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Identification and characterization of enhancer agonist human cytotoxic T-cell epitopes of the human papillomavirus type 16 (HPV16) E6/E7

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ABSTRACT

Human papillomavirus (HPV) is associated with the etiology of cervical carcinoma, head and neck squamous cell carcinoma, and several other cancer types. Vaccines directed against HPV virus-like particles and coat proteins have been extremely successful in the prevention of cervical cancer through the activation of host HPV-specific antibody responses; however, HPV-associated cancers remain a major public health problem. The development of a therapeutic vaccine will require the generation of T-cell responses directed against early HPV proteins (E6/E7) expressed in HPV-infected tumor cells. Clinical studies using various vaccine platforms have demonstrated that both HPV-specific human T cells can be generated and patient benefit can be achieved. However, no HPV therapeutic vaccine has been approved by the Food and Drug Administration to date. One method of enhancing the potential efficacy of a therapeutic vaccine is the generation of agonist epitopes. We report the first description of enhancer cytotoxic T lymphocyte agonist epitopes for HPV E6 and E7. While the *in silico* algorithm revealed six epitopes with potentially improved binding to human leukocyte antigen–A2 allele (HLA-A2)–Class I, 5/6 demonstrated enhanced binding to HLA-Class I in cell-based assays and only 3/6 had a greater ability to activate HPV-specific T cells which could lyse tumor cells expressing native HPV, compared to their native epitope counterparts. These agonist epitopes have potential for use in a range of HPV therapeutic vaccine platforms and for use in HPV-specific adoptive T- or natural killer–cell platforms.

1. Introduction

Several human cancers have been linked to high-risk types of the human papilloma virus (HPV) [1–8]. High-risk HPV types 16 and 18, in particular, are associated with genital, oropharyngeal, and head and neck squamous cell carcinomas (HNSCC) [2,5–8]. Early (E) HPV genes (E1–E8) control viral expression and replication, and late (L) genes code for viral proteins [9–11].

There has been great success in the development of vaccines to prevent HPV associated with cancer, and in particular cervical cancer [12–14]. These vaccines elicit antibody responses to late HPV genes [15,16], and thus would not be effective in treating established tumors.

The E6 and E7 genes of HPV represent potential targets for therapeutic vaccines, since they are responsible for maintenance of the malignant phenotype [17–19]. Several different epitopes of HPV capable of eliciting cytotoxic T lymphocyte (CTL) responses have previously been identified [20–22] and clinical studies employing several different HPV-specific vaccine platforms have demonstrated various degrees of success in terms of eliciting HPV-specific responses and clinical benefit [23–35]. Indeed, clear clinical responses, including complete responses in some patients, have been observed [23,26,30,31,36].

One approach in the development of more effective HPV therapeutic vaccines is the identification and use of enhancer agonist epitopes as defined HPV peptides; in particular, peptides that

Abbreviations: Ad5, adenovirus serotype 5; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; E, early gene; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; IVS, *in vitro* stimulation; L, late gene; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MOI, multiplicity of infection; NK, natural killer; ORF, open reading frame; PBMC, peripheral blood mononuclear cell.

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would elicit enhanced CTL responses capable of lysing tumor cells expressing the native HPV epitope. *In silico* algorithms, while important, often do not accurately predict which peptides will be optimal targets for T-cell–mediated lysis of tumor. Agonist peptide epitopes that strongly bind to a major histocompatibility complex (MHC), moreover, may also not necessarily induce specific T-cell responses that could lyse tumor cells.

To date, no enhancer agonist epitopes to HPV have been reported. We report here the evaluation of six potential HPV enhancer agonist CTL epitopes that were predicted via an *in silico* algorithm as potential CTL agonist epitopes: three 9-mer peptides within the HPV E6 protein and three 9-mer peptides within the HPV E7 protein. After analyses of actual binding to human leukocyte antigen (HLA)–Class I, the ability to elicit IFN-γ production by T cells, and the ability to generate T cells capable of lysing carcinoma cells expressing the native HPV epitope, three novel HPV enhancer agonist epitopes were identified: two in the E6 region, and one in the E7 region. These epitopes can potentially be employed in a range of peptide- or vector-based vaccines as well as in targeted adoptive T-cell or natural killer (NK)–cell therapies.

2. Materials and methods

2.1. Culture of human tumor cell lines

The following human tumor cell lines were used in this study: cervical carcinoma (CaSki: HLA-A2$^+$HPVE6$^+$ and E7$^+$), head and neck carcinoma (HN-4: HLA-A2$^+$HPVE6$^+$ and E7$^+$; HN-12: HLA-A2$^+$HPVE6$^+$ and E7$^+$; HN-30: HLA-A2$^-$HPVE6$^+$ and E7$^+$) [37,38], and pancreatic carcinoma (ASPC-1: HLA-A1$^+$, HLA-A26$^+$HPVE6$^-$ and E7$^-$). Cell cultures were maintained free of mycoplasma. CaSki and ASPC-1 were maintained in complete medium (RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; Mediatech, Herndon, VA). Head and neck carcinoma cell lines were cultured in DMEM (low glucose) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Mediatech). T2 cells transfected with the HLA-A2 gene [39] were provided by Dr. Peter Cresswell, Yale University School of Medicine, New Haven, CT, and maintained as previously described [40].

2.2. Peptides

The HPV E6 and E7 amino acid sequences were scanned for the match to consensus motif for HLA-A2 binding peptides. The BIMAS algorithm developed by Parker et al. [41] was used to rank potential MHC binding peptides according to predicted one-half-time dissociation of peptide/MHC complexes. Peptides were synthesized at >95% purity (American Peptide Company, Sunnyvale, CA). Agonist peptides from E6 and E7 were synthesized from the E6 and E7 native regions with amino acid substitutions in order to increase binding affinity (Table 1).

2.3. Peptide binding to HLA-A2

Binding of HPV E6 and E7 peptides and the HPV E6 and E7 analogs to HLA-A2 molecules was evaluated by the up-regulation of HLA-A2 expression on T2A2 cells, as shown by flow cytometry [42,43].

2.4. Adenovirus vectors

A previously described [44] recombinant adenovirus serotype 5 (Ad5) with additional deletions in the early 2b (E2b) gene region was utilized. This new Ad5 [E1−, E2b−] − E6$^\Delta$/E7$^\Delta$ vector with mutagenesis of HPV16 E6/E7 oncogenes as an insert and Ad5 [E1$^-$, E2b$^-$]–null (having no genetic insert) were constructed and produced as described [45] and used to infect dendritic cells (DCs).

Table 1
Binding of HPV16 E6 and E7 peptides and analog peptides to HLA-A2 molecules, with predicted and T2A2 binding assay.

	Sequence	Position	Predicted Binding[a]	T2A2 Binding[b]
E6 peptides				
E6-1	KLPQLCTEL	11–19	74.768	853
E6-A1	KLPQLCTE***V*** (L19V)		243.432	**1335**
E6-2	KISEYRHYC	72–80	53.914	761
E6-A2	KISEYRHY***V*** (C80V)		754.791	877
E6-3	QQYNKPLCDL	90–99	15.941	1020
E6-A3	Q***L***YNKPLCD***V*** (Q91L/L99V)		511.903	1280
E7 peptides				
E7-1	YMLDLQPET	11–19	375.567	1105
E7-A1	YMLDLQPE***V*** (T19V)		3505.289	1171
E7-2	TLHEYMLDL	7–15	201.447	975
E7-A2	TLHEYMLD***V*** (L15V)		655.875	**1529**
E7-3	RTLEDLLMGT	77–86	3.611	839
E7-A3	RTLEDLLMG***V*** (T86V)		2426.739	**1263**
NEG	Negative control			812
TP2a	Positive control			1540

Amino acid sequences of parental E6 and E7 peptides and analog peptides. Amino acids are shown by the single-letter code. Substitution amino acids are included in bold italic and underlined.

[a] Predicted binding on the basis of reported motif; score estimate of half time of disassociation of a molecule containing these sequences.

[b] Results are expressed in mean fluorescence intensity (MFI). A brachyury HLA-A2 binding peptide was used as a positive control.

2.5. Generation of DCs from PBMCs

Peripheral blood was collected from healthy HLA-A0201$^+$ donors, and peripheral blood mononuclear cells (PBMCs) were separated using lymphocyte separation medium (ICN Biochemicals, Aurora, VA), according to the manufacturer's instructions. DCs were generated using a modification of a previously described procedure [46]. PBMCs were resuspended in AIM-V medium (Invitrogen, Carlsbad, CA) and allowed to adhere in a 6-well plate for 2 h. Adherent cells were cultured for 5 days in AIM-V medium containing 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ). The culture medium was replenished every 3 days.

2.6. Infection of human DCs with adenovirus vectors

DCs (2×10^5) in 1 ml of AIM-V medium were infected with adenovirus vectors Ad5 [E1−, E2b−] − E6$^\Delta$/E7$^\Delta$ and Ad5 [E1−, E2b−]–null at an indicated multiplicity of infection (MOI) of 10,000 or 20,000 for 1 h in 6-well plates. AIM-V medium (4 ml) was then added to each well and incubated for an additional 2 days. DCs were harvested and analyzed for transgene expression by Western blot.

2.7. Generation of T-cell lines using adenovirus-infected DCs

A modification of the method previously described [46,47] was used to generate HPV16 E6- and E7-specific CTLs. DCs ($1–2 \times 10^5$/well in 1 ml of AIM-V medium) were infected with 20,000 MOI of Ad5 [E1−, E2b−] − E6$^\Delta$/E7$^\Delta$, as described above. Infected DCs were used as antigen-presenting cells (APCs) to stimulate autologous non-adherent cells at an effector cell:APC ratio of 10:1. Cultures

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