucosa and are the causative agents of mostly benign proliferative lesions such as common (genital) warts (1). However, persistent infection with sexually transmitted, mucosal ‘high-risk’ HPV subtypes is strongly associated with the development of anogenital malignancies such as cervical, vulvar-, penile- and anal cancer, and also a subset of oropharyngeal cancers (1-4). The association is strongest for cervical cancer, illustrated by the finding that in over 99% of cervical cancers HPV DNA can be detected (5, 6). Notably, cervical cancer is the third most common cancer in women worldwide, with an estimated death toll of almost 300,000 women annually, mostly in developing countries (7, 8). The much lower burden in the developed world is due to screening programs (most often Pap testing) that aim to detect early lesions, which can most often be cured by surgical removal of the lesion (2, 8, 9). As the immune system operates by the principle of non-self recognition, the involvement of a virus in the development of these types of malignancies provides a unique opportunity for the immune system to prevent or eradicate these types of malignancies.

**PREVENTIVE VACCINATION**

Recently two vaccines have become available for the prevention of HPV induced malignancies, namely Cervarix® and Gardasil® (10-12). Both vaccines are directed against the two most prevalent high-risk subtypes, HPV16 and 18, accounting for about 50% and 20% of cervical cancer cases respectively (13). Gardasil is also directed against the mucosal low-risk subtypes 6 and 11, together accounting for 90% of genital warts (14). Both vaccines are composed of viral like particles (VLPs) that self-assemble when the major capsid protein L1 is expressed in eukaryotic cells. These VLPs are highly immunogenic structures that resemble the virus particle, but without the genetic content of the virus and thus without the risk of inducing disease. The VLPs provoke a strong B cell mediated immune response against L1, resulting in viral capsid specific antibodies, that are believed to neutralize/shield the virions before they can infect, thereby providing sterile protection against infection with the corresponding HPV virus sub-types (11, 15). However, these vaccines have no value for the treatment of pre-existing lesions (see below) and as a consequence these vaccines need to be administered to individuals before they get infected. For optimal prophylaxis, the complete population has to be vaccinated before the onset of sexual activity (12). So far, long-term (up to 6 years of follow-up) clinical trials in young (15-26 year old) women have shown nearly 100% protection against the development of precancerous lesions, caused by HPV16 and 18, upon vaccination with these preventive vaccines (16, 17). Although this efficacy is impressive, the estimated costs involved in the prevention of a single case of cervical cancer are extremely high: approximately 5 million US dollar based on an incidence of 7 per 100,000 (the age standardized incidence of cervical cancer in Western Europe (8)) and the cost per vaccination of 360 US dollar. This is explained by the fact that only very few HPV infections will eventually result in the formation of malignancies (18, 19). It has to be noted that the prevention of precancerous lesions (that have a much higher incidence) as such already provides a significant clinical benefit as the treatment of such lesions often requires surgical intervention (16, 17).
NEED FOR THERAPEUTIC VACCINE DEVELOPMENT

Beside the poor cost-effectiveness, a major drawback is that the preventive vaccines do not generate therapeutic effects against pre-existing lesions (20, 21), as also mentioned above. This is explained by the fact that upon infection the virus is maintained inside cells where antibodies can not reach it because they cannot pass the cell membrane. Moreover, expression of the viral capsid protein L1, that is recognized by these antibodies, is lost upon malignant transformation (22, 23). Therefore, a different approach is needed in order to generate an immune response that can eradicate existing lesions. The type of immune response required to eradicate pathogen-infected cells is called a cytotoxic T cell response. Cytotoxic T cells can kill pathogen infected cells upon recognition of virus-derived peptides presented at the cell surface on MHC class I molecules (24). As it is well established that the viral proteins E6 and E7 of the high-risk sub-types play an essential role in the transformation process (25, 26), and are expressed in all HPV transformed cells, they are excellent targets for therapeutic vaccine development (21, 27). Importantly, the spontaneous clearance of HPV induced (pre-)malignancies is associated with T cell mediated immune responses against these proteins (28-30). Over the past two decades, numerous therapeutic vaccine candidates, targeting mostly HPV16 E6 and E7, have been developed in preclinical models (15, 21, 27, 31). Disappointingly clinical success has been rather limited with response rates usually not exceeding the rate of spontaneous regression (15). One recent study in patients suffering from grade 3 vulvar intraepithelial neoplasia (VIN 3) vaccinated with a vaccine consisting of E6 and E7 based long-peptides in incomplete Freunds adjuvant, showed a durable and complete regression in 47% of patients (32, 33). Also another recent study in which protein based vaccine (TA-CIN), that had no clinical effect as such (34), was combined with local immune modulation using Imiquimod (a TLR-7 agonist) in VIN 2/3 patients showed complete regression in 63% of patients (35). These two recent successes demonstrate the true value of therapeutic vaccination.

DNA VACCINATION

The therapeutic vaccines developed so far consist of broadly three categories: protein or peptide based vaccines, viral vectored vaccines or DNA vaccines (15). Among these strategies we consider DNA vaccination particularly attractive as outlined below. Uptake of the DNA by cells at the vaccination site will lead to local intracellular production of the antigen, thereby mimicking natural viral infection. As a consequence the immune system will be primed to produce predominantly cytotoxic T cells (36, 37). In contrast, injection of the proteins as such would in contrast predominantly result in the production of antibodies, which are considered useless, as E6 and E7 are intracellular proteins. An important advantage over vectored vaccines is that DNA vaccines can be administered repeatedly without the risk of inducing vector specific immunity (37). Other advantages of DNA vaccination are the fact that DNA can be relatively easily produced at large scale, the fact that DNA is stable at room temperature, the good safety profile of the DNA vaccination platform compared to for example live vector vaccines, and finally DNA can be easily manipulated in order to affect the properties of the encoded protein (37) (see also chapter 2 of this thesis for a detailed review on DNA vaccination in general). Over the past years many candidate DNA vaccines targeting E6 and E7 have been developed in rodent models (reviewed in (38, 39) and several clinical trials have been performed, or are currently ongoing (15, 38, 40, 41). Although vaccine specific immune responses could be
detected in some cases, the clinical outcome of these trials so far has been rather disappointing (15). Therefore, there is a strong need for optimization of E6 and E7 directed DNA vaccines.

**AIM OF THE THESIS AND OUTLINE**

The aim of this thesis was to develop highly immunogenic and safe candidate DNA vaccines for the treatment of HPV16 induced malignancies. Furthermore, we wanted to obtain insight in the mechanisms that contribute to the enhanced immunogenicity of so called ‘DNA fusion vaccines’. The content of the individual chapters is summarized below.

- **Chapter 2** provides a detailed review on DNA vaccination in general and DNA tattoo vaccination in particular. Among the subjects discussed in this review are: the advantages of DNA vaccination compared to conventional vaccine platforms, the mechanisms of T cell priming upon DNA vaccination, the origin of the "danger-signal" in DNA vaccine preparations and the value of DNA tattooing, a technique developed in our lab, compared to other DNA delivery methods.

- **Chapter 3** describes the development of highly effective and safe HPV16 E7 and E6 directed DNA vaccine candidates. As E6 and E7 are known oncogenes, we selected so called "gene-shuffled" versions of E6 and E7 in order to avoid cellular transformation at the vaccination site in case genomic integration might occur. The gene-shuffling results in the production of a completely rearranged protein that can be expected to have lost its oncogenic potential, while individual T cell epitopes are not altered. We found that these shuffled versions of E6 and E7 are no longer immunogenic upon DNA tattoo vaccination. Therefore, we had to develop a strategy to overcome the loss in immunogenicity. We constructed genetic fusions with Tetanus Toxin fragment C (TTFC), a bacterial protein that had been shown previously to improve the immunogenicity of C-terminally coupled antigenic peptides in DNA vaccination, and evaluated the effect of this fusion on the immunogenicity of the shuffled versions of E6 and E7.

- **Chapter 4** describes the preclinical safety studies performed to demonstrate that the vaccine candidates, TTFC-E6SH and TTFC-E7SH developed as described in chapter 2, indeed lost the oncogenic potential that is associated with E6 and E7 wild-type genes. For this purpose we selected two different model systems. In the first model system we made use of murine fibroblasts (NIH 3T3 cells) that were transfected with either our vaccine candidates, or wild-type E6 and E7 containing plasmids. Next we introduced a model system based on the viral transduction of primary human foreskin keratinocytes (HFKs). The latter model system can be regarded as more relevant as it comprises the use of the natural target cell of vaccination (the human keratinocyte). In addition, since we used retroviral vectors and grew the cells under selective pressure, we mimicked the worst-case scenario of stable integration of our vaccine candidates in the genome of keratinocytes, thereby increasing the likelihood of detecting residual oncogenic activity.

- **Chapter 5** describes the rational design of DNA vaccines encoding modified HPV16 E6 and E7. This chapter can be regarded as a follow up study of chapter 3. The exact mechanisms by which fusion with so called “carrier-proteins” (such as TTFC) enhances the immunogenicity of HPV16 E6 and E7 are not entirely clear. Often the biological function of such carrier-proteins is considered to play an important role. We hypothesized that rather more general mechanisms, such as provision of CD4+ T cell help, improvement of antigen stability or alteration of the subcellular localisation of the antigen, can explain the immune-potentiating effect observed
after fusion with such carrier-proteins. To test this hypothesis we developed modular DNA vaccines in which the presence of different components could be systemically altered.

Chapter 6 focuses on the improvement of the delivery of dermal DNA vaccines by formulating the DNA into nano-particles. It is estimated that only 1 out of $5 \times 10^4$ to $5 \times 10^6$ DNA copies is taken up after DNA tattoo vaccination. Therefore, if it would be possible to only slightly increase the efficiency of DNA uptake this could hypothetically result in an enormous increase in the amount of produced antigen. This can be expected to strongly improve the immunogenicity of DNA vaccination, as the amount of antigen expressed is considered to be a limiting factor. However, we found that complexation of DNA with cationic polymers, a method that strongly improves DNA uptake in vitro, completely blocks DNA tattoo mediated gene expression in intact human skin or in mice in vivo. We hypothesised that the positive charge of the resulting nanoparticles might lead to immobilization of the DNA in the extracellular matrix by charge interactions. Therefore we shielded the cationic charge of such particles by the addition of charge neutral PEG chains to the particles and evaluated the effect of this modification on the immunogenicity of the DNA-nanoparticles.

Finally Chapter 7 contains a summarizing discussion and provides suggestions for future research.

REFERENCE LIST


