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A novel "priming-boosting" strategy for immune interventions in cervical cancer

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ABSTRACT

Despite the encouraging development of a preventive vaccine for human papillomavirus (HPV), it cannot improve ongoing infections. Therefore, a new vaccine is urgently needed that can prevent and treat cervical cancer, and cure pre-cancerous lesions. In this study, we constructed two peptide-based vaccines. The first was a short-term, long-peptide (ST-LP) vaccine that simultaneously targeted three key carcinogenic epitopes (E5–E6–E7) on HPV16. We tested this vaccine in murine TC-1 cells infected with a recombinant adeno-associated virus (rAAV) fused with HPV16E5 DNA (rTC-1 cells), which served as a cell model; we also tested it in immune-competent mice loaded with rTC-1 cells, which served as an ectopic tumor model. The ST-LP injections resulted in strong, cell-mediated immunity, capable of attacking and eliminating abnormal antigen-bearing cells. Furthermore, to prolong immunogenic capability, we designed a unique rAAV that encoded the three predicted epitopes for a second, long-term, long-peptide (LT-LP) vaccine. Moreover, we used a new immune strategy of continuous re-injections, where three ST-LP injections were performed at one-week intervals (days 0, 7, 14), then one LT-LP injection was performed on day 120. Our in vitro and in vivo studies revealed that this strategy could boost the immune response to produce longer and stronger protection against target cells, and mice were thoroughly protected from tumor growth. Our results showed that priming the immune system with the ST-LP vaccine, followed by boosting the immune system with the LT-LP vaccine could generate a rapid, robust, durable cytotoxic T-lymphocyte response to HPV16-positive tumors.

1. Introduction

Every year, 470,000 cases of cervical cancer are diagnosed worldwide, and about half will lead to death. The World Health Organization has recognized cervical cancer as 100% attributable to persistent infections of "high-risk" human papillomavirus (HPV) (Bosch and de Sanjose, 2007). Of all the oncogenic HPVs identified, HPV serotypes 16 (HPV16) and 18 (HPV18) are the most common; they account for 50–60% and 20–25% of cervical cancers, respectively (Van den Brule et al., 1989). Recently, a preventive HPV vaccine was used to protect patients before the first encounter with HPV; however, existing infections would not benefit from the vaccine. Thus, a therapeutic vaccine is urgently needed for treating ongoing infections (Mahdavi and Monk, 2005).

To date, most HPV therapeutic vaccines have focused on the HPV oncogenes, E6 and E7; however, these vaccines cannot completely eradicate the lesions (Alberto et al., 2014). This suggested that there may be some undefined factors involved. A better, deeper understanding of HPV might suggest new insights. Recently, E5 has received increased attention. E5 is considered an oncogene, because it transformed murine fibroblasts in tissue culture, and it was implicated in cell proliferation (DiMaio and Petti, 2013). Additionally, E5 showed synergistic effects with E6 and E7 by enhancing

Abbreviations: CTL, cytotoxic T-lymphocyte; HPV, human papillomavirus; AAV, adeno-associated virus; rAAV, recombinant AAV; CIN, cervical intraepithelial neoplasm; CMI, cell-mediated immunity; CpG-ODN, CpG oligodeoxynucleotide; TAP, transporter associated with antigen processing; IR-P, irrelevant control peptide; Treg, T regulatory cell; TIL, tumor-infiltration lymphocyte; ST-LP, short-term long-peptide; LT-LP, long-term long-peptide; TDLN, tumor-draining lymph node.

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the immortalization potential. Also, the complementary efficacy is indispensable in the process of carcinogenesis (Stoppler et al., 1996). Thus, it is currently accepted that HPV expressed three key oncoproteins (E5, E6, and E7), which can transform cells in vitro and are associated with cervical carcinogenesis in vivo (Gao and Zheng, 2010). Consequently, vaccines that focused on the HPV E6 and E7 oncogenes are thought to lack the E5 "ringleader", and therefore, they could not completely overcome the lesions.

The peptide vaccine is the most serviceable antigen-specific vaccine. It is safe and easy to produce, but its immunogenic property is weak, and it is rapidly metabolized. Accordingly, in 2008, Melief and van der Burg proposed the concept of a "long-peptide vaccine". The long peptide is easy to prepare, safe to use, and it has multiple epitopes, which give it strong immunogenicity.

This study aimed to construct two kinds of long peptide (LP) vaccines, optimize the immunization technique, and test the efficacy of these vaccines for inducing a long-term immune memory response in mice re-challenged with tumor cells. We also systematically evaluated the possible mechanisms involved.

2. Materials and methods

2.1. Study design

We designed two LP vaccine with multiple epitopes derived from HPV16 oncogenes E5, E6, and E7. The efficacy of this vaccine was tested on a cell model in vitro and on tumors induced in a mouse model in vivo.

First, we synthesized a relatively short peptide vaccine that targeted HPV16 E6 and E7 epitopes, which is considered a standard vaccine. Next, we generated two forms of the LP vaccine. One was a short-term LP (ST-LP) vaccine that contained HPV16 E5, E6, and E7 epitopes. This ST-LP was coinjected with an immune adjuvant, the CpG-oligodeoxynucleotide (ODN). The unmethylated CpG motifs can activate B cells, activate monocytes, induce Th1 cytokine production, etc. (Daftarian et al., 2006). We predicted that this new synthetic ST-LP vaccine would target more key HPV carcinogenic antigen-epitopes than short-peptide vaccines, which lead to immunological tolerance rather than immunity.

The second LP vaccine was designed to improve the immunogenic properties of the LP by fusing it with a reconstituted adeno-associated virus (rAAV). The rAAV vector was predicted to amplify and prolong the vaccine effect. When viral particles are injected in mice, virally-infected cells persistently produce the vaccine peptide. Thus, we called this a long-term LP (LT-LP) vaccine. AAV is a single-stranded virus with many natural features, including non-pathogenicity, targeted integration, broad host range, and no requirement for viral gene expression. Thus, it is suitable for immunizations of naked DNA. For gene therapy applications, AAV may be superior to other viral vectors (Zhou et al., 2010).

Finally, we designed a novel, continuous re-injection, "priming-boosting" strategy. In this strategy, the ST-LP was injected multiple times to prime the system; then, the LT-LP was injected as a booster to strengthen and prolong the long-term immune response.

2.2. Epitope prediction

We applied prediction algorithms for major histocompatability (MHC) class I ligands and for binding affinity to a transporter associated with antigen processing (TAP) to identify epitopes on HPV16 E5, E6, and E7 that were most likely to be targeted by cytotoxic T-lymphocytes (CTLs). Briefly, we used four different epitope prediction databases: (1) the National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS), HLA Peptide Binding Predictions (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (Parker et al., 1994); (2) Epitope-Prediction (http://www.Syfpeithi.de/) (Rammensee et al., 1999); (3) TAPPred (http://www.imtech.res.in/cgibin/tappred/) (Bhasin and Raghava, 2004), and (4) ProPred-I (http://www.imtech.res.in/raghava/propred1/index.html) (Singh and Raghava, 2003). We chose four of the top-ranked H-2D^b binding epitopes from the four databases, respectively, as candidate peptides. After testing these in experimental assays, we selected the most effective candidate peptides, which were sequences from E5, E6, and E7. The long peptide was synthesized by joining three finally selected candidate peptides, which targeted E5, E6, and E7, with AAY spacers in-between for optimal proteosomal cleavage. Thus, the final sequence of the long peptide was **LSVSTYTSL**AAY**NKPLCDLLI**AAY**RAHYNIVTF**. At the same time, we also synthesized a control long peptide that comprised only two candidate peptides that targeted E6 and E7 (Velders et al., 2001; Daftarian et al., 2007). These peptides were synthesized and purified by Xi'an Huachen Biotech company in China at >95% purity. Their sequences and structures were confirmed with mass spectrometry. Another peptide, KIMCNSSCM, was used as an irrelevant control peptide (IR-P) (Daftarian et al., 2006).

2.3. CpG-oligodeoxynucleotide

The immunostimulatory CpG-oligodeoxynucleotide (CpG-ODN), designated 1826 (5-TCCATGACGTTCCTGA CGTT-3) (Daftarian et al., 2006), was purchased from Biobasic Inc., Canada. This ODN was synthesized with a nuclease-resistant phosphorothioate backbone.

2.4. Animals and cell lines

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Center of Experimental Animals at the Chinese Academy of Medical Science. All studies were approved by the Huazhong University of Science and Technology (HUST) Animal Care and Use Committee.

The murine TC-1 cell line was established from primary lung epithelial cells from C57BL/6 mice, which were immortalized with HPV-16 E6 + E7, and then transformed with an activated *ras* oncogene (ATCC CRL-2785^{TM}). The B16F1 cell line was a melanoma cell line derived from C57BL/6 mice (ATCC CRL-6322); these cells served as a non-HPV-infected, syngeneic control. The U14 cell line was a highly metastatic, undifferentiated mouse cervical cancer cell line derived from C57BL/6 mice (Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College). The 293 cell line was purchased from the China Typical Species Collection Center.

In this study, all the vaccines were tested on immune competent mice (C57BL/6) that were loaded with tumor-inducing cells. Mice loaded with B16F1 cells were controls (no. HPV16). To test different combinations of HPV16 epitopes, mice were loaded with TC-1 cells (which contained HPV16 E6 and E7); reconstituted TC-1 cells (rTC-1), which were TC-1 cells infected with the rAAV fused with HPV16 E5 (i.e., they contained HPV16 E5, E6, and E7); or reconstituted U14 cells (rU14), which were U14 cells infected with rAAV-HPV16 E5 (rU14 + E5 cells), with rAAV-HPV16 E6 (rU14 + E6 cells), or with rAAV-HPV16 E7 (rU14 + E7 cells).

2.5. Construction and production of rAAV-HPV16 E5/E6/E7 (separate) and rAAV-HPV16 + LP viruses

The HPV16 E5/E6/E7 genes were separately amplified by PCR from the plasmid, PBR322-HPV16, which included the complete HPV16 gene. This plasmid was a kind gift from professor zur Hausen (Heidelberg University, Germany). We performed PCR with the following primers (EcoRI and BamHI restriction sites are underlined):