M.C. Elizabeth Reynaga Hernández
Dr. sc. Hum

Generation of HBsAg(S) particles carrying combined HPV-16E7 and p16^{INK4a} epitopes and their immunogenicity in vitro)

Promotionsfach: Pathologie
Doktorvater: Prof. Dr. Med. Magnus von Knebel-Doeberitz

The aim of the present work was to generate a model of therapeutic vaccine against infection by HPV using a combination of a tumor-specific antigen (TSA) and a tumor-associated antigen (TAA), both highly expressed in HPV patients, and linked to the HBsAg(s) molecule to increase their immunogenicity.

It is well known that E7 protein encoded by high-risk serotypes of HPV is constitutively expressed in advanced stages of squamous intraepithelial lesions and cervical cancer and thus represents a tumor-specific antigen (TSA). In cervical cells whose retinoblastoma protein, pRb, is inactivated by E7, there is a strong expression of p16^{INK4a}, one of the cyclin-dependent kinase inhibitors proteins (CKIs) that regulate the cellular cycle. Due to an overexpression of p16INK4a, it can be then considered a tumor-associated antigen (TAA).

In this study our first aim was to generate chimeric proteins with the combination of the small envelop protein of hepatitis B virus, HBsAg(S), and p16INK4a or two different sequences of HPV 16 E7. HBsAg was selected because it self-assembles into non-infectious subviral particles and the immune response is directed against epitopes in the 'a' determinant region, which confers the properties of highly cytotoxic T lymphocyte-mediated immunogenicity and make from HBsAg virus like particles (VLP) potent carriers for foreign epitopes.

It was also important to consider the introduction of the codon-optimized EE7Δ1-35 in the chimeras, which lacks of amino acids 1-35 of the joint to retinoblastoma (Rb) domain, inhibing its transforming capability. The optimized codon is able to induce higher expression of the recombinant protein than the wild type sequence. Additionally, the 11-20 sequence of 16EE7 was also introduced in one chimera to test its role, since 11-20 regions induces strong CTL response.

The second aim was to evaluate the immunogenicity of the HBsAg(S) particles carrying combined $p16^{INK4a}$ and HPV-16E7 epitopes in healthy donors. We used the CD40 system in order to induce a specific CD8 cytotoxic T lymphocytes response against the epitopes of oncogenic HPV E7 proteins.

The recombinant proteins were successfully cloned in mammalian vectors as confirmed by sequencing of the inserted sequence and western blot analysis of the expressed protein. Then, the sequences coding the chimeric proteins were subcloned in yeast vectors to produce the recombinant proteins in a large scale, making easy their purification through His-tag tail. B cell antigen presenting cells were exposed several days to the purified proteins and then placed in contact with $CD3^+$ T cells. Restimulation of T cell with the recombinant proteins or peptides was carried out every seven days. CTL activation was measured by IFNγ release using an ELISpot assay. Each group of B cells and stimulated T cells were independently restimulated with 10 μg/ml of EBV, MP, HBS, E7, p16- R1 or R2 peptides, the proteins HBs-16E7Δ1-35, HBsAg-p16, HBsAg-p16-16E7Δ1-35 or HBsAg16$E7^{(11-20)}$ -p16-16EE7Δ1-35.

We have shown previously that fusion of the hepatitis B surface antigen (HBsAg) to E7 enhances the immune response to this protein, HBs-E7. In this work, HBsAg-E7 as well as HBsAg-$p16^{INK4a}$ and a triple fusion protein evoked specific cytotoxic T cell responses in HLA-A2 healthy donors. *In vitro* results showed that HBsAg-$p16^{INK4a}$ elicited a stronger response in one donor than the HBsAg-E7 fusion protein or than any other of the fusion proteins. Meanwhile two additional donors had shown a major IFN-γ release with HBs-16$E7^{(11-20)}$-p16-16E7Δ1-36 than HBsAg-$p16^{INK4a}$ recombinant proteins. One of these donors had significantly higher response to E7 (86-93), E7 (11-20) and R1 peptides.

An anti-CD4 and anti-CD8 blocking assay showed that, in cells pre-primed with HBs-E7, production of IFN-γ was apparently caused by the $CD8^+$ subset T cells, since positive spot cells were decreased blocking with anti CD8 antibody. Finally a FACS analysis showed that more than 85% of the stimulated cells are $CD8^+$ T. This result leads us to conclude that the chimeras tested in the experiments are able to stimulate $CD8^+$ T cells.

On the other hand, HBs-p16 and HBs-16$E7^{(11-20)}$-$p16^{INK4a(41-110)}$-16E7Δ1-35 had a relevance in the present study due to their major number of IFN-γ secreting cells. The difference in response against each recombinant protein observed between donors could

be due to the individual immunogenicity. The results of our work could be considered a pre-selection of the chimeras: HBs-p16 and HBs-16E7 $^{(11\text{-}20)}$-p16$^{INK4a(41\text{-}110)}$-16E7Δ1-35 to be tested in systems like Adeno-x to improve the CTL response, but also for the inclusion of patients with CIN lesions or cervical cancer.

It will be also important to considerate for furthers experiments the elucidation of the effect of HBs-p16 protein immunization in others cancers where p16 is involved.