Therapeutic Immunization strategies against cervical cancer

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Summary

The aim of the study described in this thesis is the development of a therapeutic immunization strategy against cervical cancer and pre-malignant precursor lesions of cervical cancer (CIN lesions). Cervical cancer is caused by high risk human papillomavirus (HPV). Two of the early proteins of high risk HPV, E6 and E7, interact with the cell cycle regulation proteins p53 and pRb and can cause immortalization of cells. E6 and E7 are constitutively expressed by cervical cancer cells, making these proteins attractive targets for an immunotherapy directed against HPV-induced cervical cancer. In this thesis two strategies for effective induction of immune responses against protein antigens are explored: immunization with recombinant Semliki Forest virus (rSFV) and influenza virosomes containing protein antigens. Furthermore, the underlying mechanisms of cytotoxic T lymphocyte (CTL) induction by these immunizations are investigated.

Chapter 1 presents a general introduction on HPV, the immune system, cervical cancer and the role of HPV in the etiology of the disease. As indicated above, cervical cancer is caused by high-risk HPV. The E6 and E7 proteins of HPV, which cause the transformation to cancer cells, are constitutively expressed in the cancer cells. The immune system plays an important role in clearance of HPV infections and in spontaneous regression of precursor lesions of cervical carcinoma. This role is supported by the observation that HPV-induced (pre)malignant lesions occur more frequently among immunocompromised women, like organ recipients or HIV-infected individuals. The tumor-specific nature of E6 and E7 expression and the fact that the cellular immune system plays an important role in the control of HPV underline the potential feasibility of immunotherapeutic strategies against CIN lesions and cervical cancer.

A number of immunization strategies are described in this chapter, followed by a more extensive introduction of the two vaccine candidates used in the studies of this thesis: rSFV and influenza virosomes.

Recombinant SFV is an alphavirus vector based on the single-stranded RNA genome of SFV. This expression system consists of an expression plasmid and a helper plasmid. In the expression plasmid a gene can be inserted encoding the protein to be used for immunization. On the helper plasmid the structural genes of the virus are encoded. For production of rSFV, these plasmids are translated into RNA and cotransfected into cells. The positive strand RNA ensures production of rSFV virus. Since the helper RNA encoding the structural proteins is not packaged into the viral particles, no new virus can be produced upon infection with rSFV making this expression system very safe. Infection of cells results in high expression of the encoded transgene and ultimately in apoptosis of the infected cell.

Virosomes are reconstituted viral envelopes, which lack the genetic material of the virus but do contain the membrane glycoproteins of the virus they are derived from. During the reconstitution process protein antigens may be encapsulated within the virosomal lumen. Virosomes retain the receptor-binding and membrane fusion activity
Summary of the native virus and can therefore actively deliver substances to the cytosol of cells. A number of virosomal vaccines is registered for use in humans.

Chapter 2 describes a series of initial immunization and tumor challenge studies performed with rSFV encoding the E6 and E7 proteins of HPV16 (rSFVE6E7). The rSFVE6E7 used for these murine studies was constructed and characterized in vitro. Immunization of mice with rSFVE6E7 induces specific CTL activity against E6 and E7-expressing tumor cells. The minimal effective dose of rSFVE6E7 needed for induction of CTL activity is $10^4$ virus particles. At higher doses of $10^5$ or $10^6$ virus particles, stronger specific lysis is induced. Immunization of mice with rSFVE6E7 virus generates partial protection against a subsequent tumor challenge with cells expressing HPV16 E6 and E7. Immunized mice show a delay in tumor outgrowth compared to control mice and part of the mice remain tumor-free. Thus, immunization with rSFV encoding HPV16 E6 and E7 appears to represent a promising therapeutic immunization strategy against HPV16-transformed tumor cells.

Chapter 3 describes studies performed to unravel the mechanism of rSFV-mediated induction of CTL responses. Our first hypothesis was that direct priming of antigen-presenting cells (APC) by infection of these APC with rSFV is involved. However, extensive infection studies with murine and human dendritic cells (DC) under multiple conditions show that direct infection rates of DC are extremely low. Even at high multiplicities of infection (MOI) a maximum of 0.15% of cells in DC cultures can be infected.

Since direct priming is not likely to be the mechanism behind induction of CTL responses against rSFV-encoded antigens, we next hypothesized that cross-priming could be responsible. Cells infected with rSFV produce high levels of recombinant protein and, after about 48 hours, go into apoptosis. When infected cells undergo apoptosis they can serve as a source of apoptotic bodies containing substantial amounts of the expressed antigen. DC can take up apoptotic bodies and efficiently present the enclosed antigens on MHC class I molecules. This is generally referred to as cross-priming. If cross-priming is the mechanism behind rSFV-mediated induction of CTL responses, the amount and stability of antigen present after immunization is expected to be important.

The cross-priming hypothesis was tested using three different rSFV constructs encoding the influenza virus nucleoprotein (NP) as a model antigen. Mice immunized with rSFV encoding NP show significant levels of NP-specific CTL activity. The second rSFV construct used for this study encodes enhanced NP, resulting in higher expression levels of NP. Immunization with this rSFVenhNP induces higher levels of NP-specific CTL than immunization with rSFVNP. In contrast, immunization with rSFV encoding a fusion protein of ubiquitin and NP (rSFVubNP), is disadvantageous for CTL induction. The ubiquitin moiety results in rapid antigen degradation, and thus availability of antigenic peptides in the infected cell. The amount of antigen available for APC will then be low since no protein will be present in the apoptotic bodies of cells infected with rSFVubNP.
Based on the infection studies with DC and the in vivo results obtained with rSFV constructs encoding different variants of NP, we conclude that antigen presentation after rSFV-based immunization most likely proceeds via a mechanism in which APC are not infected directly, but acquire antigen from other infected cells and present it to CTL in a process of cross-priming.

As a second immunization strategy, influenza virosomes containing protein antigens were used. Chapter 4 describes the first in vitro antigen delivery studies with influenza virosomes containing a model protein antigen (ovalbumin, OVA). For these studies OVA-containing virosomes were generated and characterized. Morphology and fusion-activity of OVA virosomes and empty virosomes are similar indicating that addition of protein antigen during reconstitution does not impair proper virosome formation.

We hypothesized that the ability of virosomes to fuse with the endosomal membrane and thereby deliver their antigen to the cytosol of APC and into the MHC class I pathway, makes them ideal vehicles for induction of MHC class I responses. Since not all of the virosomes will fuse with the endosomal membrane some will continue in the endo-lysosomal pathway, resulting in degradation of the proteins to peptides which can enter the MHC class II presentation pathway. Influenza virosomes are thus expected to deliver encapsulated protein antigen for presentation in both MHC class I and II, resulting in optimal activation of the immune system. To address this question dendritic cells (DC) were incubated with fusion-active OVA virosomes or with OVA virosomes that were fusion-inactivated by pre-incubation at low pH in the absence of target membranes.

Binding of fusion-active and fusion-inactivated OVA virosomes to DC is comparable, but fusion activity of virosomes appears to be a prerequisite for delivery of fluorescently labeled OVA to the cytoplasm of DC. Fusion-active OVA virosomes are able to induce MHC class I presentation of OVA peptide at picomolar concentrations of encapsulated OVA. By contrast, fusion-inactivated OVA virosomes are unable to induce MHC class I presentation of OVA at the concentrations tested, indicating that fusion activity is required. For MHC class II presentation of OVA peptide, no difference is observed between delivery of OVA by fusion-active virosomes or fusion-inactivated virosomes. Moreover, both virosome preparations are able to induce upregulation of expression of MHC class I and II and the maturation markers CD40, ICAM-1, B7.1 and B7.2 on DC.

Thus, influenza virosomes are efficient delivery vehicles of protein antigen for MHC class I and II presentation in vitro.

We next investigated the ability of OVA virosomes to induce an OVA-specific CTL response in vivo (Chapter 5). Immunization of mice with OVA virosomes is effective in inducing an OVA-specific cytolysis. Different routes of immunization result in strong CTL activity, with intramuscular and intraperitoneal injections being slightly superior to subcutaneous immunization. Immunization with as little as 0.75 µg of OVA in virosomes induces a modest CTL response, higher doses of antigen induce strong OVA-specific CTL activity. Immunization of mice with heat-denatured OVA admixed with virosomes
also results in significant CTL induction, indicating that virosomes have a substantial adjuvant activity.

The influence of fusion activity of virosomes on the induction of CTL was determined, since, in vitro, the presentation of OVA peptide in MHC class I by DC is dependent on the fusion activity of the virosomes (Chapter 4). It appeared that also in vivo CTL induction is lower with fusion-inactive virosomes. However, the amount of OVA-specific CTL is still about half of that induced by the same dose of fusion-active OVA virosomes. Similarly, the amount of IFN-\(\gamma\)-producing spleen cells of mice immunized with fusion-active OVA virosomes upon stimulation with OVA peptide is twice the amount induced by fusion-inactive OVA virosomes.

These in vivo results indicate that virosomes represent an ideal antigen delivery system for induction of cellular immunity against encapsulated protein antigens.

The studies described in Chapter 4 and 5 were performed with influenza virosomes containing the model protein OVA. Chapter 6 describes the preparation of influenza virosomes containing HPV16 E7 and immunization and tumor challenge studies performed with these E7 virosomes. For these studies the HPV16 E7 gene was cloned into an E. coli expression plasmid and the protein was produced and purified. After reconstitution of functional, fusion-active, E7 virosomes, immunization studies were performed in mice.

Immunization with E7 virosomes induces strong E7-specific CTL responses. When mice are immunized with empty virosomes admixed with four times the amount of E7 protein used in immunization with E7 virosomes, a low to moderate CTL response is induced, indicating again that virosomes have adjuvant activity.

The aim of these studies is development of a therapeutic immunization against an E7-expressing tumor. Tumor challenge studies where mice are immunized with E7 virosomes and challenged with E7-expressing tumor cells show that E7 virosomes can prevent tumor outgrowth. In these studies, immunization with fusion-active E7 virosomes results in tumor-free survival of the majority of mice. In concordance with CTL responses seen after immunization with fusion-active and fusion-inactive OVA virosomes (Bungener in press, Chapter 5), immunization with fusion-inactive E7 virosomes is not as effective as immunization with fusion-active E7 virosomes in preventing tumor outgrowth. Injection with fusion-inactive E7 virosomes results in a delay in tumor outgrowth in most mice and a small percentage of animals remain tumor-free. Mice receiving empty virosomes admixed with E7 show tumor outgrowth similar to mice receiving fusion-inactive E7 virosomes. These results indicate, again, that virosomes possess a substantial adjuvant effect.

Thus, virosomes represent an excellent vaccine delivery system for induction of cellular immunity against encapsulated HPV16 E7 and are a promising immunotherapeutic vaccine for treatment of (precursor lesions of) cervical cancer.

In Chapter 7, the studies performed for this thesis are discussed and placed into perspective. The study with rSFVE6E7 described in Chapter 2 was performed several years ago. Since that time, our group has developed an improved rSFV encoding a
fusion protein of E6 and E7 behind a translational enhancer. Infection with this rSFVenhE6,7 virus results in much higher and more stable expression of E6 and E7. Consequently, immunization with rSFVenhE6,7 induces very powerful CTL and anti-tumor responses. Treatment of established HPV16 E6 and E7-expressing tumor cells is possible by immunizing mice with rSFVenhE6,7. Furthermore, even in transgenic mice exhibiting strong HPV-specific CTL tolerance, immunization with rSFVenhE6,7 induces HPV-specific CTL activity. The performed studies show that rSFV encoding HPV proteins is extremely promising as a therapeutic vaccine for (precursor lesions of) cervical cancer.

The second strategy for induction of immune responses against HPV proteins described in this thesis is immunization with protein antigen-containing influenza virosomes. Results obtained by immunizing mice with influenza virosomes containing the model antigen OVA or HPV16 E7 indicate that virosomes are an excellent vaccine delivery system capable of inducing strong CTL and anti-tumor responses.

The effect of pre-existing influenza virus hemagglutinin-specific antibodies on the outcome of immunization with virosomes is discussed extensively in this chapter. There are numerous indications that pre-existing antibodies against influenza virus can mediate antibody-mediated enhancement of influenza virus infection. In our immunization studies with protein-containing virosomes booster immunization is effective. Furthermore, preliminary immunization experiments with OVA virosomes in mice pre-immunized with influenza subunit vaccine indicate that pre-existing antibodies against influenza membrane proteins could be beneficial to the CTL response to encapsulated protein antigens. These results provide strong indications that pre-existing antibodies against influenza virus hemagglutinin will not hamper the immune response to antigens encapsulated in influenza virosomes.

Finally, the potential of rSFV and influenza virosomes for therapeutic immunization against (precursor lesions of) cervical cancer is discussed. Both immunizations are effective in inducing CTL and anti-tumor responses directed against HPV antigens, although the results obtained with the improved rSFVenhE6,7 show that rSFV is a more powerful immunization than immunization with influenza virosomes in their current form. On the other hand, the practical fact that some virosomal vaccines are already approved for use in humans whereas rSFV is not yet in the phase of clinical trials may influence the choice when designing a therapeutic immunization strategy against (precursor lesions of) cervical cancer.

In conclusion, both rSFV and virosomes are promising immunization strategies for treatment of precursor lesions of cervical cancer. The next years will show if these strategies will become part of the treatment protocol for precursor lesions of cervical cancer.