Human papillomavirus type 16 L1E7 chimeric capsomeres have prophylactic and therapeutic efficacy against papillomavirus in mice

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Abstract
Genital human papillomavirus (HPV) infection is the primary cause of cervical cancer in women. Although the HPV recombinant L1 protein was recently licensed as an available vaccine, it has numerous shortcomings. New vaccination strategies should be considered. To enable the design of a prophylactic and therapeutic low-cost vaccine candidate, chimeric HPV16 L1ΔC34E7N1-60 capsomeres were produced in Escherichia coli. The immune characteristics and potential prophylactic and therapeutic effects of these capsomeres were examined in C57BL/6 mice. Following protein purification and renaturation, the majority of the recombinant chimeric proteins (L1ΔC34E7N1-60) assembled into capsomeres. These capsomeres were able to induce conformational and neutralizing antibodies against HPV virus-like particles and trigger cell-mediated specific immune responses against the L1 and E7 peptides. In vivo tumor challenge assays showed that mice immunized with the capsomeres were protected against a challenge with both C3 and TC-1 tumor cells. Furthermore, in vivo tumor rejection assays showed that capsomeres have therapeutic efficacy in mice following inoculation with C3 and TC-1 tumor cells. Chimeric capsomeres are capable of preventing and eliminating HPV16 infection. Therefore, our study has provided an economical vaccine candidate.

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only antigen used in this vaccine is L1, which is expressed exclusively in the later stages of the viral life cycle and is not involved in viral replication. The antigens of advanced vaccines should include nonstructural proteins, which are involved in and may therefore block viral replication (13, 14). Furthermore, VLP manufacturing procedures are very complex, making the cost-effectiveness of production unsuitable for worldwide application (3, 15, 16).

In this study, we explored new vaccination strategies using the nonstructural E7 protein. The E7 protein is well characterized, is involved in viral replication, and is expressed before L1. We evaluated the humoral and cellular immune responses against an L1E7 chimeric protein in C57BL/6 mice. In addition, we studied its prophylactic and therapeutic efficacy in vivo. Finally, our chimeric protein was expressed in Escherichia coli as a simple, practical strategy for producing an advanced vaccine.

**Materials and Methods**

**Experimental Animals and Cell Lines**

C57BL/6 (H2-Db, 6–8 weeks old) female mice were kept under pathogen-free conditions at the Animal Care Center of the Chinese Center for Disease Control and Prevention. All procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with national and international laws and guidelines. TC-1 cells are C57BL/6 lung tumor epithelial cells cotransformed with HPV16 E6/E7 and c-Ha-ras (kindly provided by Dr. T.C. Wu, Johns Hopkins Medical Institutions, Baltimore, MD; ref. 17); C3 cells were derived from embryonic mouse cells transformed with the full HPV16 genome (kindly provided by Professor Y. Zhang, Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Beijing, China; ref. 18). Antibody against HPV16 L1 was purchased from Chemicon, and antibody against E7 was purchased from Santa Cruz Biotechnology.

**Plasmid Construction**

A truncated HPV16 L1 gene, with 34 COOH-terminal amino acids deleted, was cloned from pUCL1-25-2 using the forward primer GGG\_AAGCTT\_TCTCTTTGGCTGCC-GCCTAGTGAGG and the reverse primer AAA\_GATTATC\_TTGTAGTAAAAATTTGCGTCCTAAAGGAAAC. The NH2-terminal 60 amino acids of the E7 gene were cloned from pUCmE7-1 using the forward primer AAAA\_GATTATC\_ATGCATGGAGATACACCTACATTGC and the reverse primer TTTT\_AAGCTT\_TTACTTGCAACAAAAGG-TTACAAT. Both primer sets included the Eco\_RV and Hind\_III restriction sites (highlighted in boldface). These two PCR fragments were digested with the restriction enzymes and ligated into pQE30 (Novagen, Germany) to form the chimeric expression vector pQE30-L1\_DC34E7N1-60 (Fig. 1A).

**Purification of Recombinant L1\_DC34E7N1-60 Protein**

*E. coli* M15 (Novagen, Germany) was transformed with pQE30-L1\_DC34E7N1-60. Following amplification, cells were collected by centrifugation, and expression levels in the soluble and insoluble fractions were analyzed by 12% SDS-PAGE. For fusion protein purification, the collected cells were incubated in buffer containing 50 mmol/L of Tris-HCl (pH 8.0), and 8 mol/L of urea for 1 h at room temperature to assure complete cell lysis. The cell lysate was sonicated on ice with high-intensity pulses and then centrifuged at 10,000 \times g for 30 min. The resulting supernatant was transferred to CM Sepharose FastFlow resin...
and subsequently analyzed. 

After drying, the IFN was stopped after 15 min by rinsing the wells with ready-to-use substrate activator I/II solution. The reaction secreting cells were visualized by adding 100 

A

were used to determine serum antibody titers against HPV. 

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were seeded on a 96-well plate at a density of 10^5 cells/

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Western blot analysis. 

SDS-PAGE. The chimeric protein was confirmed by the Bradford method, and the proteins were analyzed by 12% SDS-PAGE. The chimeric protein was confirmed by Western blot analysis.

**In vitro Assembly and Electron Microscopy**

To renature the chimeric protein, elution fractions containing the protein were pooled and slowly dialyzed against decreasing concentrations of urea as 8 mol/L of urea was diluted gradually during dialysis (50 mmol/L Tris-HCl, 1 mmol/L EDTA, and 1 mmol/L DTT; pH 8.3). The final dialysis was done overnight against PBS. All dialyses were done at 4°C. The protein solution was dialyzed against renaturation buffer, and endotoxins in the capsomere preparation were removed by polymyxin B (Sigma-Aldrich). The renatured protein was adsorbed to carbon-coated copper grids, negatively stained with 2% phosphotungstic acid, and examined by electron microscopy.

**Immunization of Mice with the Chimeric Protein**

For enzyme-linked immunospot (ELISPOT) and humoral response assays, the mice were separated into two groups of three mice each: one group was immunized i.m. at week 0 and boosted 2 weeks later with 10 μg of chimeric protein, and the other group received PBS buffer according to the same schedule. Two weeks after the booster immunization, the spleens and blood were sampled; spleen cells were isolated and used for ELISPOT assays, and blood samples were used to determine serum antibody titers against HPV.

**ELISPOT Assay**

The frequencies of IFNγ-secreting T lymphocytes specific for the L1 and E7 epitopes were determined by ELISPOT (U-CyTech; ref. 19). Briefly, mouse splenocytes were seeded on a 96-well plate at a density of 10^5 cells/well in 100 μL of medium; untreated wells served as negative controls. The cells were stimulated in triplicate with 2 μg of E749–57 (RAHYNIVTF, H-2D^d–restricted) peptide (18), L1165–173 (AGVDNRECIL, H-2D^d–restricted) peptide (20), or L1E7 protein, and incubated for 30 h at 37°C. The cells were removed by washing the plate six times with PBS containing 0.05% Tween 20 (PBST) and once with PBS alone. Following the addition of 100 μL of biotinylated antimouse IFNγ antibody, the plates were incubated for 1 h at 37°C. The wells were then washed eight times with PBS, 50 μL of α-aminobutyric acid solution were added to each well, and the plates were incubated for 1 h at 37°C. After five washes, IFNγ-secreting cells were visualized by adding 100 μL of the ready-to-use substrate activator I/II solution. The reaction was stopped after 15 min by rinsing the wells with ice-cold water. After drying, the IFNγ-reactive spots were quantified in an ELISPOT reader (Cellular Technology) and subsequently analyzed.

**ELISA for Detecting Anti-VLP Antibodies**

To determine the VLP-specific antibody titers in capsomere (chimeric protein)-immunized mice, 0.1 μg of purified VLP (kindly provided by Dr. X. Xu, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China) diluted in PBS was used to coat 96-well plates for 16 h at 4°C. After washing with PBST, serum samples (diluted from 1:200 to 1:12,800) were added in triplicate, and the plates were incubated at 37°C for 1 h. The plates were washed, goat anti-mouse IgG horseradish peroxidase–conjugated antibody (diluted 1:2,000; Invitrogen) was added, and the plates were incubated at 37°C for 1 h. After a final wash, enzyme substrate (TMB, Sigma) was added for color development, and immunoreactivity was detected by absorbance at 405 nm using an ELISA plate reader (Dynex).

**Hemagglutination Inhibition Assay**

To verify the specificity and neutralizing ability of the antibodies, hemagglutination inhibition assays were done (21). Mouse blood was collected in a heparinized tube, and the RBCs were separated by centrifugation at 1,000 rpm for 5 min at 4°C, washed twice with PBS, and suspended in dilution buffer at a concentration of 1% (vol/vol). Purified VLP (100 ng) was then incubated with various dilutions of experimental sera samples at room temperature for 2 h, after which the samples were mixed with an equal volume of the 1% RBC suspension. Aliquots (100 μL) of the mixtures were transferred to a round-bottomed, 96-well plate and incubated for 3 h at 4°C. Spots indicating VLP-neutralizing activity were documented by photography.

**In vivo Tumor Challenge and Rejection Assays**

To determine whether capsomeres protect immunized mice against a tumor challenge, mice (10 per group) were first immunized by i.m. injection of 10 μg of capsomeres without adjuvant and boosted with the same amount 2 weeks later. The control group received injections of the same volume of PBS. The mice were then challenged with either 1 × 10^5 TC-1 cells (E7-transformed) or 1 × 10^5 C3 cells (L1- and E7-transformed) 2 weeks after the booster injection. Tumor rejection assays were done to evaluate the therapeutic effect of the capsomeres.

Conversely, mice (10 per group) were inoculated with 1 × 10^5 TC-1 or C3 cells on day 0, and then therapeutic immunization with 10 μg of capsomeres or PBS was begun on day 1, with a booster 2 weeks later. The mice were evaluated for at least 2 months for the appearance and size of tumors.

**Statistical Analysis**

Significant differences between the experimental and control groups were evaluated using a two-tailed Fisher’s exact test (SPSS software, release 12.1). Differences were considered significant at P < 0.05.

**Results**

**Purification, Capsomere Assembly, and Characterization of the Chimeric Protein HPV16 L1ΔC34E7N1-60**

The chimeric protein HPV16 L1ΔC34E7N1-60 with an added His tag was expressed using pQE30 vector. After cell lysis
and centrifugation, the majority of the protein remained in the pellet. The solubilized protein was purified by ion-exchange chromatography, followed by affinity purification under denaturing conditions using urea. The purification steps were evaluated by SDS-PAGE. Using ion-exchange chromatography, the target protein was eluted in different fractions, with the 100 mmol/L NaCl buffer giving the fraction with the highest concentration. The eluant from the column contained different amounts of minor contaminating proteins, and further purification was achieved by Ni-NTA chromatography (Fig. 1C). The final protein preparation was >90% pure, as determined by high-performance liquid chromatography. Approximately 5 mg of the target protein were purified from 1 L of E. coli.

After SDS-PAGE, the purified HPV16 L1 D C34E7N1-60 protein was recognized by both L1- and E7-specific antibodies. Furthermore, the apparent molecular weight (61 kDa) was consistent with the estimated size (Fig. 1B).

To assess whether the chimeric protein was accurately assembled, the purified protein was renatured by dialysis against decreasing concentrations of urea. Using electron microscopy, we observed that HPV16 L1 D C34E7N1-60 assembled into capsomere-like structures with a diameter of 11 to 12 nm (Fig. 1D).

Antibody Response and Neutralizing Ability

Conformational and neutralizing antibodies against HPV VLPs were detected by ELISA. ELISAs of diluted (1:200 to 1:12,800) sera from mice immunized with capsomeres gave absorbance values ranging from 1.26 ± 0.15 to 0.09 ± 0.03, which were significantly higher than the control values (Fig. 2A). Although the absorbance had decreased by more than half at a 1:800 dilution (0.39 ± 0.06), antibodies were still detectable at a 1:6,400 dilution (0.13 ± 0.03; Fig. 2A).

To investigate whether these antibodies inhibit the interaction between HPV VLPs and cellular receptors, we used a hemagglutination inhibition assay. At a 1:400 dilution, the sera of capsomere-immunized mice inhibited the VLP-induced hemagglutination of mouse erythrocytes, whereas control sera did not inhibit hemagglutination (Fig. 2B).

L1 and chimeric VLPs can inhibit hemagglutination, and this correlates with cell surface binding to nucleated cells. By contrast, denatured VLPs and virions do not exhibit hemagglutination activity. Combined with our results, this indicates that capsomeres act as conformationally dependent immunodominant epitopes.

ELISPOT Assay

Cell-mediated immune responses against L1- and E7-specific peptides were measured using an IFNY ELISPOT assay. Primed T cells from capsomere-immunized mice were restimulated using E7 49–57 peptide (RAHYNIVTF, H-2Db–restricted), L1 165–173 peptide (AGVDNRECI, H-2Db–restricted), or capsomeres, and the number of IFNY-secreting T cells was determined. Mice immunized with capsomeres had significantly more IFNY-secreting T cells than the control group immunized with PBS (P < 0.01; Fig. 3). On average, capsomeres stimulated the most IFNY-secreting T cells (132 ± 21 cells), followed by L1 165–173 peptide (51 ± 13 cells; Fig. 3). Although E7 49–57 peptide showed the weakest ability to stimulate
The expression of HPV16 L1 major capsid protein in eukaryotic cells results in the formation of VLP, and immunization with the VLP efficiently induces L1-specific neutralizing antibodies. In addition, VLPs are potent inducers of a CTL response, which may activate dendritic cells and trigger the presentation of CTL epitopes (22, 23). However, the high cost of preparing the VLP vaccine prohibits its worldwide application (3). Alternatively, L1 protein can be expressed in E. coli as a capsomeric form, which is a subunit of VLP (24). Capsomeres of HPV11 L1 react with conformation-specific antibodies and induce neutralizing antibodies (25, 26). Capsomeres share strong antigenic similarities with native HPV virion and intact VLP. Furthermore, capsomeres can induce L1-specific CTLs (20). Studies using canine models have shown that canine oral papillomavirus L1 expressed in a bacterial system exist predominantly as capsomeres and that the capsomeres sufficiently protect against the infection of mucosal surfaces (26). These studies show that capsomeres display activities similar to VLPs, making capsomeres an additional vaccine candidate. The expression of recombinant proteins as inclusion bodies in bacteria is one of the most efficient ways of producing cloned proteins (27), as long as the inclusion body protein can be refolded successfully. If capsomeres were to be used instead of traditional VLPs for vaccination, the cost issue associated with vaccine production may be resolved.

Early protein E7 is required for maintaining the proliferative state in HPV-infected cells. The E7 gene products are constitutively expressed in all layers of the infected epithelium. The E7-specific CTLs provided protection against the growth of a transformed tumor cell line. Furthermore, E7 is considered a tumor antigen in the development of strategies for immune therapy (28, 29).

**Table 1. In vivo tumor challenge assays**

<table>
<thead>
<tr>
<th>L1AC34E7N1-60 (%)</th>
<th>Controls (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>0 (0)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>TC-1</td>
<td>3 (30)</td>
<td>10 (100)</td>
</tr>
</tbody>
</table>

NOTE: Mice immunized with L1AC34E7N1-60 chimeric protein or PBS (Controls; 4 groups, 10 mice for each group), followed by tumor cell line (1 x 10^6 of C3 or TC-1) challenge. The number of tumor-bearing mice is shown as a percentage (in parentheses). C3 and TC-1 are two kinds of HPV-related tumor cell lines. The data summarized in this table are for the overall development after the 2-month in vivo tumor challenge assays.

The C3 cells, which were derived from embryonic mouse cells and transformed with the full HPV16 genome, were used as a tumor cell model. To perform the in vivo tumor challenge assay, capsomere-immunized mice and control mice were challenged with C3 cells by s.c. injection at week 6 after immunization and were observed for tumor development. By day 13 post-C3 cell challenge, 50% of the control mice had palpable tumors; by day 19, all control mice had palpable tumors. This is in stark contrast to the capsomere-immunized mice, in which no palpable tumor formation was observed throughout the 2 months of the experiment (Table 1).

To assess whether capsomeres also protect against E7-related tumor formation, the E7-expressing TC-1 cell line was used in an additional tumor challenge assay. Tumor development was observed by day 9 in the control group; all control mice had developed tumors by the end of the study. In comparison, only three mice (30%) in the capsomere-immunized group developed tumors throughout the 2 months of the experiment, with the earliest tumor development by day 15. The average tumor surface in the control group ranged from 12 ± 0 (day 9) to 220 ± 34 mm² (day 60); these values were significantly higher than those in the capsomere-immunized group (range, 4 ± 2 to 38 ± 11 mm²; Table 1; Fig. 4).

**In vivo Tumor Rejection Assays**

To examine the therapeutic efficacy of capsomeres, C57BL/6 mice were injected with C3 or TC-1 cells and were then injected with either capsomeres or PBS on day 2. Two weeks after immunization, the mice received a booster with the same dose of capsomeres or PBS, and tumor rejection was observed. In the group injected with C3 cells and immunized with capsomeres, all mice survived tumor invasion; in contrast, in the group of mice inoculated with C3 cells and immunized with PBS, no mice survived tumor invasion. In the group injected with TC-1 cells and immunized with capsomeres, only one mouse failed to survive tumor invasion. In the group of mice inoculated with TC-1 cells and immunized with PBS, no mice survived tumor invasion (Table 2). These data indicate that capsomeres have therapeutic efficacy against HPV.

**Discussion**

Figure 4. Growth of TC-1 tumor cells in the tumor challenge assay. The mean tumor surface from mice immunized with capsomeres is compared with that of control mice injected with PBS on the days following tumor challenge. Tumor growth on the skin surface was monitored by caliper measurements. Points, averages for all mice in each group.
Studies have shown that the chimeric protein consisting of L1 with a COOH-terminal 34–amino acid deletion and E7 with an NH2-terminal 60–amino acid deletion can produce chimeric VLP that could induce a neutralizing antibody response (30, 31). In addition, E7 peptide (amino acids 37–54), with one or more human T-cell epitopes, induces a cell-mediated immune response that is significantly correlated with disease regression and the resolution of viral infection (32). To establish a viable prophylactic and therapeutic vaccine, we combined L1 and E7 to form capsomeres. Our results show that capsomeres display prophylactic and therapeutic efficacy. To abolish the transforming capacity of E7 protein, Cys24 and Glu26 in the E7 coding sequence were mutated to glycines in our study (33).

Previous studies have shown that various types of HPV L1 protein expressed in E. coli may self-assemble into capsid-like particles (25, 27, 34). The capsomere, which is 11 to 12 nm in size with a central stain-filled hollow under electron microscopy, is the subunit of the HPV capsid (25, 27, 34, 35). Although our electron microscopy data showed that the new protein lacks the typical pentameric structure, the majority of particles are 11 to 12 nm in size, and some of the capsomeres in our study had a central stained hollow region. This discrepancy may be explained by the 34–amino acid deletion in L1 and the fusion with E7.

In our study, the antibody titer to E7 or L1 was higher than the antibody titer induced by HPV VLP (data not shown). Conformational epitopes are very similar between VLP and native HPV virion, and our results show that antibody titers to VLP are very high in mice immunized with capsomeres. Earlier studies used hemagglutination inhibition assays to verify the specificity and neutralizing ability of antibodies to HPV VLP (21), but the hemagglutination ability of antibodies to capsomeres has rarely been investigated. In our study, sera from capsomere-immunized mice, at dilutions as great as 1:400, successfully inhibited the VLP-induced agglutination of RBCs. Although capsomeres contain only some of the HPV conformational epitopes and elicit lower titers of neutralizing antibodies, our study showed that capsomeres still have a strong prophylactic and therapeutic efficacy against HPV. Collectively, these data suggest that the epitopes on capsomeres are sufficient to protect against HPV infection in mice (36).

As stated previously, C3 cells express both L1 and E7, whereas TC-1 cells express E7 only. Mice challenged with C3 cells were completely protected by chimeric capsomeres, but mice challenged with TC-1 cells were only 70% protected. This result illustrates the strength of the L1E7 combination.

A complete immunogenic evaluation would include data for humoral responses, cellular responses, and tumor challenge responses. We have not yet studied T cell proliferation or the CTL response. Humoral responses are not always consistent with cellular immune responses and may affect the final functional efficacy. Nevertheless, our in vivo tumor challenge and rejection assays showed that E. coli–expressed chimeric capsomeres display prophylactic and therapeutic efficacy against papillomavirus in mice. Therefore, capsomeres may provide a low-cost vaccine strategy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

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