Identification of Novel Immunogenic Human Leukocyte Antigen-A*2402-Binding Epitopes of Human Papillomavirus Type 16 E7 for Immunotherapy Against Human Cervical Cancer

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BACKGROUND: A study was undertaken to identify new immunogenic human leukocyte antigen (HLA)-A*2402-restricted epitopes from human papillomavirus (HPV) type 16 E7 protein for immunotherapy against cervical cancer.

METHODS: Synthetic overlapping peptides were screened by measuring the frequency of CD8+ cytotoxic T lymphocytes (CTLs) producing intracellular interferon-γ (IFN-γ) using flow cytometry and were validated in SiHa cells with a Cr release cytotoxicity assay. In vivo antitumor effects of peptide-sensitized peripheral blood mononuclear cells (PBMCs) and isolated CD8+ CTLs were evaluated using BALB/c nude mice with SiHa cell xenotransplants.

RESULTS: Among 14 overlapping 15-amino acid peptides, E761-75(CDSTLRLCVQSTHV) and E767-81(LCVQSTHVDIRTLED) induced significantly higher IFN-γ production (P < .05) and showed higher in vitro cytotoxicity against SiHa cells than did cells sensitized with the negative control. To determine the exact HLA-A*2402-restricted epitopes, a total of 25 overlapping 9- or 10-amino acid peptides spanning E761-75 and E767-81 were synthesized. E761-69(CDSTLRLC) and E767-76(LCVQSTHV) induced significantly greater IFN-γ production as well as increased in vitro cytotoxicity against SiHa cells compared with those of other peptides and the negative control (P < .01), and the antitumor effects of these peptide-sensitized PBMCs were induced by CD8+ CTLs. E761-69-sensitized and E767-76-sensitized PBMCs and isolated CD8+ CTLs showed a much greater suppression of tumor growth in vivo compared with that of control groups treated with PBS (P < .01). The authors also confirmed the synergistic antitumor effect of cisplatin followed by E767-76-sensitized PBMCs in vivo.

CONCLUSIONS: E761-69 and E767-76 were identified as novel HPV type 16 E7 epitopes for HLA-A*2402, which could be used for immunotherapy against cervical cancer. Cancer 2011;000:000–000. © 2011 American Cancer Society.

KEYWORDS: HLA-A24, HPV 16 E7, immunotherapy, cervical cancer, epitopes.

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide and is highly associated with persistent human papillomavirus (HPV) infection.1,2 HPV is detected in 99.7% of cervical cancers,3 and HPV type 16 (HPV 16) is the most prevalent HPV type, present in approximately 50% of invasive cervical cancers.4 In addition, HPV 16 is detected in about 85% to 90% of anal cancers.5 Currently available prophylactic vaccines for HPV have been shown to effectively reduce the incidence of HPV-associated anogenital disease in young women and men6 and have also been shown to protect against HPV 16/18-related persistent infections and CIN2 lesions7 as well as anal cancer.8 However, these vaccines have not been shown to provide therapeutic effects against pre-existing HPV infections or cervical cancer,8 and it will take decades to develop preventive vaccines that significantly reduce the prevalence of cervical cancer because of the
considerable number of pre-existing HPV infections. Conventional treatment modalities for recurrent/advanced cervical cancer have low success rates. Therefore, there is a need for research into the development of therapeutic HPV vaccines for the current treatment of HPV-related anogenital disease, especially cervical cancer and anal cancer.

The importance of the cellular immune response in controlling the pathogenesis of HPV and associated cervical lesions in humans is well established, and cytotoxic T lymphocytes (CTLs) are considered the major eradicators of both HPV-infected cells and cervical cancer through the adaptive immune response. E6 and E7 are nuclear proteins of HPV that are constitutively retained and expressed in cervical cancer cells and that have immortalizing and transforming properties. Therefore, E6 and E7, which are not present in normal cells, have been considered attractive targets for specific immunotherapy against cervical cancer. Identification and characterization of CTL epitopes of HPV 16 E6 and E7 proteins have facilitated the development of peptide vaccines against cervical cancer. Although the immune dominance of E6 and E7 proteins among HPV 16 antigens has been reported, previously identified CTL epitopes derived from E7 protein were limited to human leukocyte antigen (HLA)-A*0201. The wide clinical application of HPV peptide vaccine requires the identification of at least 1 immune dominant E6 or E7 epitope for each class I HLA antigen, especially for epitopes such as HLA-A*2402, 1 of the most common HLA-A alleles in many different races, including United State, European, and South American populations (80%), and Asian populations (80% ~ 90%).

In this study, we report new HLA-A*2402-restricted epitopes, E761-69(CDSTLRLCV) and E767-76(LCVQSTHVDI), from HPV 16 E7 protein that can be used to stimulate CD8+ CTLs for adoptive immunotherapy against cervical cancer; these epitopes also might be used in a therapeutic vaccine platform.

MATERIALS AND METHODS

Donors and Peripheral Blood Mononuclear Cells
Peripheral blood mononuclear cells (PBMCs) were collected from 24 HLA-A*2402 healthy donors who were human cytomegalovirus (HCMV) seropositive. The presence of immunoglobulin (Ig) G and IgM HCMV antibodies in each donor was analyzed using passive latex agglutination (CMVSCAN kit; Becton-Dickinson Microbiology System, Cockeysville, Md). Cytomegalovirus (CMV)-specific epitopes were used as the positive controls for T-cell immune response in CMV seropositive donors. Major histocompatibility complex (MHC) class I genotypes were determined by the HLA laboratory at the Seoul Medical Science Institute (Seoul, South Korea) via sequence-specific polymerase chain reaction using genomic DNA. PBMCs were isolated via density-gradient centrifugation using Ficoll-Hypaque 1.077 (Pharmacia, Wilkstrom, Sweden). The mononuclear cells were cryopreserved at −160°C in human AB+ serum containing 10% dimethylsulfoxide (DMSO; Sigma, St Louis, Mo). This research was approved by the institutional review board of Yonsei University Health System, and all participants provided written informed consent (Federalwide Assurance #00001684).

Synthesis of HPV 16 E7 Peptides
A total of fourteen 15-amino acid peptides for HPV 16 E7 protein that overlapped by 9 residues were synthesized commercially (purity >95%; A & Pep, Yeongi-gun, South Korea; Table 1). After selection of the immunedominant candidate 15-amino acid peptides from the peptide library through the screening and confirmation test described below, 9- or 10-amino acid peptides spanning the candidate 15-amino acid peptides were synthesized (Table 2). The peptides were diluted to working solution concentrations (1 µg/µL) in diethylpyrocarbonate-treated water (Invitrogen, Carlsbad, Calif)

### Table 1. Fifteen-Amino Acid Overlapping Peptides Spanning HPV Type 16 E7 Protein

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Abbreviation: HPV, human papillomavirus.

*HPV 16 E761-69 was not synthesized due to low purity (<50%) by the high-performance liquid chromatography method.
containing 1% DMSO and stored at −80°C before testing.

**Generation of Autologous Dendritic Cells and Peptide-Specific CTLs**

Autologous dendritic cells (DCs) were generated as previously described, with minor modifications.17,18 PBMCs were incubated for 2 hours at 37°C using complete RPMI medium containing 10% fetal bovine serum (FBS). Adherent monocytes were resuspended at a concentration of 5 × 10^6 cells/mL in complete RPMI medium with granulocyte-macrophage colony-stimulating factor (1500 IU/mL; PeproTech, Rocky Hill, NJ) and interleukin (IL)-4 (1200 IU/mL; PeproTech). On days 2, 4, and 6 of culture, fresh cytokines were added. On day 5 of culture, 10 ng/mL tumor necrosis factor-α (R & D Systems, Minneapolis, Minn) was added for DC maturation. After maturation, autologous DCs were pulsed with peptides for at least 5 hours.

PBMCs were plated at a concentration of 2 × 10^6 cells per well in a 24-well culture plate (Nunc, Rochester, NY) with 2 mL complete RPMI medium. PBMCs were sensitized with synthetic E7 peptides (10 μg/mL/well), and 1000 IU/mL/well of recombinant human IL-2 (rhlL-2; PeproTech) was added. Then, rhIL-2 (1000IU/mL/well) was added to the culture every other day. For 1-week expansion, peptide-pulsed autologous DCs (4-10 × 10^6/well) were added to the PBMCs on day 7, incubated for 5 hours, and analyzed with flow cytometry. DC-treated PBMCs were cultured for another 7 days, and cytotoxicity assays were performed. CD8^+ and CD8^− fractions were separated using Dynabeads FlowComp Human CD8 (Invitrogen) cytotoxicity assay after 14 days of sensitization.

**Intracellular Flow Cytometric Analysis**

Fluorescein isothiocyanate-conjugated anti–interferon-γ (IFN-γ), PerCP-conjugated anti-CD69, PerCP-conjugated anti-CD3, and phycoerythrin–conjugated anti-CD8 were purchased from BD Biosciences (San Jose, Calif). For each sample, 75,000 events were gated on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). For data analysis (CELLQuest software; Becton Dickinson, Franklin Lakes, NJ), positive cells were expressed as a percentage of the respective reference population. Assessment of responses was previously described in more detail.18 Briefly, peptide-sensitized PBMCs (1 × 10^6 cells/mL) stimulated with phytohemagglutinin (Sigma) and PBMCs pulsed with autologous DCs that were not loaded with any peptide were used as positive and negative controls, respectively. HCMV pp65<sub>341-350</sub> (QYDP-VAALFF, HLA-A*2402), pp65<sub>495-503</sub> (NLVPMVATV, HLA-A*0201), or pp65<sub>91-100</sub> (SVNVHNPTGR, HLA-A33) were used as positive or negative controls according to the donor’s HLA type. One hour after stimulation, 10 μg Brefeldin A (Sigma) was added to each well. After 5 additional hours of incubation, PBMCs were washed once with phosphate-buffered saline (PBS) and then incubated in PBS containing 1 mM ethylenediaminetetraacetic acid for 10 minutes. After 2 additional washes with PBS containing 5% FBS, the cells were incubated with fluorescence-labeled monoclonal anti-CD3^+, anti-CD8^+, anti-CD69, and anti–INF-γ^+ antibodies for 15 minutes on ice in the dark before analysis.

**Cancer Cells and Cell Culture**

The human cervical cancer cell line SiHa (HLA-A*2402/2402) was purchased in 2010 from and has been tested and authenticated by the Korean Cell Line Bank (Seoul, Korea)19; it was expanded and frozen in aliquots within 4 weeks of purchase. SiHa cells were thawed and cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) containing 10% FBS and 1% DMSO and stored at −80°C before testing.

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Novel HLA-A*2402-Binding Epitopes/Jang et al

Table 2. Nine- or Ten-Amino Acid Overlapping Peptides Spanning E761-75 and E761-81
antibiotics for no more than 8 passages. Immortalized Epstein-Barr virus-B lymphoblastoid cell lines (EBV-BLC, HLA-A*2402/0207; a gift from Dr. David Stroncek, NIH, Bethesda, Md) were cultured at 37°C and 5% CO2 in RPMI-1640 with 10% FBS. Cells were routinely tested for absence of mycoplasma.

In Vitro Cytotoxicity Assay
Cytotoxicity assays were performed using the 51Cr release assay as previously described, with minor modification.20,21 Briefly, SiHa cells were labeled for 45 minutes with 51Cr (100 mCi/10^6 cells; Perkin Elmer, Waltham, Mass), washed in PBS, and dispensed in triplicate into 96-well U-bottom plates (Nunc) at 4 × 10^3 cells/well. Peptide-sensitized CTLs were added at an effector:target ratio of either 10:1, 30:1, 50:1, or 100:1. The cells were pelleted and incubated for 5 hours, and the supernatant was analyzed using a WIZARD^2 Automatic Gamma Counter (Perkin Elmer). Spontaneous and total releases for each target were used to calculate the percentage of specific release according to the following formula: % specific release = (experimental counts per minute – spontaneous counts per minute)/(total counts per minute – spontaneous counts per minute) × 100.

Mouse Xenografts
Female 4- to 6-week-old BALB/c nude mice were purchased (Central Lab. Animal Inc., Seoul, South Korea) and cared for under the guidelines of the institutional animal care and use committee. 1 × 10^6 of SiHa cells were washed twice with PBS and injected into the flanks of mice via subcutaneous injection once to initiate tumorigenesis. When the tumors had a mean volume of approximately 40 to 50 mm^3, mice were injected intravenously with 5 × 10^6 peptide-sensitized PBMCs or 3 × 10^6 CD8^+ CTLs per injection 3× in a final volume of 200 µL PBS. Mice were monitored 3× per week for tumor growth. Tumor size was measured in millimeters using a caliper and was recorded as mean volume (tumor volume = maximum length (mm) × perpendicular width (mm)^2)/2). Tumor samples were fixed overnight in 4% paraformaldehyde at 4°C and paraffin-embedded for immunohistochemical analyses.

Cisplatin Treatment
BALB/c nude mice were treated once intratumorally with cisplatin at a dose of 12.5 mg/kg body weight. Cisplatin was diluted with PBS to the required concentration and injected in a volume of 200 µL.

Immunohistochemistry
All samples were paraffin-embedded, sliced into 4- to 5-mm sections, and placed on a slide for immunostaining. Slides were deparaffinized in xylene (20 minutes, 2×) and 100% ethanol (5 minutes, 3×). The slides were washed in TBS (10 mM Tris-HCl [pH 7.4], 0.85% NaCl). The antigen was retrieved by boiling the slides with 10 mM citrate buffer (pH 6.0). The slides were subsequently incubated with mouse anti-CD8 (1:100; Dako, Carpinteria, Calif) or mouse anti-Fas (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif) primary antibody at 4°C. After incubation for 24 hours, the slides were washed in TBS, and the appropriate secondary antibody (EnVision+ system; Dako) was incubated at room temperature for 30 minutes. After washing, the slides were color-reacted using the DAB substrate chromogen system (Dako) and counterstained with Gill hematoxylin solution (Merck, Whitehouse Station, NJ). All images of representative fields were captured at ×400 magnification using DP controller software with a BX40 microscope (Olympus, Tokyo, Japan). All slides were evaluated by 2 experienced observers who were blinded to the conditions of the mice (S.J. and J.-B.L.).

Statistical Analysis
Data presented as mean ± standard deviation are representative of at least 2 different experiments. For nonparametric comparisons between the test group and control group, the Mann-Whitney U test was used. For all statistical analyses, the social sciences software package (version 13.0; SPSS, Chicago, Ill) was used, and a P value <.05 was considered statistically significant.

RESULTS
Identification of Specific 15-Amino Acid Candidate Epitopes Using In Vitro Sensitization
To determine which of the synthetic HPV 16 E7 peptides have potential immunogenicity for generation of CTLs, intracellular IFN-γ production of PBMCs from various HLA-A*2402 donors was measured after a week of in vitro sensitization with each of the 14 candidate 15-amino acid peptides. HPV 16 E761-75 (CDSTLRCLCVQSTHVD) and HPV 16 E767-81 (LCVQSTHVDRTLED) induced significantly higher IFN-γ production than did the other peptides or the negative control (nonpeptide; P = .46, P = .16, respectively; Fig. 1A, B). To substantiate the immunogenicity of the E761-75 and E767-81 peptides
selected by flow cytometry, HLA-A*2402-restricted cytotoxicity was evaluated using a \(^{51}\)Cr release assay. E7\(_{61-75}\) and E7\(_{67-81}\)-sensitized PBMCs demonstrated significantly higher \(^{51}\)Cr release compared with that of the negative control. In particular, E7\(_{67-81}\)-sensitized PBMCs showed higher cytotoxicity than did the negative control (Xs) in a cytotoxicity assay. Points, mean (n = 10); bars, SD. CMV pp65495-503 (CMV A02, exes) was used as a negative control.

**Figure 1.** Quantification is shown of intracellular interferon-\(\gamma\) (IFN-\(\gamma\)) production and cytotoxicity assays of CTLs sensitized with candidate 15-amino acid peptides. (A) Flow cytometric analysis showed that E7\(_{61-75}\) and E7\(_{67-81}\) induced significantly greater IFN-\(\gamma\) production in peripheral blood mononuclear cells (PBMCs) from human leukocyte antigen (HLA)-A*2402 subjects compared with that in negative controls (nonpeptide). Data are representative of 10 independent experiments using PBMCs from HLA-A*2402 subjects. (B) Bar graph depicts the number of peptide-specific INF-\(\gamma\)\(_+\)CD8\(_+\)T cells per 7.5 x 10\(^4\) PBMCs for each peptide. Columns, mean (n = 10); bars, standard deviation (SD). Statistically significant differences between the tested group and control group (nonpeptide) were determined using the Mann-Whitney \(U\) test. ***\(P < .001\); *\(P < .05\). (C) E7\(_{67-81}\) (diamonds)-sensitized and E7\(_{61-75}\) (squares)-sensitized PBMCs lysed significantly more SiHa cells than did the negative control (Xs) in a cytotoxicity assay. Points, mean (n = 10); bars, SD. Cytomegalovirus (CMV) pp65495-503 (CMV A02, exes) was used as a negative control. FITC, fluorescein isothiocyanate.

**Ex Vivo Sensitization of 9- and 10-Amino Acid Peptides Spanning E7\(_{61-75}\) and E7\(_{67-81}\)**

To determine the exact HLA-A-restricted HPV 16 E7 epitopes that were immunogenic in HLA-A*2402 subjects, a total of 25 overlapping 9- or 10-amino acid peptides spanning E7\(_{61-75}\) and E7\(_{67-81}\) were synthesized and tested. INF-\(\gamma\) protein production was measured in PBMCs from HLA-A*2402 donors that had been in vitro sensitized for a week with each of the 25 candidate peptides.
peptides. Among the peptides, E761-69 (CDSTLRLCV) and E767-76 (LCVQSTHVDI) induced significantly greater IFN-γ production than that of the other peptides (P = .01, P = .001, respectively; Fig. 2A, B). To provide further evidence of the immunogenicity of E761-69 and E767-76, HLA-A*2402-restricted cytotoxicity was evaluated using a 51Cr release assay. E761-69+ and E767-76-sensitized PBMCs lysed significantly more SiHa cells than did the negative control (Fig. 2C). To further demonstrate the epitope-specific cytotoxicities of these peptides, a cytotoxicity assay was performed using EBV-BLC cells expressing HLA-A*2402 as the target cell. PBMCs from HLA-A*2402 subjects sensitized with E761-69 and E767-76 lysed the peptide-loaded EBV-BLC cells but not the same EBV-BLC cells loaded with pp65495-503 (CMV A02) or any of the negative controls (Fig. 2D). E761-69 and E767-76 peptides also consistently induced higher expression of CD69 in CD8+ INF-γ+ CTLs than did the negative control (Fig. 2E).

**E761-69** and **E767-76**-Specific Cytotoxicities Were Induced by CD8+ CTLs

To determine whether peptide-specific cytotoxicity was induced through the cytolytic effect of CD8+ CTLs in PBMCs, SiHa cell-specific cytotoxicity of isolated CD8+ CTLs and CD8+ T lymphocyte-depleted PBMCs was measured using the 51Cr release assay. E761-69-sensitized isolated CD8+ CTLs lysed significantly more SiHa cells than did CD8+ T lymphocyte-depleted PBMCs or the negative control (Fig. 3A). E767-76-sensitized PBMCs and CD8+ CTLs also showed significantly higher cytotoxicities against SiHa cells than did CD8+ T lymphocyte-depleted PBMCs or the negative control (Fig. 3B).

**In Vivo Therapeutic Effects of Transplanted HPV 16 E761-69-Sensitized and E767-76-Sensitized CTLs**

To evaluate the in vivo immunotherapeutic effects of transplanted E761-69-sensitized and E767-76-sensitized CD8+ CTLs against human cervical cancer, we established a nude mouse model that allows outgrowth of an SiHa cell xenograft. Injection of 3 x 10^6 of isolated E761-69-sensitized and E767-76-sensitized CD8+ CTLs for 3 x caused a much greater suppression of tumor growth than that in the control group treated with PBS from 35 days after injection of the CD8+ CTLs (P = .028, P = .009 at day 42, respectively; Fig. 4A, B). At 42 days post-treatment, we evaluated the tumor weights of sacrificed mice and found a greater reduction in tumor weight in mice treated with E767-76-sensitized CD8+ CTLs compared with that in control mice (Fig. 4H). Immunohistochemical analysis with anti-CD8+ antibody showed that CD8+ CTLs successfully infiltrated into the cancer tissues in mice treated with both E761-69-sensitized and E767-76-sensitized CD8+ CTLs. In contrast, tumor tissues from untreated mice revealed no tumor-infiltrating human CD8+ CTLs (Fig. 4D, Top). Immunohistochemical staining with anti-Fas showed that Fas was more highly expressed in the cytoplasm of cancer cells in mice injected with both E761-69-sensitized and E767-76-sensitized CD8+ CTLs compared with that in control mice (Fig. 4D, Bottom).

Transfer of 1 x 10^7 of E767-76-sensitized PBMCs for 3 x into nude mice bearing SiHa cell xenografts caused a much greater delay in tumor growth compared with that in the control group treated with PBS from 31 days after injection of PBMCs (P = .002 at day 70; Fig. 4E, F). We also observed greater decreased in tumor weight in mice treated with E767-76-sensitized PBMCs compared with that in the control mice (P = .004; Fig. 4G).

**Treatment With Cisplatin Followed by Peptide-Sensitized PBMCs Generated a Potent and Long-Term Antitumor Effect**

To investigate the synergistic effect of combined chemotherapy and immunotherapy, tumor-bearing mice were injected intratumorally with 12.5 mg/kg cisplatin, a widely used chemotherapeutic agent for cervical cancer (5 mice per group). Treatment of mice with cisplatin alone induced complete extinction of the tumor burden from 26 days after cisplatin injection. However, tumor progression resumed 47 days after the cisplatin treatment in 2 of the control group mice. Injection of mice with 1 x 10^7 of E767-76-sensitized PBMCs at 30 days after cisplatin treatment completely suppressed tumor growth, and this antitumor effect lasted for at least 4 weeks after PBMC injection. We rechallenged mice with complete extinction of tumor burden with SiHa cells 40 days after cisplatin treatment and observed a greater delay in tumor growth in the group treated with peptide-sensitized PBMCs 30 days after cisplatin treatment than there was in the group that had received cisplatin treatment alone (data not shown). Thus, chemotherapy followed by immunotherapy potentiated the antitumor effects in a model in which each individual modality had antitumor activity.
Figure 2. Quantification is shown of intracellular interferon-γ (IFN-γ) production and cytotoxicity assay of cytotoxic T lymphocytes (CTLs) sensitized with candidate 9- or 10-amino acid peptides. (A) Flow cytometric analysis showed that E761-69 and E767-76 induced significantly higher IFN-γ production in peripheral blood mononuclear cells (PBMCs) from human leukocyte antigen (HLA)-A*2402 subjects than did the negative control (nonpeptide). Data are representative of 10 independent experiments using PBMCs from HLA-A*2402 subjects. (B) Bar graph depicts the number of peptide-specific INF-γ+ CD8+ T cells per 7.5 x 10^4 PBMCs. Columns, mean (n = 10); bars, standard deviation (SD). Statistically significant differences between the tested group and nonpeptide group were determined using the Mann-Whitney U test. ***P < .001; **P < .01. (C) E761-69 (squares)-sensitized and E767-76 (diamonds)-sensitized PBMCs lysed significantly more SiHa cells than did the negative control (nonpeptide, circles). Cytomegalovirus (CMV) pp65341-350 (CMV A24, triangles) was used as a positive control, and CMV pp65495-503 (CMVA02, exes) was used as a negative control. Points, mean (n = 10); bars, SD. (D) PBMCs sensitized with E761-69 (squares) and E767-76 (diamonds) lysed significantly more EBV-BLC cells than did the negative control (nonpeptide, circles). CMV pp65341-350 (CMV A24, triangles) was used as a positive control, and nonpeptide sensitized-PBMCs (nonpeptide, circles) or nonpeptide pulsed EBV-BLC cells (exes, *) were used as negative controls. Points, mean (n = 10); bars, SD. (E) CD69 expression by CD8+ T cells was measured using flow cytometry to determine the activation of CD8+ CTLs after a 1-week sensitization with each candidate peptide. E761-69 and E767-76 induced higher expressions of CD69 in CD8+ CTLs than did the negative control. CMV pp65341-350 (CMV A24) was used as a positive control, and nonpeptide was used as a negative control. Data are representative of 2 independent experiments using PBMCs from HLA-A*2402 subjects. FITC, fluorescein isothiocyanate.
DISCUSSION

Various forms of therapeutic vaccines against anogenital cancer, mainly targeting HPV 16 E6 and E7, have been tested in preclinical and clinical trials, and each has its own advantages and disadvantages. Recently, Kenter et al reported that HPV 16 E6 and E7 long peptide-based vaccines showed clinical effectiveness against HPV 16-positive vulvar intraepithelial neoplasia.22 These peptide-based vaccines are safer, easier to produce, and more stable than other therapeutic vaccines. Moreover, the low cost of peptide-based vaccines has led many investigators to initiate development of tumor-specific peptide antigens.23,24 However, these peptide-based vaccines are MHC restricted, and one of the most important steps in a peptide-based vaccine approach is the identification of immunogenic epitopes within HPV proteins that bind to HLA class I or II molecules expressed by a major proportion of the population. Previously identified CTL epitopes derived from HPV 16 E7 protein were limited, and most were restricted to HLA-A*0201. Although HLA-A*2402 is another prevalent HLA-A molecule among the entire human race, HLA-A*2402-restricted HPV 16 E7 epitopes have not yet been reported. Thus, we focused on the identification of novel HLA-A*2402-restricted epitopes derived from HPV 16 E7 protein using overlapping 15-amino acid peptides. These HPV 16 E7-specific HLA-A*2402-restricted epitopes will be useful for vaccination, adoptive immunotherapy using CTLs as well as DCs, and monitoring the cellular immune response in cervical cancer patients.

In this study, a total of fourteen 15-amino acid peptides that overlapped by 9 residues and that covered the full length of the HPV 16 E7 protein were screened using flow cytometric quantification of INF-γ production in PBMCs from HLA-A*2402 individuals. Among the 14 peptides, HPV E761-75 and E767-81 induced significantly more INF-γ in PBMCs than did the other peptides and also induced in vitro cytotoxicity against SiHa cells in a 51Cr release assay (Fig. 1). Virus-infected human cells or human cancer cells can be recognized by CD8+ T lymphocytes (CTLs) in peripheral blood mononuclear cells (PBMCs). (A) E767-81-sensitized isolated CD8+ CTLs (squares) and E767-81-sensitized PBMCs (diamonds) lysed significantly more SiHa cells than did CD8+ T lymphocyte-depleted PBMCs (triangles) or the nonpeptide-sensitized PBMCs (exes) used as a negative control. Points, mean (n = 10); bars, standard deviation (SD). (B) E767-76-sensitized isolated CD8+ CTLs (diamonds) also showed significantly higher cytotoxicity against SiHa cells compared with those of CD8+ T lymphocyte-depleted PBMCs (triangles) or the negative control (nonpeptide, exes). Points, mean (n = 10); bars, SD.

Figure 3. Human papillomavirus (HPV) type 16 E7 peptide-specific cytotoxicity is caused by the cytolytic effect of CD8+ cytotoxic T lymphocytes (CTLs) in peripheral blood mononuclear cells (PBMCs): (A) E767-81-sensitized isolated CD8+ CTLs (squares) and E767-81-sensitized PBMCs (diamonds) lysed significantly more SiHa cells than did CD8+ T lymphocyte-depleted PBMCs (triangles) or the nonpeptide-sensitized PBMCs (exes) used as a negative control. Points, mean (n = 10); bars, standard deviation (SD). (B) E767-76-sensitized isolated CD8+ CTLs (diamonds) also showed significantly higher cytotoxicity against SiHa cells compared with those of CD8+ T lymphocyte-depleted PBMCs (triangles) or the negative control (nonpeptide, exes). Points, mean (n = 10); bars, SD.
induced significantly more IFN-γ from PBMCs (Fig. 2A, B). These 2 peptides were also successfully presented by the HLA-A*2402 alleles of SiHa cells and exhibited strong in vitro cytotoxicity against SiHa cells and peptide-pulsed EBV-BLC cells (Fig. 2C, D). These results suggest that the 2 peptides are processed naturally, presented successfully in human cervical cancer cells, and recognized by peptide-specific CTLs that induce a cancer-killing effect in vitro. To determine whether this peptide-specific cytotoxicity was induced through a cytolytic effect of CD8+ CTLs present in PBMCs, isolated peptide-sensitized CD8+ and CD8-depleted cells were tested for cytotoxicity against SiHa cells. The peptide-sensitized CD8+ CTLs showed significantly greater cytotoxicity against SiHa cells than did CD8-depleted PBMCs (Fig. 3), confirming the crucial role of CD8+ CTLs in cytotoxicity against human cervix cancer cells in vitro.

In previous studies, the critical role of CD8+ CTLs in the obliteration of tumors has been reported in several animal cervical cancer models.26,27 In this study, we
constructed a human cervical cancer mouse model using SiHa cell xenografts in BALB/c nude mice and tested the antitumor effect of CD8$^+$ CTLs sensitized with the previously identified epitopes. Our data demonstrated significant suppression of tumor growth after intravenous injection of E7$_{61-69}$-sensitized and E7$_{67-76}$-sensitized CD8$^+$ CTLs (Fig. 4A-C), successful infiltration of the injected peptide-sensitized CD8$^+$ CTLs into the cancer tissues (Fig. 4D), and induction of significant expression of Fas, a well-known marker of apoptosis, within the cancer tissue (Fig. 4D). We also confirmed that E7$_{61-69}$-sensitized and E7$_{67-76}$-sensitized PBMCs could delay tumor growth in our mouse model and could be effectively used for adoptive immunotherapy against cervical cancer (Fig. 4E-G). However, a possible limitation of this preclinical study is that the tumor microenvironment in this model lacks T regulatory cells and other nonlymphoid suppressor cells that interfere with effective killing and control of tumor by tumor-specific CD8$^+$ T cells; these cells can influence the results of clinical study.

For adaptive immunotherapy, peptide-sensitized PBMCs have several advantages over CTLs, as they can be more easily produced and handled and are more cost effective than isolated CD8$^+$ CTLs. Therefore, peptide-sensitized PBMCs could easily be applied in clinical studies of adaptive immunotherapy against cervical cancer. We also tested the efficacy of combination therapy using cisplatin and peptide-sensitized PBMCs, and our data suggested that treatment with cisplatin followed by peptide-sensitized PBMCs generated a more potent and long-term antitumor effect compared with treatment with cisplatin alone or that of peptide-sensitized PBMCs alone (Fig. 5). These results are similar to previous observations, and the enhanced antitumor effect of combined treatment with cisplatin and peptide-sensitized PBMCs could be caused by several mechanisms.$^{28-30}$

We think that treatment with cisplatin may render E7-expressing tumors more susceptible to death because of E7-specific CD8$^+$ CTLs and may reduce the number of immune suppressor cells, including myeloid suppressor cells, in tumor-bearing mice. However, overestimation of PBMCs’ effect after cisplatin injection in this result is possible, because tumors recurred in only 2 among 5 control mice, and it is difficult to differentiate the treatment effect between cisplatin and PBMCs in the remaining 3 control mice.

In conclusion, we identified E7$_{61-69}$ and E7$_{67-76}$ as novel HPV 16 E7 epitopes for HLA-A*2402 and demonstrated that PBMCs and CD8$^+$ CTLs sensitized with these peptides have cytolytic activity and antitumor effects against cervical cancer. In addition, our data suggest that treatment with cisplatin followed by peptide-sensitized PBMCs generates a more potent and long-term antitumor effect than treatment with cisplatin or peptide-sensitized PBMCs alone.

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REFERENCES