Induction of Specific CD8+ T-Lymphocyte Responses Using a Human Papillomavirus-16 E6/E7 Fusion Protein and Autologous Dendritic Cells

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Abstract

When intracellular viral proteins are degraded, only a limited number of peptide epitopes are capable of eliciting specific CD8+ cellular immune responses for a given human leukocyte antigen (HLA) haplotype. We sought to induce CD8+ T-lymphocyte (CTL) responses to human papillomavirus-16 (HPV-16) E6 and E7 proteins using a recombinant E6/E7 fusion protein and autologous human dendritic cells (DCs). CTLs were generated by in vitro stimulation using a recombinant HPV-16 E6/E7 fusion protein and autologous DCs from a healthy HLA-A*0201 donor. CTL specificity was assessed by cytokine release assays when the cells were reacted with autologous DC targets coincubated with the E6/E7 fusion protein. These CTLs were also reacted with the immunodominant E7 peptides (E711-20 and E786-93) and DCs as a target. As a negative control, DCs were incubated with or without an irrelevant control protein (Helicobacter pylori) as target for the E6/E7-induced CTLs. The E6/E7-induced CTLs were capable of specific recognition of target DCs coincubated with E6/E7 but not the control protein. When E6/E7-specific CTLs were reacted with DCs and either E711-20 or E786-93 specific peptide recognition was also detected. These data demonstrate that specific CTLs can be elicited using autologous human DCs and a HPV-16 E6/E7 fusion protein. Therefore, extracellular viral proteins seem to be engulfed and processed by DCs; then the immunodominant HLA-A2-restricted peptides become available for CD8+ T-lymphocyte recognition. These data suggest that vaccine strategies using recombinant viral proteins may overcome the limitation of peptide epitopes for specific HLA haplotypes and may, therefore, permit more generalized clinical application.

Introduction

The HPV2 has been causally linked with cervical cancer, and over 90% of invasive cervical tumors harbor HPV DNA (1). Recent investigations emphasize the importance of the host’s immune response in the development of HPV-associated cervical lesions (2, 3) and provide a compelling rationale for the development of novel immunotherapies targeted at HPV. Because the HPV-16 genes E6 and E7 are selectively retained and expressed in cervical tumors (4), they are attractive targets for novel immunotherapies. HPV-16 E6 and E7 oncoproteins undergo intracellular degradation, and the peptide by-products have been shown to induce potent antitumor immune responses. This has inspired the development of peptide-based vaccines for the treatment of patients with advanced cervical cancer (5, 6). However, for any specific patient, it is necessary to know a priori which by-product peptides are appropriate for their particular HLA haplotype. We seek to induce specific cellular immune responses to HPV-16 E6 and E7 proteins in all patients with cervical cancer, regardless of their haplotype, by using autologous antigen-presenting cells, DCs, and a recombinant E6/E7 fusion protein. Studies have clearly shown that peptide-pulsed DCs are significantly more efficient in inducing antitumor protection than immunization with peptide alone (7) or with peptide emulsified in incomplete Freund’s adjuvant (8). In the current study, we investigated whether HPV-specific CTLs could be induced from the human PBMCs by in vitro stimulation using a recombinant full-length E6/E7 fusion protein and autologous DCs. We sought to examine whether extracellular HPV proteins could be engulfed by DCs and then undergo intracellular processing in the class I pathway, making the peptide fragments available for CD8+ T-lymphocyte recognition. Because autologous DCs are used to induce the CTLs, this approach theoretically permits all of the possible epitopes of the E6/E7 fusion protein to be processed and presented for CTL recognition.

Materials and Methods

Preparation of PBMCs and Lymphocytes. Autologous DCs were prepared from the PBMCs and used as antigen presenting cells. PBMCs (4 × 10^6) were obtained from a HLA-A*0201 donor by leukapheresis and separated in Ficoll-Hypaque gradients (LSM, Organon Teknika, Durham, NC). The PBMC preparations were frozen in human AB serum with 10% DMSO (Sigma Chemical Co, St. Louis, MO) and stored in liquid nitrogen.

HLA Typing and Subtyping. HLA class I type was established on PBMCs as described previously (9). HLA-A2 subtyping was performed using a high-resolution nested sequence PCR set to resolve the HLA-A*0201 through the HLA-A*02017 alleles.

E6/E7 Fusion Protein and Helicobacter pylori Protein. Good laboratory practice grade HPV-16 E6/E7 fusion protein and H. pylori protein was produced by CSL, Parkville, Victoria, Australia. The HPV protein was produced to good laboratory practice standards using the following procedure:

(a) E6 and E7 coding regions were amplified by PCR using appropriate HPV-16 DNA primers with appropriate restriction sites for assembly as an “in frame” fusion and subsequent cloning into the pDS56 (Roche) expression vector, which has an IPTG inducible promoter and a COOH-terminal region encoding his residues;

(b) The E6/E7hh fusion sequence including the hexa-histidine region was isolated from the pDS56 vector, then this cassette was placed into a modified pGEX-4T-1 expression vector;

(c) Recombinant Escherichia coli was grown in large-scale batch ferment using Terrific broth, and cultures were sparged at pH 7.0;

(d) E6/E7hh production was induced with isopropyl-1-thio-β-D-galactopyranoside at mid-exponential growth phase;

(e) Recombinant protein was expressed at 2.5% of total cell protein at a concentration of 8 mg/ml culture;

(f) Cells were concentrated and washed and then lysed in homogenization buffer;

(g) Inclusion bodies containing E6/E7hh were then washed in homogeni-
zation buffer containing 2 mM urea and 1% Triton X-100 and then solubilized with 20 volumes of buffer containing 8 mM urea; and (h) E6/E7h was purified from solubilized inclusion bodies by metal chelate chromatography followed by size-exclusion chromatography in urea on Superdex 200. A similar process was used to produce the H. pylori protein. This protein was used as a specificity control in cytokine release assays.

**Peptides.** The HPV-16 E7̵95̵95–93̵95 (TLGIVCPI) peptide, HPV-16 E7̵11̵11–20̵11 (YMLDLQPETT) peptide, and the control influenza matrix M1̵58̵58–66̵66 peptide (GILGFVFTL) used for the in vitro analysis were synthesized by a solid-phase method and purified by high-pressure liquid chromatography (>95% pure). The binding affinity of E7̵86̵86–93̵83 and E7̵11̵11–20̵11 to HLA-A*0201 has been reported previously (10). All of the peptides were diluted from aliquots dissolved in 100% DMSO and stored at −70°C. E7̵86̵86–93 and E7̵11̵11–20 were used usually at a final concentration of 20 μM; M1̵58̵58–66 was used at a final concentration of 1 mM. These peptides, all of which are HLA-A*0201-restricted, were used to assess in vitro CTL responses.

**Preparation of DCs.** After Ficoll-Hypaque separation, 2 × 10⁸ PBMCs were processed for preparation of DCs as described previously (11). The PBMCs were cultured in 75-cm² culture flasks for 3 days at 37°C. The nonadherent cells were removed, and the adherent cells were cultured for 5–7 days in sterile conditions in 20 ml of complete medium consisting of Iscove’s modified Dulbecco’s medium (Biofluids, Rockville, MD) plus 10% heat-inactivated human AB serum (Biofluids), 0.03% L-glutamine, 100 units/ml penicillin-streptomycin (both from NIH media unit), and 0.5 mg/ml amphotericin B (Biofluids). hr-GM-CSF (2000 IU/ml, Pepro Tech, Inc., Rocky Hill, NJ) and hr-IL-4 (2000 IU/ml, Pepro Tech, Inc.) were added every 2–3 days from day 0.

**Peptide and Protein Pulsing of DCs.** The recovered DCs were coinubated, or pulsed, with either 10 or 20 μg/ml of peptide, 10 or 100 μg/ml of E6/E7 fusion protein, or 25 μg/ml H. pylori protein for 2 h in 15-ml conical tubes at 37°C at a concentration of 1 × 10⁶ cells/ml.

**In Vitro Sensitization of Peripheral Blood Lymphocytes with DCs.** CD8+ enrichment of T cells was achieved by positive selection on biomagnetic separation beads (Dynal Corp., New York, NY). CD8+ lymphocyte cells (4–5 × 10⁶/well) were cocultured with 1 × 10⁶ protein-pulsed or peptide-pulsed DCs in 24-well plates and were restimulated after 1 week with 1 × 10⁶ protein-pulsed or peptide-pulsed DCs. IL-2 (300 IU/ml) was added 24 h after each stimulation and every 2–3 days thereafter. The effectors were tested for specificity 7–9 days after the restimulation.

**Target Cells.** In addition to using autologous DCs as targets for assessing CTL reactivity, T2 cells were used in cytokine release assays for CTLs induced with the HPV E7 peptides. The T2 cell line was selected as a target because it expresses only the HLA-A*0201 allele, which is the most prevalent class I allele expressed among humans in general and Caucasians in particular. This cell line is defective in endogenous processing, which enhances the effectiveness of exogenous peptide loading.

**Assessment of CTL Reactivity Using Cytokine Release Assay.** Effector cells (1 × 10⁵) were coinubated with 1 × 10⁵ stimulator cells in the presence or absence of the peptide or protein for 24 h at 37°C in 200 μl of complete medium (5 × 10⁴ effector cells/ml). Duplicate supernatant samples from these cocultures were tested for specific secretion of IFN-γ by human IFN-γ Quantikine enzyme-linked immunosorbant assay kits (R&D Systems, Minneapolis, MN). To normalize the data, background IFN-γ secretion (defined as IFN-γ released by effector cells exposed to DC-only targets) was subtracted from each of the observed measurements. Measurements are presented as picograms of IFN-γ released by 5 × 10⁵ effectors/24 h. The data are representative results from five independent experiments.

**Results**

**HLA Typing.** Analysis of HLA class I antigen expression identified the following haplotype: HLA-A*0201,0205, HLA-B*13,44, and HLA-C*03,07.

**DCs and CD8+ Lymphocytes.** DCs were prepared from PBMCs by using hr-GM-CSF and rh-IL-4. After 5–7 days incubation, the cells showed the characteristic morphological appearance of DCs with veiled edges and multiple processes. In all of the experiments, the T-cell population after CD8+ selection with biomagnetic separation beads revealed greater than 95% CD8+ and included <5% contamination with CD4+ cells by FACS analysis.

**Induction Peptide-specific CTLs.** PBMCs were stimulated with the HPV peptides in vitro and tested for peptide-specific CTL reactivity. CD8+ T cell reactivity was tested for IFN-γ release in a HLA-A*0201-restricted assay by pulsing the peptides onto T2 cells that express HLA-A*0201 molecules (Fig. 1). This assay excludes non-HLA-A*0201-restricted secretion of IFN-γ because the T2 cell line does not express any other HLA class I or class II alleles. Therefore, this assay is aimed at analyzing specifically HLA-A*0201-restricted secretion of IFN-γ. To enhance the specificity of the CTLs, we did a second stimulation on each CTL 7 days after the first stimulation. CTLs induced with E7̵11̵11–20̵11 secreted increased amounts of IFN-γ when T2 cells were pulsed with E7̵11̵11–20̵11, whereas CTLs induced with E7̵86̵86–93̵83 secreted IFN-γ when T2 cells were pulsed with E7̵86̵86–93. The CTL cultures induced with E7̵86̵86–93 did not secrete IFN-γ when the T2 target cells presented irrelevant peptide (i.e., peptide not used to induce the CTLs). However, CTLs induced with E7̵11̵11–20̵11 exhibited some cross-reactivity with T2 target cells pulsed with M1̵58̵58–66. CTL responses occurred at every peptide dosage studied with no clear dose-response relationship. Because virtually all humans are exposed to the influenza matrix protein, we used M1̵58̵58–66 as a control epitope to assess each patient’s capacity to generate anti-M1̵58̵58–66 CTLs. CTLs induced with M1̵58̵58–66 recognized only T2 cells pulsed with M1̵58̵58–66 but not unpulsed target cells. These results demonstrate both the efficiency of DCs in presenting peptide antigens and the epitope specificity of the CTLs for HLA-A*0201-restricted target cell lysis.

**Induction HPV E6/E7 Fusion Protein-specific CTLs.** We have attempted to induce HPV-specific CTLs using proteins rather than peptides because this strategy overcomes barriers imposed by peptide-based approaches that require a priori knowledge of both the HLA haplotype and the relevant HPV-16 E6 or E7 peptide epitope for any given HLA allele. For this purpose, CTLs were generated by in vitro stimulation using a recombinant HPV-16 E6/E7 fusion protein or the H. pylori control protein and autologous DCs. CD8+ T cell reactivity was tested for IFN-γ release by coincubating the target cells (DCs) with either the E7 fusion protein or the immunodominant HLA-A*0201-restricted, E7 peptides (E7̵86̵86–93 or E7̵11̵11–20). Specific secretion of IFN-γ resulted when the CTLs that were induced with the
E6/E7 fusion protein were reacted with target DCs that were pulsed with the fusion protein. In addition, these CTL cultures also recognized the immunodominant HLA-A*0201-restricted E7 86–93 epitope and, to a lesser degree, the E7 11–20 peptide. CTLs induced with the H. pylori protein did not secrete IFN-γ when reacted with target DCs pulsed with the E6/E7 fusion protein, although some nonspecific recognition of the E7 peptides was observed. These data indicate that HPV-specific CTLs can be induced using a recombinant E6/E7 fusion protein and that certain of these CTL cultures also recognize an immunodominant HLA-A*0201-restricted E7 epitope (E7 86–93). Therefore, extracellular viral proteins seem to be engulfed by DCs, then undergo intracellular processing in the class I pathway, making the peptide fragments available for CTL recognition.

To further characterize the specificity of the E6/E7 fusion protein-induced CTL, CTLs were induced with either no protein, the E6/E7 fusion protein, or the H. pylori protein. These cultures were then reacted with DCs alone or pulsed with either one of the proteins (Fig. 3). Consistent with the previous experiment, CTLs induced with the E6/E7 fusion protein recognized DCs target cells cocultured with the same fusion protein. Considerably less IFN-γ secretion resulted when the DCs cells were used alone as targets or when these cells were cocultured with the H. pylori protein. In contrast, CTLs induced with the H. pylori protein did not secrete IFN-γ when the DCs target cells were pulsed with the E6/E7 fusion protein and only secreted negligible amounts of IFN-γ when the DC target cells were cocultured with the H. pylori protein. CTLs induced in the absence of either protein did not secrete IFN-γ under any of the culturing conditions. Taken together, these results suggest that the CTL responses generated with the viral E6/E7 fusion protein are specific and that the nonviral H. pylori protein is not effective in generating a specific CTL response.

Discussion

Cervical cancer represents a model tumor system for vaccine development because of the firmly established causal association of HPV. Our goal is to develop and evaluate the clinical application of a therapeutic or prophylactic vaccine for the treatment or prevention of HPV-induced cervical cancer. Vaccine strategies aimed at augmenting cellular immune responses may ultimately be shown to have an important impact on both the prophylaxis and treatment of HPV-associated cervical neoplasia. For example, in experiments performed using HLA-A*0201 transgenic mice, 15 of 15 mice receiving the E7 86–93 peptide were protected from subsequent challenge with a lethal dose HPV-16 E7-induced tumor (10), which suggests that cellular immune responses may have prophylactic capacities in preventing the progression of HPV-induced preinvasive cervical precursor lesions. In addition, we recently evaluated the efficacy of a lipified form of this same peptide (E7 86–93) as a candidate vaccine for patients with invasive cervical cancer (6) and found that specific E7 86–93 CTL reactivity could be enhanced in the peripheral circulation. Although this enhanced reactivity was not sufficient to induce a clinical response during short-term follow-up (3 months), one of the three patients in whom a primary CTL response was observed subsequently achieved a clinical complete response in the absence of additional treatment.3

We have shown that specific anti-HPV-16 CD8 + CTLs can be induced in vitro using autologous human DCs to process and present either immunodominant HLA-A*0201 peptide epitopes of HPV-16 E7 or a recombinant HPV E6/E7 fusion protein. The results of these experiments emphasize the efficiency of DCs for presenting peptide antigens and also suggest that DCs may be capable of engulfing extracellular viral proteins that can then undergo intracellular processing in the class I pathway to permit the peptide fragments to become available for CD8 + T-lymphocyte recognition. Our findings are consistent with those of other investigators who have documented vigorous T-cell responses when DCs were loaded with protein antigens (7, 12). Because autologous DCs are used to induce the CTLs, this approach permits all of the possible epitopes of the E6/E7 fusion protein to be processed and presented for CTL recognition. Thus, the limitations of peptide-based vaccines, such as a priori knowledge of the patient’s HLA haplotype to chose appropriate peptides compatible with that particular haplotype, can be overcome and may, therefore, permit a more potent immune response involving the presentation of epitopes from all of the possible restriction elements (the molecules expressed by both of the HLA A, both of the HLA B, and both of the HLA C alleles for any given subject). To date, HLA-A*0201-restricted CTL responses represent the majority of those described, reflecting the prevalence of this class I allele among humans in general and Caucasians in particular (nearly 50% of Caucasians express HLA-A2; Ref. 13). The use of a protein-based vaccine may, therefore, be more readily translated into clinical application because no patient would be excluded because of HLA haplotype restrictions. This approach also has distinct advantages over strategies using viral vectors to express the E6 and E7 genes because these genes have well-recognized tumorigenic potential, and the viral expression vectors themselves may cause substantial morbidity, especially in immunosuppressed patients.

By using autologous DCs cultured with recombinant human IL-4 and GM-CSF, we have sensitized PBMCs in vitro from a healthy

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HLA-A*0201 donor against both a recombinant full-length E6/E7 fusion protein and certain MHC class I restricted epitopes of E7. Because the PBMCs were enriched for CD8+ cells before inducing the CTLs with the fusion protein, our results suggest that this extracellular viral protein is engulfed and processed via the MHC class I pathway. To exclude the possibility that the main effectors in our studies might be CD4+ CTLs, we tested the cells treated with the biomagnetic separation beads for CD8+ selection by FACS analysis. The T-cell population was greater than 95% CD8+ and included <5% contamination with CD4+ cells (data not shown). FACS analysis also confirmed that the autologous DCs used in our study had an abundance of molecules necessary for T-cell binding and costimulation (14). To confirm antigen specificity, we used autologous DCs and T2 cells as target cells pulsed exogenously with relevant or irrelevant synthetic peptides. In general, endogenously produced antigens are presented in association with MHC class I molecules, whereas engulfed exogenous antigens, such as the fusion protein, are to be presented in association with MHC class II molecules. The discrimination does not seem to be absolute because the mechanisms of regulation on antigen uptake, processing, and presentation are not yet defined (15). Recently, Bachmann et al. (16) demonstrated that DCs can process exogenous viral proteins for class I presentation to CD8+ CTLs in mice. Consistent with Bachmann’s in vivo study, the nonviral H. pylori protein used in our in vitro study was not effective in eliciting CTL responses. The unique characteristics of viral, as opposed to nonviral, proteins to elicit specific cellular immune responses is currently under intensive investigation.

The objective of our work is to develop a vaccine strategy applicable to all patients with cervical cancer that can induce an effective antitumor response. Although there are examples of protracted survival in patients receiving anti-HPV therapeutic vaccines (6, 17), the vast majority of patients failed to demonstrate any objective clinical responses. It is conceivable that the CTL reactivity by HPV-specific vaccines may not be quantitatively sufficient to cause tumor regression in patients with advanced disease. Indeed, the generation of effective immune responses in tumor-bearing hosts has proven to be more difficult than induction of protective immunity in normal animals (18). Additional studies in patients who may be capable of rejecting a considerably smaller burden of HPV-infected cells, such as those with preneoplastic cervical lesions or patients at high risk for recurrence after primary therapy, may yield more encouraging clinical outcomes. To further emphasize the plausibility of using protein-based vaccines against HPV-associated malignancies, De Bruin et al. (19) recently described the successful in vivo use of DCs loaded with soluble E7 protein for the induction of MHC class I-restricted protection to HPV-16-induced tumor cells. Taken together with the results of the current study using autologous human DCs and a HPV-16 E6/E7 fusion protein, these studies suggest that protein-based vaccination strategies may be worthy of further development for the treatment of patients with HPV-associated cervical neoplasms.

The development of an effective therapeutic anti-HPV vaccine could have a profound impact on the worldwide morbidity and mortality of cervical cancer, which remains a leading cause of cancer-related death in women from developing countries, in which cervical cytology screening programs are not widely available. In addition, a therapeutic anti-HPV vaccine could have enormous economic implications because existing cervical cytology screening programs cost nearly six billion dollars annually in the United States alone (20). Because most sexually active women are eventually exposed to HPV (21), it is unlikely that a prophylactic HPV vaccine will confer protection to women who have already been infected. Therefore, a novel immunological therapy to treat existing HPV infection as well as HPV-associated cervical cancer may represent an attractive and cost-effective treatment alternative that may reduce the need for expensive screening and surveillance prevention programs and substantially reduce the worldwide morbidity from this disease.

References

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