Enhancing Major Histocompatibility Complex Class I Antigen Presentation by Targeting Antigen to Centrosomes

Chien-Fu Hung, Wen-Fang Cheng, Liangmei He, Morris Ling, Jeremy Juang, Cheng-Tao Lin, and T-C. Wu

ABSTRACT

Several strategies that increase proteosomal degradation of antigen have been shown to improve MHC class I presentation of antigen. Because recent studies have demonstrated that the centrosome is a subcellular compartment rich in proteasomes, we hypothesized that targeting a tumor antigen to centrosomal compartments would enhance both the MHC class I presentation of antigen and the vaccine potency. We, therefore, created a chimerica of γ-tubulin, an established centrosomal marker, with a model tumor antigen, human papillomavirus type 16 (HPV-16) E7, in a DNA vaccine. The linkage of γ-tubulin to E7-targeted antigen to centrosomal compartments, resulted in enhanced MHC class I presentation of E7, and led to a marked increase in the number of E7-specific CD8+ T-cell precursors as well as a potent CD4-independent antitumor effect against an E7-expressing tumor cell line, TC-1. In addition, vaccination with γ-tubulin/E7 DNA in transporter associated with antigen presentation (TAP)-1−/− mice revealed that the enhancement of E7-specific CD8+ T-cell immune responses is TAP-1-dependent. Our data suggest that the centrosome may be an important locus for MHC class I antigen processing and that targeting antigen to the centrosome can improve DNA vaccine potency.

INTRODUCTION

DCs are the most potent professional antigen presenting cells that prime helper and killer T cells in vivo for review see Refs. (1–3). Vaccine strategies that target DCs provide an opportunity to enhance T-cell mediated immunity. Efficient delivery of DNA directly into professional antigen-presenting cells in vivo can be achieved through the use of the gene gun, which sends DNA-coated gold beads into the epidermis (4). Our lab has successfully used the gene gun to test several strategies for enhanced antigen presentation (5–9). One novel strategy to enhance the MHC class I presentation of antigen is to target antigen to the centrosome. The centrosome, which is used as the microtubule organizing center, is a perinuclear organelle that contains a high density of proteasomes (10–12). Several other proteins, including γ-tubulin and β-tubulin, are also localized in the centrosome. The centrosome has been implicated as an important intracellular compartment for proteosomal degradation of proteins into antigenic peptides (10). We hypothesize that a strategy that targets and concentrates antigens to the centrosome may be able to facilitate proteosomal degradation of antigen and to enhance MHC class I presentation of antigen, resulting in improved vaccine potency.

MATERIALS AND METHODS

Plasmid DNA Construction. For the generation of pcDNA3-E7, the DNA fragment encoding HPV-16 E7 was amplified by PCR using the template, pCMVneoBam-E7 (13) and a set of primers: 5′-gggaaatcggatgagacaaagaat-3′ and 5′-gggatccttcagacctaca-3′ and 5′-ggggtggatccttcagacctaca-3′ and 5′-ggggtggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-ggggtggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′. The amplified product was further cloned into the EcoRI and BamHI sites of pcDNA3 (Invitrogen, Carlsbad, CA). For construction of pcDNA3-γ-tubulin, the DNA fragment containing γ-tubulin was amplified with a set of primers, 5′-ggtggatccttgagaacagatgg-3′ and 5′-gggatccttcagacctaca-3′, and with the pH3-16 plasmid (14) as DNA template. The pH3-16 plasmid was a gift kindly provided by Dr. Beil R. Oakley (The Ohio State University, Columbus, OH). The PCR product was further cloned into the XhoI and EcoRI sites of pcDNA3. For construction of pcDNA3-γ-tubulin/E7, the PCR product of γ-tubulin was further cloned into the XhoI and EcoRI sites of pcDNA3-E7. For the generation of pcDNA3-E7/GFP, the DNA fragment encoding GFP was first amplified with PCR using the template, pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers as follows: 5′-atcggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′ and 5′-gggatccttcagacctaca-3′. The amplified product was then cloned into the BamHI and HindIII sites of pcDNA3-E7. To generate pcDNA3-γ-tubulin/E7/GFP, the DNA fragment encoding γ-tubulin was amplified and cloned into the Xhol/EcoRI sites of pcDNA3-E7/GFP. For the generation of pSC11CMS2-γ-tubulin/E7, the pH3-16/E7 was cut with XhoI/HindIII and cloned into the pGEM vector. pGEM-γ-tubulin/E7 was then cut with SphI/HindIII and cloned into the pSC11CMS2 vector. The accuracy of all of the constructs was confirmed by DNA sequencing.

Generation of Recombinant Vaccinia Virus. Recombinant γ-tubulin/E7 vaccinia virus was generated using pSC11CMS2-γ-tubulin/E7 vector according to a previously described protocol (13). Plaque-purified recombinant vaccinia viruses were tested for the presence of HPV-16 E7 genome by PCR and for the expression of HPV-16 E7 protein by immunofluorescence staining.

Mice and Murine Tumor Cell Line. Six- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and stored in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). TAP-1 KO C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All of the animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. The production and maintenance of TC-1 cells has been described previously (15). In brief, HPV-16 E6, E7, and ras oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate TC-1.

DNA Vaccination. Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to a previously described protocol (6). DNA-coated gold particles (1-μg DNA/bullet) were delivered to the shaved abdominal region of C57BL/6 mice or TAP-1 KO mice (5 mice/group) using...
for CD4+ T cells, MiCK-1 (PharMingen, San Diego, CA), were used as a positive control.

Cell surface marker staining of CD8 or CD4+ T cells and intracellular cytokine staining for IFN-γ were performed.

After the last vaccination, mice were s.c. challenged with 5×10^6 TC-1 tumor cells s.c. 1 week after the last vaccination. Depletions were started 1 week before tumor challenge. Monoclonal antibody GK1.5 was used for CD4 depletion, monoclonal antibody 2.43 was used for CD8 depletion, and monoclonal antibody PK136 was used for NK1.1 depletion. The completeness of depletion was checked by flow cytometry analysis. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the other subsets. Depletion was terminated on day 90 after tumor challenge.

Intracellular Cytokine Staining with Flow Cytometry Analysis

To detect IFN-γ secretion by E7-specific CD8+ T cells, cell surface marker staining of CD8+ and intracellular IFN-γ staining followed by FACScan analysis were performed using conditions described previously (6). The immortalized DC line was kindly provided by Dr. Kenneth Rock (University of Massachusetts, Worcester, MA; Ref. 17). With continued passage, we have generated subclones of DCs that are easily transfected using LipofectAMINE. DCs were transfected with γ-tubulin/E7, E7, or empty plasmid DNA using LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). DCs were collected 24 h after transfection, 2×10^5 transfected DCs (stimulators) were mixed with 2×10^5 D^8-restricted E7-specific CD8+ T cells (Ref. 18; responders) for 16 h before intracellular cytokine staining and flow cytometry analysis were performed.

We also characterized the ability of γ-tubulin/E7 vaccinia-infected bone marrow-derived DCs from either wild-type or TAP-1 KO C57BL/6 mice to activate an E7-specific CD8+ T cell line using intracellular cytokine staining followed by flow cytometry analysis. Bone marrow-derived DCs were obtained by culturing bone marrow cells in the presence of granulocyte-macrophage colony-stimulating factor as described previously (19). 2×10^5 DCs from wild-type or TAP-1 KO C57BL/6 mice were infected with wild-type vaccinia or γ-tubulin/E7 vaccinia at a multiplicity of infection of 10 overnight. An E7-specific T cell line served as responder cells. Vaccinia-infected DCs were washed twice and incubated with 2×10^5 E7-specific CD8+ T cells for 16 h before intracellular cytokine staining and flow cytometry analysis were performed.

Intracellular Cytokine Staining and Flow Cytometry Analysis of T-Cell Precursors in Vaccinated Mice

To assess the effect of γ-tubulin/E7 on antigen presentation, we transfected DCs with empty plasmid, γ-tubulin, E7, or γ-tubulin/E7 DNA and incubated the cells with an H-2D^b-restricted E7-specific CD8+ T-cell precursor clone (18) followed by flow cytometry analysis. As shown in Fig. 2A, DCs transfected with γ-tubulin/E7 DNA generated a significantly greater number of IFN-γ-secreting E7-specific CD8+ T cells than did DCs that were transfected with wild-type E7 DNA (one-way ANOVA on pooled splenocytes, P < 0.0001). Our results suggested that the linkage of γ-tubulin to E7 leads to the enhanced presentation of E7 antigen through the MHC class I pathway to CD8+ T cells.

Enhanced Presentation of E7 through the MHC Class I Pathway in Cells Transfected with γ-Tubulin/E7 DNA. We reasoned that the ability of γ-tubulin/E7 to target antigen to the centrosome may enhance MHC class I presentation of E7 antigen. To assess the effect of γ-tubulin/E7 DNA on antigen presentation, we transfected DCs with empty plasmid, γ-tubulin, E7, or γ-tubulin/E7 DNA and incubated the cells with an H-2D^b-restricted E7-specific CD8+ T-cell line (18) followed by flow cytometry analysis. As shown in Fig. 2A, DCs transfected with γ-tubulin/E7 DNA generated a significantly greater number of IFN-γ-secreting E7-specific CD8+ T cells than did DCs that were transfected with wild-type E7 DNA (one-way ANOVA on pooled splenocytes, P < 0.0001). Our results suggested that the linkage of γ-tubulin to E7 leads to the enhanced presentation of E7 antigen through the MHC class I pathway to CD8+ T cells.

Vaccination with Chimeric γ-Tubulin/E7 DNA Significantly Enhances E7-specific CD8+ T Cell-mediated Immune Responses in Vaccinated Mice. We evaluated whether the observed enhancement of MHC class I presentation in vitro was indicative of augmentation of CD8+ T-cell immune responses in vivo. To determine whether vaccination of mice with pcDNA3-γ-tubulin/E7...
DNA can enhance E7-specific CD8⁺ T-cell activity, we performed intracellular cytokine staining for IFN-γ-secreting E7-specific CD8⁺ T-cell precursors using splenocytes from vaccinated mice. Mice vaccinated with γ-tubulin/E7 DNA exhibited a marked increase in E7-specific IFN-γ CD8⁺ T-cell precursors compared with mice vaccinated with wild-type E7 DNA (Fig. 2B). These results indicated that the linkage of γ-tubulin to E7 significantly enhanced E7-specific CD8⁺ T-cell-mediated immune responses and that fusion of E7 to γ-tubulin was essential for this observed enhancement because γ-tubulin mixed with E7 (γ-tubulin+E7 DNA) did not enhance CD8⁺ T cell activity. Furthermore, the linkage of irrelevant proteins (such as GFP) to E7 did not lead to the enhancement of E7-specific CD8⁺ T-cell activity (data not shown).

Although linkage of γ-tubulin to E7 led to enhanced E7-specific CD8⁺ T-cell activity, we did not detect a significant difference in the number of E7-specific IFN-γ-secreting CD4⁺ T cells (Fig. 2C) among the various vaccination groups (one-way ANOVA on pooled splenocytes, P = 0.2367). When the splenocytes collected from γ-tubulin/E7-vaccinated mice were pulsed with the E7₃₀₋₆₇ peptide, we observed a significant population of CD4-negative IFN-γ-secreting cells by flow cytometry analysis. Because the E7₃₀₋₆₇ peptide also contains the E7 H-2Db-restricted epitope (aa 49–57), the majority of the CD4 negative IFN-γ-secreting cells are likely E7-specific IFN-γ-secreting CD8 T⁺ cells.

Protection against the Growth of E7-expressing Tumors in Mice Vaccinated with Chimeric γ-Tubulin/E7 DNA. To determine whether the observed enhancement in E7-specific CD8⁺ T-cell-mediated immunity translated to a significant E7-specific antitumor effect, we performed an in vivo tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1 (15). As shown in Fig. 3A, 100% of mice receiving the γ-tubulin/E7 DNA vaccine remained tumor free 63 days after the TC-1 challenge. In contrast, all of the unvaccinated mice and mice receiving pcDNA3 (no insert), γ-tubulin alone, or wild-type E7 developed tumor growth within 14 days of tumor challenge. We also observed that the fusion of E7 to γ-tubulin was required for antitumor immunity, because γ-tubulin mixed with E7 (γ-tubulin+E7 DNA) failed to enhance tumor protection compared with E7 DNA alone (log-rank test, P < 0.0001). These data suggested that the physical linkage of γ-tubulin to E7 was important for the observed enhancement of E7-specific tumor protection.

Treatment with Chimeric γ-Tubulin/E7 DNA Eradicates Established E7-expressing Tumors in the Lungs. We also investigated the therapeutic potential of the chimeric γ-tubulin/E7 DNA construct in treating TC-1 tumor metastases in the lungs. As shown in Fig. 3B, mice treated with γ-tubulin/E7 DNA exhibited the lowest mean number of pulmonary nodules (0.75 ± 0.95) compared with mice treated with wild-type E7 DNA (62.6 ± 3.5), or γ-tubulin DNA (94 ± 2.5; one-way ANOVA, P < 0.001). The results from the tumor
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DISCUSSION

In this study, we demonstrated that the direct linkage of γ-tubulin to E7 dramatically enhanced antitumor effects against the growth of TC-1 tumors.

CD8+ T Cells but not CD4+ T Cells or NK Cells Are Essential for the Antitumor Effect Generated by Chimeric γ-Tubulin/E7 DNA Vaccine. We performed in vivo antibody depletion experiments to determine the subset of lymphocytes important for the antitumor effect (24). As shown in Fig. 3C, all of the naïve mice and all of the mice depleted of CD8+ T cells grew tumors within 15 days of tumor challenge. In contrast, all of the nondepleted mice and the mice depleted of CD4+ T cells or NK1.1 cells remained tumor-free 60 days after tumor challenge (log-rank test, \( P < 0.0001 \)). These results suggested that the antitumor effect generated by the γ-tubulin/E7 DNA vaccine was dependent on CD8+ T cell immune responses but independent of CD4- or NK-associated responses.

Enhancement of E7-specific CD8+ T-Cell Immune Responses in Vaccinated Mice Is TAP-1 Dependent. To elucidate the role of TAP-1-dependent processing on the observed E7-specific CD8+ T-cell-mediated immune response in γ-tubulin/E7 DNA-vaccinated mice, we used TAP-1 KO C57BL/6 mice. TAP-1 KO C57BL/6 mice failed to generate appreciable E7-specific CD8+ T-cell immune responses (Fig. 4A). To further evaluate the importance of TAP-1 for the presentation of E7 antigen to E7-specific CD8+ T cells in vaccinated mice, we isolated bone marrow-derived DCs from wild-type or TAP1 KO C57BL/6 mice and infected these cells with either wild-type vaccinia or γ-tubulin/E7 vaccinia for use as stimulators. Vaccinia-infected DCs were incubated with an E7-specific CD8+ T cell line. We found that γ-tubulin/E7 vaccinia-infected DCs from wild-type C57BL/6 mice were able to efficiently induce E7-specific CD8+ T cells to secrete IFN-γ; in contrast, γ-tubulin/E7 vaccinia-infected DCs from TAP-1 KO C57BL/6 mice were unable to induce E7-specific CD8+ T cells to secrete IFN-γ (Fig. 4B). These data indicated that the observed enhancement of E7-specific CD8+ T-cell immune responses in γ-tubulin/E7 DNA-vaccinated mice was dependent on the TAP-1-dependent MHC class I presentation pathway.

Our data indicate that the centrosome is a potentially important locus for the processing of antigenic peptides for MHC class I presentation. This perinuclear structure is a site for the accumulation of proteasomes and associated regulatory proteins including ubiquitin (32, 33) and HSPs-70 and -90 (11, 12) and facilitates intracellular protein degradation. Studies have demonstrated that proteasomal inhibitors hinder processing by cytosolic (34, 35) or centrosomal proteasomes (10) and encumber the stable assembly of MHC class I molecule/peptide complexes. Another study found that proteasomal degradation of misfolded antigen (denatured influenza nucleoprotein), and, presumably, generation of antigenic peptides, occurs in the centrosome and promyelocytic leukemia oncogenic domains (10). It is likely that proteasomes in the centrosome catalyzed the degradation of γ-tubulin/E7 protein and the generation of epitopes for MHC class I protection and treatment experiments indicated that the linkage of γ-tubulin to E7 dramatically enhanced antitumor effects against the growth of TC-1 tumors.

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presentation. Because of its abundance of proteasomes and proximity to the ER, the centrosome may be a key site for the processing of certain antigens and subsequent presentation through the MHC class I pathway.

The success of the γ-tubulin/E7 DNA vaccine warrants the consideration of strategies that use other proteins that also target the centrosome to enhance vaccine potency. Biochemical analysis of the centrosome has revealed that a variety of proteins including γ-tubulin (22), pericentrin (36), HSP-70 (11), HSP-90 (11), human TOGp (37, 38), p115 (39), and the homologues of these proteins are targeted and concentrated to the centrosome. Understanding the targeting nature of these proteins would facilitate their application in vaccine developments in a manner similar to that described in the present study.

We have used gene gun immunization to test several intracellular targeting strategies that enhance MHC class I and/or class II presentation of antigen. In addition to the γ-tubulin/E7 DNA vaccine strategy, MHC class I presentation of HPV-16 E7 can be significantly enhanced by linkage with *Mycobacterium tuberculosis* HSP (HSP-70; Ref. 6) or the translocation domain (domain II) of *Pseudomonas aeruginosa* exotoxin A [ETA(dII); Ref. 8] in the context of a DNA vaccine. A comparison of γ-tubulin/E7 DNA, E7/HSP70 DNA, and ETA(dII)/E7 DNA revealed that the enhancement of E7-specific CD8+ T-cell immune responses was slightly greater in mice vaccinated with γ-tubulin/E7 DNA compared with the other vaccinated mice and wild-type control (data not shown). Whereas chimeric γ-tubulin/E7, E7/HSP70, ETA(dII)/E7, or CRT/E7 DNA generates...
potent CD8+ T-cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T-cell responses. For example, a DNA vaccine encoding a signal sequence linked to E7 and the sorting signal of the lysosome-associated membrane protein (LAMP-1; creating the Sig/E7/LAMP-1 chimera) targets E7 to endosomal and lysosomal compartments and enhances MHC class II presentation to CD4+ T cells compared with DNA encoding wild-type E7 (5). The availability of these strategies raises the notion of coadministration of vaccines such as of γ-tubulin/E7 DNA and Sig/E7/LAMP-1 DNA in a synergistic fashion. Such an approach may directly enhance both MHC class I and class II presentation of E7 and may lead to significantly enhanced E7-specific CD4+ and CD8+ T-cell responses and potent antitumor effects.

In summary, our findings indicate that the linkage of γ-tubulin to antigen is able to target antigen to the centrosome and elicit potent antigen-specific CD8+ T-cell activity and antitumor effects in vivo. This approach may be promising for future vaccine development to control other cancers or infectious diseases and may be particularly useful in patients with diminished CD4+ T-cell immune responses, particularly HIV-infected patients.

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