Recognition of a cervical cancer derived tumor cell line by a human papillomavirus type 16 E6 52-61-specific CD8 T cell clone

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The E6 and E7 proteins of high-risk human papillomavirus (HPV) types are thought to be the ideal sources of antigens for immunotherapy for cervical cancer since they are expressed by the tumors and not by normal cells. We recently described new HPV 16 epitopes, including the E6 52-61 peptide restricted by HLA class I molecule B57. Primary tumor cell lines were established from three HLA-B57 positive, HPV 16 positive cervical cancer patients, and their recognition by an E6 52-61 specific CD8+ T cell clone was determined using a chromium release assay and an IFN-γ enzyme-linked immunospot (ELISPOT) assay. The recognition of homologous epitopes contained in other high-risk HPV types (18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73) was also examined at the peptide level. A low level of killing of two of the tumor cell lines derived from the three patients was demonstrated using a chromium release assay. The level of killing of one of these tumor cell lines was enhanced upon treatment with IFN-γ and/or the addition of antigen. This tumor cell line also induced measurable IFN-γ secretion. The recognition of homologous epitopes from HPV 35, 39, 45, 51, and 73 was detected in an ELISPOT assay. Therefore, the HPV 16 E6 52-61 epitope appears to be at least weakly expressed by tumor cell lines derived from cervical cancer, and the HPV 16 E6 52-61-specific T cell clone can recognize homologous peptides derived from other high risk HPV sequences.

Keywords: human, cervical cancer, cultured tumor cells, HPV type 16, cytotoxic T lymphocytes, epitope analysis

Introduction

Cervical cancer is the second most common malignancy among women worldwide, and the link between human papillomavirus and the development of cervical cancer is well known. HPV infection is also associated with squamous intraepithelial lesion (SIL), the precursor lesion of cervical cancer. Transformation to a malignant phenotype by HPV is mediated by two early gene products, E6 and E7. Because their expression is required for the maintenance of a transformed phenotype, they are thought to be ideal sources of antigens for use in dendritic cell immunotherapy.

Previously, we described a human papillomavirus type 16 (HPV 16) E6 52-61 CD8 T cell epitope (FAFRDLICIVY) (1). Its selection was based on the largest magnitude of the CD8 T cell immune response to the HPV 16 E6 and E7 proteins in a woman who was able to clear her HPV 16 infection (1). In this study, we examined the recognition of three cell tumor lines derived from cervical cancer tissues, and that of homologous peptides derived from other high risk HPV sequences, by the HPV 16 E6 52-61 specific T cell clone.

Results

Recognition of HLA-B57-positive primary tumor cell lines by an HPV 16 E6 52-61-specific T cell clone

Primary tumor cell lines were established from patients with cervical cancer as described in the Materials and Methods section. Three primary tumor cell lines from HLA-B57-positive patients (Table 1) were labeled with chromium and were used as targets in chromium release assays. A low but discernable level of lysis was observed with tumor cell lines #1 and #3, but not with tumor cell line #2 (Figure 1, upper left). These differences between tumor cell lines were not explainable on the basis of HLA-B57 subtypes since subject #1 from whom the T cell clone was isolated is HLA-B5703-positive and tumor cell lines #1, #2, and #3 are HLA-B5701-, B5703-, and B5701-positive, respectively (Table 1). Upon treatment of the tumor cell lines with IFN-γ, increased lysis of tumor cell line #1 was observed, whereas the level of lysis of tumor cell lines #2 and #3 was unchanged (Figure 1, upper right). Similarly, addition of exogenous peptide (HPV 16 E6 52-61) increased lysis of tumor cell line #1, but not of tumor cell lines #2 and #3 (Figure 1, middle left). A high percentage of Epstein-Barr virus-transformed B-lymphoblastoid cell line (EBV-LCL) cells from patient #1 were lysed when pulsed with peptide, demonstrating the strong antigenicity of the epitope (Figure 1, middle left). Interestingly, lysis of tumor cell line #1 increased significantly when it was treated with IFN-γ and was pulsed with the peptide (Figure 1, middle right). A similar level of lysis was observed when the E6 epitope was introduced by infecting the IFN-γ-treated tumor cell line #1 with recombinant vaccinia virus expressing E6 (Figure 1, lower right), suggesting that both the increased level of MHC expression by IFN-γ treatment (see next section) and the increased presentation of the HPV 16 E6 epitope are necessary to significantly enhance killing by the T cells.
Table 1
HLA types of cervical cancer patients from whom HPV-16-positive primary tumor cell lines were derived and of subjects with a history of HPV 16 infection

<table>
<thead>
<tr>
<th>Source</th>
<th>Histology</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>squamous cell</td>
<td>A1, A2, 3501, 5701, Cw6</td>
<td>DR1, DR3, DR7, DR53, DQ5, DQ9</td>
</tr>
<tr>
<td>Patient 2</td>
<td>squamous cell</td>
<td>A30, 5301, 5703, Cw4, Cw7</td>
<td>DR13, DR15, DQ2, DQ6, DR51, DR52</td>
</tr>
<tr>
<td>Patient 3</td>
<td>squamous cell</td>
<td>A1, A2, 5001, 5701, Cw6</td>
<td>DR3, DR7, DR53, DQ2, DQ9</td>
</tr>
<tr>
<td>Subject 1</td>
<td>NA</td>
<td>A2, A68, 4001, 5703, Cw3, Cw6</td>
<td>ND</td>
</tr>
<tr>
<td>Subject 2</td>
<td>NA</td>
<td>A2, A32, 1501, 5701, Cw3, Cw6</td>
<td>ND</td>
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<tr>
<td>Subject 3</td>
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<td>A1, A2, 0702, 5701, Cw6, Cw7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not applicable; ND, not done.

Figure 1

Chromium release assay examining the expression of the E6 52–61 CD8 T cell epitope by primary tumor cell lines derived from cervical cancer patients. The cells were untreated (upper left), treated with IFN-γ (upper right), pulsed with the E6 52–61 peptide (middle left), pulsed with the peptide and treated with IFN-γ (middle right), infected with E6-vac (lower left), or infected with E6-vac and treated with IFN-γ (lower right). Assays were performed in triplicate wells, with error bars representing the standard error of the means. A representative experiment, of two experiments performed, is shown.

Next, the recognition of tumor cell line #1 by the HPV 16 E6 52-61-specific T cell clone was measured using an IFN-γ ELISPOT assay (Figure 2). With 30,000 and 50,000 T cell clone cells, the entire range of tumor cells used (1,000, 3,000, 5,000, and 10,000 cells) demonstrated spot forming units above the negative control in a dose-dependent manner. Therefore, the HPV 16 E6 52-61-specific T cell clone cells were able to lyse tumor cell line #1 cells and to secrete IFN-γ upon recognition.
**Figure 2**

ELISPOT assay demonstrating the dose-dependent IFN-γ secretion by HPV 16 E6 52-61-specific T cell clone cells upon stimulation by tumor cell line #1. The experiment was performed in triplicate wells. The wells contained 1 x 10^5 EBV-LCL cells from patient #1, the T cell clone (10,000, 30,000, or 50,000 cells), and tumor cell line #1 cells (1,000, 3,000, 5,000, 10,000, or none).

**Figure 3**

Surface marker expression of HPV-16-positive primary tumor cell lines with and without IFN-γ treatment. The cells were stained with anti-HLA class I antibody (W6/32), anti-HLA-A2 antibody (BB7.2), anti-HLA class II antibody (IVA12), anti-HLA class II antibody (9.3F10), or anti-CD54 (ICAM-1). The results from two experiments are shown because the expression levels of some antigens were different. (A) First experiment. (B) Second experiment.
molecules, either with or without IFN-γ enhanced by treatment with IFN-γ. None of the tumor cell lines expressed HLA-class II antigens (including HLA-A2), HLA class II antigens, and ICAM-1 was examined on the three primary tumor cell lines, with and without IFN-γ treatment. The EBV-LCL from patient #1 was also studied. The expression of all antigens examined was demonstrated. The results of two experiments are shown in Figure 3 since the expression levels of some antigens were different. The expression of HLA Class I molecules was enhanced by treatment with IFN-γ for tumor cell lines #1 and #3, but not for tumor cell line #2. Patients #1 and #3 are HLA-A2 positive (Table 1) but tumor cell line #1 appears to have lost HLA-A2 expression although her EBV-LCL cells were HLA-A2 positive. None of the tumor cell lines expressed HLA-class II molecules, either with or without IFN-γ treatment, while all of them showed expression of ICAM-1 at varying levels.

Table 2

| HPV Type | Amino Acid Residues | Sequence
<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>16</td>
<td>52–61</td>
<td>VAFRDLC1TVY</td>
</tr>
<tr>
<td>18</td>
<td>47–56</td>
<td>VAFK0L1D1TVY</td>
</tr>
<tr>
<td>31</td>
<td>45–54</td>
<td>VAPTD1LTVY</td>
</tr>
<tr>
<td>33</td>
<td>45–54</td>
<td>VAPAD1LTVV</td>
</tr>
<tr>
<td>35</td>
<td>45–54</td>
<td>VAPCD1LTVY</td>
</tr>
<tr>
<td>39</td>
<td>47–56</td>
<td>VAFSDK1TVY</td>
</tr>
<tr>
<td>45</td>
<td>47–56</td>
<td>VAFSDK1TVY</td>
</tr>
<tr>
<td>51</td>
<td>45–54</td>
<td>VAFTE1X1TVY</td>
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<td>52</td>
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<tr>
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<td>58</td>
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<td>68</td>
<td>47–56</td>
<td>VAFTE1X1TVY</td>
</tr>
<tr>
<td>73</td>
<td>45–54</td>
<td>VAFTE1X1TVY</td>
</tr>
</tbody>
</table>

*Amino acid residues different from those in the HPV-16 E6 52–61 epitope are highlighted.

Surface expression of HLA class I and class II antigens on primary tumor cell lines

Since the expression of some HLA antigens has been reported to be defective in cervical cancer (2), the expression of HLA class I antigens (including HLA-A2), HLA class II antigens, and ICAM-1 was examined on the three primary tumor cell lines, with and without IFN-γ treatment. The EBV-LCL from patient #1 was also studied. The expression of all antigens examined was demonstrated. The results of two experiments are shown in Figure 3 since the expression levels of some antigens were different. The expression of HLA Class I molecules was enhanced by treatment with IFN-γ for tumor cell lines #1 and #3, but not for tumor cell line #2. Patients #1 and #3 are HLA-A2 positive (Table 1) but tumor cell line #1 appears to have lost HLA-A2 expression although her EBV-LCL cells were HLA-A2 positive. None of the tumor cell lines expressed HLA-class II molecules, either with or without IFN-γ treatment, while all of them showed expression of ICAM-1 at varying levels.

Recognition of homologous peptides from other high risk HPV types by the HPV 16 E6 52-61-specific T cell clone

As the first step in examining the extent of the utility of the E6 52-61 epitope as a target for immunotherapy for cervical cancer, the recognition of homologous peptides (Table 2) from other high risk types (HPV 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 72) was determined. Homologous peptides were defined as 10-mer sequences located within 20 amino acids from the HPV 16 E6 52-61 epitope, and that share the same amino acids as the E6 52-61 epitope as a target for immunotherapy for cervical cancer cell lines including CaSki, and SiHa transfected with HPV 16 E6 52-61-specific T cell clone. The difference in susceptibility of the primary tumor cell lines to lysis by the HPV 16 E6 52-61-specific T cell clone could not be explained by the cell surface expression of HLA class I antigens and ICAM-1 since both tumor cell lines #1 and #3 showed expression which was enhanced by IFN-γ treatment, but only tumor cell line #1 lysis was demonstrably increased. Also, a low level expression of the HLA class I antigens was demonstrated.

Discussion

The goal of this project was to assess the feasibility of the HPV 16 E6 52-61 epitope restricted by the HLA-B57 molecule as a target of immunotherapy for treating cervical cancer. Small but discernable killing of two out of three primary tumor cell lines derived from HLA-B57-positive cervical cancer patients (Figure 1) was observed. This cytotoxicity could be enhanced slightly in one tumor cell line by increasing the availability of the E6 epitope by adding the peptide (Figure 1, middle left), by adding the peptide and treating with IFN-γ (Figure 1, middle right), or by infecting the cells with E6-expressing recombinant vaccinia virus and treating with IFN-γ (Figure 1, lower right). In addition, positive recognition of tumor cell line #1 cells by the HPV 16 E6 52-61-specific T cell clone was demonstrated using an IFN-γ ELISPOT assay (Figure 2). Our results are similar to those reported by Evans et al. who studied the HPV 16 E6 29-38 epitope (TIHDILEVC) restricted by the HLA-A2.1 molecule (3). They have shown that the HLA-A2-positive and HPV 16-positive cervical cancer tumor cell lines CaSki and C33A-HPV 16 were not recognized by the E6 29-38-specific T cell clone. Pulsing with the E6 29-38 peptide, but not infection with HPV 16/18 E6/E7 expressing recombinant vaccinia virus (TA-HPV), resulted in demonstrable killing by the T cell clone suggesting that the tumor cell lines were defective in endogenous processing of the antigen. However, they also performed an experiment which showed that CaSki cells treated with IFN-γ and infected with TA-HPV were killed by the T cell clone, but not C33A-HPV 16 cells treated with IFN-γ and infected TA-HPV. Therefore, treatment with IFN-γ (which they have demonstrated results in increased surface HLA-A2 expression) and the increased availability of the appropriate E6 antigen by TA-HPV infection resulted in killing of one cervical carcinoma cell line but not another. Although we were able to demonstrate the recognition of tumor cell line #1 by the HPV 16 E6 52-61-specific T cell clone, both E6 epitopes (29-38 and 52-61) may be difficult to use as targets of cervical cancer immunotherapy due to the absence or low level of recognition.

Youde et al. have recently shown that a polyclonal CTL line monospecific for the HPV 16 E7 11-20 epitope known to be restricted by HLA-A2.1 molecule (but not the HPV 16 E6 29-38 epitope also restricted by HLA-A2.1 molecule) was able to kill cervical cancer cell lines including CaSki, and SiHa transfected to express HLA-A2.1 molecule (4). Our group has previously demonstrated killing of autologous cervical cancer derived tumor cell lines by CD8 T cell lines stimulated with HPV 16/18 E7 pulsed autologous dendritic cells (5). Therefore, it is possible that the E7 protein may be a better target for cervical cancer immunotherapy and that our future investigations should include using T cell clones specific for HPV 16 E7 epitopes.
ELISPOT analysis to determine recognition of peptide antigens homologous to the HPV 16 E6 52-61 epitope. One thousand T cell clone cells were plated along with 1 x 10^5 EBV-LCL cells from subject #1, subject #2, subject #3, and patient #1 (from left to right) for each peptide tested. The experiment was performed in triplicate wells, with error bars representing the standard error of the means. Each peptide was tested at least twice.

for tumor cell line #2 in one of the experiments, although no lysis of this tumor cell line has been shown. It is possible that expression of the HLA-B57 molecule has been lost while that of other HLA class I antigens is preserved.

The relevance of the HPV 16 E6 52-61 epitope to other high risk HPV types was demonstrated by the recognition of homologous HPV 35, 39, 45, 51, and 73 peptides by the HPV 16 E6 52-61-specific T cell clone. Whether these homologous peptides are endogenously processed would need to be addressed in the future.

In short, we have demonstrated the recognition of cervical cancer derived tumor cell lines and of homologous peptides derived from other high risk HPV types by the HPV 16 E6 52-61-specific T cell clone. However, the use of this epitope as a source of immunotherapy for treating cervical cancer patients does not appear to be promising due to the weak recognition of the tumor cells by the T cell clone.

Abbreviations

EBV-LCL, Epstein-Barr virus-transformed B-lymphoblastoid cell line

Acknowledgements

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References


Materials and methods

Cells

The cloning and characterization of HPV 16 E6 52-61-specific CD8 T cells have been described previously (1). Briefly, CD8 T cells from a woman who had acquired and cleared an HPV 16 infection, as detected by polymerase chain reaction analysis of cervical samples, were stimulated with autologous mature dendritic cells infected with recombinant vaccinia viruses expressing the HPV 16 E6 and E7 proteins. The presence of HPV-specific T cells was demonstrated in an IFN-γ enzyme-linked immunospot (ELISPOT) assay with a series of pooled overlapping 15-mer peptides covering the entire length of the E6 and E7 proteins. The strongest response was seen in the E6 46-70 region, and specific T cell clones were selected magnetically on the basis of IFN-γ secretion. Multiple clones were isolated, characterized, and were found to have identical characteristics. For consistency, only clone #2 was used in this study; it was expanded on a feeder cell mixture (Yssel's medium (Gemini Bioproducts, Inc., Calabasas, CA) containing 1% pooled human serum, penicillin G (100 units/mL), streptomycin (100 µg/mL), 1 x 10^6/ml irradiated allogeneic PBMCs, 1 x 10^6/ml irradiated JY cells, and 0.1 µg/ml PHA).

Primary tumor cell lines were established from three HLA-B57-positive and HPV 16-positive patients (tumor cell lines #1, #2, and #3 are from patients #1, #2, and #3 respectively) after sterile processing of the samples from surgical biopsy specimens as previously described (5). Fresh tumor biopsy specimens from patients diagnosed with frankly invasive stage IIa–IIa cervical cancer (staged according to the FIGO operative staging system) were obtained at the time of surgery and/or staging through the Gynecologic Oncology Division and the Pathology Department at the University of Arkansas for Medical Sciences under the approval of the Institutional Review Board. Briefly, single-cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature. Viable tumor tissue was mechanically minced in RPMI 1640 to fragments no larger than 1 to 3 mm² and washed twice with RPMI 1640. These were then placed into 250-ml flasks containing 30 mL of an enzyme solution [0.14% collagenase type I (Sigma–Aldrich, St. Louis, MO) and 0.01% DNAse (Sigma–Aldrich)] in RPMI 1640 and incubated on a magnetic stirring apparatus either for 2 hours at 37°C or overnight at 4°C. Enzymatically dissociated tumor material was filtered through a 150-µm nylon mesh to generate a single-cell suspension. The resultant cell suspension was washed twice in RPMI 1640 plus 10% fetal bovine serum (Gemini Bioproducts Inc.) before being seeded in tissue culture flasks in serum-free keratinocyte medium supplemented with 5 ng/mL epidermal growth factor and 35 to 50 µg/mL bovine pituitary extract (Invitrogen, Grand Island, NY) and cultured at 37°C.

Epstein-Barr virus-transformed B-lymphoblastoid cell lines (EBV-LCLs) were available from one of the HLA-B57-positive patients from whom a primary tumor cell line was established (patient #1) and from three HLA-B57-positive women with a history of HPV 16 infection, including subject #1 from whom the CD8 T cell clone was isolated (6).

Peptides

The HPV-16 E6 52–61 peptide, as well as peptides from high-risk HPV types homologous to this CD8 T cell epitope (Table 2), were synthesized by SynPep (Dublin, CA).

Chromium release assay

The primary tumor cell lines (with and without treatment with 200 U/ml of IFN-γ for 48 h prior to assay set up) and the EBV-LCL from patient #1 were radiolabeled using 100 µCi sodium chromate (Na_2^{51}CrO_4). After washing, the cells were plated in triplicate in 96 well plates at 3 x 10^5 cells per well. The peptides were added to the medium in the appropriate wells at 10 µM. Effector cells (HPV 16 E6 52-61-specific T cell clone cells) were added at four different effector:target cell ratios starting from 40:1. The plated cells were pelleted by centrifugation, followed by incubation for 5 hours at 37°C in a humidified 5% CO_2 incubator. The supernatants were harvested using a Skatron harvesting press and the ^{51}Cr was counted using a gamma counter (PerkinElmer, Shelton, CT). The percentage of specific lysis was calculated as described previously (7).

Fluorescence-activated cell sorter analysis

The primary tumor cell lines (with and without treatment with 200 U/ml of IFN-γ for 48 h) and an EBV-LCL from subject #1 were stained for surface markers with anti-HLA class I antibody (W6/32), anti-HLA class II antibody (IVA12 or 9.3F10), anti-HLA-A2 antibody (BB7.2) (all from the American Type Culture Collection, Manassas, VA). FITC-conjugated goat anti-mouse IgG (Sigma–Aldrich) was used for detection. PE-conjugated mouse anti-human CD54 (ICAM-1) antibody (Caltag, Burlingame, CA) was also used. Cells were analyzed using a COULTER EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

IFN-γ enzyme-linked immunospot (ELISPOT) assay

The method described by Larsson and colleagues (8) was used with minor modifications. For the ELISPOT assay to assess the recognition of tumor cell line #1 by the HPV 16 E6 52-61-specific T cell clone (Figure 2), tumor cell line #1 cells were treated with 200 U/ml of IFN-γ 48 hours prior to assay set up. On the day of the assay, increasing numbers of HPV 16 E6 52-61-specific T cell clone cells (10,000, 30,000 or 50,000), and increasing numbers of tumor cell line #1 cells (1,000, 3,000, 5,000, 10,000 or none) were added in triplicate wells. One-hundred thousand EBV-LCL cells from patient #1 were also added to all wells along with 20 U/ml of rIL-2. The negative control wells contained EBV-LCL cells from patient #1 and T cell clone cells (10,000, 30,000, or 50,000), but no tumor cells. The incubation period was 48 hours.

To test homologous peptides derived from high risk HPV types, 1,000 HPV 16 E6 52-61-specific T cell clone cells were plated with 1 x 10^5 EBV-LCL cells from subjects #1, #2, and #3, as well as from patient #1. Peptide antigens were added at a concentration of 10 µM, along with 20 U/ml of rIL-2, and the cells were incubated for 20 hours.
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